ADVANCES IN THE BIOSCIENCES, Volumes 22 & 23

Marihuana: Biological Effects

Analysis, Metabolism, Cellular Responses, Reproduction and Brain

Editors: G. G. NAHAS and W. D. M. PATON

PERGAMON PRESS

MARIHUANA
BIOLOGICAL EFFECTS

ANALYSIS, METABOLISM, CELLULAR RESPONSES, REPRODUCTION AND BRAIN

Proceedings of the Satellite Symposium of the
7th International Congress of Pharmacology, Paris, 22–23 July 1978

Editors:

GABRIEL G. NAHAS, *New York and Paris*
SIR WILLIAM D. M. PATON, *Oxford*

Co-editors:

MONIQUE BRAUDE, *Rockville*
JEAN CLAUDE JARDILLIER, *Reims*
DAVID J. HARVEY, *Oxford*

PERGAMON PRESS

OXFORD · NEW YORK · TORONTO · SYDNEY · PARIS · FRANKFURT

U.K.	Pergamon Press Ltd., Headington Hill Hall, Oxford OX3 0BW, England
U.S.A.	Pergamon Press Inc., Maxwell House, Fairview Park, Elmsford, New York 10523, U.S.A.
CANADA	Pergamon of Canada, Suite 104, 150 Consumers Road, Willowdale, Ontario M2J 1P9, Canada
AUSTRALIA	Pergamon Press (Aust.) Pty. Ltd., PO Box 544, Potts Point, N.S.W. 2011, Australia
FRANCE	Pergamon Press SARL, 24 rue des Ecoles, 75240 Paris, Cedex 05, France
FEDERAL REPUBLIC OF GERMANY	Pergamon Press GmbH, 6242 Kronberg-Taunus, Pferdstrasse 1, Federal Republic of Germany

Copyright © 1979 Pergamon Press Ltd.

All Rights Reserved. No part of this publication may be reproduced, stored in a retrieval system or transmitted in any form or by any means, electronic, electrostatic, magnetic tape, mechanical, photocopying, recording or otherwise, without permission in writing from the publishers.

First edition 1979

British Library Cataloguing in Publication Data
Advances in the biosciences.
22 & 23: Marihuana
1. Biology
I. Nahas, Gabriel G II. Paton, William Drummond MacDonald III. Symposium on Marihuana, *2nd, Reims, 1978*
574 QH307.2 78–41169

ISBN 0–08–023759–2

Printed and bound at William Clowes & Sons Limited Beccles and London

LIST OF PARTICIPANTS

ABUZZAHAB, F. S., Clinical Associate Professor, Departments of Psychiatry and Pharmacology, University of Minnesota Medical School, Minneapolis, MN.
AGURELL, S., Professor, Department of Pharmacognosy, Faculty of Pharmacy, University of Uppsala, Sweden.
BANCHEREAU, J., Research Associate, School of Pharmacy, Chatenay Malabry, France.
BARKOV, N. Vice Chairman, U.N. International Narcotics Control Board.
BINDER, M., Institute of Physiological Chemistry, Ruhr-University, Bochum, Germany.
BOURDON, R., Professor, School of Pharmacy, Université René Descartes, Paris, France.
BRAUDE, M. C., Chief, Preclinical Pharmacology, Division of Research, NIDA, Rockville, MD.
CARCHMAN, R. A., Assistant Professor, Department of Pharmacology, Medical College of Virginia, Richmond, VA.
CHAPMAN, L., Associate Professor, Department of Behavioral Biology, University of California School of Medicine, Davis, CA.
CHESHER, G. B., Reader in Pharmacology, University of Sydney, Australia.
CHOISY, H. Professor of Pharmacology, University of Reims Medical School, Reims, France.
CLARK, W. C., Associate Professor of Psychiatry, Columbia University, College of Physicians and Surgeons, New York, NY.
COZENS, D., Deputy Head, Department of Reproductive Toxicology, Huntingdon Research Centre, Huntingdon, England.
DESOIZE, B., Research Associate, University of Reims School of Pharmacy, Reims, France.
DEWEY, W. L., Professor, Department of Pharmacology, Medical College of Virginia, Richmond, VA.
ETEVENON, P., Research Associate (INSERM), Department of Psychiatry, St. Anne Hospital, Paris, France.
FEENEY, D. M., Professor of Psychology and Physiology, University of New Mexico, Albuquerque, NM.
FUJIMOTO, G. I., Associate Professor, Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY.
GARRETT, E. R., Graduate Research Professor, University of Florida College of Medicine, Gainesville, FL.
GILLESPIE, H. K., Department of Psychiatry, Veterans Administration Hospital, Palo Alto, CA.
HARCLERODE, J., Professor & Chairman, Department of Biology, Bucknell University, Lewisburg, PA.
HARDY, N., Research Fellow, Unité de Toxicologie 26 (INSERM), Hôpital Fernand Widal, Paris, France.
HARVEY, D. J., Department of Pharmacology, Oxford, England.
HEATH, R. G., Chairman, Department of Psychiatry & Neurology, Tulane University School of Medicine, New Orleans, LA.
HEMBREE, W. C., Associate Professor, Department of Obstetrics & Gynecology, Columbia University, College of Physicians & Surgeons, New York, NY.
HERSHKOWITZ, M., Professor, Department of Isotope Research, The Weizmann Institute of Science, Rehovot, Israel.
HOLLISTER, L. E., Director, Geriatric Center, Veterans Administration Hospital, Palo Alto, CA.
HUANG, H., Research Associate, Department of Obstetrics & Gynecology, Columbia University, College of Physicians & Surgeons, New York, NY.
HUBER, G., Chief, Division of Respiratory Diseases, Department of Medicine, Harvard Medical School, Boston, MA.
ISSIDORIDES, M. R., Professor, Neurobiology Research Laboratory, Department of Psychiatry, University of Athens Medical School, Athens, Greece.
JACUBOVIC, A., Professor, Department of Psychiatry, Kinsman Laboratory of Neurological Research, University of British Columbia, Vancouver, B.C., Canada.
JARDILLIER, J. C., Professor of Biochemistry, University of Reims School of Pharmacy, Reims, France.
KALANT, H., Professor, Department of Pharmacology, University of Toronto, Toronto, Canada.
KARLER, R., Professor, Department of Pharmacology, University of Utah Medical Center, Salt Lake City, UT.

KAYMAKCALAN, S., Professor of Pharmacology, Ankara University Medical School, Ankara, Turkey.
LEGER, C., Research Associate, Unité de Toxicologie 26 (INSERM), Hôpital Fernand Widal, Paris, France.
LEUCHTENBERGER, C., Director, Department of Cytochemistry, Swiss Institute for Experimental Cancer Research, Lausanne, Switzerland.
LUTHRA, Y. K., Scientific Regulatory Consultant, Clement Associates, Washington, DC.
MALOR, R., Reader in Pharmacology, University of Sydney, Australia.
MARKS, V. Professor of Clinical Biochemistry, University of Surrey, Guildford, England.
MARTIN, B. R., Assistant Professor of Pharmacology, Medical College of Virginia, Richmond, VA.
MCGEER, P. L., Professor of Neurological Sciences, Kinsman Laboratory of Neurological Research, University of British Columbia, Vancouver, B.C., Canada.
MECHOULAM, R., Professor, Laboratory of Natural Products, School of Pharmacy, The Hebrew University, Jerusalem, Israel.
MELLORS, A., Associate Professor, Department of Chemistry, University of Guelph, Ontario, Canada.
MILLER, L. I., Assistant Professor in Psychiatry, University of Kentucky, Albert B. Chandler Medical Center, Lexington, KY.
MORISHIMA, A., Associate Professor of Pediatrics, Columbia University, College of Physicians & Surgeons, New York, NY.
NAHAS, G. G., Professor, Department of Anesthesiology, Columbia University, College of Physicians & Surgeons, New York, NY. (and INSERM U 26, Paris, France).
OHLSSON, A., Department of Pharmacology, University of Uppsala, Sweden.
OSUMI, Y., Professor, Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan.
PATON, W. D. M., Professor, Department of Pharmacology, University of Oxford, England.
PRADHAN, S. N., Professor, Department of Pharmacology, Howard University College of Medicine, Washington, DC.
ROSELL, S., Associate Professor, Department of Pharmacology, Karolinska Institute, Stockholm, Sweden.
ROSENKRANTZ, H., Director of Biochemical Pharmacology, Mason Research Institute, Worcester, MA.
ROSENTHAL, F., Professor of Near Eastern Languages, Yale University New Haven, CT.
SASSENRATH, E. N., Associate Professor in Residence, Department of Behavioral Biology, University of California School of Medicine, Davis, CA.
SCHERMANN, J. M., Research Associate, Unité de Toxicologie 26 (INSERM), Hôpital Fernand Widal, Paris, France.
SMITH, C. G., Assistant Professor, Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD.
SMITH, M. T., Department of Neuropathology, Armed Forces Institute of Pathology, Washington, DC.
STEFANIS, C. N., M.D., Chairman, Department of Psychiatry, Athens University Medical School, Athens, Greece.
STEIN, G., Associate Professor, Department of Biochemistry and Molecular Biology, University of Florida, Gainesville, FL.
SZEPSENWOL, J., Professor of Anatomy, University of Puerto Rico School of Medicine, San Juan, Puerto Rico.
TAYLOR, D., Department of Pharmacology, University of Melbourne, Parkville, Australia.
TORO-GOYCO, E., Professor of Biochemistry, University of Puerto Rico School of Medicine, San Juan, Puerto Rico.
TUCHMANN-DUPLESSIS, H., Professor of Pathology, University of Paris, France.
VAN NOORDWIJK, J., Director of Pharmacology, National Institute of Public Health, Utrecht, The Netherlands.
WALL, M. E., Vice-President and Director, Research Triangle Institute, Research Triangle Park, NC.
WIDMAN, M., Assistant Professor, Department of Pharmacognosy, Faculty of Pharmacy, University of Uppsala, Sweden.
ZIMMERMAN, A., Professor and Associate Chairman, Department of Zoology, University of Toronto, Canada.
ZIMMERMAN, S. B., Division of Natural Science, Glendon College, York University, Toronto, Canada.

FOREWORD

This symposium, the second satellite symposium on Marihuana, like the first satellite meeting in Helsinki in 1975, was the result of international collaboration. Its inclusion as a satellite of the VIIth Congress of the International Union of Pharmacological Sciences (IUPHAR) was approved following a recommendation of the Congress Programme Committee (Professors J. P. Boissier and P. Lechat). In France, initial grants for the organization of the meeting in Reims were awarded by the Institut National de la Santé et de la Recherche Médicale (INSERM, Professor Camille C. Burg, Director General and Professor P. Laudat, Scientific Director) and the Ministère de la Santé (Madame Simone Weil, Ministre; Professor P. Denoix, Director), by the "Délégation Générale à la Recherche Scientifique (DGRST)" and by the University of Reims (President, L. Bernard). In the United States, the National Institute on Drug Abuse (NIDA: Dr. Robert Dupont, Director; Dr. William Pollin, Director of Research) awarded a conference grant to defray the travelling expenses of some of the American participants. The Medical Research Council provided travel expenses from Britain. In addition, grants were provided by the International Business Machines Company, American Telephone and Telegraph Company, Rhodia Incorporated and the American Council on Marihuana and other Psychoactive Drugs. The International Medical Council on Drug Use, the Alice and Gabrielle Nahas Foundation, and the Myrin Institute provided additional support.

Special thanks are due to the University of Reims which hosted the meeting, and especially to the late Professor J. LeMen, Deans J. Levy and S. Kochman, and Professor H. Choisy. Professor J.-C. Jardillier, who was in charge of local arrangements, performed this task superbly.

The programme was arranged after consultations between Gabriel Nahas, W. D. M. Paton and Monique Braude. In the event, it was not possible for all those on the programme to attend; and there was such a pressure on the symposium time available that some papers were given in much abbreviated form. For this volume, however, full papers have been used, including papers from scheduled participants unable to attend. A feature of the programme brochure was the poem "Cannabis and Alcohol, the Green and the Red" translated by Dr. F. Rosenthal. The opportunity has been taken to include this poem, together with a historical account of it, as an appendix to the scientific papers. We are much indebted to Mr. Martin Richardson of Pergamon Press for his helpfulness in seeing the book through the press, and particularly grateful to Dr. D. J. Harvey for preparing both an author and a subject index.

G. G. Nahas
W. D. M. Paton

INTRODUCTION
MARIHUANA AND THE CELL

A REVIEW of the literature indicates that a great deal of studies on the biological effects of cannabis have been performed since the last symposium held in Helsinki in 1975. Many of the results which were presented then, have been confirmed and extended in the area of quantitation of cannabinoids, mechanisms of their action, and of their cellular effects on lung, brain and reproductive organs.

An increasing number of studies have focused on long term effects of cannabis administration: since one is dealing with substances with a half-life of the order of one week; their daily consumption should result in tissue levels of the order of the micromole, quite different from the nanomolar concentration required to induce acute intoxication. If cannabinoids have a toxic effect, it would become more apparent as a result of their cumulative storage in the cells.

It is a credit to Harris Rosenkrantz to have established correlation between dosages smoked or ingested by man and those administered to animals (Table 1). The relevancy of these correlations was proven when blood levels of cannabinoids were measured under such a schedule of administration, and proved to be quite similar in man and different species studied. *Therefore, one may state that the dosage used in the animal experiments reported in the present monograph are comparable to the doses currently taken by cannabis users.*

If one wishes to find a common target for all of the multiple effects of cannabinoids described in this volume, one would be tempted to think first of the plasma membrane. The presently accepted view describes this membrane as consisting for the most part of a bimolecular layer of amphipathic lipids and cholesterol overlaid and penetrated by protein molecules, more or less embedded in the lipid matrix. The protein molecules of the membrane are dotted with ion-selective pores and receptor sites with which biologically active molecules will interact.

All pharmacologically active substances must pass through the plasma membrane and thereafter modify intracellular processes in the cytoplasm or some organelles. And many exert their immediate effects by interacting with one of the two major components of the cell membrane envelope: proteins or lipids.

Seeman was one of the first pharmacologists to systematize the way in which the properties of the neuroleptic fat soluble drugs may be classified into specific and non-specific, which might be related to their point of impact on the membrane: on the lipid portion or on the protein fraction.

The specific effects of these neuroleptics are based on their stereoselectivity and on their activity recorded with nanomolar concentrations of the drug which will attach to a protein receptor. The nanomolar concentration criterion for specific neuroleptic action refers to

TABLE 1. RELEVANCY OF Δ^9-TETRAHYDROCANNABINOL DOSES AND ROUTES OF ADMINISTRATION USED IN ANIMALS AS COMPARED TO MAN

	mg/kg Δ^9-THC (marihuana, 1% THC; hashish, 5% THC)		
Route[a]	Man[b]	Rat[c]	Mouse[c]
Inhalation, 1 cig/day	0.1–0.5	0.7–3.5	1–6
3 cigs/day	0.3–1.5	2–10	4–18
6 cigs/day	0.6–3.0	4–20	7–36
Approx. LD50	[d]	36–42	40–60[e]
Oral	0.3–1.5	2–10	4–18
	0.9–4.5	6–30	10–54
	1.8–9.0	12–63	20–100
Approx. LD50	[d]	800–1200	1400–2200

[a] In man, oral route requires 3 times the inhalation dose.
[b] Assumes 50 kg mean body weight and 50% loss of THC during smoking.
[c] Dose based on body surface area; conversion factor of 7 and 12 for rat and mouse, respectively.
[d] As a guide, IV LD50 in monkey was about 100 mg/kg and orally it was estimated to be approximately 15,000 mg/kg.
[e] N value; however, IV and inhalation values in rat shown to be nearly identical.
(Reprinted with permission from: H. Rosenkrantz, in *Marihuana: Chemistry, Biochemistry & Cellular Effects*, p. 444. G. Nahas, W. D. M. Paton, and J. Idanpaan-Heikkila, Springer Verlag, New York, 1976.)

the fact that the active concentration of the neuroleptic in plasma water is between 0.1 and 50 nM. Hence of the many neuroleptic sites proposed, only those affected by nanomolar concentrations (up to 100 nM) would have any likehood of being truly specific.

Of all the natural cannabinoids, delta-9 (and perhaps delta-8 THC) are the only molecules which appear to have a specific activity on or close to a receptor site, for instance a protein embedded in the lipid matrix of the membrane: they do act in nanomolar concentrations, and stereospecificity is essential for displaying their effects. These specific effects are primarily, if not exclusively, exerted on the neuronal membranes of the brain and of those of the conductive system of the heart.

In addition to these specific effects, neuroleptics also have non-specific sites of action on the double lipid layer of the plasma membrane. Since neuroleptics are highly fat soluble and surface active, they readily partition into biomembranes. In the case of delta-9-THC, if the aqueous concentration is 10^{-7} M and the octanol water partition coefficient is 3000, concentration in the plasma membrane phase might be 30 micromolar. It could be expected therefore that such high concentrations within the membrane phase could elicit some non-specific actions such as membrane expansion and membrane fluidization.

Membrane expansion underlies the antihemolytic action of neuroleptics, which in concentrations of 10^{-6} to 10^{-4} M protect erythrocytes from hypotonic hemolysis by increasing the area to volume ratio of the cells. Delta-9-THC and other cannabinoids possess such properties, the concentration of THC required to protect against 50% hemolysis (AH 50) is of the order of 10^{-5} M.

TABLE 2. DAILY DOSE OF DIFFERENT PSYCHOTROPIC DRUGS, THEIR MAXIMAL MOLAR (M) PLASMA CONCENTRATION, AND THRESHOLD CONCENTRATIONS REQUIRED TO INHIBIT, IN VITRO, THYMIDINE INCORPORATION INTO CULTURED LYMPHOCYTES

	Daily dose (mg)	Max. Plasma Concentration (M)	"Threshold" IC (M)*	I.C. 50 (M)†	A.H. 50 (M)‡	Log Octanol/ Water Partition Coefficient
Haloperidol	2–30	0.6×10^{-8}	3×10^{-7}	1.1×10^{-5}	2.2×10^{-5}	5.74
Chlorpromazine	200–2000	2.0×10^{-6}	5×10^{-6}	1.3×10^{-5}	1.0×10^{-5}	5.32
Imipramine	50–300	2.5×10^{-6}	3×10^{-5}	4.3×10^{-5}	1.0×10^{-5}	4.62
Delta-9-THC	4–400	2.0×10^{-7}	5×10^{-6}	3.0×10^{-5}	1.0×10^{-5}	3.20
Diazepam	5–30	3.5×10^{-6}	4×10^{-5}	7.8×10^{-5}	4.0×10^{-5}	2.82
Chlordiazepoxide	20–160	6.0×10^{-6}	4×10^{-5}	1.4×10^{-4}	3.0×10^{-4}	2.44
Hydantoin	100–500	9.0×10^{-5}	1×10^{-5}	8.2×10^{-5}	1.5×10^{-3}	2.47
Phenobarbital	30–60	1.3×10^{-4}	8×10^{-4}	1.2×10^{-3}	5.7×10^{-3}	1.42
Meprobamate	400–1200	0.8×10^{-4}	5×10^{-4}	1.7×10^{-3}	—	0.70

* "Threshold" inhibiting concentration (producing significant inhibition of thymidine incorporation).
† Concentration of drug required to inhibit by 50% incorporation of ^3H thymidine.
‡ Concentration of drug to protect against 50% hemolysis.
(Reprinted with permission from *Proc. Soc. Exptl. Biol. Med.* **160**, 344 (1979).)

Concomitant with membrane expansion, neuroleptics fluidize the membrane, loosening the lipid protein interactions, increasing the mobility of lipids within the plane of the membrane as well as permitting water to permeate more readily. As a result, membrane bound enzyme function may be altered. Several examples of such alterations are included in this monograph. I will present just one instance of a biochemical event which is altered in a similar fashion by neuroleptics: the incorporation of thymidine into cultured lymphocytes. This alteration observed in our laboratory with some cannabinoids and their metabolites was also observed to be a property of other psychotropic drugs (Table 2). The concentration of these drugs required to inhibit by 50% incorporation of thymidine into cultured lymphocytes is well correlated with their octanol water partition coefficient ($p < 0.001$): the more lipophilic a substance, the greater its inhibitory effect. Such a non-specific effect of these drugs is exerted with micromolar concentrations which may be reached only in daily consumption.

This is especially true for THC and its metabolites which have a prolonged half-life, because of their liposolubility and their enterohepatic recirculation. A daily consumption of 100 to 400 mg THC equivalent is not uncommon in areas of cannabis cultivation and free availability such as the Blue Hills of Jamaica, the mountains around Ketama in Morocco, or even Ashbury Heights in San Francisco. It would appear that when tolerance develops to psychotropic drugs, the nanomolar concentration required to activate receptor sites is associated with micromolar concentration of these compounds or their metabolites in tissues where they are stored.

However, one should not limit the cellular effects of THC (and other cannabinoids) to an inhibition of precursor transport. They are much more far reaching as emphasized in this monograph by Stein, Carchman and Issidorides who report that the cannabinoids interact with the nucleus, and alter the biosynthesis of chromosomal proteins, such as histones and non-histones, thereby modifying the expression of the genome. Cannabinoids also interact with hormone mediated cellular mechanisms.

Such interactions of psychoactive and non-psychoactive cannabinoids with the fundamental mechanisms of macromolecular synthesis might well account for the biological effects of chronic cannabis administration reported in this monograph: damage to the lung, impairment of spermatogenesis and alteration of brain ultrastructure.

LA MARIHUANA ET SES EFFETS SUR LA CELLULE

De nombreux travaux ont été effectués sur les effets biologiques du cannabis depuis le dernier symposium international sur ce sujet qui se tint à Helsinki en 1975. Il s'agit surtout d'études décrivant la détection quantitative des cannabinoïdes dans les humeurs ansi que les effets de leur consommation chronique sur la division cellulaire, le poumon, la fonction de reproduction et le cerveau. En effet, il est établi que le delta-9-THC ayant une demi vie d'environ huit jours, est stocké dans les tissus d'où il est lentement éliminé sous forme de métabolites. La consommation journalière de cannabis resultera dans l'accumulation de cannabinoïdes en concentrations micromolaire fort différente de la concentration nanomolaire qui crée l'intoxication aigue. L'effet toxique des cannabinoïdes deviendra donc plus apparent à la suite de leur accumulation dans les cellules.

C'est Harris Rosenkrantz qui à été le premier à établir une corrélation entre les doses consommées par l'homme et celles administrées expérimentalement à l'animal (Tableau I). La validité de ces corrélations fut établie lorsque la concentration plasmatique des cannabinoïdes mesurée dans de telles conditions était du même ordre que celle atteinte chez l'homme. *Par conséquent, il est maintenant avéré que les doses administrées dans les modèles expérimentaux décrits dans ce volume sont semblables à celles utilisées par les fumeurs et les utilisateurs de cannabis.*

En recherchant une cible commune à toutes les manifestations des cannabinoïdes décrites dans ce volume, on serait tenté de choisir la membrane plasmique. Il s'agit d'une double couche moleculaire de lipide dans laquelle sont inserées des molecules protéiques, douées de sites récepteurs sur lesquels les molécules biologiquement actives vont s'insérer. Toutes ces substances doivent pénétrer dans la membrane plasmiques pour ensuite modifier des mécanismes intracellulaire, et beaucoup de ces substances exercent leurs effets immédiats par l'intermédiaire d'une des deux composantes de la membrane plasmique protéinique ou lipidique. Seeman fut l'un des premiers à systématiser les propriétés des neuroleptiques liposolubles en "spécifiques" et "non-spécifiques" selon leur point d'impact sur la portion lipidique ou protéinique de la membrane.

Les effets spécifiques des neuroleptiques sont basés sur leur steréo-selectivité et s'exercentà des concentrations nanomolaires: la drogue en concentration nanomolaire s'attachera à une protéine "réceptrice". Le critère de l'action spécifique des neuroleptiques est défini par leur concentration nanomolaire ce que corréspond à une concentration plasmatique de l'ordre de 0.1 à 100 nanomoles.

Parmi tous les cannabinoïdes naturels, le delta-9-THC (et peut être le delta-8) sont les seules molécules qui paraissent avoir une activité spécifique sur un site récepteur: ils exercent leurs effets à des concentrations nanomolaires et ont une action stéréo-specifique. Ces effets spécifiques sont limités aux membranes des neurones du cerveau et du système de conduction cardiaque.

En plus de leurs effets spécifiques, les neuroleptiques liposolubles ont aussi des effets "non-spécifiques" sur la partie lipidique de la membrane plasmique. Par example, si la

concentration du delta-9-THC dans l'eau plasmatique est de l'ordre de 10^{-7} M., sa concentration dans la membrane plasmique pourra atteindre 30 micromoles puisque le coéfficient de partage octanol -eau de cette drogue est de l'ordre de 3000. Une telle concentration pourait entrainer un effet non-spécifique tel une modification de l'expansion ou de la fluidité membranaires. L'expansion de la membrane explique l'action antihémolytique des neuroleptiques qui en concentration de 10^{-6} à 10^{-4} M protègent les érythrocytes contre l'hémolyse hypotonique, en augmentant le rapport entre la surface et le volume de la cellule. Le delta-9-THC et les autres cannabinoïdes possèdent une telle propriété: la concentration de THC (et d'autres cannabinoïdes) nécessaire pour proteger l'hematie contre 50% d'hémolyse (AH 50) est de l'ordre de 10^{-5} M.

Les neuroleptiques vont également augmenter la fluidité de la membrane modifiant les interactions entre lipides et protéines ainsi que leur structure. Il en résultera une altération de la fonction des enzymes liés à la membrane. Plusieurs exemples de telles altérations sont illustrés dans cette monographie. Je voudrai à cet égard donner un exemple de l'altération d'un processus biochimique qui parait être altéré d'une façon "non-spécifique" par les neuroleptiques: l'incorporation de la thymidine par les lymphocytes en culture. Les cannabinoïdes et certains de leurs métabolites, ainsi que d'autres drogues psychotropes courantes inhibent l'incorporation de la thymidine par les lymphocytes; la concentration nécessaire pour inhiber de 50% cette incorporation est correlée avec le coefficient de partage octanol-eau de ces drogues ($p < 0{,}001$). Plus la drogue est liposoluble plus l'inhibition est grande (Tableau 2). Un tel effet non-spécifique requirt des concentrations micromolaires qui ne peuvent être atteintes qu'au cours d'une consommation chronique. Cela est particulièrement vrai pour le THC et ses métabolites à cause de leur demi vie prolongée: une consommation chronique de cannabie correspondant à 100–400 mg de THC. S'observe dans tous les régions où le cannabis est aisément disponible.

Toutefois on ne devrait pas limiter les effets céllulaires du THC et des autres cannabinoïdes à une inhibition du transport membranaire des précurseurs des macromolécules. Ces effets sont en effet beaucoup plus étendus comme le montre dans cette monographie les travaux de Stein, de Carchman et d'Issidorides. Ces auteurs rapportent que les cannabinoïdes exercent aussi leurs effets au niveau de la membrane nucléaire, interfèrent avec les mécanismes cellulaires controles par les steroides et alterent la synthèse des protéines chromosomiques, histones et non histones, modifiant ainsi l'expression du génome. De telles interactions des cannabinoïdes psychoactifs et non psychoactifs avec les mécanismes fondamentaux de la synthèse macromoléculaire pourraient expliquer les effets biologiques de l'administration chronique du cannabis décrits dans ce volume: détérioration de la fonction de reproduction mâle et femelle, lésions pulmonaires et altération de l'ultrastructure de la région limbique du cerveau.

GABRIEL NAHAS

INTRODUCTION TO QUANTIFICATION OF CANNABINOIDS AND THEIR METABOLITES IN BIOLOGICAL FLUIDS

S. Agurell, J.-E. Lindgren and A. Ohlsson

Department of Pharmacology, Karolinska Institute, 104 01 Stockholm 60, Astra Läkemedel AB, 151 85 Södertälje, Faculty of Pharmacy, Biomedical Center, 751 23 Uppsala, Sweden

Up to 1973 the biological fate of Δ^1-tetrahydrocannabinol (Δ^1-THC; or Δ^9-THC using the alternate numbering system) itself and its metabolites could be followed only by the use of radio-labelled compound. The first report of a quantitative, analytical procedure for non-labelled Δ^1-THC in human blood plasma after smoking Δ^1-THC (Agurell *et al.*, 1973) was rapidly followed by other publications using varying analytical methodology.

It is not the purpose of this introduction to extensively review the different methods, since the proceedings of recent conferences at the National Institute of Drug Abuse (Willette, 1976) and the American Chemical Society (Vinson, 1978) are devoted to the assay of cannabinoids in biological fluids. Also, the subsequent papers by Drs. Wall, Harvey, and Marks in the present volume will review progress in specific areas as well as presenting new findings.

The difficulties in quantifying Δ^1-THC and its metabolites are dependent upon the potency of Δ^1-THC itself and its pharmacokinetic and metabolic properties.

Δ^1-THC is a lipid soluble compound having a phenolic group with a pKa of 10.6 and rather unstable to light and oxygen. Being the major active component of marihuana (usually 1–2% THC-content) and hashish (4–10%), Δ^1-THC is generally inhaled by smoking often in a dose of 10–50 μg/kg, viz. 0.5–2.5 mg calculated as the amount absorbed by a person via the lungs. Under controlled conditions Δ^1-THC has also been given i.v. to volunteers as a 2–30 min. infusion in a microsuspension with albumin or as an alcoholic solution into a rapidly flowing dextrose infusion. Under such conditions up to 4 to 5 mg of Δ^1-THC produces an immediate and intense " high " (cf. Hollister and Gillespie, 1973). By the oral route Δ^1-THC—then often incorporated as hashish into sweets—is less potent with a dose of 10–30 mg being required. Orally it has a slow and sometimes unpredictable onset of action. As pointed out by Garrett in a subsequent paper in this volume the low potency of Δ^1-THC orally is partly due to its instability in the acidic pH of the stomach.

PHARMACOKINETICS AND METABOLISM RELATED TO EFFECT

The pharmacokinetics of Δ^1-THC show after smoking a very rapid distribution of the extensively lipoprotein-bound drug out into the tissues. It is uncertain whether at present any true elimination phase has been determined in man and earlier estimates of an elimination half-life in man of 24–48 hrs may be underestimated (Lemberger, 1972). From experiments in dogs carried out by Garrett and Hunt (see Willette, 1976) it seems likely that the rate limiting step in the elimination of Δ^1-THC is the redistribution from tissues back to the blood for further metabolism in the liver. As a consequence of these factors, Δ^1-THC metabolites continue to be excreted long after the administration of Δ^1-THC (e.g. Hollister *et al.*, 1972).

It appears that of all the cannabinoids present in cannabis essentially only Δ^1-THC causes the desired psychotomimetic effects in man with cannabinol being about 1/10 as active and cannabidiol being inactive (e.g. Hollister, 1973). The latter compound is *in vitro* a drug metabolism inhibitor but whether this is of clinical importance in man is not clear.

Thus, there is a general agreement that Δ^1-THC is the active compound in cannabis but it is still a matter of some controversy whether Δ^1-THC is active *per se* or first after metabolism to mono-hydroxylated derivatives. It has been shown in man and, for example, in the rhesus monkey (cf. Leander *et al.*, 1978) that 7-hydroxy-Δ^1-THC and its Δ^6-isomer as well as metabolites of THC hydroxylated in the side chain in the 3″-, 4″- or 5″-positions are at least equipotent with THC with regard to psychoactive effects. The plasma levels of monohydroxylated metabolites after administration of Δ^1-THC are little known in man, but Wall and co-workers (see Willette, 1976) have shown a rapid formation of 7-hydroxy-Δ^1-THC after i.v. infusion of Δ^1-THC in volunteers. The plasma concentration of the 7-hydroxy metabolite remains throughout the first 1.5 hrs post-infusion at a level of about 1/10 of that of Δ^1-THC itself. Nevertheless, the relative tissue concentrations of 7-hydroxy-Δ^1-THC, and hence the contribution to the effects of Δ^1-THC, may be somewhat greater since animal experiments by Schou (see Nahas, 1976) indicate a more preferential brain uptake of the metabolite than of Δ^1-THC.

As shown in recent reviews (Mechoulam, McCallum and Burstein, 1976; Nahas, 1976) the metabolic patterns of Δ^1-THC and related cannabinoids are very complex and numerous monohydroxylated derivatives of Δ^1- have been isolated and their biological activity assessed. The pattern of monohydroxylated metabolites in man is as stated rather unknown, but experiments by Widman and collaborators in our laboratory (to be published) show that human liver microsomes *in vitro* predominantly hydroxylate Δ^1-THC in the 7-position. Thus, in addition to previous knowledge, there seems at present to be no indications that other metabolites than 7-hydroxy-Δ^1-THC contribute to the effects of Δ^1-THC in man.

Thus, one may at this stage make a preliminary conclusion that the psychotomimetic effects of Δ^1-THC in man are mainly due to Δ^1-THC itself. After oral administration the metabolites may possibly be of greater significance but evidence in either direction is lacking. It has also been shown that the effects are reasonably well correlated to the plasma levels of Δ^1-THC (cf. Lemberger, 1972; Agurell *et al.* in Braude and Szara, 1976).

IMPORTANCE OF IDENTIFYING AND ASSAYING PLASMA LEVELS OF Δ^1-THC OR URINARY METABOLITES OF Δ^1-THC

Interest in determining plasma levels of Δ^1-THC is—as for many other drugs—related to the necessary knowledge about absorption, distribution and elimination. There also appears to be a good relation between the "high" and the Δ^1-THC blood level whereas some psychomotor functions are affected longer than the plasma levels would indicate (cf. Agurell *et al.* in Braude and Szara, 1976). Plasma level determinations of THC may also be important for the correct clinical diagnosis of cannabis intoxication and for drug interaction studies. Due to the complexity of assaying THC, plasma and tissue level information is unfortunately scarce in pharmacological experiments with the drug. It has been shown that Δ^1-THC and particularly some related new compounds have potential therapeutic interest, e.g. antiemetic and bronchodilating effects, and here plasma level determinations are important for the safety evaluation.

The forensic interest is in two areas. One area is epidemiological studies in selected population groups where the extent of *use* of cannabis needs to be investigated. Since cannabis has effects on time perception and short-time memory, and has been implicated as a cause of road traffic accidents, drivers are obviously one such group of concern. For epidemiological purposes it would generally be sufficient with screening of urine samples for THC-metabolites, using less specific but high-capacity methods. More specific requirements are necessary when not only the use but the *intoxication* has to be shown by chemical analysis. For the latter purpose a blood plasma level of at least a few ng Δ^1-THC per ml and measured by a specific analytical technique may be appropriate although, as mentioned, certain psychomotor functions are negatively affected also at lower levels.

METHODOLOGY FOR ASSAYING Δ^1-THC

There are various principal methods for the determination of Δ^1-THC in blood and other biological fluids:

- thin-layer chromatography (TLC)
- gas chromatography (GC)
- radioimmunoassay (RIA)
- gas chromatography—mass spectrometry (GC—MS)

These basic methods in combination with auxiliary extraction and separation steps, fluorimetry, use of radioisotopes, derivatization etc. yield methods of varying capacity, specificity and sensitivity.

Subsequently an attempt will be made to evaluate the use and value of some methods. It should, however, be borne in mind that both the experience and the equipment of a laboratory as well as the analytical object determines the selection of analytical method.

TLC

The first plasma level studies carried out in man (cf. Lemberger, 1972) used radiolabelled Δ^1-THC. Following extraction, the unchanged Δ^1-THC was assayed after TLC by scintillation counting. It has later been shown that this is a rather accurate technique for assaying Δ^1-THC (Rosenthal *et al.*, 1978). The limitations for using radioactive compounds in man are, however, severe.

Dansylation has been used as a means of detecting in the saliva Δ^1-THC—presumably released from the compound absorbed in mucous membranes rather than by glandular secretion—up to two hours after smoking cannabis. This technique has now been developed further by Scherrmann *et al.* (1977). By the use of tritiated Δ^1-THC as internal standard and C^{14}-labelled dansyl chloride the final fluorimetric assay could be quantitative and allowed a sensitivity limit of a few ng Δ^1-THC per ml blood plasma. The potential of this assay method and a related method proposed by Vinson *et al.* (cf. Vinson, 1978) remains to be explored.

GC

As in other assay systems, the separation of Δ^1-THC from interfering plasma compounds is a necessity to achieve sensitivity. Simple GC in combinations with electron capture (EC) sensitive derivatives have apparently not been found satisfactory to analyze Δ^1-THC in blood from cannabis smokers, except as described by Fenimore *et al.* (see Willette, 1976). To obtain a sufficient clean-up, the authors first use a GC column for the initial separation followed by a second capillary GC column for the final resolution prior to the EC-detection of the heptafluorobutyrate derivative. The sensitivity probably allows quantitation down to 2 ng/ml and the selectivity is satisfactory when compared to a GC—MS-method (Rosenthal *et al.*, 1978).

Reverse-phase high-performance liquid chromatography (HPLC) has been suggested by Garrett and Hunt as a prior step to GC analysis with EC detection (see Willette, 1976).

It is obvious that none of the above methods has high capacity compared to RIA methods.

RIA

RIA-methods are available for the assay of Δ^1-THC and its metabolites. Now when the technical problems are largely solved, RIA-methods offer quick and convenient determination of "THC cross-reacting" compounds. (Cook *et al.*, Chase *et al.* and Gross and Soares in Willette, 1976). The sensitivity is perhaps at 1–2 ng/ml but could be improved. The specificity and response for different cannabinoids varies both with the structure and how Δ^1-THC or a related cannabinoid has been attached to the carrier protein when preparing the antiserum. It is not clear to what extent claims for specificity for a single cannabinoid in RIA-methods can be substantiated. Thus, in general RIA lacks the specificity and precision of other methods, used to quantify Δ^1-THC. However, this is an advantage in, for example, epidemiological studies where the presence of Δ^1-THC metabolites (less than 0.02% of Δ^1-THC is excreted unchanged) in urine can be detected due to their cross reactivity in the

RIA-method. Since Δ^1-THC metabolites continue to be excreted for up to a week after intake (cf. Hollister *et al.*, 1972), the existence of "THC cross reacting" substances can be detected with RIA during this time. Conversely, the existence of "THC cross reacting" compounds in a urine sample does not imply an intoxication at the time the sample was taken. With present knowledge, it merely shows intake of cannabis any time during the preceding days.

Williams, Moffat and King (1978) have recently suggested a system where Δ^1-THC and three groups of metabolites characterized as "monohydroxylated", "dihydroxylated" and more "polar metabolites" in human blood plasma were separated by HPLC before RIA. This combination would considerably increase the specificity of the RIA method. Their results showed that a GC-MS method and RIA in combination with HPLC gave similar Δ^1-THC blood concentration, whereas there was little similarity with a "direct" RIA method. The addition of the HPLC obviously limits the capacity of the RIA method. Teale *et al.* (1977) have recently used this method to investigate the presence of cannabinoids in fatally injured drivers. The presence of "THC cross reacting" compounds were indicated in 6 cases out of 66 and definitely proven by the combined technique in three of the cases.

In connection with the RIA methods, one should also mention the homogeneous enzyme immunoassay or EMIT (Rodgers *et al.* 1978). This technique may also be useful for the rapid screening of urine specimens. As in the conventional RIA methods major cannabinoids as well as some tested metabolites cross react in the assay. In agreement with Williams *et al.* (1978) the investigators showed hardly any cross reactivity with non-cannabinoid drugs except two steroids at high concentrations.

GC-MS, MASS FRAGMENTOGRAPHY

GC-MS methods seems at present to provide the most specific and sensitive assays for THC in blood with a lower sensitivity level of 0.1–2 ng/ml, largely depending upon the work-up procedure. The original method consisted of addition of deuterium labelled Δ^1-THC as internal standard to the plasma sample, subsequent extraction with a non-polar solvent followed by chromatographic purification on a Sephadex LH-20 column and collection of the appropriate THC-fraction. The final quantification was made by mass fragmentography (Agurell *et al.*, 1973). Similar standard mass fragmentographic procedures have been used also by other laboratories (cf. Willette, 1976). The chromatographic clean-up is time consuming and has been replaced in other methods with aqueous acid and base washes. It would appear, however, that some of the sensitivity is lost when the chromatographic step is replaced. Also, the Sephadex LH-20 column can be used to specifically collect monohydroxylated derivatives eluted after THC as described by Ohlsson *et al.* (see Willette, 1976). It should also be noted that silylation before the mass fragmentographic quantitation decreases interference and increases the sensitivity (Ohlsson *et al.* in Vinson, 1978).

Routine methods worked out by the Battelle Columbus Laboratories (using chemical ionization) and the Research Triangle Institute (electron impact mass spectrometry) are available in detailed manual form (Fentiman *et al.*, 1978). See also Rosenthal *et al.* (1978) and Detrich and Foltz in Willette (1976).

The simultaneous determination of Δ^1-THC and 7-hydroxy-Δ^1-THC in plasma using mass fragmentography was recently described by Rosenfeld (1977). The method includes

extractive alkylation of the phenolic group with ethyl iodide and uses the corresponding compounds with perdeuterated ethyl groups as internal standard. The sensitivity for Δ^1-THC in this method needs to be improved.

Another approach to the mass spectrometric assay of Δ^1-THC has been used by Valentine *et al.* (1977). Their initial clean-up of the plasma extract was carried out by HPLC. The final assay, however, was carried by high resolution mass spectrometry using repetitive scannings of Δ^1-THC and the deuterated Δ^1-THC as internal standard.

PRELIMINARY REPORT

PLASMA LEVELS OF Δ^1-THC AFTER SMOKING, I.V. AND ORAL ADMINISTRATION TO VOLUNTEERS

In collaboration with L. Hollister, H. Gillespie and K. Knopes, Veterans Administration Hospital, Palo Alto, Calif. 94304, USA.

Plasma levels of Δ^1-THC after smoking have now been estimated in many laboratories and show surprisingly small interindividual variation (Willette, 1976). Immediately after smoking a few mg of THC, plasma levels of 50–200 ng/ml are reached dropping within 1

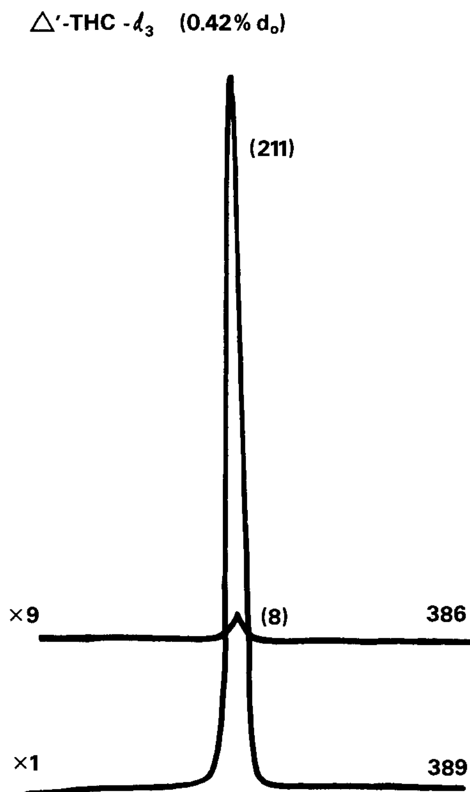


FIG. 1. Mass fragmentogram of the silylated internal standard Δ^1-THC-d_3(m/e 389). The channel for contaminating Δ^1-THC-d_0 (m/e 386) is magnified 9 × . Calculated Δ^1-THC-d_0 content 0.42%.

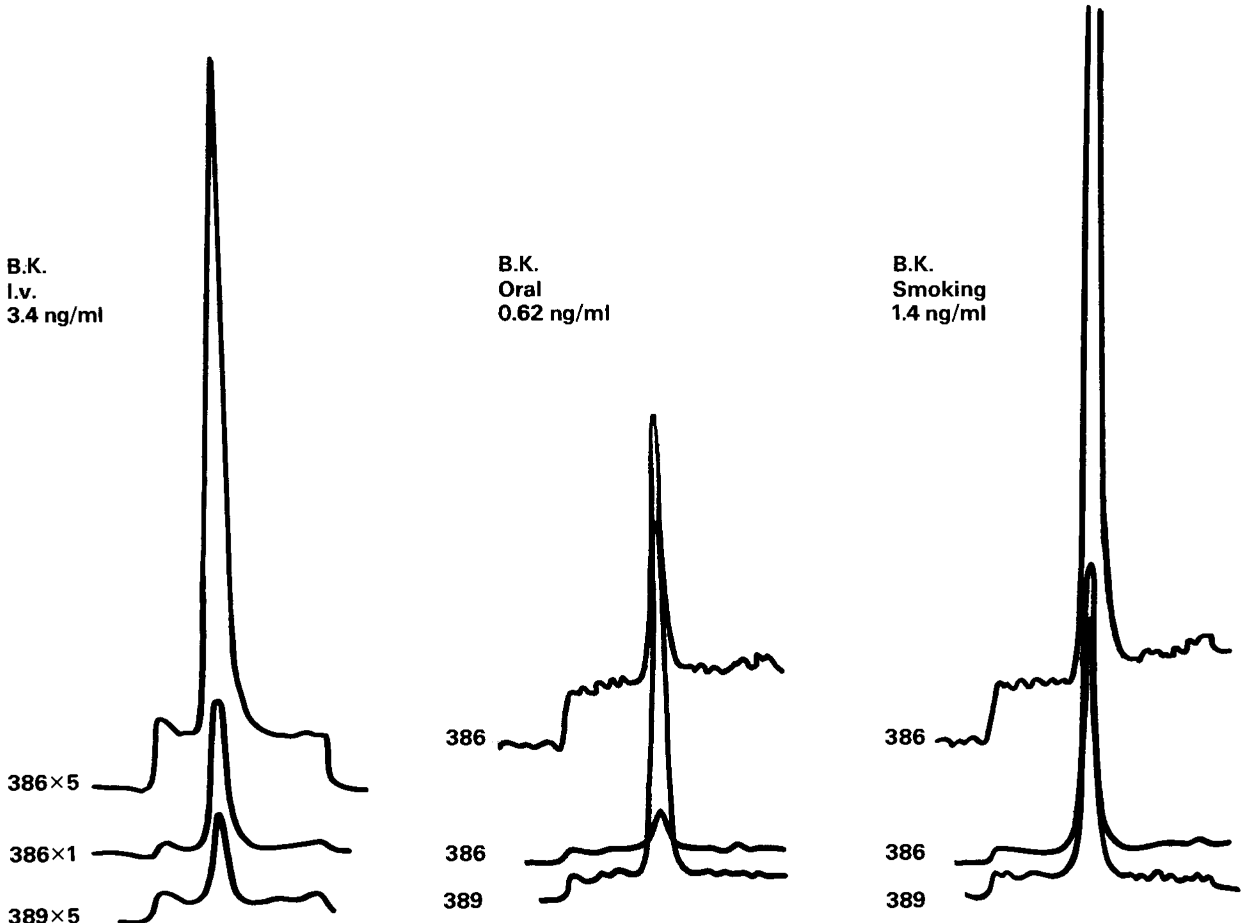


FIG. 2. Mass fragmentograms of purified and silylated plasma extracts from subject B.K. Route of administration and Δ^1-THC concentrations are shown in the figure. Two channels (m/e 386) at varying magnifications were used for Δ^1-THC in blood plasma, m/e 389 for the internal standard Δ^1-THC-d_3.

hour to 5–10 ng/ml and in the range 1–5 ng/ml at 4 hours. Twenty-four hours' values are in the range of 1 ng/ml or less. THC levels after i.v. infusion are roughly of similar magnitude (Willette, 1976; Rosenthal *et al.*, 1978). Little information is available on the Δ^1-THC blood levels after oral administration.

In an on-going study we are comparing the plasma levels of Δ^1-THC in the same subjects after smoking up to 10 mg Δ^1-THC as a cigarette, after i.v. injection during 2 min. of 5 mg Δ^1-THC and after oral administration of 20 mg Δ^1-THC in a chocolate cookie. Blood samples were taken as indicated in the figures up to 5 hours after administration.

Plasma levels of Δ^1-THC were assayed by mass fragmentography as described previously (Agurell *et al.*, 1973; Ohlsson *et al.* in Willette, 1976). Since trimethylsilylation increased the sensitivity, Δ^1-THC and the internal standard Δ^1-THC-d_3 were silylated in acetonitrile at 60°C for at least 10 min. with BSTFA before mass fragmentography (Ohlsson *et al.* in Vinson, 1978). Figure 1 shows the minute amount (0.42%) of non-labelled (d_0) Δ^1-THC in the deuterated internal standard (Δ^1-THC-d_3). The minimal content is necessary to obtain high sensitivity in our method.

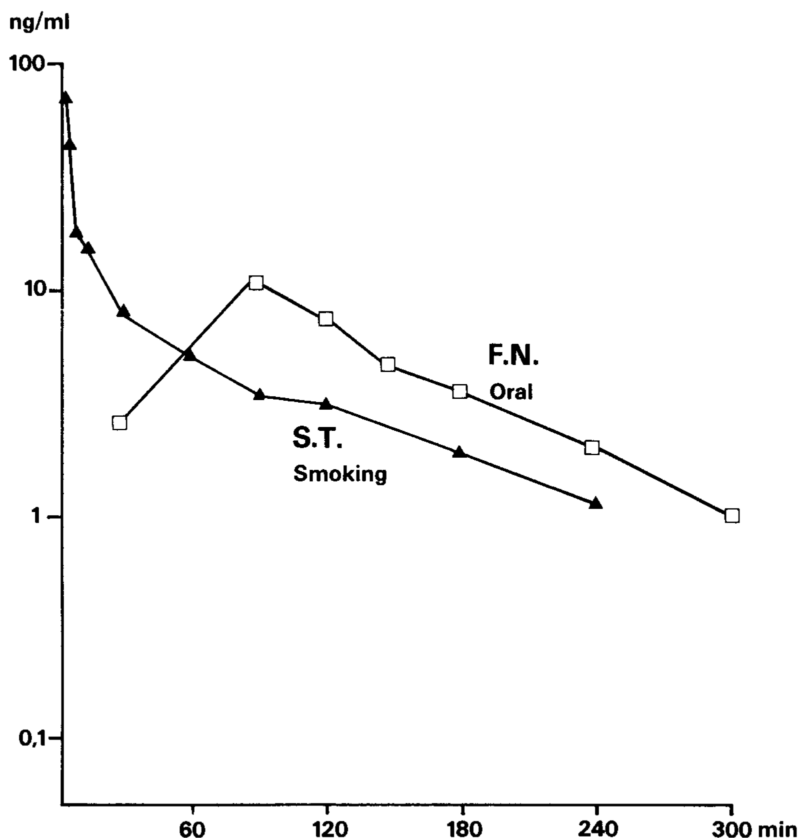


FIG. 3. Plasma levels of Δ^1-THC in subject S.T. after smoking 4.4 mg of Δ^1-THC of which 2.2 mg was assumed to be absorbed. Subject F.N. received 20 mg Δ^1-THC orally as a chocolate cookie. Each sampling time is indicated by a symbol. Each subject smoked a marihuana cigarette containing a known amount of Δ^1-THC to a desired level of "high". The amount of Δ^1-THC remaining in the butt was assayed. Of the difference between the original amount in the cigarette and the amount remaining in the butt—for subject S.T. 4.4 mg—half was assumed to have been absorbed by the smoker.

Mass fragmentograms of Δ^1-THC in blood plasma after different routes of administration are shown in Fig. 2.

Figure 3 shows the result from two subjects. Subject S.T. smoked 4.4 mg of Δ^1-THC—about half of which may be absorbed (for further information see legend to Fig. 3). The plasma levels are as expected with Δ^1-THC levels of about 70 ng/ml at 3 min., 5 ng/ml at 1 hour and 1 ng/ml at 4 hours.

Subject D.C. received Δ^1-THC by all three routes. Assuming a 50% transfer of Δ^1-THC by smoking, he may have absorbed 2.7 mg Δ^1-THC by this route. The i.v. administration of 5 mg Δ^1-THC yielded plasma levels which were 3–5 times that reached by smoking (Fig. 4). In the other subjects (e.g. R.R., Fig. 5) there was a more direct correlation between plasma levels of Δ^1-THC and dose, independent of whether Δ^1-THC was given i.v. or smoked. We have then assumed that 50% of the Δ^1-THC lost from the smoked marihuana cigarette was absorbed via the lungs, while the remainder was lost by pyrolysis and

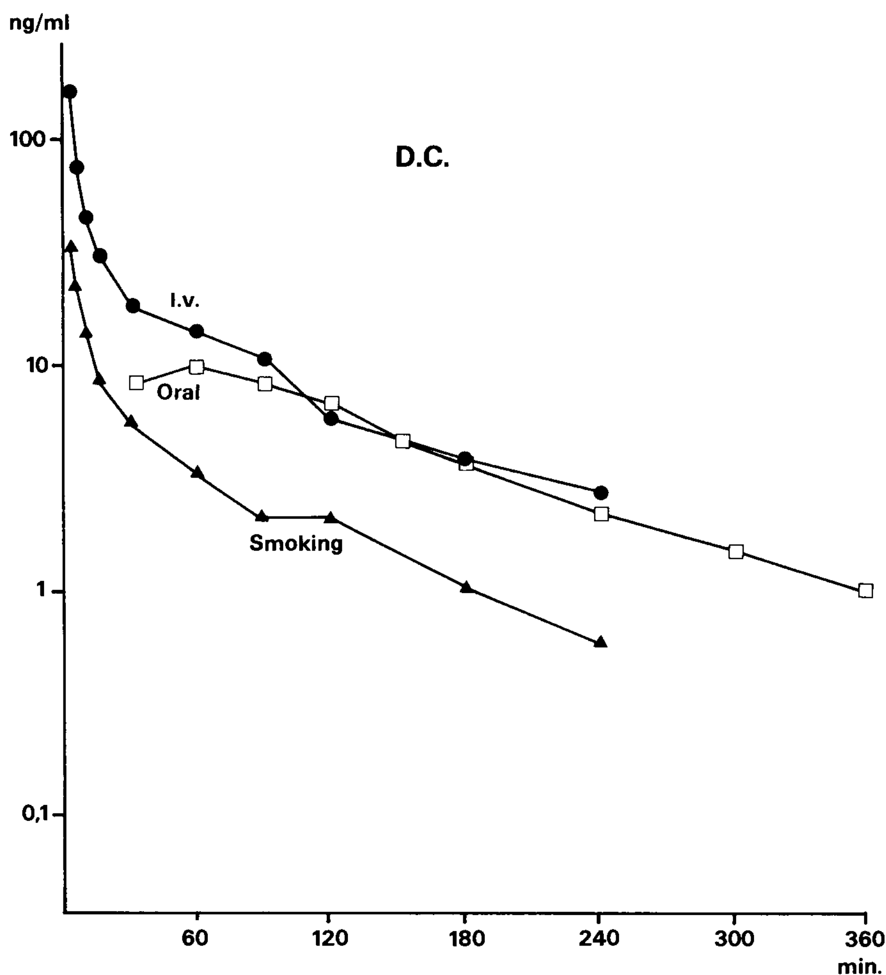


Fig. 4. Plasma levels of Δ^1-THC in subject D.C. after smoking 5.5 mg Δ^1-THC (approx. 2.7 mg absorbed), i.v. injection of 5.0 mg and oral administration of 20 mg Δ^1-THC.

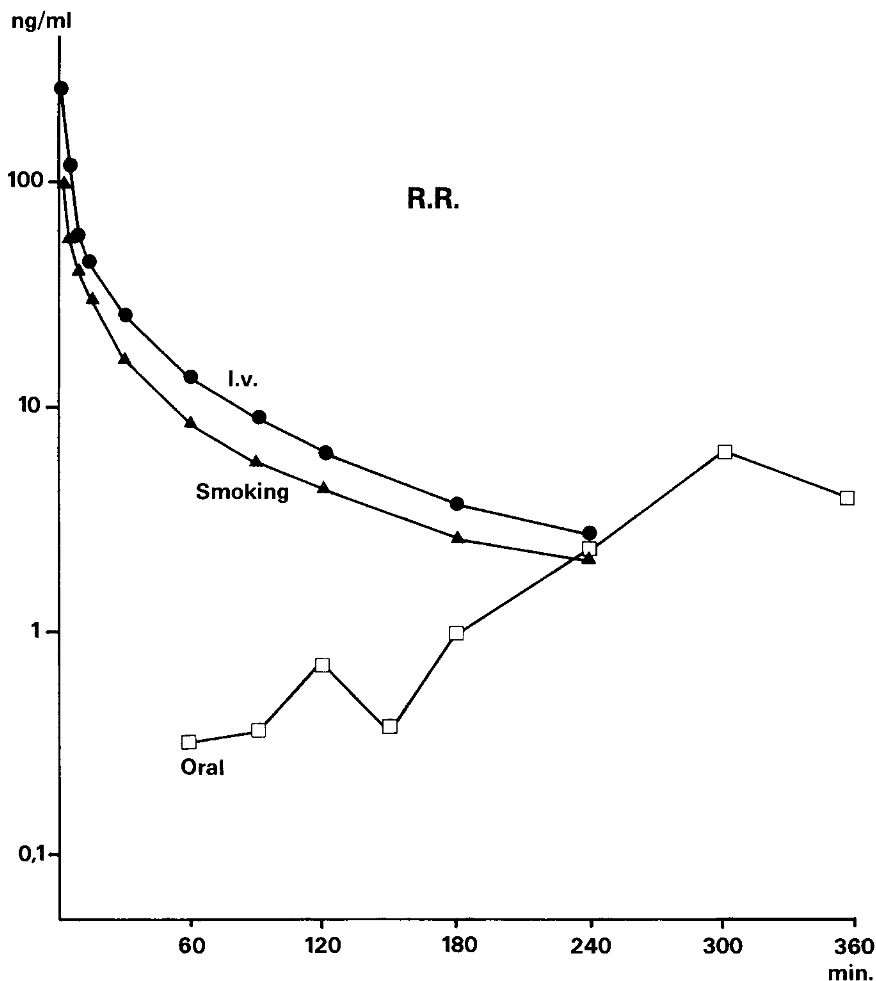


FIG. 5. Plasma levels of Δ^1-THC in subject R.R. after smoking 5.7 mg Δ^1-THC (approx. 2.8 mg absorbed), i.v. injection of 5.0 mg and oral administration of 20 mg Δ^1-THC.

side-stream smoke. Oral administration of 20 mg gave a peak plasma level of 8–10 ng/ml between 0.5 and 1.5 hour with a decline very similar to that after i.v. and smoking in this subject. Subject R.R. (Fig. 5) showed very similar plasma decay curves from i.v. and smoke administration and quite directly related to the dose. The oral administration, however, gave rise to very slowly increasing plasma levels of Δ^1-THC with one small peak at 2 hours but reaching a maximum of 6 ng/ml as late as after 5 hours. The clinical experience that oral administration of Δ^1-THC is often less predictable is borne out by this plasma curve.

Full details including plasma levels in all subjects, bioavailability, and relation to clinical effect will be presented in a later publication.

Acknowledgements: The work in Sweden was supported by grants from the Medical Research Council and a travel grant from the Swedish Academy of Pharmaceutical Sciences.

SUMMARY

The pharmacokinetics of Δ^1-THC are briefly summarised and the relative importance of measuring THC or one of its metabolites is discussed. In man only THC and the 7-hydroxy metabolite appear significant. TLC, GLC, radioimmunoassay, and GC-MS methods for the measurement of cannabinoids are then reviewed briefly. Radioimmunoassay has the highest capacity for rapid results but lacks the specificity of GC-MS for blood assays. Plasma levels of Δ^1-THC are compared in the same subject after smoking up to 10 mg of Δ^1-THC, after i.v. injection of 5 mg Δ^1-THC over 2 min., and after oral administration of 20 mg Δ^1-THC. THC is quantitated by GC-MS as TMS derivatives using a deuterated internal standard. The oral route was found to give the least predictable results and to result in lower plasma levels than the other routes of administration.

REFERENCES

AGURELL, S., GUSTAFSSON, B., HOLMSTEDT, B., LEANDER, K., LINDGREN, J.-E., NILSSON, I., SANDBERG, F. and ÅSBERG, M., Quantitative assay of Δ^1-THC in plasma from Cannabis smokers. *J. Pharm. Pharmac.*, **25**,554–558 (1973).

BRAUDE, M., SZARA, S. (Eds.), The pharmacology of marihuana, Raven Press, New York (1976).

FENTIMAN, A. F., FOLTZ, R. B., FOLTZ, R. L., Preliminary version of the manual on quantitation of Δ^9-THC in biological media by GC/MS. Report to NIDA. Battelle Columbus Laboratories, King Avenue, Columbus, Ohio (1978).

HOLLISTER, L., Cannabidiol and cannabinol in man. *Experientia*, **29**,825–826 (1973).

HOLLISTER, L., GILLESPIE, H. K., Δ^8- and Δ^9-THC, Comparison in man by oral and i.v. administration. *Clin. Pharmacol. Ther.*, **14**,353–357 (1973).

HOLLISTER, L. E., KANTER, S. L., MOORE, F., GREEN, D. E., Marihuana metabolites in urine of man. *Clin. Pharmacol. Ther.*, **12**,849–855 (1972).

LEANDER, K., DAHMÉN, J., AGURELL, S., OHLSSON, A., EDERY, H., PORATH, G., LEVY, S., MECHOULAM, R., Synthesis and psychotropic activity of side chain hydroxylated Δ^6-THC metabolites. *Arzneimittelforsch.* (in press).

LEMBERGER, L., The metabolism of tetrahydrocannabinols. *Advan. in Pharmacol. Chemother.*, **10**,221–255 (1972).

MECHOULAM, R., MCCALLUM, N. K., BURSTEIN, S., Recent advances in the chemistry and biochemistry of cannabis. *Chem. Rev.*, **76**,75–112 (1976).

NAHAS, G. (Ed.), Marihuana: Chemistry, biochemistry and cellular effects, Springer Verlag, New York (1976).

RODGERS, R., CROWL, C. P., EIMSTAD, W. M., HU, M. W., KAM, J. K., RONALD, R. C., ROWLEY, G. L., ULLMAN, E. F., Homogenous enzyme immunoassay for cannabinoids in urine. *Clin. Chem.*, **24**,95–100 (1978).

ROSENFELD, J., The simultaneous determination of Δ^9-THC and 11-hydroxy-Δ^9-THC in plasma. *Anal. Lett.*, **10**,917–930 (1977).

ROSENTHAL, D., HARVEY, T. M., BURSEY, J. T., BRINE, D., WALL, M. E., Comparison of gas chromatography mass spectrometry methods for the determination of Δ^9-THC in plasma. *Biomed. Mass Spectr.*, **5**,312–316 (1978).

SCHERRMAN, J., HOELLINGER, H., NGYEN-HOANG-NAM, BOURDON, R., FOURNIER, E., Detection and quantitation of Δ^9-THC in plasma by dansylation and double labelling. *Clin. Chem. Acta*, **79**,401–409 (1977).

TEALE, J. D., CLOUGH, J. M., KING, L. J., MARKS, V., WILLIAMS, P. L., MOFFAT, A. C., The incidence of cannabinoids in fatally injured drivers. *J. Forens. Sci. Soc.*, **17**,177–183 (1977).

VALENTINE, J. L., BRYANT, P. J., GUTSHALL, P. L., GAN, O. H. M., LOREGREN, P. D., THOMPSON, E. D., NIU, H. C., HPLC—MS determination of Δ^9-THC in human plasma following marihuana smoking. *J. Pharm. Sci.*, **66**,1263–1266 (1977).

VINSON, J. A. (Ed.), Proceedings from symposium on Cannabis. 173rd Meeting of American Chemical Society. Academic Press (in press).

WILLETTE, R. E. (Ed.), Cannabinoid assays in humans. NIDA Research Monograph 7, National Institute of Drug Abuse, Rockville, Maryland (1976).

WILLIAMS, P. L., MOFFAT, A. C., KING, L. J., A combined HPLC and RIA method for the quantitation of Δ^9-THC and some of its metabolites in human plasma. To be published.

APPLICATIONS OF MASS SPECTROMETRY IN CANNABINOID RESEARCH

MONROE E. WALL and DOLORES R. BRINE

Chemistry and Life Sciences Group, Research Triangle Institute, Post Office Box 12194, Research Triangle Park, NC 27709

Summary. GC—MS methods are described for the quantitation of Δ^9-THC, 11-hydroxy-Δ^9-THC, 11-nor-Δ^9-THC-9-oic acid and CBN in blood and urine using deuterated internal standards. Linear calibration curves were obtained and levels as low as 0.2–0.5 ng/ml could be measured. Results obtained on different instruments and using both electron-impact and chemical ionization showed good reproducibility. Good correlation was also found with measurements made by GLC or radio-TLC. 11-nor-Δ^9-THC-9-oic acid could be measured with reasonable linearity from 1 ng/ml to 50 ng/ml. A biphasic elimination pattern was observed following i.v. infusion of 4–5 mg Δ^9-THC. The peak concentration of 50–60 ng/ml declined rapidly for 15–40 min and then more slowly to a value of 3–5 ng/ml after 24 hours. 11-nor-Δ^9-THC-9-oic acid also showed biphasic elimination but with a longer half-life. 3″-Hydroxy-Δ^9-THC was also measured by GC—MS using the 11-hydroxy isomer as internal standard. The elimination of 11-[$^2H_3$]Δ^9-THC was also studied and found to be more rapid than elimination of the unlabelled drug. Finally a GC—MS method was developed for the measurement of paraquat in marihuana smoke as its sodium borohydride reduction product.

IN RECENT years there has been a great increase in interest in the pharmacology, metabolism and biodisposition of the cannabinoids (for recent reviews cf. Mechoulam[1], Paton and Crown[2], Wall[3], and Wall *et al.*[4]). Until recently quantitation of the various cannabinoids in blood, urine, feces and other biological tissues could be carried out only by the use of appropriately radiolabeled analogs of the cannabinoids under study[4, 5]. Because of the widespread and increasing opposition to the use of radiolabeled isotopes in studies involving man, and because many of the studies currently being conducted with various cannabinoids involve large-scale experiments in which radiolabeled cannabinoids are not used, the need for the development of non-radiolabeled quantitative methodology for certain key cannabinoids has become increasingly apparent. In addition, radiolabeled thin-layer chromatography techniques, while useful in initial studies, lack sufficient accuracy. When biological extracts are studied by radiolabeled means, separation of Δ^9-THC from cannabinol and of 11-hydroxy-Δ^9-THC from other monohydroxylated analogs is poor. If such interfering substances are present in considerable quantity, one will obtain erroneously high values. This will increasingly be the case when one is analyzing biological materials obtained from marihuana smokers which contain Δ^9-THC, cannabinol, cannabidiol, and 11-hydroxylated analogs of these compounds.

Quantitative gas liquid chromatography combined with mass spectrometry

(GLC—MS) has been used with excellent results for the quantitative analysis of drugs in biological materials, combining as it does the separative powers of GLC and the inherent sensitivity of MS detection. Pioneer studies by Hammar and Holmstedt[6] introduced the concept of mass fragmentography (since termed multiple ion detection (MID) and more recently selective ion monitoring (SIM)) which greatly increased the sensitivity of MS methodology so that it could be applied to the nanogram and picogram levels. The concept has been applied to many drugs, including its pioneer use by Agurell, Holmstedt and co-workers[7] in the determination of Δ^9-THC in blood and plasma. The application of GLC—MS techniques to the *in vitro* and *in vivo* metabolism of Δ^9-THC[4, 7–9] laid the groundwork for the quantitative analysis of other cannabinoid metabolites. The sites of metabolic hydroxylation for many cannabinoids are shown in Fig. 1.

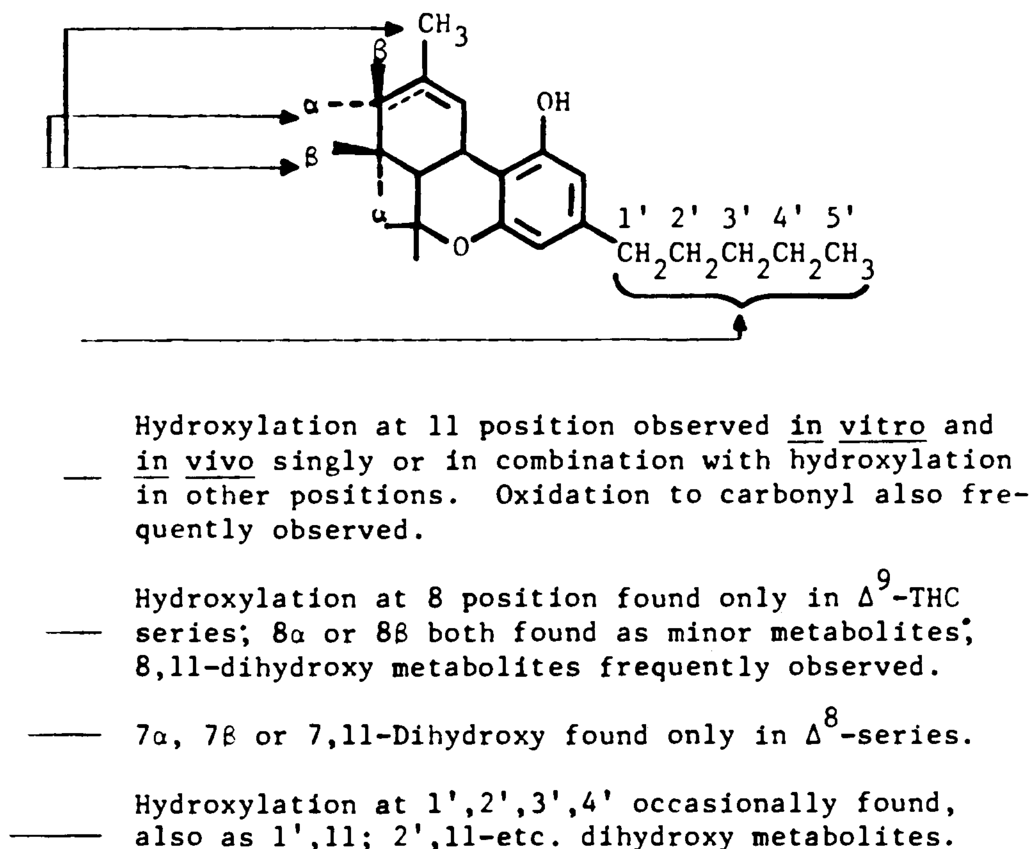


FIG. 1. Hydroxylation Sites of Cannabinoids Observed *in Vitro* or *in Vivo*.

A detailed discussion of many alternate methods for the quantitative determination of Δ^9-THC and some of its metabolites has been presented previously[10]. This paper will deal with the GLC—MS determination of Δ^9-THC, 11-hydroxy-Δ^9-THC and cannabinol in blood with one extraction. In addition, details will be presented for the GLC—MS determination of 11-nor-Δ^9-THC-9-carboxylic acid in blood and urine. The latter metabolite is gaining increased importance in forensic determinations.

Other applications of mass spectrometry to pertinent cannabinoid problems will be discussed briefly as they will be presented elsewhere in detail. These experiments include studies of the brain levels of unlabeled 3′-hydroxy-Δ^9-THC(11), *in vitro* metabolism of 11-$^2H_3$-Δ^9-THC(12), and the determination of the levels of paraquat in marihuana smoke(13).

MATERIALS AND METHODS

CLINICAL PROTOCOL

Human, male volunteers who were experienced marihuana users were administered 4.0–5.0 mg of Δ^9-THC by the intravenous method of Perez-Reyes *et al.*(14). The volunteers were kept under medical supervision for 24 hours in the Clinical Research Unit of the University of North Carolina, School of Medicine. Blood samples (approximately 10 ml) were collected at periodic intervals over 24 hours. Plasma was obtained by centrifugation, frozen immediately and stored in frozen condition until analyzed.

INTERNAL STANDARDS

A key feature of our quantitative procedures was the use of appropriate deuterated analogs of the cannabinoids under study both as carriers for the small quantity of cannabinoids expected to be present in many cases and as internal standards for quantitation by mass spectrometry. The structures of the cannabinoids and their deuterated analogs used in these studies are shown in Fig. 2. All of the compounds used were synthetic and were made available by the National Institute on Drug Abuse Synthesis Program.* Synthetic methods for the various deuterated cannabinoids utilized in these studies have been presented by Pitt *et al.*(15).

GENERAL PRECAUTIONS

Close attention must be paid to the procedural details presented below in order to obtain reproducible and quantitative data. In general, in working with cannabinoids, exposure of samples or extracts to light or air should be minimized. All solvent evaporations should be conducted *in vacuo* or under nitrogen at low temperature. Cannabinoids in

* Research Triangle Institute Contract HSM-42-71-95. Appropriately qualified investigators may obtain a variety of labeled and unlabeled cannabinoids by application to Dr. Robert Willette, Chief Research Technology Branch, Division of Research, National Institute on Drug Abuse, Parklawn Building, 5600 Fishers Lane, Rockville, Maryland 20857.

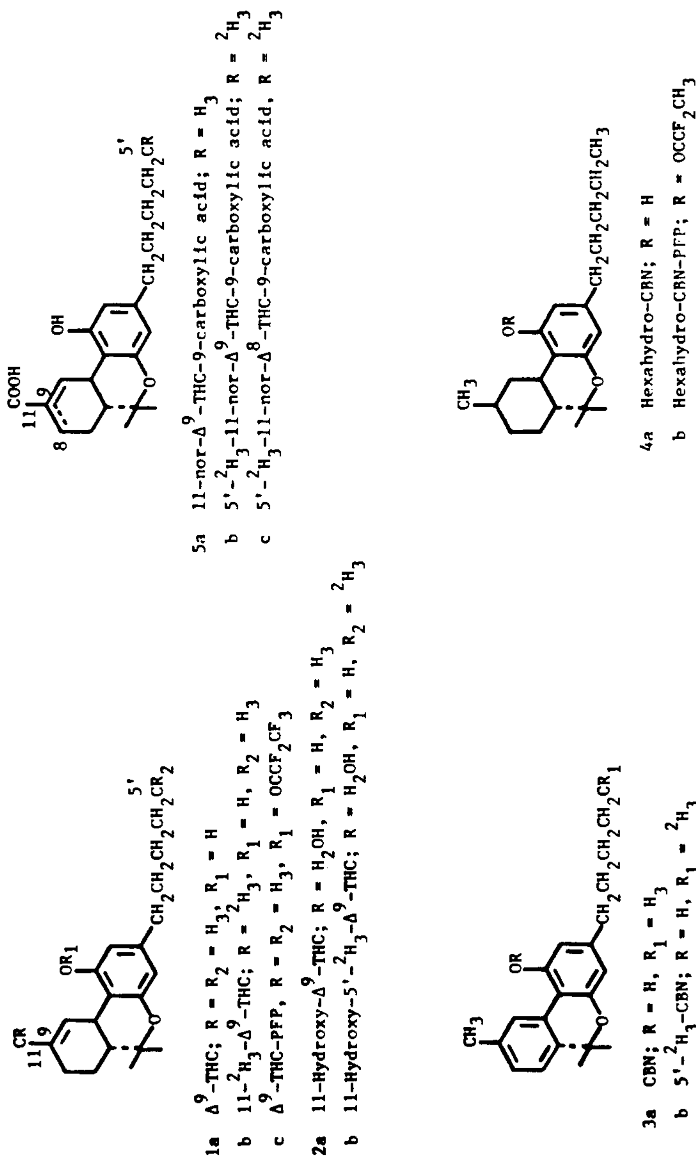


FIG. 2. Structure of Cannabinoids and Internal Standards.

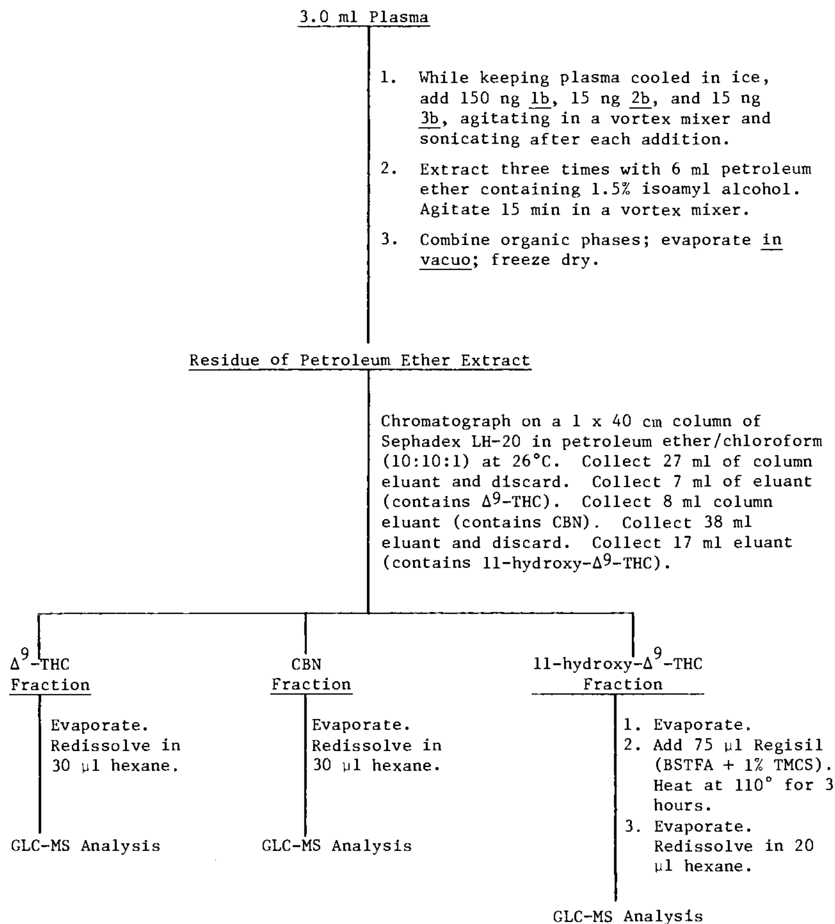
nanogram levels are subject to adsorption on the surface of glassware. In order to minimize this problem all glassware, including chromatography columns, was silanized using 5% DMCS in toluene.

EXTRACTION AND PURIFICATION PRIOR TO GLC—MS ANALYSIS

For the GLC—MS determination of Δ^9-THC, cannabinol, and 11-hydroxy-Δ^9-THC in the same plasma sample, preliminary studies with plasma extracts indicated that interference from endogenous plasma constituents would be encountered. (When the mass spectrometers were operated in the EI mode the molecular ions or charged fragments utilized for the quantitative analysis of underivatized cannabinoids were in a range of *m/e* 320 or lower.) Interference from endogenous plasma components could be avoided by carrying out a preliminary cleanup using Sephadex LH-20 chromatography prior to the GLC—MS step. The methods which are presented are for the combined determination of Δ^9-THC (*1a*), 11-hydroxy-Δ^9-THC (*2a*), and cannabinol (*3a*). The methods, of course, are equally utilizable for the determination of individual constituents. Deuterated internal standards (Fig. 2) were added to a sample of 3.0 ml of cold (not frozen) plasma as follows: *1b*, 150.0 ng; *2b*, 15.0 ng; and *3b*, 15.0 ng. Each internal standard was added in 15–30 μl ethanol. Following addition of each internal standard the plasma sample was stirred for 3–5 seconds in a vortex agitator and then subjected to sonication (Cole-Parmer ultrasonic cleaner) for the same time. The plasma samples (contained in a screw-capped centrifuge tube) were then extracted 3 times with 6.0 ml petroleum ether (bp 30–60°, Mallinckrodt Nanograde or Burdick and Jackson) containing 1.5% isoamyl alcohol. The tubes were agitated 15 minutes each time in a vortex agitator and the layers separated by centrifugation after each extraction. The petroleum ether extracts were combined, evaporated *in vacuo* at room temperature, and freeze dried overnight to remove water and isoamyl alcohol. The dried residue was dissolved in a minimal volume of petroleum ether/chloroform/ethanol (10 : 10 : 1) and chromatographed in the same solvent mixture on 1 × 40 cm water jacketed Sephadex LH-20 columns at 26°C. Twenty-seven ml of column eluant was collected and discarded. Seven ml of eluant was then collected as the fraction containing Δ^9-THC. The next 8 ml of eluant was collected as the CBN-containing fraction. Thirty-eight ml of column effluent was then collected and discarded. Finally, 17 ml of eluant was collected as the fraction containing 11-hydroxy-Δ^9-THC. The Δ^9-THC and CBN fractions were evaporated to dryness and dissolved in 30 μl hexane. The 11-hydroxy-Δ^9-THC fraction was evaporated to dryness under vacuum and heated with 75 μl of Regisil (BSTFA + 1% TCMS) in a closed vial at 110° for 3 hours. The reagent was removed in vacuum and the residue dissolved in 20 μl hexane. The procedure is summarized in Table 1.

A summary of the extraction and purification procedure for the analysis of 11-nor-Δ^9-THC-9-COOH is shown in Table 2. Following addition of 300 ng of deuterated internal standard *5c* to 3.0 ml of cold plasma or urine, the sample was sonicated with 30 ml acetone for 30 minutes. The sample was centrifuged to remove precipitated proteins, the acetone decanted, and the pellet reextracted with an additional 30 ml acetone. Acetone extracts were combined and concentrated *in vacuo* to an aqueous residue. The volume was adjusted to 3.0 ml with distilled water and the pH to 3. The cannabinoid acids were

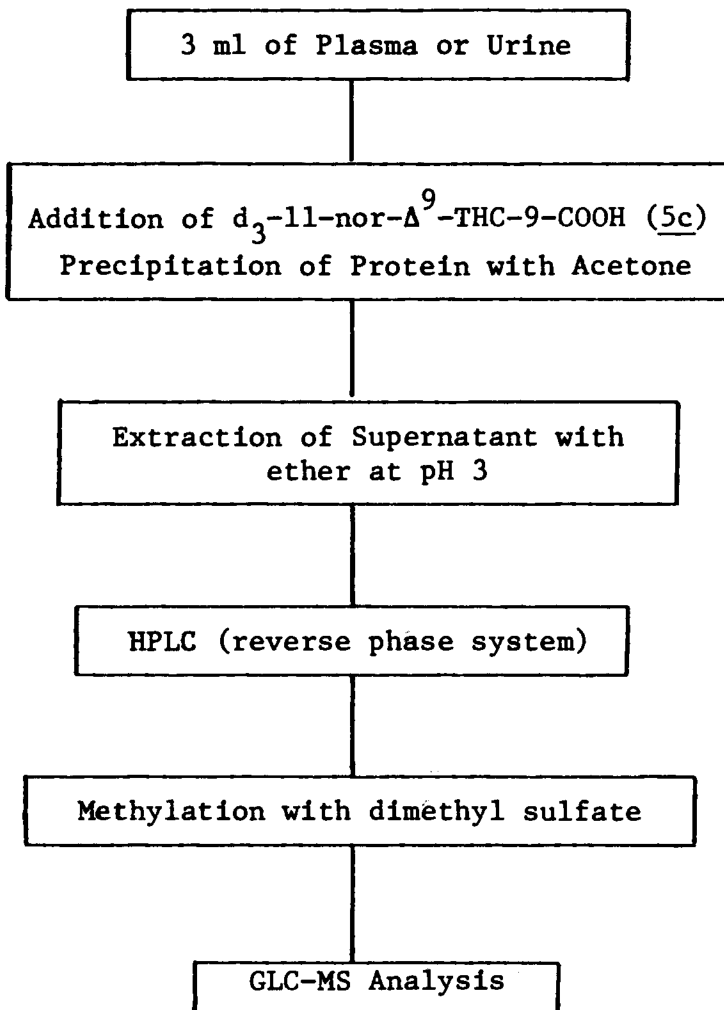
TABLE 1. EXTRACTION AND PRELIMINARY PURIFICATION FOR THE ANALYSIS OF Δ⁹-THC, 11-HYDROXY-Δ⁹-THC, AND CANNABINOL IN HUMAN PLASMA.



extracted from the aqueous phase by two partitions with 10 ml of diethyl ether. This was accomplished by agitating the centrifuge tubes 15 minutes each time in a vortex agitator and separating the phases by centrifugation. The diethyl ether extracts were combined, evaporated *in vacuo*, redissolved in 0.5 ml methanol, and filtered through a 5 micron teflon filter.

The extracts thus obtained were purified by high performance liquid chromatography (HPLC) on a reverse phase Partisil-20/ODS column (50 cm × 4.6 mm ID) using a methanol/water mobile phase (65 : 35, containing 0.05% ammonium acetate). At a flow rate of 4 ml/min the mixture of *5a* and deuterated internal standard *5c* were eluted at a time interval of 8–13 minutes. This fraction of eluant was collected and freeze dried. The residue was transferred to a half dram vial and dried *in vacuo* overnight with 10 mg of potassium carbonate. Under anhydrous conditions 0.2 ml dry acetone and 8 μl dimethyl sulfate were added to the sample. The vial was tightly capped and heated with shaking at 50°C for 6 hours. Following the addition of 0.5 ml distilled water to each sample, the

TABLE 2. EXTRACTION AND PURIFICATION OF PLASMA AND URINE SAMPLES CONTAINING 11-NOR-Δ^9-THC-9-COOH.



derivatized cannabinoids were removed by two extractions with 0.5 ml chloroform. The chloroform was evaporated under nitrogen and the residue redissolved in 30 μl chloroform.

GAS CHROMATOGRAPHY—MASS SPECTROMETRY CONDITIONS

Plasma samples for the quantitation of Δ^9-THC, CBN, and 11-hydroxy-Δ^9-THC were analyzed on the LKB-9000 or Finnigan 3300 mass spectrometers. On the LKB-9000 GLC—MS a 3′ or 6′ × $\frac{1}{4}$″ glass column of 2% OV-17 on Chromosorb W-HP (80/100 mesh) was utilized, the former length being used for 11-hydroxy-Δ^9-THC-bis-TMS ether (at 220°C) and the latter length for both Δ^9-THC (at 220°C) and CBN (at 240°C). Helium was used as the gas phase at a rate of 35 ml/min. Under the conditions stipulated above retention times of 4–6 minutes were observed for each compound. Only Δ^9-THC and CBN were analyzed on the Finnigan 3300 instrument in the electron impact (EI) mode. Six-foot glass columns containing 1% SE-30 on 100/120 mesh Chromosorb W-HP were used at column temperatures of 200–230°C and He flow of 30–35 ml/min.

For the analysis of 11-nor-Δ^9-THC-9-COOH, the dimethylated derivatives of the mixture of *5a* and *5c* were submitted to analysis on the LKB-9000 GLC—MS. Samples were chromatographed at 250°C on a 6′ column of OV-17, 2%, on Supelcoport (80/100 mesh) with a He carrier gas flow rate of 30 ml/minute.

The mass spectrometers and associated equipment have been described in detail[10]. In brief, an LKB 9000 GLC—MS (a magnetic sector instrument) was utilized with a modified accelerating voltage alternator (AVA) (Klein *et al.*[16]). For Δ^9-THC the mass spectrometer was set to focus alternately on the ions m/z 314 and 317 which correspond to the molecular ions of the compound and its trideutero analog. For CBN the molecular ions were m/z 310 and 313. For analysis of 11-hydroxy-Δ^9-THC as the bis-TMS ether, the strong M-103 ions[9,17] at m/z 371 and 374 was selected. For 11-nor-Δ^9-THC-9-COOH analysis the ratio of the peak heights at m/z 372 and 375 was determined. The AVA accessory measures the two peak heights or areas utilized for each analysis as described above. Alternatively the Finnigan 3300 GLC—MS (a quadrupole instrument) with a dedicated PDP-12 computer[10] was utilized, and the ratios of peak heights or areas were determined using computer software.

TLC-RADIOLABEL PROCEDURE

The volunteer subjects described in Clinical Protocol received 100 μCi of tritium labeled Δ^9-THC along with the standard 4.0–5.0 mg intravenous dose. Two to three ml aliquots of plasma were analyzed by the procedure described by Wall *et al.*[4].

RESULTS

ANALYSIS OF Δ^9-THC, CANNABINOL, AND 11-OH-Δ^9-THC

Plasma calibration curves obtained with the LKB and Finnigan GLC—MS instruments were quite similar for both Δ^9-THC and cannabinol. For Δ^9-THC linear calibration curves

in the range of 1–100 ng/ml of plasma were obtained with both instruments. Figure 3 shows data for the Finnigan. Detection of Δ^9-THC down to 0.1 ng/ml could be attained, but 0.5 ng/ml is regarded as the minimal concentration at which reliable data could be obtained. Plasma calibration curves for CBN are exemplified for the Finnigan in Fig. 4; the LKB data was similar. Linear curves on both instruments were obtained between 0.2–10.0 ng/ml with detection limits about 0.1 ng/ml. Only the LKB 9000 MS was used for the 11-hydroxy-Δ^9-THC determinations. The data are shown in Fig. 5. The curve was linear in the range of 0.2–10.0 ng/ml plasma of 11-hydroxy metabolite.

Figure 6 presents the average values with standard error obtained for Δ^9-THC, 11-OH-Δ^9-THC and cannabinol from plasma of male volunteers receiving Δ^9-THC by intravenous infusion. The measurements covered a 24-hour period. Δ^9-THC values obtained with the LKB-9000 EI source were in close agreement with the data obtained on the Finnigan 3300 chemical ionization (CI) source. Comparison of values for LKB *vs.* Finnigan EI source also showed excellent agreement. Δ^9-THC values increased rapidly during the first 10–20 minutes, the peak values in the range of 50–60 ng/ml coinciding with the maximal psychomimetic activity. A typical biphasic elimination pattern was noted; the Δ^9-THC plasma levels decreased rapidly between 15 and 40 minutes and then fell at a much slower rate. With a particular group of volunteers (3 subjects) levels after 24 hours were between 3 and 5 ng/ml. Spot checks at lower levels utilizing the Finnigan MID program confirmed that the substance being evaluated was indeed Δ^9-THC and not instrument "noise". In the case of 11-hydroxy-Δ^9-THC much lower levels were found. Peak values in the neighborhood of 2.0 ng/ml were noted between 30 and 40 minutes. The maximal values declined in a more gradual manner than was the case for Δ^9-THC, falling to a level of 1.0 ng/ml in 60–90 minutes and 0.5 ng/ml after 24 hours. The values for CBN shown in Fig. 6 have no pharmacokinetic significance as most of the data falls below the level of analytical reliability.

Figure 7 compares the results obtained from the average of four subjects analyzed by GLC—MS(EI), TLC-radiolabel, and electron capture GLC. Correlation coefficients are calculated in Fig. 8. The results are in reasonable agreement, and in particular the GLC—MS and electron capture GLC procedures gave good agreement for most points over the whole curve.

ANALYSIS OF 11-NOR-Δ^9-THC-9-CARBOXYLIC ACID

A plasma calibration curve for 11-nor-Δ^9-THC-9-carboxylic acid (*5a*) is shown in Fig. 9. There was reasonable linearity from 1.0 to 50 ng/ml plasma with detection limits of 0.5 ng or less per ml. Figure 10 presents similar data for a urine calibration curve. The method showed reasonable linearity between 2.0 and 100 ng/ml urine. Figure 11 presents pharmacokinetic data for plasma levels of a human volunteer, BS, over a 0.5-hour to 48-hour period comparing Δ^9-THC and 11-nor-acid levels after a dose of 5.0 mg of Δ^9-THC by the intravenous route. Both parent compound and acid metabolite exhibited a biphasic elimination pattern although the levels of the acid did not fall as rapidly as parent compound. Elimination of the acid metabolite *5a* in urine is shown in Fig. 12. It is evident that urinary elimination proceeded rapidly as 80% of the total 11-nor-acid excreted was eliminated in the urine during the first 6 hours after administration of parent compound; further elim-

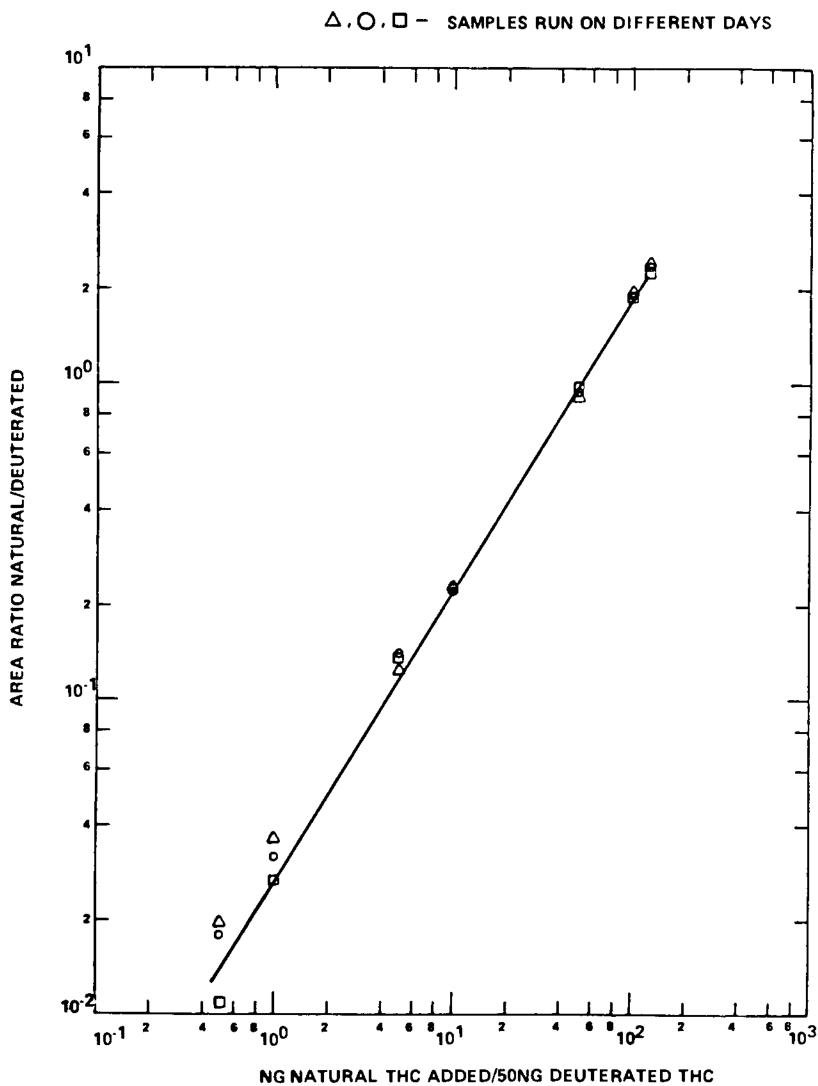


FIG. 3. Finnigan 3300-EI Plasma Calibration Curve for Δ^9-THC.

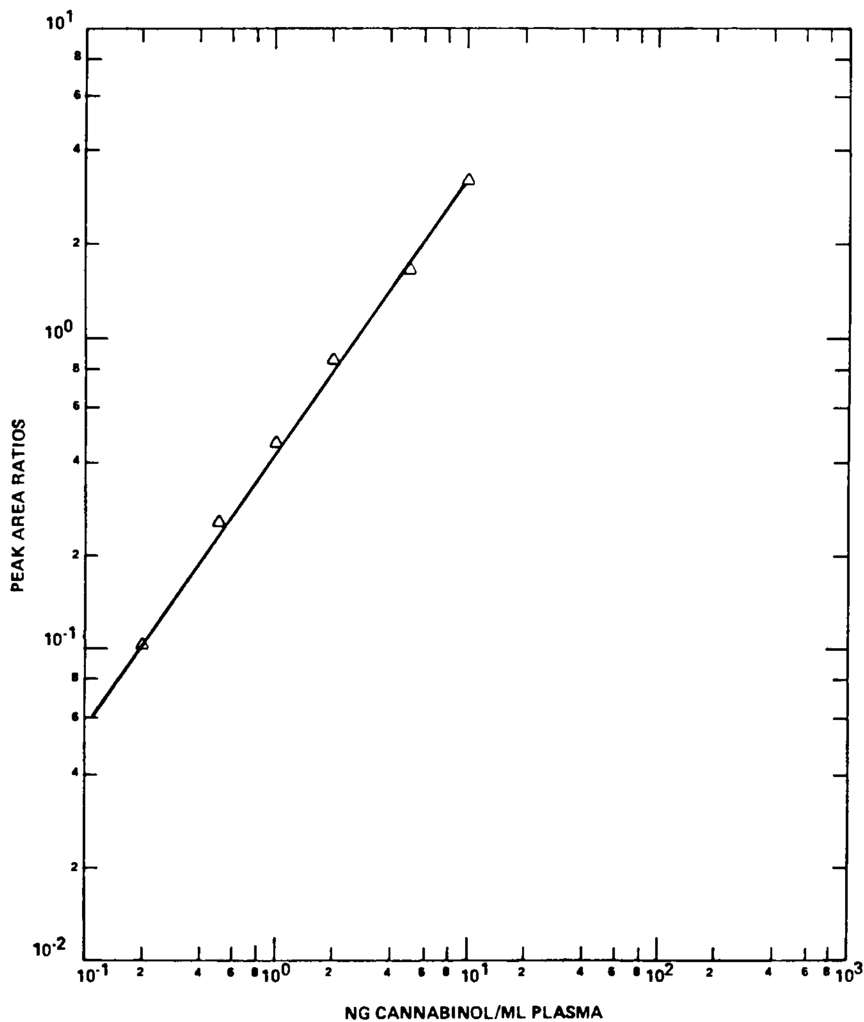


FIG. 4. Finnigan 3300-EI Plasma Calibration Curve for CBN.

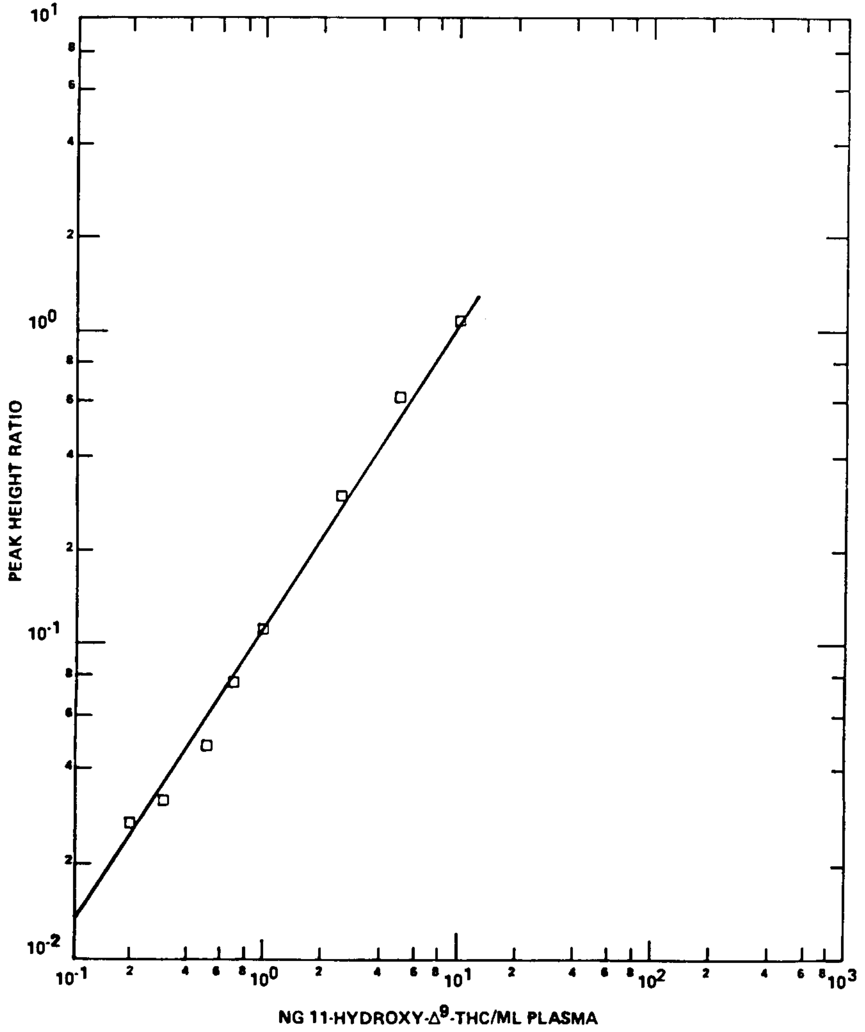


FIG. 5. LKB 9000-EI Plasma Calibration Curve for 11-Hydroxy-Δ^9-THC.

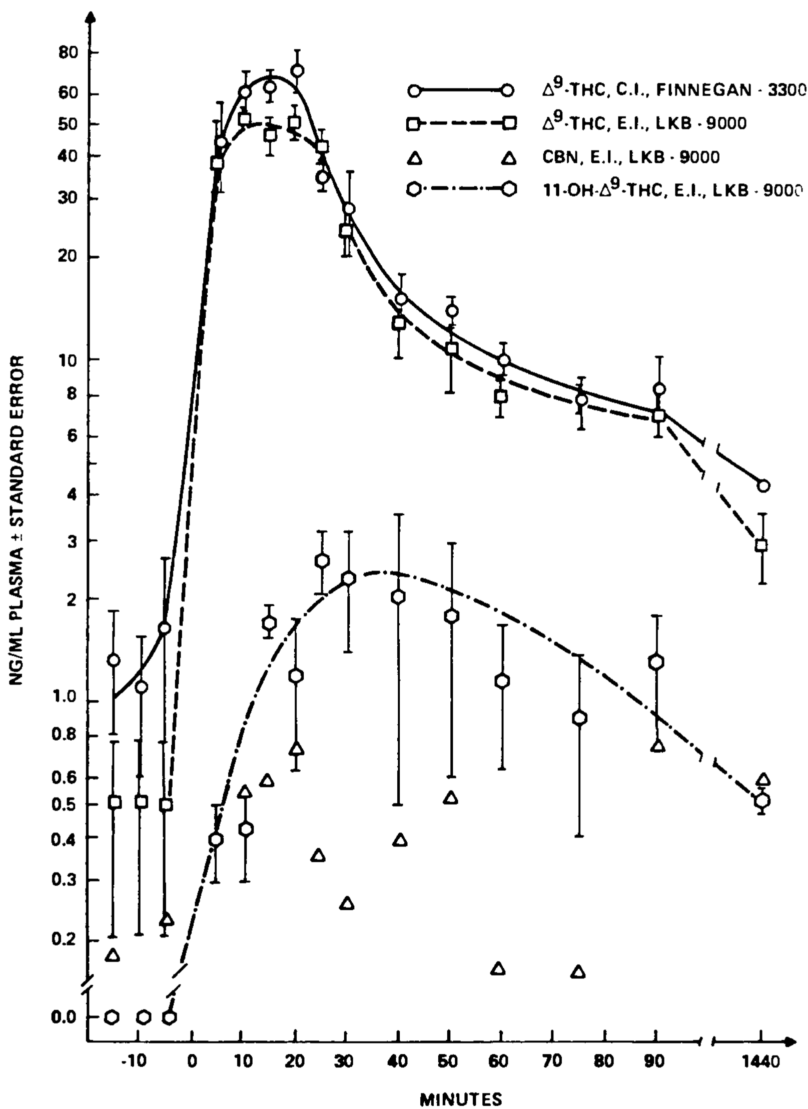


FIG. 6. Plasma Levels of Δ^9-THC, 11-Hydroxy-Δ^9-THC and CBN Found Over a 24-Hour Period in Human Plasma from Volunteers Receiving Δ^9-THC by IV Administration.

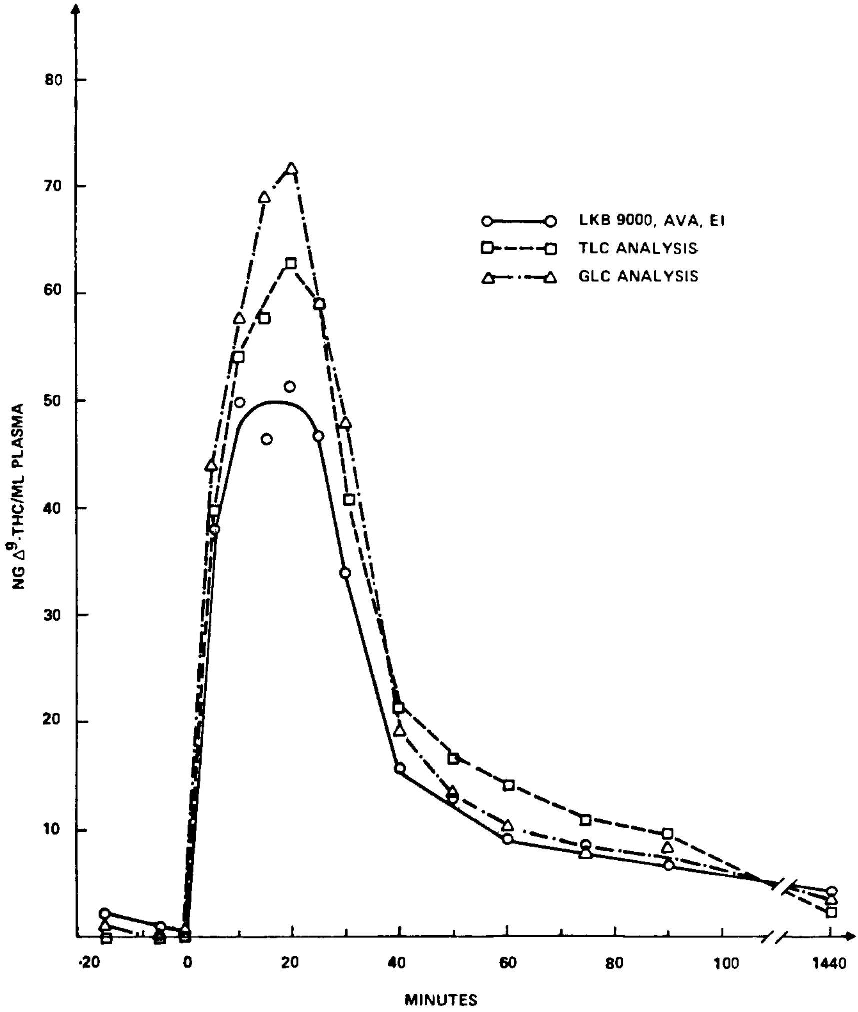


FIG. 7. Δ^9-THC Found in the Plasma Following Intravenous Administration of 5 mg Δ^9-THC, Average of 4 Subjects.

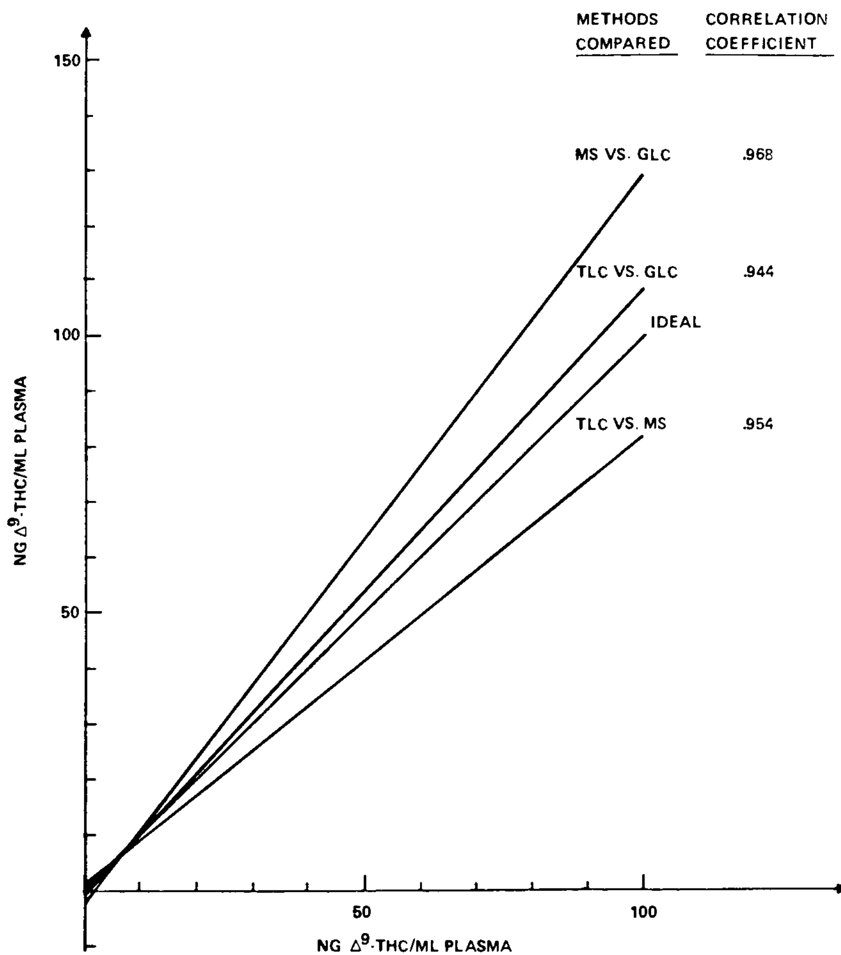


FIG. 8. Least-Squares Best Lines Comparing All Data Obtained by Each Two Methods of Analysis.

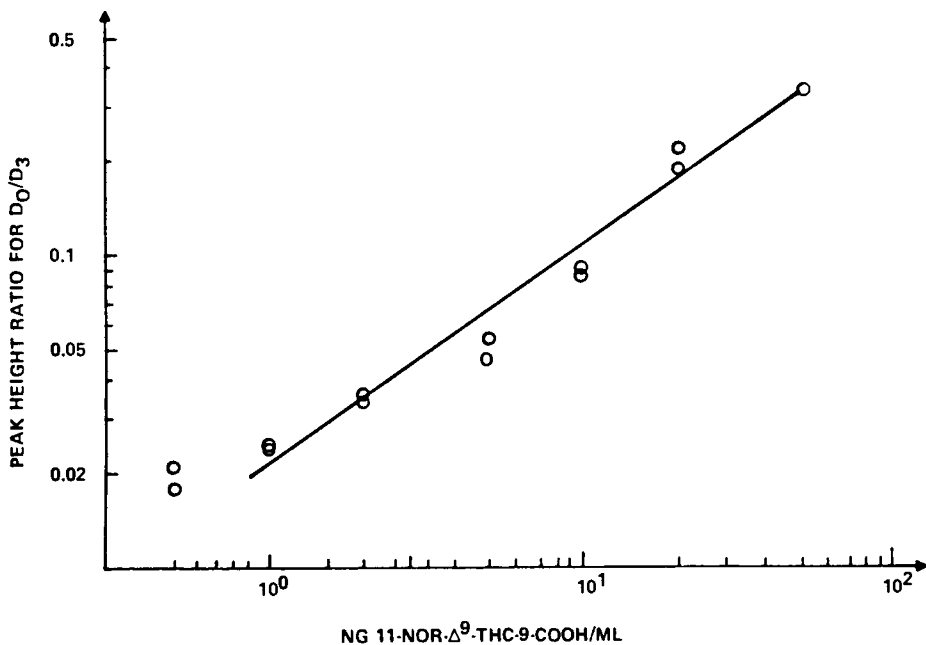


FIG. 9. Instrument Calibration for 11-nor-Δ^9-THC-9-COOH Extracted from Human Plasma.

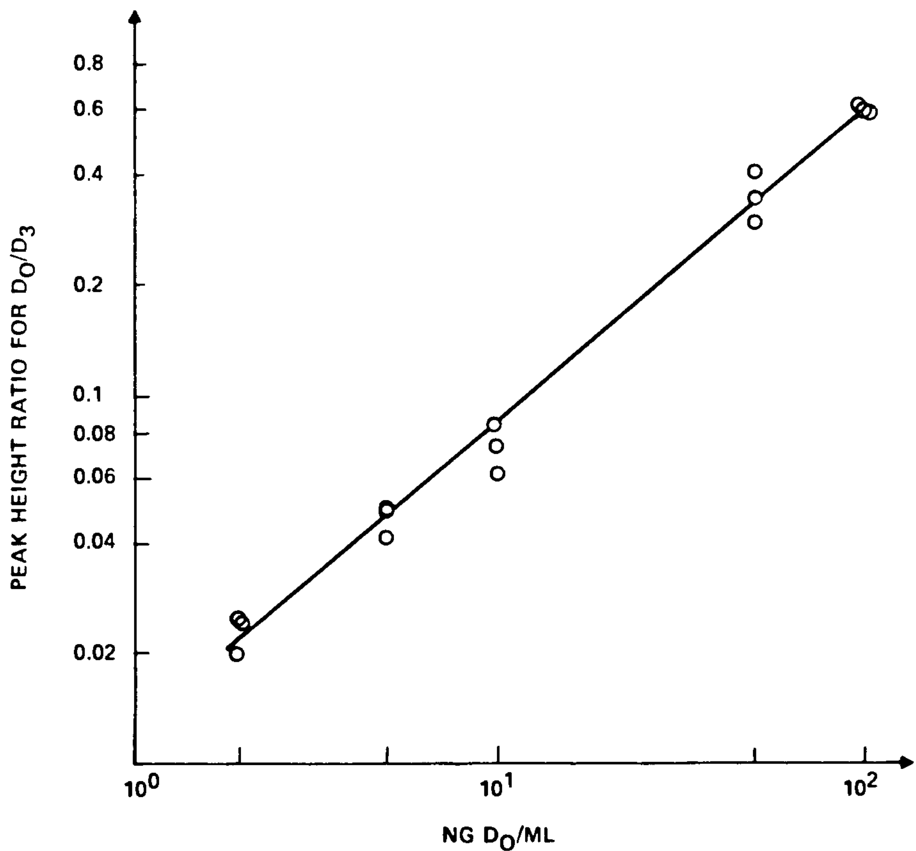


FIG. 10. Instrument Calibration Curve for 11-nor-Δ^9-THC-9-COOH in Human Urine.

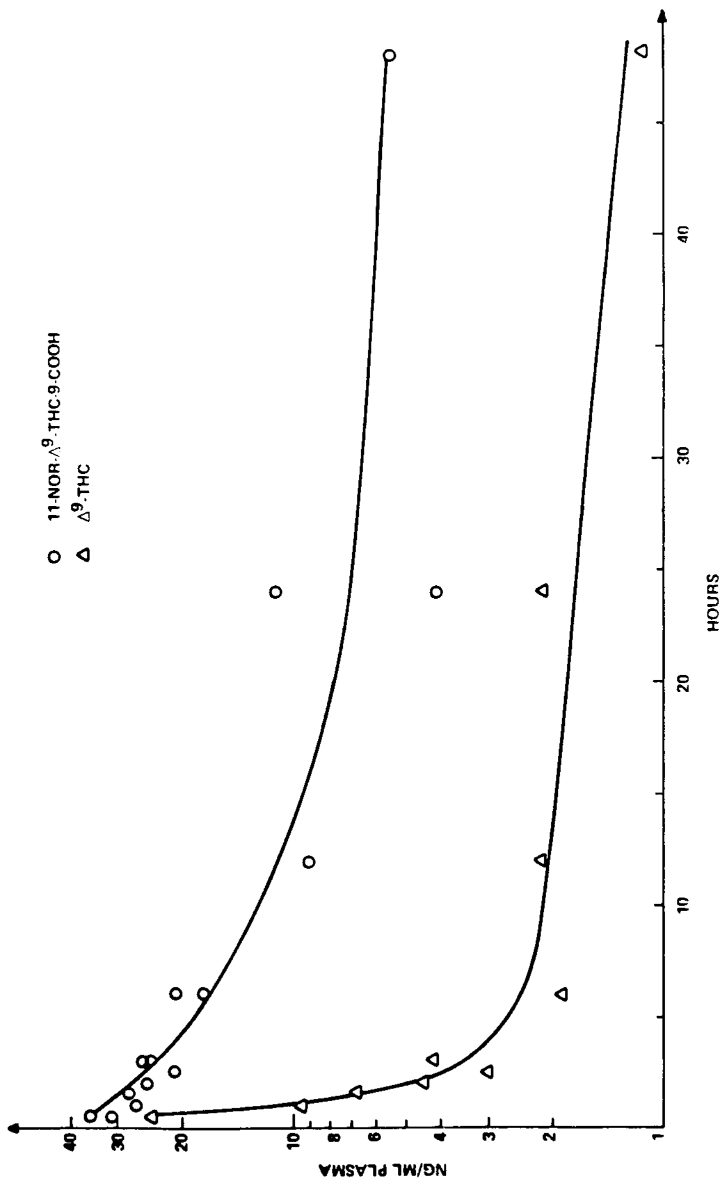


FIG. 11. Δ^9-THC and 11-nor-Δ^9-THC-9-COOH Present in the plasma of Subject BS after Intravenous Infusion of 4 mg Δ^9-THC.

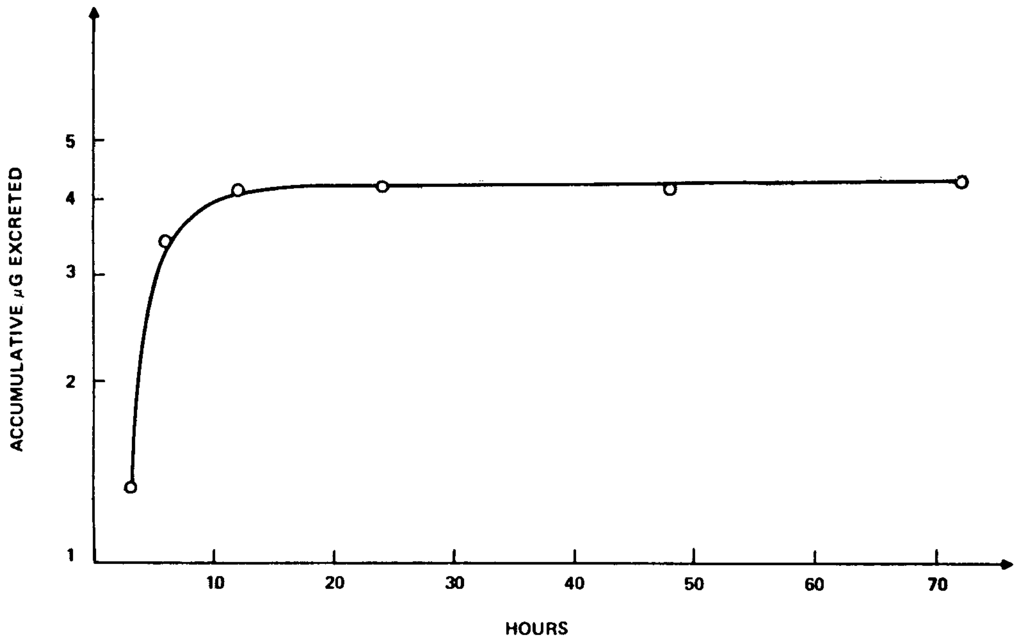


FIG. 12. 11-nor-Δ^9-THC-9-COOH Excreted in the Urine of Subject BS after Intravenous Infusion of 4 mg Δ^9-THC.

TABLE 3. COMPARISON OF GLC-MS AND TLC ANALYSIS OF 11-NOR-Δ^9-THC-9-COOH IN THE PLASMA AND URINE OF SUBJECT BS.

minutes	ng/ml plasma found by	
	TLC	GLC-MS
30	36	36
60	30	27
90	32	28
120	35	25

hours	ng/ml urine found by	
	TLC	GLC-MS
3	7.4	5.4
6	15	22
12	6.7	6.2
24	2.1	.2
48	1.0	--
72	0.89	.2

ination proceeded much more slowly. Table 3 compares results obtained by TLC assay (Wall *et al.*[4]) and the GLC—MS procedure for urine and plasma levels of the 11-nor-acid in subject BS. Agreement was excellent in most of the cases.

DISCUSSION

The basic objective of this investigation was to establish sensitive methodology which would not depend on radiolabeling for the quantitative estimation of Δ^9-THC, its primary metabolite (11-hydroxy-Δ^9-THC[3]), and cannabinol, which has been reported to be a metabolite of Δ^9-THC in the rat[18, 19]. This objective has been realized, utilizing GLC—MS with a variety of techniques and instruments. In addition, the quantitative estimation of 11-nor-Δ^9-THC-9-carboxylic acid has been achieved. Several aspects of our results merit further discussion.

CHOICE OF INSTRUMENT

Two completely different types of mass spectrometers coupled with different means for quantitation of data were utilized. One instrument was a relatively old (1968) magnetic sector MS, the LKB-9000, which was coupled with an accelerating voltage alternator which permitted measurement of the ratio of the peak height of the unknown as compared with that of the internal standard. The other was a newer (1974) quadrupole MS, the Finnigan 3300, which was interfaced to a PDP-12 computer. The Finnigan MS has both EI and CI sources. As shown in the Results section, both instruments in the EI mode gave virtually identical plasma calibration curves with identical linear range and quite similar standard error of estimation. Figure 6 gives pharmacokinetic data in man obtained on the LKB in the EI mode and the Finnigan in the CI mode. The results are quite similar. It is thus evident that a wide variety of mass spectrometers can be used with comparable results provided appropriate internal carriers and standards are added. Before concluding this discussion one word of caution should be given. The nature of the separators is most important; the LKB with the Ryhage separator and the Finnigan with a silylated glass jet separator gave appropriate sensitivity. On the other hand, another mass spectrometer which utilized a Watson-Biemann separator showed poor sensitivity and could not be utilized for cannabinoid studies.

INTERNAL STANDARDS

As indicated previously, the final mass spectrometric measurements can be conducted with great accuracy. The key to success in the various analytical studies was the utilization of appropriate compounds which could be employed as both carriers and internal standards. For this purpose deuterium labeled cannabinoids identical to the parent compound except for the label are ideal and were utilized for all of the EI studies. It is possible to use with equal success an internal carrier which is not isotopically labeled; its properties, however, must be very similar to that of the cannabinoid being studied but permit separation by GLC. Hexahydrocannabinol (*4a*) was excellent for this purpose and was used in CI studies of Δ^9-THC and cannabinol.

THE ANALYSIS FOR 11-NOR-Δ⁹-THC-9-CARBOXYLIC ACID

The analysis for this "end" metabolite of Δ⁹-THC caused particular problems. Especially baffling for a considerable time was the analysis of *5a* in plasma. This was finally solved by the realization that *5a* in plasma or urine containing blood (in cases of illness or injury) is probably non-covalently bonded to a protein. As a result, although this combination is *ether* extractable, it remains bound and passes through the requisite HPLC purification step at a retention time markedly different than pure *5a*. It was found that

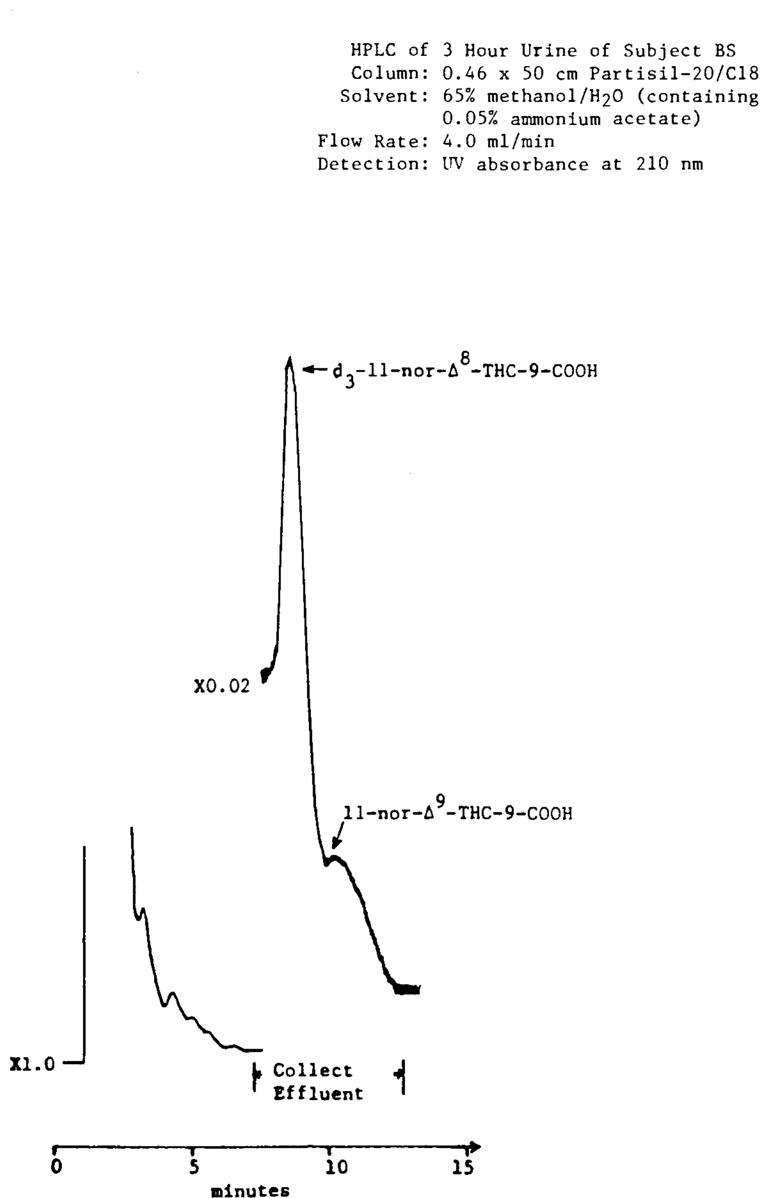


FIG. 13. Reverse phase HPLC Purification of 11-nor-Δ⁹-THC-9-COOH in Urine.

treatment of the "protein"-*5a* complex with acetone precipitated the plasma proteins and broke up the "protein"-*5a* complex. Purification of the 11-nor-acid (*5a*) by reverse phase HPLC was a requisite for its subsequent quantification by GLC—MS. Figure 13 shows the separation of the deuterated Δ^8-11-nor acid (*5c*) used as a carrier and internal standard and its Δ^9- analog, (*5b*) from polar impurities by this technique. It should be noted that there can be a slight separation of the two isomers, but the effluent collection volume is adjusted to collect both compounds.

Other points of interest were the requirement for derivatization of *5a* as the methyl ester of the carboxylic acid and the methyl ether of the phenolic hydroxyl moiety. Bis-silylation of these functions was not quantitative. The Δ^8-deuterated analog (*5c*) is more readily available than the Δ^9-deuterated analog (*5b*). Recently the latter has been synthesized[15, 20]. No advantage in precision or sensitivity was noted in using *5b* in preference to *5c*.

METABOLIC AND PHARMACOKINETIC DATA

The development of sensitive and accurate GCL—MS methodology permitted a preliminary study in man utilizing these techniques for the precise determination of Δ^9-THC, 11-hydroxy-Δ^9-THC and CBN in plasma. Previously we have made an extensive study[4] of the metabolism of Δ^9-THC in man using radiolabeled tracers and thin layer chromatography. The procedures utilized (in addition to the undesirability of a radiolabeled tracer in man) suffer from two potential sources of error. The method would not permit separation of Δ^9-THC from CBN, and in the case of 11-hydroxy-Δ^9-THC, would not permit separation from all other monohydroxy metabolites which might be present[9].

The data in Fig. 6 for Δ^9-THC are quite comparable to pharmacokinetic data obtained in earlier studies[4]. In both instances a biphasic elimination curve was noted. After the initial maximum level, a sharp decline was followed by a more gradual decrease. Maximal values in the current studies were 50–60 ng/ml. After 24 hours, 3–5 ng/ml of Δ^9-THC were still found in the plasma. Our results for 11-hydroxy-Δ^9-THC are probably the most accurate data yet reported in man. The concentration of this active metabolite (Fig. 6) was only 2–3 ng/ml at peak levels declining at a slower rate than Δ^9-THC to 0.5 ng/ml after 24 hours. Although 11-hydroxy-Δ^9-THC is readily produced from Δ^9-THC in the liver and is heavily excreted in the feces of man[4], only small quantities find their way into the blood.

Our interest in CBN was aroused by reports[18, 19] which indicate that CBN might be a transitory metabolite found at very early time periods after administration of Δ^9-THC to the rat. As shown in Fig. 6, the level of CBN in human plasma was below the reliability limits in the EI mode. Other studies we have carried out by electron capture GLC or GLC—MS in the CI mode indicate the virtual absence of this substance at all time periods. Since we have found that CBN has the same general pharmacokinetic pattern as Δ^9-THC in man[4], we must conclude that CBN can be *disregarded* in terms of its importance as a metabolite in man.

We present in this paper for the first time pharmacokinetic data in plasma and urine obtained by GLC—MS for the important acid metabolite 11-nor-Δ^9-THC-9-carboxylic acid. This and related acids constitute the major means by which Δ^9-THC is excreted in the urine. The data indicate rapid elimination of the acid in the urine during the first 3–6 hours after administration of Δ^9-THC.

COMPARISON OF GLC—MS WITH OTHER PROCEDURES

As shown in Figs. 7 and 8, the GLC—MS procedures show reasonable agreement in the case of Δ^9-THC with data obtained by two independent procedures involving, respectively, thin layer chromatography of radiolabeled cannabinoids and a dual GLC-electron capture procedure. In preliminary studies on cannabinol levels of subjects who received Δ^9-THC, good agreement was found between the GLC—mass spectrometric CI method and electron capture GLC. Cannabinol could not be found by either method, reinforcing our belief that CBN is not a significant metabolite of Δ^9-THC in man. Finally, Table 3 shows excellent agreement between the TLC-radiolabel procedure and GLC—MS analysis for the 11-nor-acid (*5a*).

OTHER GLC—MS EXPERIMENTS

Space permits us to touch only briefly on a number of interesting utilizations of mass spectrometry in our current cannabinoid program. Recently, as part of a systematic program designed to study the brain levels of a number of cannabinoids in mice[11], we made a study of the interesting side chain metabolite 3′-hydroxy-Δ^9-THC. This compound produces profound effects in mice and has greater potency than any of a wide variety of cannabinoid compounds which we have studied. It is available in unlabeled form as a result of a synthesis developed by Pitt and coworkers in our laboratory[21]. A procedure was developed (Table 4) for the extraction and purification of the 3′-hydroxy analog. Since the deuterated analog was not available to use as a carrier and internal standard, 11-hydroxy-Δ^9-THC was used for this purpose. This compound was added at the initial stage of the brain extraction. Extensive purification requiring both a reverse phase HPLC and a normal phase HPLC treatment was necessary because of the large amount of extraneous compounds found in the brain tissue. Subsequent to the final HPLC purification, the samples were converted to the trimethylsilyl ethers. Area ratios for 3′-hydroxy-Δ^9-THC/11-hydroxy-Δ^9-THC were plotted against the μgs of 3′-hydroxy-Δ^9-THC, and a linear calibration curve was obtained. Studies were carried out on a new mass spectrometer, the LKB 2091 GLC—MS equipped for SIM studies. This instrument was used to obtain quantitation of the data using both the ratio of the parent ions at m/z 474 and the strong fragments at m/z 330 and 371 for the 3′-hydroxy and 11-hydroxy compounds, respectively. Figure 14 shows the data obtained in the overall study. Particularly noteworthy is the large 3′-hydroxy-Δ^9-THC concentration found in the brain.

Another interesting utilization of GLC—MS may be noted in a study we have recently conducted in which our objective was to compare the metabolism of 11-$^2H_3$-Δ^9-THC *vs.* its unlabeled analog, Δ^9-THC. Table 5 presents the experimental conditions of this study. A typical calibration curve is shown in Fig. 15, which demonstrates the use of Δ^9-THC as an internal standard and carrier for its 11-deuterated analog. When the metabolism of Δ^9-THC was being studied, the procedure was reversed; the 11-deutero analog was used as a carrier and internal standard. Figure 16 shows some of the results obtained in the study. Rather surprisingly, the 11-deuterated analog metabolized more rapidly in this study than Δ^9-THC itself. The experiment will have to be repeated at shorter time intervals, but it is of

TABLE 4. EXTRACTION AND PURIFICATION OF 3'-HYDROXY-Δ^9-THC FROM MICE BRAINS.

Brains of Five Mice

1. Homogenize with 40 ml distilled water.
2. While keeping sample cold, add 1 μg 11-OH-Δ^9-THC as internal standard. Mix and sonicate.
3. Extract 3 times with 60 ml petroleum ether (containing 1.5% isoamyl alcohol).

Petroleum Ether Extract

1. Evaporate *in vacuo*. Freeze dry. Redissolve in methanol and filter.
2. Chromatograph on HPLC on a C_{18}/Partisil-20 column in a methanol/0.019M NH_4OAc solvent system.

HPLC Fraction Containing 11-OH-Δ^9-THC and 3'-OH-Δ^9-THC

Chromatograph on HPLC on a Partisil-10/PAC column in a methanol/butyl chloride solvent system.

HPLC Fraction Containing 11-OH-Δ^9-THC and 3'-OH-Δ^9-THC

Derivatize to trimethylsilyl ethers. Subject to SIM analysis on GLC-MS.

Data in % Dose/Mouse Brain as 3'-OH-Δ^9-THC

interest as it possibly indicates that a metabolic shift may be taking place. That is, instead of hydroxylation at the 11-position, it is possible that more rapid hydroxylation of the deuterium analog occurred at the 8-position or in the side chain.

Finally, we wish to discuss a very interesting facet which, while not directly related to cannabinoid chemistry, is certainly quite topical. In recent years the spraying of illicit marihuana with the herbicide paraquat dichloride and a subsequent introduction of the sprayed marihuana into the United States has caused considerable concern. The concern, in particular, has grown after the finding that picogram quantities of paraquat introduced into the lungs of mice produce lung lesions. We have undertaken a study to analyze for paraquat in marihuana smoke. In order to carry out this analysis, considerable purification of the extracts was necessary. In addition it was required that paraquat, which is a quaternary ammonium salt, be reduced with sodium borohydride to an unsaturated amine in order to have a product which could be handled by GLC—MS. The structures of some of these compounds are shown in Fig. 17. Bipyridine is the major product formed by pyrolysis of paraquat. For an internal vehicle carrier and calibration standard, we carried

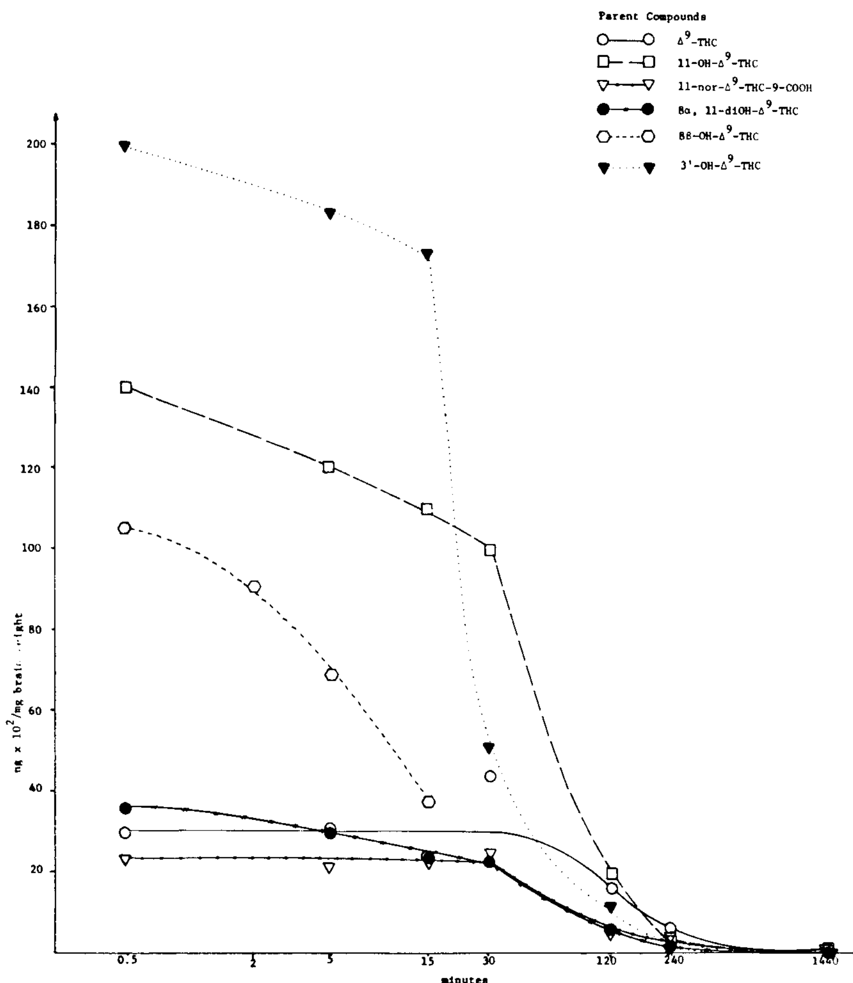


FIG. 14. Cannabinoid Brain Levels in Mice After Tail Vein injection of 100 μg Parent Compound.

out a synthesis of deuterated paraquat bis-methylsulfate which was converted to the corresponding $^2H_6$-deuterium labeled analog by sodium borohydride reduction. Again, prior to GLC—MS, it was necessary to carry out HPLC chromatography. The deuterated carrier was of great value in facilitating the following of the course of the HPLC purification (Fig. 18). The analysis was carried out on the LKB 2091 GLC mass spectrometer using a 1% SE-30/$BaCO_3$ SCOT capillary column. The ions which were monitored were m/z 192 (M^+ for reduced paraquat) and m/z 198 (M^+ for reduced $^2H_6$-paraquat). An excellent linear calibration curve was obtained from 0.01 to 10 μg of paraquat using 10 μg of the deuterium labeled paraquat as a carrier and internal standard (Fig. 19). Table 6 shows some of the results obtained in this study. The data indicate that although the bipyridine is produced in linear fashion (greater than 95% or more), paraquat is found in the smoke in a nonlinear manner with more of the substance being found from marihuana coated at lower concentrations than would be expected.

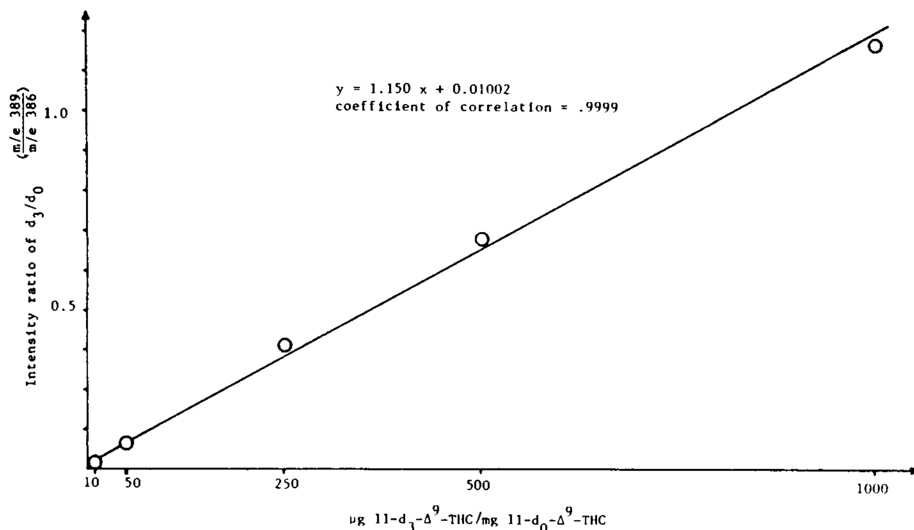


FIG. 15. GLC—MS Calibration Curve for the Analysis of $11\text{-}^{2}H_{3}\text{-}\Delta^{9}$-THC Using Δ^{9}-THC as Internal Standard.

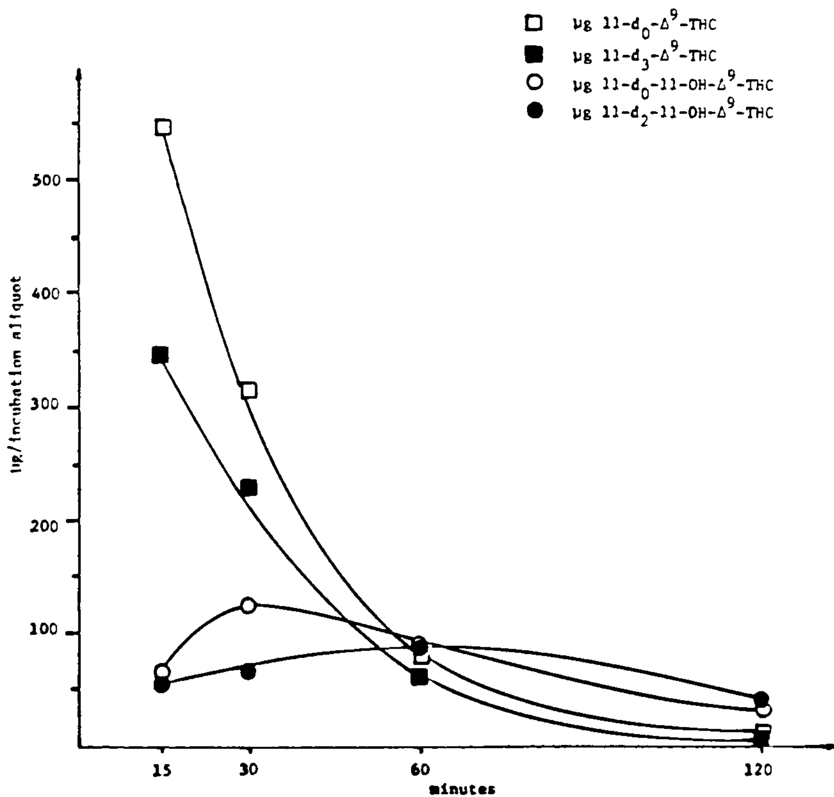


FIG. 16. *In Vitro* Metabolism of $11\text{-}^{2}H_{3}\text{-}\Delta^{9}$-THC and Δ^{9}-THC.

TABLE 5. RAT LIVER INCUBATIONS OF 11-$^{2}H_3$-Δ^9-THC AND Δ^9-THC.

Incubation of	Internal Standard added to each 20% aliquot	Incubation Mixture Volume
5 mg 11-d_3-Δ^9-THC	1 mg 11-d_0-Δ^9-THC and 100 μg 11-OH-CBN	25 ml
5 mg each of: 11-d_3-Δ^9-THC and 11-d_0-Δ^9-THC	None	50 ml
5 mg 11-d_0-Δ^9-THC	0.7 mg 11-d_3-Δ^9-THC and 100 μg 11-OH-CBN	25 ml

TABLE 6. COMBUSTION OF MARIHUANA COATED WITH ^{14}C-PARAQUAT

Level of Coating	g burned	Moisture Content (%)	μg paraquat in the smoke from 1 g marihuana	mg bipyridine in the smoke from 1 g marihuana
1.0%	17	15	4.4	7.1
0.1%	20	15	0.26	0.59
0.01%	21	15	0.17	0.074

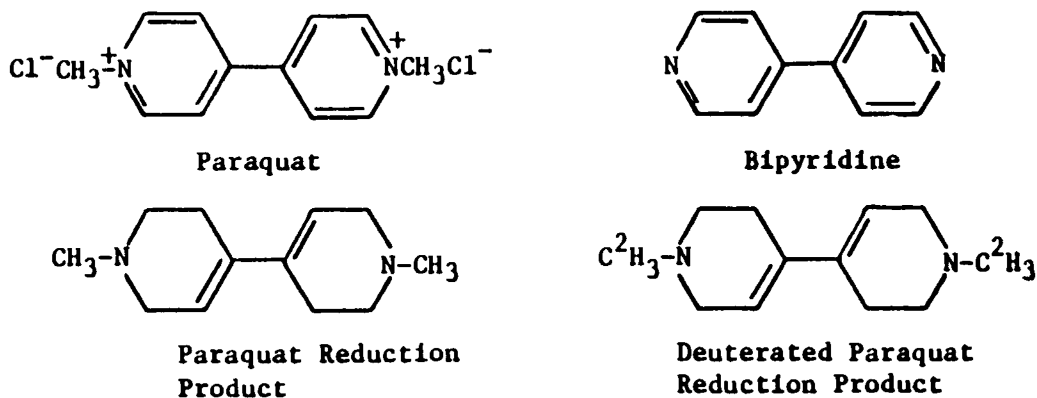


FIG. 17. Structures of Paraquat and Related Compounds.

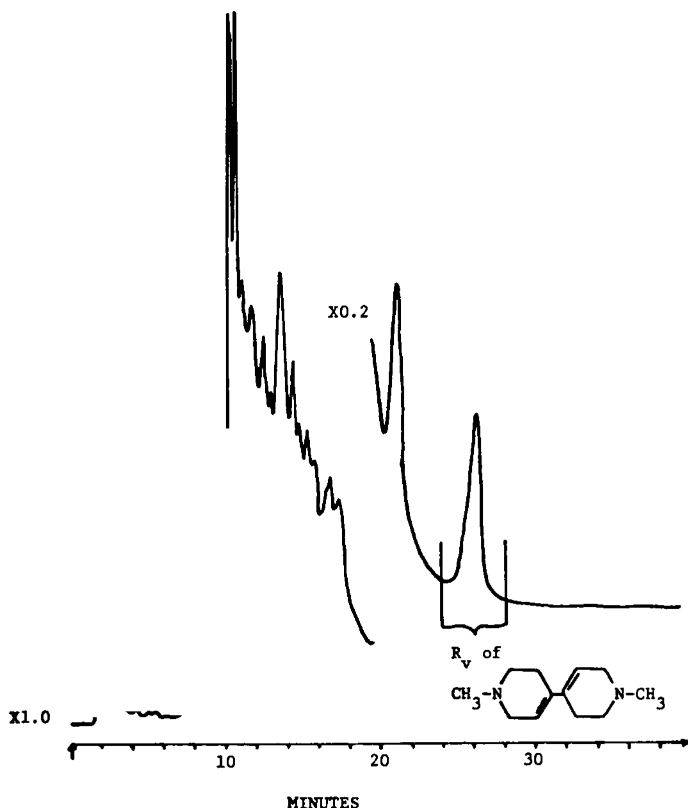


FIG. 18. HPLC Purification of Reduced Aqueous Soluble Fraction of Smoke Condensate from Combustion of Marihuana Coated with 1% Paraquat.

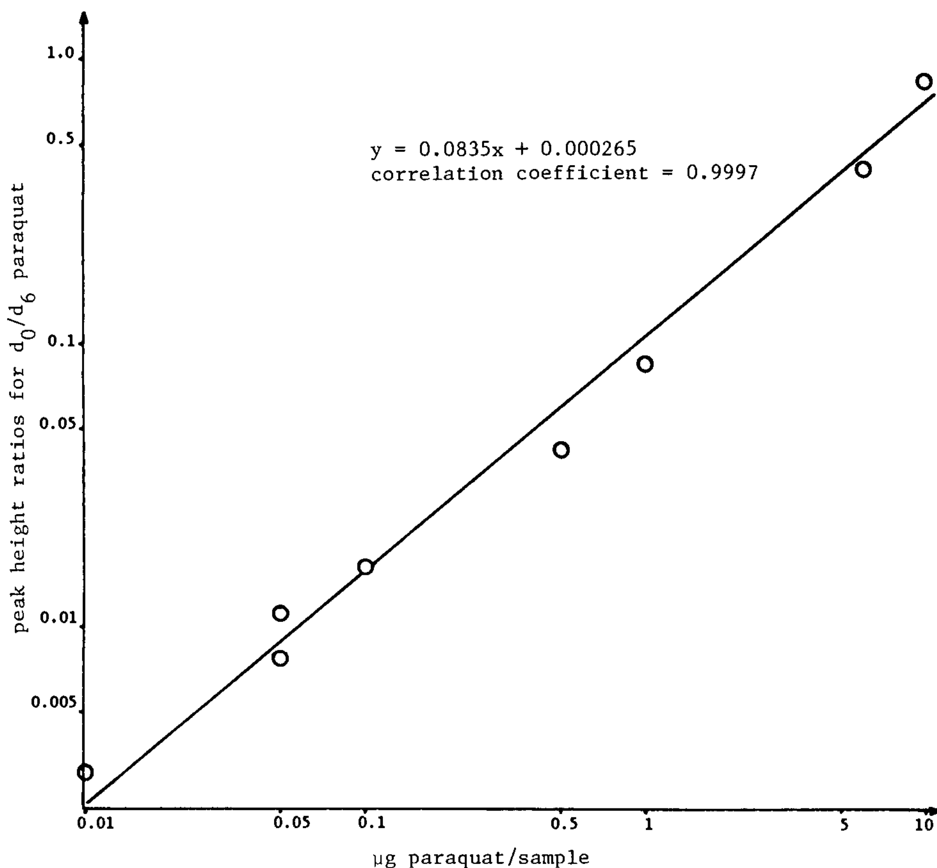


FIG. 19. GLC—MS Calibration Curve for Paraquat.

ACKNOWLEDGEMENTS

These studies were conducted with the support of the National Institute on Drug Abuse under contracts HMS-41-71-95 and ADM-45-74-109. We wish to thank Mario Perez-Reyes, M.D. for clinical material used in some of these studies and express to Mrs. Carolyn Parker, Mrs. Valerie H. Schindler, Mr. Philip Brown Belt, and Mr. Javey M. Taylor our appreciation for their technical assistance. We thank Drs. Joan Bursey, David Rosenthal, and Kenneth Tomer for mass spectrometric analyses.

REFERENCES

1. MECHOULAM, R., ed., *Marihuana*. Academic Press, New York (1973).
2. PATON, W. D. M. and CROWN, J., eds., *Cannabis and its derivatives*, Oxford University Press, London, England (1972).
3. WALL, M. E., Recent advances in the chemistry and metabolism of the cannabinoids. *Recent Advances in Phytochemistry* (V. C. Runeckles, ed.). Plenum Publishing Co., New York (1975).
4. WALL, M. E., BRINE, D. R., and PEREZ-REYES, M., Metabolism of cannabinoids in man. *Pharmacology of Marihuana* (M. Braude and S. Szara, eds.). Raven Press, New York (1976).

5. LEMBERGER, L., The metabolism of the tetrahydrocannabinoids. *Advances in Pharmacology and Chemotherapy* (S. Garratini, F. Hawking, A. Golden and I. Kopin, eds.). Academic Press, New York (1972).
6. HAMMAR, C.-G. and HOLMSTEDT, B., Mass fragmentography identification of chlorpromazine and its metabolites in human blood by a new method. *Anal. Biochem.* **22**,532 (1968).
7. AGURELL, S., GUSTAFSSON, B., HOLMSTEDT, B., LEANDER, K., LINDGREN, J.-E., NILSSON, I., SANDBERG, F., and ASBERG, M., Quantitation of Δ^9-tetrahydrocannabinol in plasma from cannabis smokers. *J. Pharm. Pharmac.* **25**,554 (1973).
8. WALL, M. E. and BRINE, D. R., Application of mass spectrometry to structure of metabolites of Δ^9-tetrahydrocannabinol. Presented at the International Symposium on Mass Spectrometry in Biochemistry and Medicine. Milan, Italy (1973).
9. WALL, M. E., BRINE, D. R., and PEREZ-REYES, M., Identification of cannabinoids and metabolites in biological materials by combined gas-liquid chromatography—mass spectrometry. *Marihuana: Chemistry, Biochemistry, and Cellular Effects* (G. G. Nahas, ed.). Springer-Verlag, New York (1976).
10. WALL, M. E., HARVEY, T. M., BURSEY, J. T., BRINE, D. R., and ROSENTHAL, D., Analytical methods for the determination of cannabinoids in biological media. *Cannabinoid Assays in Humans* (Robert E. Willette, ed.). NIDA Research Monograph Series. **7**,107 (1976).
11. WALL, M. E., and BRINE, D. R., Blood-brain levels of Δ^9-tetrahydrocannabinol and metabolites. Presented at the Seventh International Congress of Pharmacology, Paris, France (1978).
12. WALL, M. E., and BRINE, D. R., *In vitro* metabolism of $11\text{-}d_0\text{-}\Delta^9$-THC and $11\text{-}d\text{-}_3\text{-}\Delta^9$-THC. Quarterly Report for NIDA Contract No. HSM-42-71-95 (March, 1978).
13. WALL, M. E., BRINE, D. R., BRINE, G. A., and TOMER, K., Determination of paraquat in marihuana pyrolysis products. Presented at Third International Conference on Stable Isotopes. Chicago, Illinois (1978).
14. PEREZ-REYES, M., TIMMONS, M., LIPTON, M., DAVIS, K., and WALL, M., Intravenous injection in man of Δ^9-tetrahydrocannabinol and 11-OH-Δ^9-tetrahydrocannabinol. *Science* **177**,633 (1972).
15. PITT, C. G., HOBBS, D. T., SCHRAN, H., TWINE, C. E., and WILLIAMS, D. L., The synthesis of deuterium, carbon-14 and carrier-free tritium-labeled cannabinoids. *J. Label. Comp.* **11**,551 (1975).
16. KLEIN, P. D., HAUMAN, J. R., EISLER, W. J., Gas chromatograph-mass spectrometer-accelerating voltage alternator system for the measurement of stable isotope ratios in organic molecules. *Anal. Chem.* **44**,410 (1972).
17. WALL, M. E., BRINE, D. R., BRINE, G. A., PITT, C. G., FREUDENTHAL, R. I., and CHRISTENSEN, H. D., Isolation, structure and biological activity of several metabolites of Δ^9-tetrahydrocannabinol. *J. Am. Chem. Soc.* **92**,3466 (1970).
18. MCCALLUM, N. K., The effect of cannabinol on Δ^1-tetrahydrocannabinol clearance from the blood. *Experientia*, **31**,957 (1975).
19. MCCALLUM, N. K., YAGEN, B., LEVEY, S., and MECHOULAM, R., Cannabinol: a rapidly formed metabolite of Δ^1- and Δ^6-tetrahydrocannabinol. *Experientia* **31**,520 (1975).
20. PITT, C. G., FOWLER, M. S., SATHE, S., SRIVASTAVA, S. C., and WILLIAMS, D. L., Synthesis of metabolites of Δ^9-tetrahydrocannabinol. *J. Am. Chem. Soc.* **97**,3798 (1975).
21. PITT, C. G., SELTZMAN, H. H., SAYED, Y., TWINE, C. E., and WILLIAMS, D. L., A general synthesis of side chain derivatives of Δ^9-THC. *Tetrahedron Lett.* **1**,37 (1978).

IDENTIFICATION AND MEASUREMENT OF CANNABINOIDS AND THEIR *IN VIVO* METABOLITES IN LIVER BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

D. J. HARVEY, B. R. MARTIN and W. D. M. PATON

University Department of Pharmacology, South Parks Road, Oxford, OX1 3QT.

Summary. *In vivo* metabolites of Δ^1-THC, CBD and CBN have been examined in mouse, rat and guinea-pig by combined gas liquid chromatography—mass spectrometry. Extensive metabolism with considerable species variation was found. The major metabolic pathways involved allylic and aliphatic hydroxylations, oxidation of the alcohols to ketones and acids, β-oxidative degradation of the pentyl side chain and conjugation with glucuronic acid (especially with CBN and CBD). Most metabolites showed transformation at two or three sites leading to compounds such as diols and dihydroxy acids. When administered together, as in a cannabis tincture sample, all cannabinoids underwent biotransformation to give similar products to those found in the metabolic profiles of individual compounds. Δ^1-THC was quantitated in liver using selected ion monitoring with 1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC as an internal standard; various factors affecting sensitivity are assessed. Similar quantitation of metabolites was not possible as suitable internal standards were unavailable, but selected ion monitoring of the major metabolites of Δ^1-THC in mouse following IP injections showed rapid formation and decay of all major metabolites identified. Metabolism of deuterium labelled analogues of Δ^1-THC was examined in the context of the identification of metabolites by the isotope doublet technique and the switching of metabolic pathways.

INTRODUCTION

THE tissue distribution and biotransformation of the cannabinoids has received considerable attention in recent years[27] but although it is known that Δ^1-tetrahydrocannabinol (Δ^1-THC), for example, accumulates in such tissues as liver, lung, heart and spleen[1], there is little further information on the nature of the material present in each tissue especially during chronic cannabis use. Much is, however, now known about the major biotransformations undergone by the cannabinoids as reflected in urinary and faecal metabolites or in the metabolites produced *in vitro* from liver homogenates. Our own work on *in vivo* liver metabolites has revealed a large range of polar metabolites, mainly carboxylic acids[14] but even so, a large amount of material found in liver remains unidentified. It would appear from these studies and from papers from other workers, that little of the polar material reaches tissues other than the liver in any substantial quantity. For example, compounds found in brain following Δ^1-THC administration seem to be confined to Δ^1-THC itself and its 7-hydroxy metabolite[5]. A little of the material which accumulates in tissues has been

proposed to be in the form of long chain fatty esters of hydroxy metabolites[22]; these compounds are reported to occur in liver and spleen after administration of both Δ^1- and Δ^6-THC[22] and in urine following CBN administration.

This paper summarises our work on the identification of the *in vivo* liver metabolites of the three major cannabinoids, Δ^1-THC, cannabidiol (CBD) and cannabinol (CBN), briefly considers the metabolism of the cannabinoids in an extract of cannabis tincture, and discusses problems associated with quantitation of the cannabinoids and their *in vivo* metabolites by combined gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

MATERIALS

Δ^1-THC, CBD and CBN were obtained from the National Institute for Drug Abuse and were checked for purity by GLC before use. The THC sample was found to contain about 2% CBN.

Tincture of cannabis BPC, (1949), an ethanolic extract of the flowering tops of *Cannabis Sativa* L., grown in Pakistan, was obtained from W. Ransom (Hitchin). Cannabinoids were extracted by ethyl acetate-water partition and the cannabinoid content was analysed by GLC as TMS derivatives on 3% SE-30 with 5α-cholestane as an internal standard; the results are listed in Table 1.

TABLE 1. CANNABINOIDS PRESENT IN CANNABIS TINCTURE.

Cannabinoid	Quantity (mg/ml)	Relative amount (Δ^1-THC = 1.00)
Pr-CBD	0.285[a]	0.20
Pr-CBC	trace	trace
Pr-Δ^1-THC	0.48[a]	0.34
Pr-CBN	0.135[a]	0.096
CBD	1.26	0.90
CBC	0.28[b]	0.20
Δ^1-THC	1.40	1.00
CBN	0.40	0.29
CBG	trace	trace

a. Response factor based on the pentyl homologues as no standards available.
b. Response factor based on Δ^1-THC.

1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC was prepared as follows: the α-hydrogens of 3,5-dimethoxy-1-pentanone were exchanged with deuterium under basic conditions (D_2O, NaOD, dioxane) and the ketone group was reduced with deuterium and a 10% Pd/C catalyst to give dimethoxyolivetol. This was demethylated and condensed with (+)-*trans*-mentha-2,8-dien-1-ol to give 1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC[29] of isotopic purity: [$^2H_0$], 2.9%; [$^2H_1$], 2.9%; [$^2H_2$], 7.2%; [$^2H_3$], 17%; [$^2H_4$], 70%. Full details will be published later.

IDENTIFICATION OF METABOLITES

The cannabinoid, suspended in Tween-80 and isotonic saline was administered intraperitoneally at a dose of 100 mg/kg to male Charles River CD1 mice (23–25g), male Dunkin Hartley quinea-pigs (400–500g) or male Wistar rats (200–250g). The animals were killed at one or two hours after treatment, and the livers were removed and frozen until required.

For experiments with cannabis tincture, similar mice in groups of three were treated as above with a dose equivalent to 100 mg/kg Δ^1-THC. In parallel experiments, mice were also treated with Δ^1-THC or CBD at the same dose. The animals were killed at one or two hours after drug administration.

For the isotope doublet and metabolic switching experiments, three groups of three mice were treated with Δ^1-THC, 1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC, or a 1 : 1 molar mixture of both compounds at a dose of 100 mg/kg. The animals were killed after one hour.

The livers from three mice (1.5–2g each) or 2g samples of liver from each of the guinea-pigs or rats were homogenized in isotonic saline and the metabolites and non polar lipids were extracted with 3 x 5 ml portions of ethyl acetate (each sample). Separation of the metabolites from the lipids was achieved by chromatography on Sephadex LH-20 in chloroform and chloroform-methanol mixtures as already described[9, 11]. Six or fourteen fractions were collected and equivalent fractions from the three liver samples from each experiment were pooled. The conditions are listed in Table 2, together with the contents of each fraction. Extraction efficiency and column recovery were checked with tritium labelled Δ^1-THC. With animals treated with tritiated Δ^1-THC, 50% of the label, probably consisting of tissue bound THC, water soluble conjugates and tritium removed metabolically, was not extracted by ethyl acetate. All of the label in samples applied to the column was recovered; the per cent recovered in each fraction is given in Table 2.

TABLE 2. FRACTIONATION ON SEPHADEX LH-20 OF LIVER EXTRACTS FROM MICE TREATED WITH Δ^1-THC.

Fraction	Solvent	Vol (ml)	Recovery[a]	Contents
1	$CHCl_3$	18	50	triglycerides cholesterol
2	$CHCl_3$	8	10	Δ^1-THC
3	$CHCl_3$	10	0	fatty acids
4	$CHCl_3$	35	40	Mono-OH, keto-OH
5	20% MeOH:$CHCl_3$	50		polar metabolites
6	50% MeOH:$CHCl_3$	50	0	—

a. Percentage radioactivity applied to column.

Aliquots of each fraction, equivalent to 0.1–0.5g of liver were converted into trimethylsilyl (TMS), [$^2H_9$]TMS[26], methyl ester-TMS (Me TMS), methyloxime-TMS (MO-TMS), or methane boronate-TMS derivatives[9, 11], or were reduced with lithium aluminium deuteride and converted into TMS derivatives for examination by GC—MS.

QUANTITATION OF Δ^1-THC IN LIVER

The above procedure was modified as follows: (a) all glassware was silanized (dimethyldichlorosilane followed by methanol) before use; (b) extractions were performed with

hexane in experiments where only unmetabolised THC was being examined; (c) 1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC at a concentration roughly equivalent to that of the expected Δ^1-THC level was added to the homogenized liver as an internal standard before extraction of the THC. Where levels were unknown, a pilot run without internal standard was carried out. Recoveries were measured by GC—MS using selected ion monitoring.

COMPARISON OF DERIVATIVES FOR QUANTITATION

Stock solutions of Δ^1-THC (1 mg/ml) and its TMS, acetate (prepared by adding a 10% solution of acetic anhydride in pyridine), *t*-butyldimethylsilyl (TBDMS, prepared by adding TBDMS-imidazole in dimethylformanide)[(7)] or pentafluorophenyldimethylsilyl (flophemesyl, F_5PhDMS, prepared from flophemesyl diethylamine and flophemesyl chloride–Lancaster Synthesis Ltd.)[(4)] were prepared and successively diluted with solvent (ethyl acetate for the acetates and underivatized THC, acetonitrile with about 10% of silylating reagents for the silyl derivatives) to give concentrations of 500 ng, 50 ng, 500 pg, 50 pg and 5 pg/μl (100 μl of each solution was made up). Aliquots (1 or 2 μl) of these solutions were injected into the chromatograph. The mass spectrometer was set for single ion recording of the base peak (Table 3) with the column temperature set to give approximately the same retention time for each derivative. Peak heights were measured for multiple injections of each concentration. Experimental conditions and results are listed in Table 3.

TABLE 3. COMPARISON OF DERIVATIVES FOR THE QUANTITATION OF Δ^1-THC.

Derivative	Ion Monitored[a]	Column Temp (°C)	Elution Time (min)	Detection Limit (pg)[b]	Noise (mm)
Free	314 (M^+)	260	3	500	15
OAc	297 ($M-COCH_3$)	260	2.45	5	5
F_5PhDMS	538 (M^+)	280	3.45	10	6
TBDMS	371 (M-*t*-Bu)	270	2.15	5	9
TMS	386 (M^+)	250	2.10	4	4
TMS	386 (M^+)	280	1.10	4	4

a. Base peak.
b. Three times the noise level.

STUDIES ON TISSUE LEVELS OF METABOLITES

As internal standards were not available, Δ^1-THC was used as an external standard and was added to fractions 4 and 5 from the Sephadex LH-20 column. Metabolites were examined by selected ion monitoring of their Me-TMS derivatives. For studies on the kinetics of metabolism by mouse liver, Δ^1-THC was administered to male CD-1 mice as described above, at a dose of 10 mg/kg. The animals were killed at ½, 1, 2, 4, 6, 8, 12, 18 and 24 hours after dosing and the metabolites were extracted as described above. 500 ng of Δ^1-THC was added as external standard.

GAS CHROMATOGRAPHY

Gas chromatography was performed with a Varian 2400 gas chromatograph fitted with flame ionization detectors and either 2 m × 2 mm glass columns packed with 3% SE-30 or 3% OV-17 on 100–200 mesh Gas Chrom Q (Applied Science Laboratories Inc., State College, PA, USA) or a 50 m × 0.5 mm SCOT column coated with OV-1[6]. The injector and detector temperatures were 300° and the column oven was temperature programmed from 150 to 330° at 4°/min. Nitrogen at 30 ml/min was used for the packed columns and helium at 4.5 ml/min for the SCOT column.

GC—MS

Low resolution GC—MS was performed with a V.G. micromass 12 B mass spectrometer, interfaced to a V.G. 2040 data system and via a glass jet separator to a similar chromatographic system to that described above (SE-30 and OV-17 packed columns). The injector, interface and ion source temperatures were 300, 270 and 260° respectively and the column oven was temperature programmed from 170° to 300° at 2°/min. The mass spectrometer was operated at 25 eV with a trap current of 100 μA and an accelerating voltage of 2.5 kV (mass range *m/e* 40–700) spectra were acquired repetitively at 3 sec/decade with an inter-scan delay of 2 sec and stored on magnetic disc for future processing. Acquisition was started when the column oven reached 190°. In addition to the plotting of normalized spectra, the GC—MS runs were processed by the data system to give total and single-ion chromatograms and "Massmax" chromatograms,[28] the latter technique greatly improved the apparent resolution of the GLC column.

GC—MS of high molecular weight metabolites (glucuronides) were recorded with a V.G. micromass 70/70F mass spectrometer interfaced to the data system and a similar chromatographic system to that on the 12 B instrument.

High resolution spectra were recorded with the 70/70F instrument under the following conditions; resolution, 7000; accelerating voltage 4 KV; electron energy, 70 eV. Samples were introduced via the GLC or direct insertion probe and mass measurement was performed with the data system.

Single and multiple ion detection were performed with the 70/70F instrument with the slits open to give flat top peaks; resolution about 500 for THC and 800 for the metabolites, other parameters unchanged. Multiple ion monitoring of the metabolites was achieved with an 8-channel peak selector, cycle time 0.3 sec with the GLC oven temperature programmed from 220–280° at 4°/min. The ions monitored are listed in the text.

RESULTS AND DISCUSSION

METABOLITE IDENTIFICATION

Most metabolites of drugs of the cannabis type, with the exception of highly polar conjugates such as sulphates, can be separated and identified by GC—MS. With high resolution GLC columns, extensive pre-GLC clean-up procedures are usually unnecessary and methods which remove ionic compounds, compounds of high molecular weight, and endogenous compounds whose concentration far exceeds that of the metabolites, are

usually sufficient. This minimises problems associated with degradation or absorption. Metabolites can then be suitably derivatized and examined. Varying the derivative gives valuable information on functional groups[15] and can also be used to achieve selective shifts in the retention times of compounds with the same functional group, e.g. carboxylic acids.

We have been using these and related methods employing deuterium labelling[18] in order to extract the maximum information from GC—MS analysis of complex samples. This is particularly necessary since the concentrations of many metabolites are too low for separation and study by techniques such as NMR to be practicable. We have, in fact been able, by GC—MS methods, to identify most of the compounds present in fractions 4 and 5 (Table 2) of livers from mice, rats and guinea-pigs treated with Δ^1-THC, CBD or CBN as outlined below.

Δ^1-THC

Figure 1 illustrates the GLC profile of the TMS derivatives of Δ^1-THC extracted from mouse liver. 7-Hydroxylation followed by oxidation to a carboxylic acid was the major

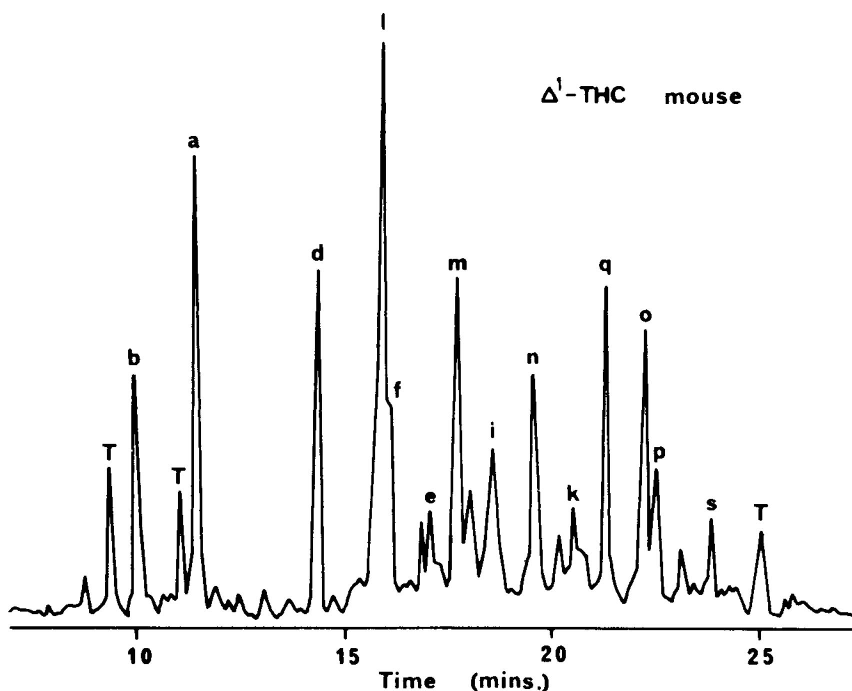


FIG. 1. "Massmax" chromatogram (*m/e* 300–700) of the metabolites (TMS derivatives) of Δ^1-THC extracted from mouse liver and separated on a 2 m × 2 mm 3% SE-30 column, temperature programmed from 190° to 300° at 2°/min. Peaks labelled T are tissue constituents. Identified peaks are: (a) 7-hydroxy-Δ^1-THC; (b) 6α-hydroxy-Δ^1-THC, (d) 6α,7-dihydroxy-Δ^1-THC; (e) 6β,7-dihydroxy-Δ^1-THC; (f) 2″,7-dihydroxy-Δ^1-THC; (i) 2″,6α,7-trihydroxy-Δ^1-THC; (k) 3″- and 4″,6α,7trihydroxy-Δ^1-THC; (l) Δ^1-THC-7-oic acid; (m) 6α-hydroxy-Δ^1-THC-7-oic acid; (n) 2″-hydroxy-Δ^1-THC-7-oic acid; (o) 3″-hydroxy-Δ^1-THC-7-oic acid; (p) 4″-hydroxy-Δ^1-THC-7-oic acid; (q) 2″,6α-dihydroxy-Δ^1-THC-7-oic acid; (s) 3″- and 4″,6α-dihydroxy-Δ^1-THC-7-oic acid.

metabolic route giving 7-hydroxy-Δ^1-THC (a) and Δ^1-THC-7-oic acid (1). Hydroxylation in the other allylic position (6) was also prominent giving 6α-hydroxy-Δ^1-THC (b). 6β-Hydroxylation was a minor pathway. Oxidation of the 6-hydroxy groups gave a ketone[9, 14] (Fig. 2). Probably the most interesting feature of the biotransformation of Δ^1-THC in the mouse was the high abundance of poly-substituted metabolites,[9, 11, 14–16, 18] often containing hydroxylation in the 2″-, 3″- and 4″-, positions of the pentyl side-chain; side-chain mono-hydroxy metabolites have only been observed in trace quantities in this species. Diol metabolites, except for 6α,7-dihydroxy-Δ^1-THC (peak d) were generally of low abundance and were represented by 6β,7- (e), 2″,7- (f), 3″,7- and 4″,7-Δ^1-THC. Separation of the 3″- and 4″-hydroxy metabolites was best achieved on OV-17 columns. Triols containing substitution at the same positions, namely 6α and 7 together with the third hydroxy group at either the 2″-, 3″-, or 4″-positions (peaks i and k)

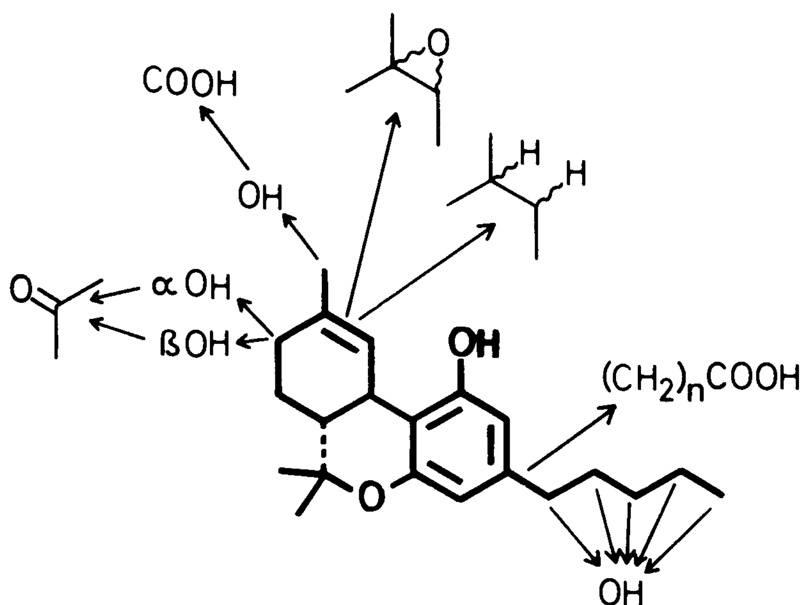


FIG. 2. Outline of the major metabolic transformation shown by Δ^1-THC.

were also found. These positions of substitution were common to all other poly-functional metabolites; thus hydroxy acids were represented by 6β (m), 2″- (n), 3″- (o) and 4″- (p) hydroxy-Δ^1-THC-7-oic acids. 6-oxo metabolites, again with mono- or di-hydroxylation at equivalent positions produced another series of metabolites. Minor metabolic routes in the mouse, outlined in Fig. 2 included hydrogenation of the double bond of the 7-oic acid metabolite to give a pair of hexahydrocannabinol-7-oic acids[10] and conjugation of the phenol group with glucuronic acid.[12] Epoxidation of the double bond followed by hydroxylation has not been seen in our extracts although this is a significant metabolic route for other THC isomers such as Δ^6-THC.[17]

The rat showed a very similar metabolic profile but with lower concentrations of the more highly substituted metabolites.

The guinea-pig, on the other hand, showed a different pattern of metabolism.[14] 6β- rather than 6α-hydroxylation was favoured to give the 6β,7-diol as a major metabolite,

side-chain monohydroxy metabolites were produced, 1″-hydroxylation occurred and major metabolites were produced by oxidative degradation of the side-chain; 4″, 5″-*bis*-nor-Δ^1-THC-3″-oic acid was the major metabolite of this type.[23] Diols containing 6β- together with side-chain hydroxylation were observed, but polysubstituted metabolites were generally of low abundance. These differences are summarized in Table 4. Thirty-six metabolites have now been identified from Δ^1-THC; thirty of these occur in the mouse. A few minor metabolites have still not been identified.

TABLE 4. COMPARISON OF THE IN VIVO LIVER METABOLISM OF Δ^1-THC IN MOUSE, RAT AND GUINEA-PIG.

Metabolic route	Mouse	Rat	Guinea-pig
1. *Route*			
7-hydroxylation	+ + +	+ + +	+ + +
6α-hydroxylation	+ + +	+ + +	+
6β-hydroxylation	+	+	+ + +
side-chain-hydroxylation	2″, 3″, 4″	2″, 3″, 4″	1″, 2″, 3″, 4″, (5″)
7-acid formation	+ + +	+ + +	+
6-ketone formation	+ +	+	+
double bond reduction	+	–	–
glucuronide formation	+	+	+ +
β-oxidation	+	+	+ + +
2. *Metabolite type*			
mono OH (allylic)	+ + +	+ + +	+ + +
mono OH (aliphatic)	+	+	+ +
diol	+ +	+ +	+ + +
triol	+ +	+	+
7-acid	+ + +	+ + +	+
hydroxy acid	+ + +	+ +	+
dihydroxy acid	+ + +	+ +	+

Key. + + + major metabolite;
+ minor metabolite;
– not observed.

Deuterium labelling has been used quite extensively in these studies. In addition to the use of $[^2H_9]$TMS derivatives and reduction of metabolic fractions with lithium aluminium deuteride, discussed earlier,[11, 14, 15] we have now used the isotope doublet technique[20, 21] to identify which peaks of a GC—MS run are produced by metabolites. Either 3-$[^2H_1]\Delta^1$-THC or 1″, 1″, 2″, 2″-$[^2H_4]\Delta^1$-THC, administered as 1 : 1 molar mixtures with the unlabelled analogues were used and the best results were obtained with the latter compound. As the doublet peaks were only separated by one mass unit in the 3-deuterated analogue, it was often difficult to distinguish the doublets in complex fractions. However, as discussed below, this derivative gave different results with metabolites substituted at C-2″ as formation of such compounds involved replacement of one of the deuterium atoms.

Metabolic displacement of deuterium can theoretically be used to identify all metabolites containing hydroxylation at the site of deuteration in a single GC—MS run, both by

observing the loss of the deuterium atom, or by metabolic switching.[19] The latter effect, which has been reported with compounds metabolized by multiple alternate pathways, is a direct result of the slowing of metabolism at the site of deuteration by a primary kinetic isotope effect[3] and results in low concentrations of metabolites otherwise expected. In order to see if this effect was significant in cannabinoid metabolism, 1″, 1″, 2″, 2″-[$^2H_4$]Δ^1-THC, Δ^1-THC and a 1 : 1 molar mixture of both compounds were administered to three sets of three mice and metabolites containing 2″-hydroxylation were examined. Pronounced decreases in the concentrations of these compounds were observed in the chromatograms from the mice treated with the deuterated drug; 2″-hydroxy-Δ^1-THC-7-oic acid and 2″,6α-dihydroxy-Δ^1-THC-7-oic acid were reduced to 10 and 20% respectively of their concentrations in the mice treated with unlabelled drug. This effect could possibly be exploited to obtain further information on both the pharmacological activity of various hydroxylated species and the metabolic routes producing each metabolite.

CBD

CBD metabolism was studied in mouse[24] and guinea-pig and similar metabolic profiles were produced by both species. Major differences in the biotransformation routes were observed in mouse from those occurring with Δ^1-THC. Although 7-hydroxylation was still a major pathway, a large number of metabolites were formed via oxidative degradation (probably β-oxidation) of the side-chain; CBD-5″-oic acid and particularly its 3″- and 1″-homologues were abundant. Derivatives of these acids, containing either a 7-hydroxy or a 6-oxo group were also present. It has not been determined if 6β-hydroxylation predominated in the guinea-pig, as it did with Δ^1-THC, as the mass spectra and retention times of the TMS derivatives of 6α- and 6β-hydroxy-CBD are very similar.[25] Glucuronide formation, particularly in the mouse was a major metabolic pathway;[8, 12] the conjugates of CBD and its hydroxy metabolites were found. These results are summarized in Table 5.

TABLE 5. COMPARISON OF THE IN VIVO LIVER METABOLITES OF CBD FOUND IN MOUSE AND GUINEA-PIG.

Metabolic route	Mouse	Guinea-pig
7-hydroxylation	+ + +	+ + +
6-hydroxylation	+ +	+
side-chain hydroxylation (mono)	–	+
7-acid formation	+ + +	+ +
diol formation	+	+
hydroxy-acid formation	+ + +	+ + +
β-oxidation	+ + +	+ + +
β-oxidation + hydroxylation	+ + +	+ +
β-oxidation + 6-oxo formation	+ +	–
glucuronide formation	+ + +	+

Key. + + + major metabolite;
\+ minor metabolite;
– not observed.

CBN

CBN appeared to be metabolized less extensively and more slowly than the other cannabinoids[13]; this was probably due to the absence of allylic hydroxylation caused by the aromatization of the terpene ring. Nevertheless 7-hydroxylation, as with the other two cannabinoids, was the major oxidative route. Indeed, in the mouse, 7-hydroxy-CBN and CBN-7-oic acid together with their glucuronides and the glucuronide of CBN itself, were the only abundant metabolites. More extensive metabolism was observed for CBN in rat liver (Table 6), particularly in the 1″-position, the 1″, 2″, 3″ and 4″-hydroxy derivatives of both 7-hydroxy-CBN and CBN-7-oic acid were found. β-oxidation of the side-chain was a minor pathway—only 4″, 5″-*bis*-nor-CBN-7-oic acid was observed in low concentration.

TABLE 6. COMPARISON OF THE IN VIVO LIVER METABOLITES OF CBN FOUND IN MOUSE, RAT AND GUINEA-PIG.

Metabolic route	Mouse	Rat	Guinea-pig
7-hydroxylation	+ + +	+ + +	+ + +
7-acid formation	+ + +	+ + +	+
1″-hydroxylation (mono)	−	+	+ + +
3″-hydroxylation (mono)	−	−	+
4″-hydroxylation (mono)	−	+	+
Diol formation	+	+ +	+
hydroxy acid formation	+	+ + +	−
β-oxidation	−	+	−
glucuronide formation	+ + +	+ +	+

Key. + + + major metabolite;
+ minor metabolite;
− not observed.

Considering the high levels of the side-chain acid metabolites of both Δ^1-THC and CBD in guinea-pig liver, it was somewhat surprising to find no evidence of oxidative degradation of the side-chain of CBN by the guinea-pig. Metabolite levels were generally low, and monosubstituted compounds were dominant. 1″- and 7-hydroxy-CBN were the major metabolites, 3″-, and 4″-hydroxy-CBN and CBN-7-oic acid were present in low concentrations. The only polysubstituted metabolite appeared to be 1″, 7-dihydroxy-CBN. It must be emphasised, however, that the concentrations of metabolites observed in these experiments represent only the levels of the metabolites in liver and do not take into account any different rates of excretion. The absolute amount of metabolites produced by attack at various sites could be quite different.

CANNABIS TINCTURE

The major cannabinoids present in the sample of cannabis tincture were identified and quantitated by GC—MS and GLC respectively and are listed in Table 1. This particular sample contained a relatively high concentration of the propyl homologues, the metabolism of which does not appear to have been studied. The tincture was administered at a dose equivalent to 100 mg/kg THC and the metabolites were extracted as for the indi-

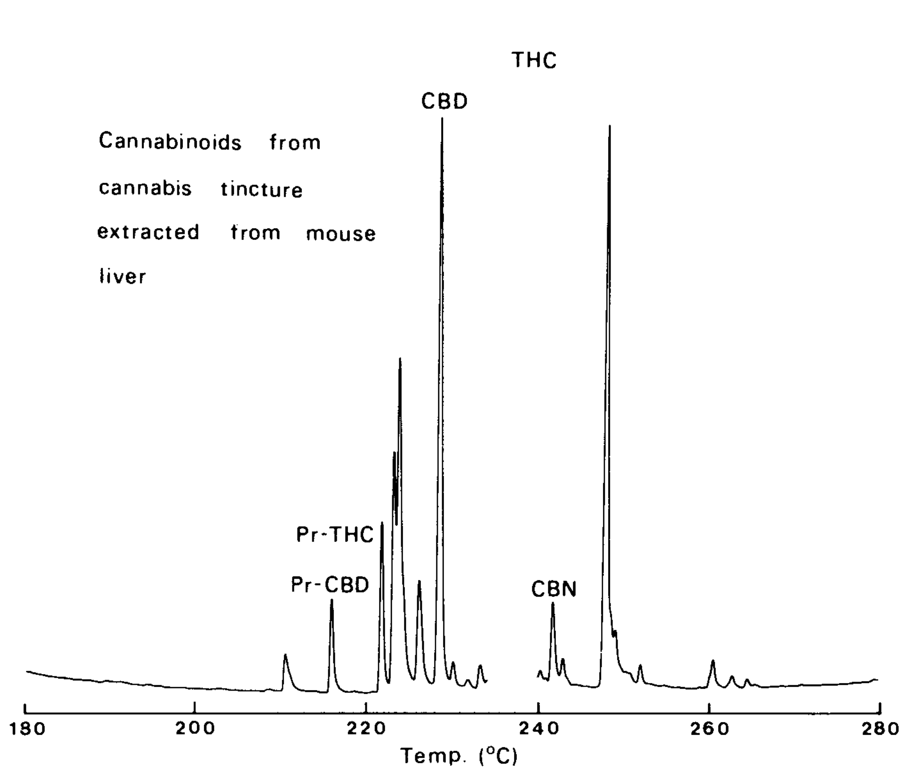


FIG. 3. Cannabinoids extracted from mouse liver (Sephadex LH-20 column, fraction 2) one hour after the administration of an extract of cannabis tincture (100 mg/kg THC equivalent, IP) separation as TMS derivatives on a 50 m × 0.5 mm SCOT column coated with OV-1 and temperature programmed from 150° at 4°/min.

vidual cannabinoids. Unchanged cannabinoids were recovered in fraction 2 from the Sephadex LH-20 column. CBD and THC were present in the same ratio as the original tincture (Fig. 3), the concentration of CBN was lower as it eluted slightly later from the column. Quantities of the cannabinoids were measured by GLC (OV-1 SCOT column with 5α-cholestane as external standard) and Δ^1-THC was also measured by GC—MS (selected ion monitoring, see below, using 1″, 1″, 2″, 2″-$[^2H_4]\Delta^1$-THC as external standard) from two sets of mice killed at one hour and two hours after drug administration. Results are given in Table 7.

The metabolic profiles from fractions 4 and 5 were similar after one and two hours with slightly higher concentrations of the more highly substituted metabolites present after two hours. The profiles were obtained with an OV-1 SCOT column (Fig. 4) and with a 3% SE-30 packed column on the GC—MS instrument with computer processing (Fig. 5). Accurate measurements of the concentrations of each metabolite have not yet been made. However, the profiles show that all the major metabolites from the three main cannabinoids were present in roughly the same ratio as was found with the individual compounds. The levels of metabolite from each compound reflected the concentrations of the original cannabinoids. Concentrations in the order of 8 μg/liver for 7-hydroxy-Δ^1-THC were found.

TABLE 7. QUANTITIES OF UNCHANGED CANNABINOIDS FOUND IN THE LIVERS OF MICE TREATED WITH CANNABIS TINCTURE. (100 mg/kg THC EQUIVALENT).

Cannabinoid	1 hour	2 hours
Pr-CBD	11 μg/liver[a]	9.3 μg/liver
Pr-Δ^1-THC	25	17.8
CBD	88	62
Δ^1-THC	103	75
CBN	10	10.5

a. Average liver weight 1.8 g.

For single doses, therefore, it appears that the magnitude of possible metabolic interactions between the cannabinoids is relatively small so that the metabolite output approximates that expected from an arithmetical sum of metabolites from each cannabinoid.

The metabolism of the propyl homologues was similar to that of the pentyl cannabinoids. Thus, for example, propyl-Δ^1-THC gave the 7-hydroxy-(base peak *m/e* 343) and

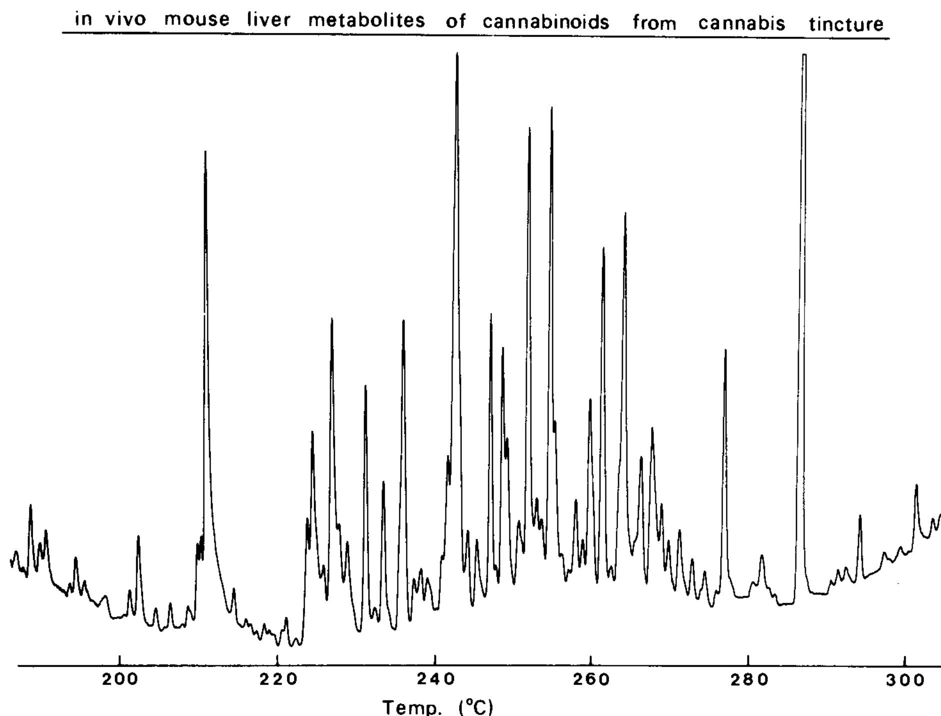


FIG. 4. Cannabinoid metabolites extracted from mouse liver (LH-20 fractions 4 and 5) two hours after the administration of an extract of cannabis tincture and separated as TMS derivatives on a 50 m × 0.5 mm OV-1 SCOT column temperature programmed at 4°/min. Peaks eluting between 230 and 250° are produced mainly by metabolites of the propyl homologues. The two largest peaks are tissue constituents, the next largest is 7-hydroxy-Δ^1-THC. Most metabolites elute between 250 and 280°.

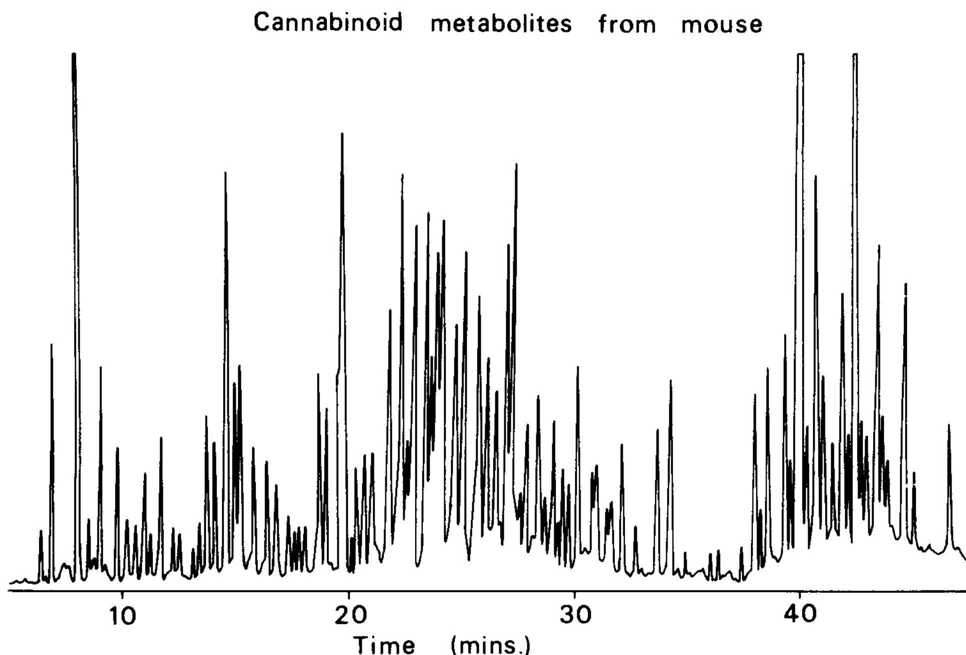


FIG. 5. Cannabinoid metabolites extracted from mouse liver (LH-20 fractions 4 and 5) two hours after the administration of cannabis tincture and separated as TMS derivatives on a 2 m × 2 mm 3% SE-30 column, temperature programmed from 190° at 2°/min. The chromatogram was produced by the data system using the "Massmax" program on ions above *m/e* 300. The peaks eluting between 10 and 35 min consist mainly of oxygenated metabolites; those eluting between 35 and 45 mins are glucuronides.

6α-hydroxy metabolites (base peak *m/e* 356, $[M\text{-}TMSOH]^+$), pr-CBD gave the 7-hydroxy metabolite [*m/e* 415 (100%, $M\text{-}CH_2OTMS$), 450 (91%, $M\text{-}C_4H_8$), 309 (57%, tropylium ion)] as did pr-CBN [*m/e* 427 (100%, $M\text{-}CH_3$), 442 (14%, M^+)].

QUANTITATION OF Δ^1-THC AND METABOLITES IN LIVER

Several mass fragmentographic and GLC methods have recently been published[30] for the quantitation of Δ^1-THC and its 7-hydroxy and 7-acid metabolites in body fluids—mainly plasma. All involve solvent extraction and most rely on additional chemical clean-up and quantitation using a deuterated internal standard. The detection limits for all the methods lie in the range 0.3–1 ng/ml. No work appears to have been reported on similar methods for the quantitation of cannabinoids in tissues. We have used 1″, 1″, 2″, 2″-$[^2H_4]$ Δ^1-THC as an internal standard to measure Δ^1-THC in liver using the ethyl acetate: LH-20 extraction method outlined above but found it less sensitive than the published methods with plasma. With a 3% SE-30 column, the detection limit was only in the order of 5 ng/g. The reason for the low sensitivity was interference by residual endogenous constituents, which were present in much higher quantities than in plasma. Changing to a 3% OV-17 column improved the sensitivity to around 2 ng/g and using a lower polarity solvent (hexane) gave a limit of about 0.5 ng/g. However, the latter method was less attractive when measurements needed to be made on Δ^1-THC and its metabolites in the same sample.

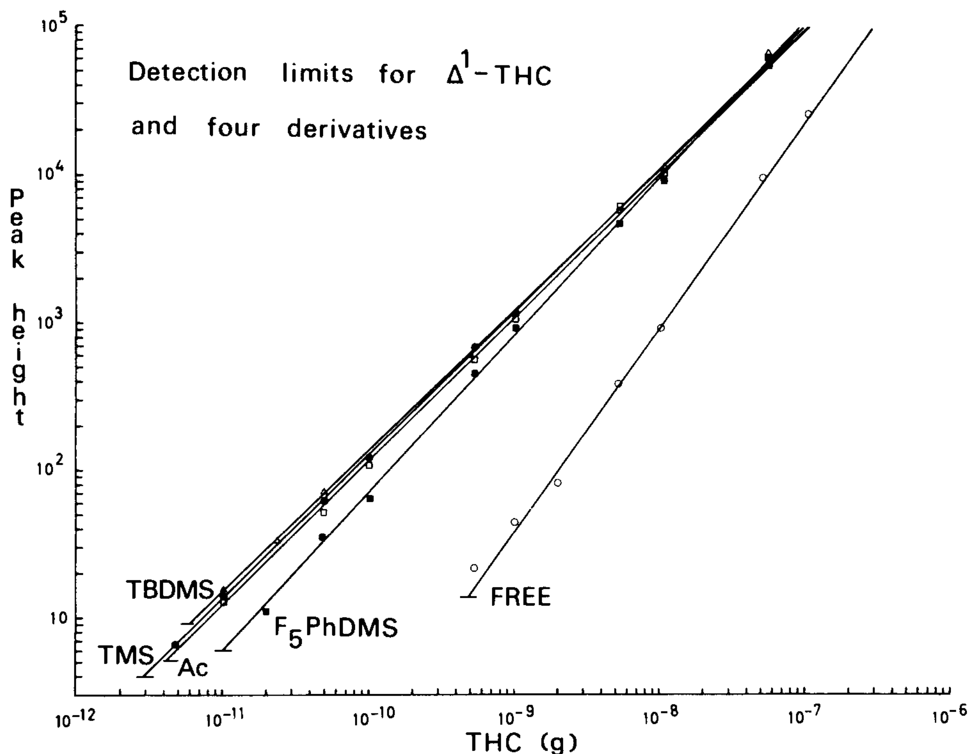


FIG. 6. Plots on a double log scale of concentration/peak height for Δ^1-THC, and its TMS, acetate (Ac) TBDMS and F_5PDMS derivatives obtained by single ion monitoring of the base peaks, (Table 3). The horizontal line at the lower end of each line shows the instrument noise level at the mass monitored.

Several derivatives were examined in an attempt to improve absolute detection limits. These are shown in Table 3 and Fig. 6. The sensitivity of detection by selected ion monitoring depends on:

a. the stability and non-polar nature of the compound. This gives low or negligible adsorption and a sharp peak.
b. the extent of ionization in the mass spectrometer.
c. the amount of ion current carried by the ion or ions measured.
d. instrument noise (column bleed, ion statistics).
e. "noise" from biological components.

From Fig. 6 it is apparent that derivatization of Δ^1-THC greatly improved the limit of detection presumably by reducing adsorption effects, but also because the instrument noise at *m/e* 314 (the ion monitored for free Δ^1-THC) was higher than at the masses of the base peaks of the derivatives. As much as two orders of magnitude was gained when derivatives were prepared but there was little variation between the four derivatives studied. The mass spectra of most derivatives of Δ^1-THC were similar as ionization appeared to be remote from the derivatized function[7], thus the limit of detection was roughly correlated with the percentage of the ion current in the ion monitored. With the acetate derivative, however,

ionization occurred at the acetate group to give an abundant ion at $[M\text{-}OCOCH_3]^+$. Even so, the GC—MS characteristics of this derivative were similar to those of the other derivatives. The TBDMS derivative gave an abundant $[M\text{-}t\text{-}Bu]^+$ ion but again this did not significantly alter the detection limit. More important was the instrument noise level, shown in Fig. 6. This resulted in the TMS derivative giving the best overall performance with a detection limit of 9 pg (three times the noise level). However, as stated above, in the presence of a liver extract, "biological noise" reduced this considerably. The effect of biological noise on the other derivatives has not yet been determined. The use of a higher column temperature to give a sharper peak had little effect on the detection limit as shown by the last two entries in Table 3. Typical results obtained for Δ^1-THC levels in liver were 1.42 μg/g one hour after an IP dose of 10 mg/kg and 3.15 μg/g one hour after a 20 mg/kg IP injection. At these levels, recovery was in the region of 95%.

Internal standards were not available for the Δ^1-THC metabolites and consequently no attempt was made to obtain accurate quantitative data on tissue levels. However, some qualitative results were obtained in mice dosed with Δ^1-THC intraperitoneally at a level of 10 mg/kg. The metabolites were extracted as above and converted into Me-TMS derivatives; the TMS derivatives of the acid metabolites were not sufficiently stable for the quantitation of nanogram quantities. An eight channel peak selector was used and tuned to the ions indicated in Fig. 7. This figure shows a typical trace from fraction 5 obtained from the LH-20 column. Δ^1-THC (500 ng/liver, 50 ng/sample injected) was used as an external standard and was monitored using the ion at *m/e* 371. This ion was also the base peak for the major metabolite, Δ^1-THC-7-oic acid. The ions used for monitoring the other

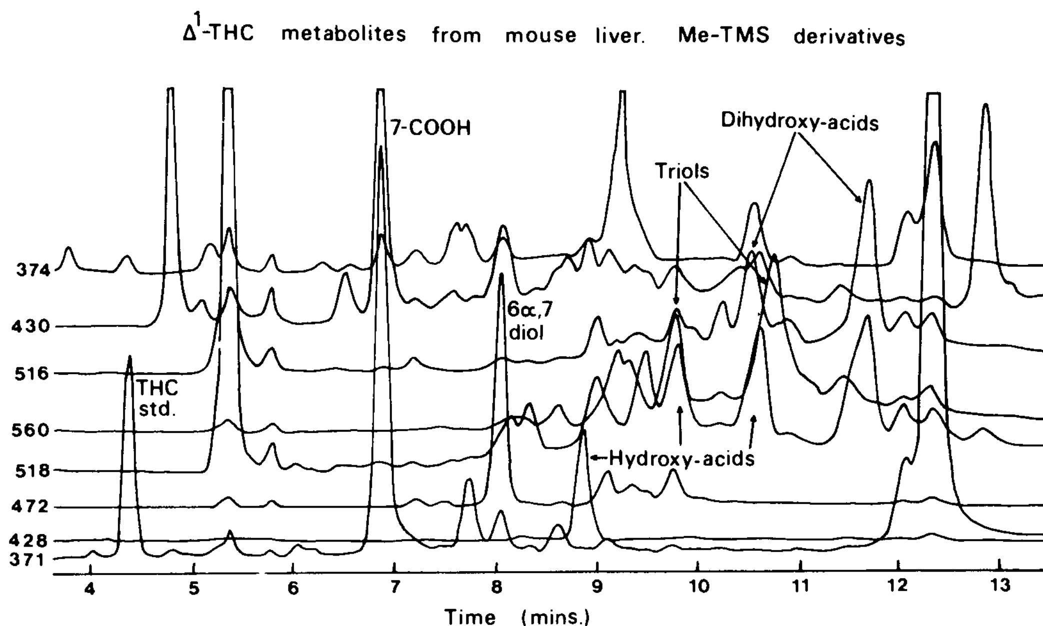


FIG. 7. Multiple ion trace obtained from the metabolites extracted from mouse liver (Sephadex LH-20, fraction 5) 30 mins. after I.P. administration of Δ^1-THC (10 mg/kg) and separated on a 2 m × 2 mm 3% SE-30 column. The trace represents 10% of the liver sample and the THC external standard corresponds to 50 ng. Metabolites monitored and channel gains are listed in Table 8.

metabolites are listed in Table 8. Other ion combinations have been used for monitoring metabolites, but the ions shown in Fig. 7 generally gave the most satisfactory results. As this chromatogram was obtained on SE-30, there was poor separation of the 3″- and 4″-hydroxy acids; thus to monitor the former compound, the characteristic ion at *m/e* 374 ($[M\text{-}144]^{+}$)[2] was used.

TABLE 8. IONS USED FOR MONITORING Δ^1-THC METABOLITES IN FRACTION 5 FROM THE LH-20 COLUMN. (FIG. 7).

Channel	Ion	Gain	Compounds monitored
1	371	3	Δ^1-THC, THC-7-oic acid
2	428	3	6α-OH-Δ^1-THC ($[M\text{-}TMSOH]^+$ ion)
3	472	10	6α,7-di-OH-Δ^1-THC
4	518	100	side-chain OH-acids (M^+)
5	560	100	Triols ($[M\text{-}TMSOH]^+$ ion)
6	516	100	Di-OH-acids ($[M\text{-}TMSOH]^+$ ion)
7	430	30	THC-7-oic acid (M^+)
8	374	10	3″-OH-Δ^1-THC-7-oic acid ($[M\text{-}144]^+$)

This method has been used to monitor the metabolites present after the administration of Δ^1-THC (IP 10 mg/kg). Mice were killed at various times as given in the experimental section. After 30 mins (the shortest time taken) all the metabolites listed in Table 8 were present. Their levels declined in successive samples and could no longer be detected after 2 hours. The relative amounts of each compound remained the same. In the absence of reference compounds or deuterated standards it was not possible to quantitate the metabolites. However, assuming similar response factors between Δ^1-THC and its metabolites, it would appear that tissue levels in the range 200–400 ng/liver for Δ^1-THC-7-oic acid are reached 30 mins after a 10 mg/kg IP injection.

CONCLUSIONS

GC—MS, particularly when combined with methods involving selective derivative preparation or deuterium labelling has enabled much qualitative and quantitative information to be obtained on the metabolites of Δ^1-THC present in the livers of several species. Hydroxylation, mainly allylic and aliphatic, or benzylic in the case of CBN, initiated most of the major metabolic routes in the three species studied. The position and extent of the hydroxylation showed considerable species variation. Oxidation of these alcohols to ketones and particularly acids produced the major metabolites. Polysubstitution was found to be particularly widespread. Other major metabolic routes included conjugation with glucuronic acid and β-oxidation of the side-chain. Minor pathways included reduction of the double bond. The major cannabinoids could be quantitated in tissues by GLC using an external standard or Δ^1-THC could be measured by selected ion monitoring using 1″, 1″, 2″, 2″-$[^2H_4]\Delta^1$-THC as internal standard with a detection limit of around 0.5 ng/g. Quantitation of metabolites was limited by the lack of suitable standards but useful semi-quantitative data could nevertheless be obtained by selected ion monitoring.

ACKNOWLEDGEMENTS

We thank the Medical Research Council for a Programme Research Grant and the Wellcome Trust for the support of B.R.M. We also thank Dr. E. W. Gill and Mr. M. Slater for the preparation of the deuterated cannabinoids and Miss J. Hughes and Mr. D. Perrin for expert technical assistance.

REFERENCES

1. Agurell, S., Dahmen, J., Gustafsson, B., Johansson, U.-B., Leander, K., Nilsson, I., Nilsson, J. L. G., Nordqvist, M., Ramsay, C. H., Ryrfeldt, A., Sandberg, G., Widman, M., Metabolic fate of Tetrahydrocannabinol. *Cannabis and its derivatives.* (W. D. M. Paton and J. Crown, eds.), Oxford University Press, London, pp. 16–36, 1972.
2. Binder, M., Agurell, S., Leander, K., Lindgren, J.-E., Zur identifikation potentieller metabolite von cannabis-inhaltstoffen: kernresonanz und massenspektroskopische untersuchungen an seitenkettenhydroxylierten cannabinoiden. *Helv. Chim. Acta* **57**,1626 (1974).
3. Blake, I. M., Crespi, H. L., Katz, J. J., Studies with deuterated drugs. *J. Pharm. Sci.* **64**,367 (1975).
4. Burkinshaw, P. M., Morgan, E. D., Poole, C. F., Detection of alcohols at the femtogram level as pentafluorophenyldimethylsilyl ethers. *J. Chromatogr.* **132**,548 (1977).
5. Erdmann, G., Just, W. W., Thel, S., Werner, G., Wiechmann, M., Comparative autoradiographic and metabolic study of Δ^8- and Δ^9-tetrahydrocannabinol in the brain of the marmoset. *Callithrix jacchus. Psychopharmacology* **47**,53 (1976).
6. German, A. L., Horning, E. C., Thermostable open tube capillary columns for the high resolution gas chromatography of human urinary steroids. *J. Chromatogr. Sci.* **11**,76 (1973).
7. Harvey, D. J., Comparison of fourteen substituted silyl derivatives for the characterization of alcohols, steroids and cannabinoids by combined gas-liquid chromatography and mass spectrometry. *J. Chromatogr.* **147**,291 (1978).
8. Harvey, D. J., Martin, B. R., Paton, W. D. M., Identification of glucuronides of cannabidiol and hydroxycannabidiols in mouse liver. *Biochem. Pharmacol.* **25**,2217 (1976).
9. Harvey, D. J., Martin, B. R., Paton, W. D. M., Identification of di- and tri-substituted hydroxy and ketone metabolites of Δ^1-tetrahydrocannabinol in mouse liver. *J. Pharm. Pharmacol.* **29**,482 (1977).
10. Harvey, D. J., Martin, B. R., Paton, W. D. M., Identification of metabolites of Δ^1- and $\Delta^{1(6)}$-tetrahydrocannabinol containing a reduced double bond. *J. Pharm. Pharmacol.* **29**,495 (1977).
11. Harvey, D. J., Martin, B. R., Paton, W. D. M., Characterization of *in vivo* liver metabolites of Δ^1-tetrahydrocannabinol by EI and CI mass spectrometry. *Mass spectrometry in Drug Metabolism* (A. Frigerio and E. L. Ghisalberti, eds.). Plenum, New York, pp. 403–428, 1977.
12. Harvey, D. J., Martin, B. R., Paton, W. D. M., Identification of glucuronides as *in vivo* liver conjugates of seven cannabinoids and some of their hydroxy and acid metabolites. *Res. Commun. Chem. Path. Pharmacol.* **16**,265 (1977).
13. Harvey, D. J., Martin, B. R., Paton, W. D. M., *In vivo* metabolism of cannabinol by the mouse and rat and a comparison with the metabolism of Δ^1-tetrahydrocannabinol and cannabidiol. *Biomed. Mass Spectrom.* **4**,364 (1977).
14. Harvey, D. J., Martin, B. R., Paton, W. D. M., Comparative *in vivo* metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC), cannabidiol (CBD) and cannabinol (CBN) by several species. *Recent Developments in Mass Spectrometry in Biochemistry and Medicine, Vol. 1.* (A. Frigerio, ed.), Plenum, New York, pp. 161–184, 1978.
15. Harvey, D. J., Paton, W. D. M., Examination of the metabolites of Δ^1-tetrahydrocannabinol in mouse liver, heart and lung by combined gas chromatography and mass spectrometry. *Marihuana: Chemistry, Biochemistry, and cellular effects.* (G. G. Nahas, W. D. M. Paton and J. E. Idänpään-Heikkilä, eds.). Springer-Verlag, New York, pp. 93–109, 1976.
16. Harvey, D. J., Paton, W. D. M., Characterization of three monohydroxyacid and two dihydroxyacid metabolites of Δ^1-tetrahydrocannabinol in mouse liver. *Res. Commun. Chem. Path. Pharmacol.* **13**,585 (1976).
17. Harvey, D. J., Paton, W. D. M., *In vivo* metabolites of $\Delta^{1(6)}$-tetrahydrocannabinol produced by the mouse via the epoxide-diol pathway. *J. Pharm. Pharmacol.* **29**,498 (1977).
18. Harvey, D. J., Paton, W. D. M., The metabolism of deuterium-labelled analogues of Δ^1-, Δ^6-, and Δ^7-tetrahydrocannabinol and the use of deuterium labelling for the elucidation of metabolic structures. *Recent developments in Mass Spectrometry in Biochemistry and Medicine, Vol. 2.* (A. Frigerio, ed.). Plenum, New York (in press).

19. HORNING, M. G., THENOT, J.-P., HAEGELE, K., NOWLIN, J., STAFFORD, M., SOMMER, K. R., Metabolic switching of drug pathways as a consequence of deuterium substitution. *Fed. Proc.* **35**,488 (1976).
20. KNAPP, D. R., GAFFNEY, T. E., MCMAHON, R. E., Use of stable isotope mixtures as a labelling technique in drug metabolism studies. *Biochem. Pharmacol.* **21**,425 (1972).
21. KNAPP, D. R., GAFFNEY, T. E., MCMAHON, R. E., KIPLINGER, G., Studies of human urinary and biliary metabolites of nortriptyline with stable isotope labelling. *J. Pharmacol. Exptl. Therap.* **180**,784 (1972).
22. LEIGHTY, E. G., FENTIMAN, A. F., JR., FOLTZ, R. L., Long-retained metabolites of Δ^9- and Δ^8-tetrahydrocannabinols identified as novel fatty acid conjugates. *Res. Commun. Chem. Path. Pharmacol.* **14**,13 (1976).
23. MARTIN, B. R., HARVEY, D. J., PATON, W. D. M., Identification of new *in vivo* side-chain acid metabolites of Δ^1-tetrahydrocannabinol. *J. Pharm. Pharmacol.* **28**,773 (1976).
24. MARTIN, B. R., HARVEY, D. J., PATON, W. D. M., (1977). Biotransformation of cannabidiol in mice. Identification of new acid metabolites. *Drug Metab. Dispos.* **5**,259 (1977).
25. MARTIN, B. R., NORDQVIST, M., AGURELL, S., LINDGREN, J.-E., LEANDER, K., BINDER, M., Identification of monohydroxylated metabolites of cannabidiol formed by a rat liver. *J. Pharm. Pharmacol.* **28**,275 (1976).
26. MCCLOSKEY, J. A., STILLWELL, R. N., LAWSON, A. M., Use of deuterium-labelled trimethylsilyl derivatives in mass spectrometry. *Analyt. Chem.* **40**,233 (1968).
27. MECHOULAM, R., MCCALLUM, N. K., BURSTEIN, S., Recent advances in the chemistry and biochemistry of cannabis. *Chem. Rev.* **76**,75 (1976).
28. POWERS, P., WALLINGTON, M. J., HOPKINSON, J. A. V., KEARNS, G. L., An extension to the role of the dedicated data system in GC—MS. Paper presented at the 23rd Annual Conference on Mass Spectrometry and Allied Topics. Houston, Texas, May 25–30. American Society for Mass Spectrometry. Abstracts pp. 499–502 (1975).
29. RAZDAN, R. K., DALZELL, H. C., HANDRICK, G. R., Hashish. A simple one-step synthesis of (−)-Δ^1-tetrahydrocannabinol (THC) from *p*-mentha-2,8-dien-1-ol and olivetol. *J. Amer. Chem. Soc.* 96:5860 (1974).
30. See *Res. Monogr. Ser.*—NIDA, (R. E. Willette, ed.) **7**,33, 42, 48, 64, 70, 88, 96, 107 (1976).

RELATION BETWEEN EFFECTS OF CANNABINOID DERIVATIVES ON THE TWITCH RESPONSE OF THE ISOLATED GUINEA-PIG ILEUM AND THEIR PSYCHOTROPIC PROPERTIES

S. ROSELL, U. BJÖRKROTH, S. AGURELL, K. LEANDER, A. OHLSSON, B. MARTIN and R. MECHOULAM

Department of Pharmacology, Karolinska Institute, 104 01 Stockholm; Astra Läkemedel, 151 85 Södertälje; Department of Organic Chemistry, University of Stockholm, 106 91 Stockholm; Department of Pharmacology, Medical College of Virginia, Richmond, Virginia 23298, USA and Department of Natural Products, Hebrew University School of Pharmacy, Jerusalem, Israel.

Summary. The ability of Δ^6-THC, five of its hydroxy metabolites and a number of synthetic cannabinoids were tested for their ability to reduce the response of a guinea-pig ileum to electrical stimulation. Good correlation was found between high activity in this test and observed psychotropic activity. Thus Δ^6-THC and its 3″- and 7-hydroxy metabolites showed equal potency in the guinea-pig test with 5″-hydroxy-Δ^6-THC showing lower activity. In behavioural tests, 3″-, 4″-, 5″- and 7-hydroxy-Δ^6-THC showed high activity. THC analogues containing a methylated or acetylated phenol group or an aromatic carboxyl group were inactive in both tests.

IN A previous publication (Rosell, Agurell, Martin, 1976) we reported the effect of eight cannabinoids on isolated smooth muscle preparation. It was shown that the electrically stimulated guinea pig ileum was very sensitive to those cannabinoids which are psychoactive.

The metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC; I)—the psychotropic compound in cannabis—has been extensively investigated (cf. Mechoulam, McCallum and Burstein, 1976). Particular interest was created by the fact that Δ^1-THC was found to be hydroxylated in various positions of the monoterpene moiety and the pentyl side chain and that 7-hydroxy-Δ^1-THC retained, as shown in several species including man (e.g. Perez-Reyes *et al.*, 1973; the psychotropic activity of Δ^1-THC. The metabolite 6 β-hydroxy-Δ^1-THC was less potent and the 6α-epimer was devoid of activity in man. As discussed in a recent paper (Leander *et al.*, 1978) numerous metabolites of Δ^1-THC, cannabinol (CBN) and cannabidiol (CBD) hydroxylated in various positions of the side chain have been isolated in a number of laboratories. In summary, all three major cannabinoids can be hydroxylated on all five carbons of the side chain but the 3″- and 4″-positions

are preferred. Side chain hydroxylation is usually a minor metabolic route but favoured, for example, in the rabbit and the guinea pig, whereas 7-hydroxylation is preferred, for example, in the rat (see also Agurell *et al.*, 1976). The patterns of primary metabolites have often been investigated using liver microsomes and this may differ from the *in vivo* situation. Thus, a more pronounced side chain hydroxylation of CBN was found in the rat *in vivo* than when compared with the *in vitro* results (Yisak *et al.*, 1977).

To investigate the psychotropic activity of THC-metabolites hydroxylated in each of the five positions of the side chain, these compounds were synthesized in the Δ^6-series (Leander *et al.*, 1978). The Δ^6-THC derivatives are more readily synthesized and show activities which are parallel with the Δ^1-series (Edery *et al.*, 1972). Their cannabinoid-type activity was evaluated in the rhesus monkey (Edery *et al.*, 1972; Leander *et al.*, 1978).

To further elucidate, whether the isolated guinea-pig ileum (Paton, 1954; Gill & Paton, 1970) is a suitable model for investigations of cannabinoid type activity, we have now investigated the ability of the same side chain hydroxylated Δ^6-THC metabolites to inhibit the twitch response of the electrically stimulated guinea pig ileum. To further extend the comparison, we have also explored the activity of other cannabinoids some of which possess psychotropic or analgesic effects.

EXPERIMENTAL

The terminal portion of the guinea pig ileum was used except for the 5 cm nearest the ileocecal junction. The isolated ileum was suspended in 5 ml Krebs solution in a silanized glass vessel bubbled with 95% O_2 : 5% CO_2 at 36–37°C. Rectangular, electrical pulses of 0.5 msec. 50 V and 0.1 Hz were applied to the electrodes. Contractions were recorded with transducers (Rosell, Agurell and Martin, 1976).

The synthesis of side chain hydroxylated metabolites of Δ^6-THC was described by Leander *et al.* (1978). Compounds 4, 5 and 7 were synthesized by Wilson *et al.* (Wilson *et al.*, 1976; Wilson and Dewey, 1978). Information on compound 3 and other naturally occurring cannabinoids or analogues can be found in Mechoulam, McCallum and Burstein (1976) and Edery *et al.* (1972). The purity of the tested compound was checked by thin-layer chromatography and when necessary preparative thin-layer chromatography was used for purification.

The evaluation of the psychotropic activities of side chain hydroxylated and other cannabinoids in the rhesus monkey is reported in Leander *et al.* (1978) and Edery *et al.* (1972). Behavioural changes consistent with cannabis type activity was evaluated in two animals for each dose level after i.v. administration of the compound. The analgesic effects and dog ataxia produced by compounds 4 and 5 is published by Wilson *et al.* (1976).

RESULTS AND DISCUSSION

We have previously found (Rosell, Agurell and Martin, 1976) that (−)-7-hydroxy-Δ^1-THC and its Δ^6-isomer (2) are very potent in inhibiting the electrically stimulated twitch response of the guinea pig ileum. A concentration of approximately 7 ng/ml of (−)-7-hydroxy-Δ^6-THC inhibits the twitch response by 50% after 10–15 min. The (+)-enantiomer of 7-hydroxy-Δ^1-THC is devoid of activity showing a stereoselective

requirement congruent with the requirement for psychotropic effect (Edery *et al.*, 1972). The non-hydroxylated compounds (−)-Δ^1-THC and (−)-Δ^6-THC (1) were 1/5–1/10 as potent as (−)-7-hydroxy-Δ^1-THC and the inhibition was more gradual in onset, probably due to less and slower penetration into the tissue. The inhibitory effect was of presynaptic origin. It was further found that CBN and CBD were without effect.

SIDE-CHAIN HYDROXYLATED Δ^6-THC METABOLITES

In Fig. 1 we show the effect of all the side-chain hydroxylated Δ^6-THC derivatives on the twitch response of the guinea pig ileum. For comparison, also (−)-7-hydroxy-Δ^6-THC was included. At a bath concentration of 20 ng/ml the twitch response was most effectively inhibited by (−)-7-hydroxy-Δ^6-THC and (−)-3″-hydroxy-Δ^6-THC with (−)-5″-hydroxy-Δ^6-THC being less potent. At this concentration the biological activity of both 1″-, 2″- and 4″-hydroxy-Δ^6-THC was marginal.

For comparison we show in Table 1 the behavioural effects of the same compounds in rhesus monkeys (Leander *et al.*, 1978). (−)-7-hydroxy-Δ^6-THC is in this model equipotent

TABLE 1. BEHAVIORAL EFFECTS ELICITED BY SIDE-CHAIN HYDROXYLATED Δ^6-THC DERIVATIVES IN RHESUS MONKEYS. (FROM LEANDER ET AL., 1978).

Compound	Dose, (mg/kg i.v.)	Activity*
Δ^6-THC	0.1–0.3	+
	0.4–0.9	+ +
	1–2	+ + +
1″-Hydroxy-	1	−
-Δ^6-THC	5–7	+
2″-Hydroxy-	1	+
-Δ^6-THC	2	+ +
	5	+ + +
3″-Hydroxy-	0.05–0.1	−
-Δ^6-THC	0.2	+ +
	1	+ + +
4″-Hydroxy-	0.1	−
-Δ^6-THC	0.25–0.5	+ +
	1	+ + +
5″-Hydroxy-	0.25	−
-Δ^6-THC	0.5	+ +
	1–2	+ + +

* (−) indicates no changes; (±) tranquility; (+) drowsiness, decreased motor activity, occasional partial ptosis, occasional head drop; (+ +) stupor, ataxia, full ptosis, suppression of motor activity, typical crouched position ("thinker position") for up to 3 hours, presence of reaction to external sensorial stimuli; (+ + +) severe stupor and ataxia, full ptosis, immobility, "thinker position" lasting for more than 3 hours, and absence of reaction to external stimuli.

7-Hydroxy-Δ^6-THC is in this model equipotent with Δ^6-THC (Edery *et al.*, 1972).

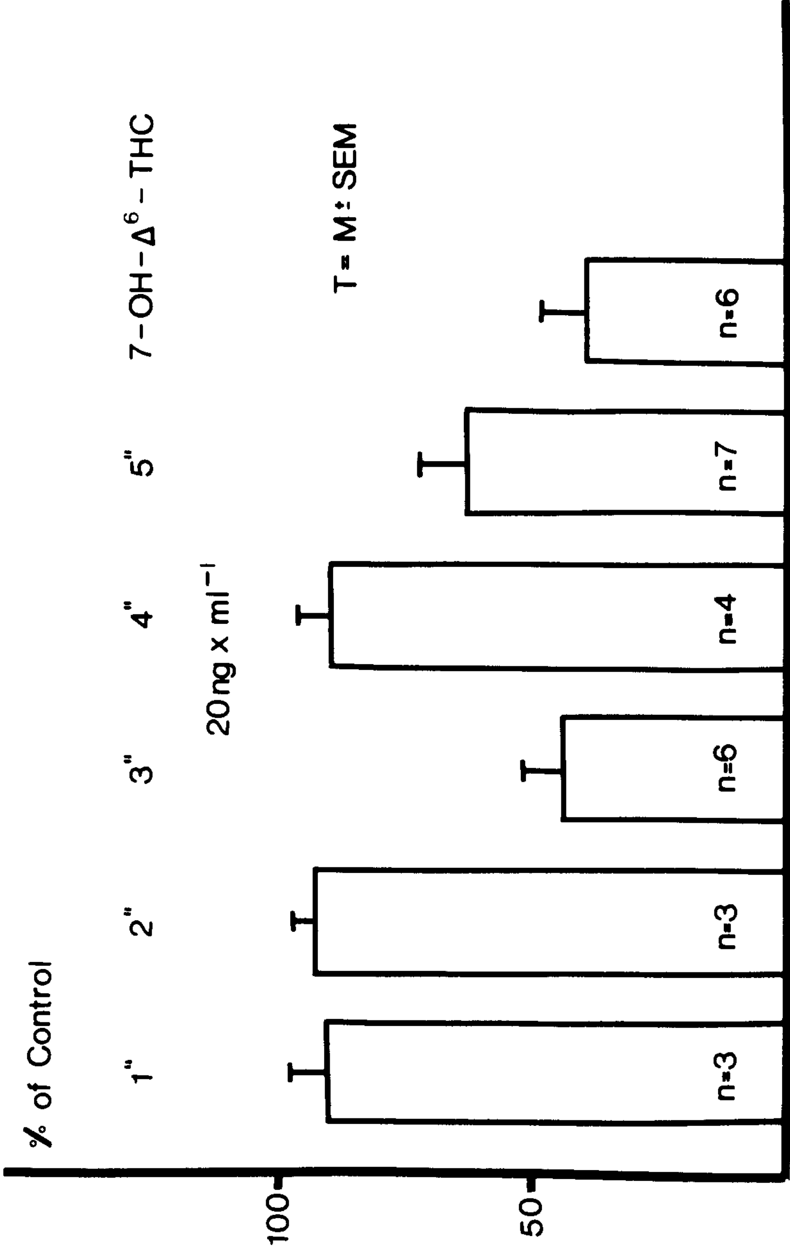


FIG. 1. Effect of side chain hydroxylated THC derivatives and (−)-7-Hydroxy-Δ^6-THC on the twitch responses of the guinea pig ileum to electric field stimulation.

1" = 1"-Hydroxy-Δ^6-THC; 2" = 2"-Hydroxy-Δ^6-THC; etc.

with Δ^6-THC. Although the monkey model affords a somewhat rough estimate of cannabis type effect, it is clear that of the monohydroxylated derivatives, 3″-, 4″-, 5″- and 7-hydroxy-Δ^6-THC were most potent and equipotent with Δ^6-THC *in vivo*. The *in vitro* activity is almost parallel, the only exception being 4″-hydroxy-Δ^6-THC. Similar to the *in vitro* situation, 1″- and 2″-hydroxy-Δ^6-THC possess weak psychotropic activity.

OTHER CANNABINOIDS

To further test whether psychotropic derivatives also are potent inhibitors of the guinea pig ileum twitch response *in vitro*, we have carried out a preliminary study of the compounds 3–14 shown in Fig. 2. For a number of compounds test results from the rhesus monkey model were available (Table 2). Besides the rhesus monkey model also the dog ataxia test is considered reasonably predictive of cannabis type effects in man (e.g. Wilson *et al.*, 1976). Compounds 4, 5 and 6 had been tested in the dog. For compounds 3 and 7 only information about their analgesic effect in the mouse hot plate test was available. It should be pointed out that there is not necessarily a good correlation between the psychotropic and the analgesic effects of cannabinoids (cf. Wilson *et al.*, 1976).

The results of the preliminary study, where 7-hydroxy-Δ^6-THC (2) served as reference, are shown in Table 2. The table also contains very brief information about the known biological activity of each compound, in what test this activity was assayed and the pertinent literature references.

Compound 3 shows analgesic and hypnotic/sedative properties (Mechoulam, McCallum and Burstein, 1976) but it is not known whether it is psychoactive in man. The compound is a nitrogen analogue with a hydrophilic ester grouping and was equipotent

TABLE 2. EFFECTS OF CANNABINOID DERIVATIVES ON THE TWITCH RESPONSE OF THE GUINEA PIG ILEUM TO ELECTRIC FIELD STIMULATION.

Compound No.	Conc. in bath, ng × ml^{-1}	Twitch response, % of control (n)	Biological activity (type of test, lit. ref.)
2	20	10 (6)	High (cannabinoid action in rhesus monkey; Edery *et al.*, 1972)
3	20	10 (2)	High (analgesic effect; Mechoulam, McCallum and Burstein, 1976)
4	20	20 (2)	High (analgesic effect mouse, dog ataxia, Wilson *et al.*, 1976)
5	50	80 (2)	Low-intermediate (dog ataxia; Wilson *et al.*, 1976)
6	40	100 (2)	Low (analgesic effect mouse, dog ataxia, Wilson and Dewey, 1978)
7	20	20 (2)	High (analgesic effect mouse, 1/3 of compound 4; Wilson, unpublished)
8	50	50 (3)	High (rhesus monkey, Edery *et al.*, 1972)
9	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)
10	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)
11	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)
	100	80 (1)	Inactive (rhesus monkey, Edery *et al.*, 1972)
12	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)
13	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)
14	40	100 (2)	Inactive (rhesus monkey, Edery *et al.*, 1972)

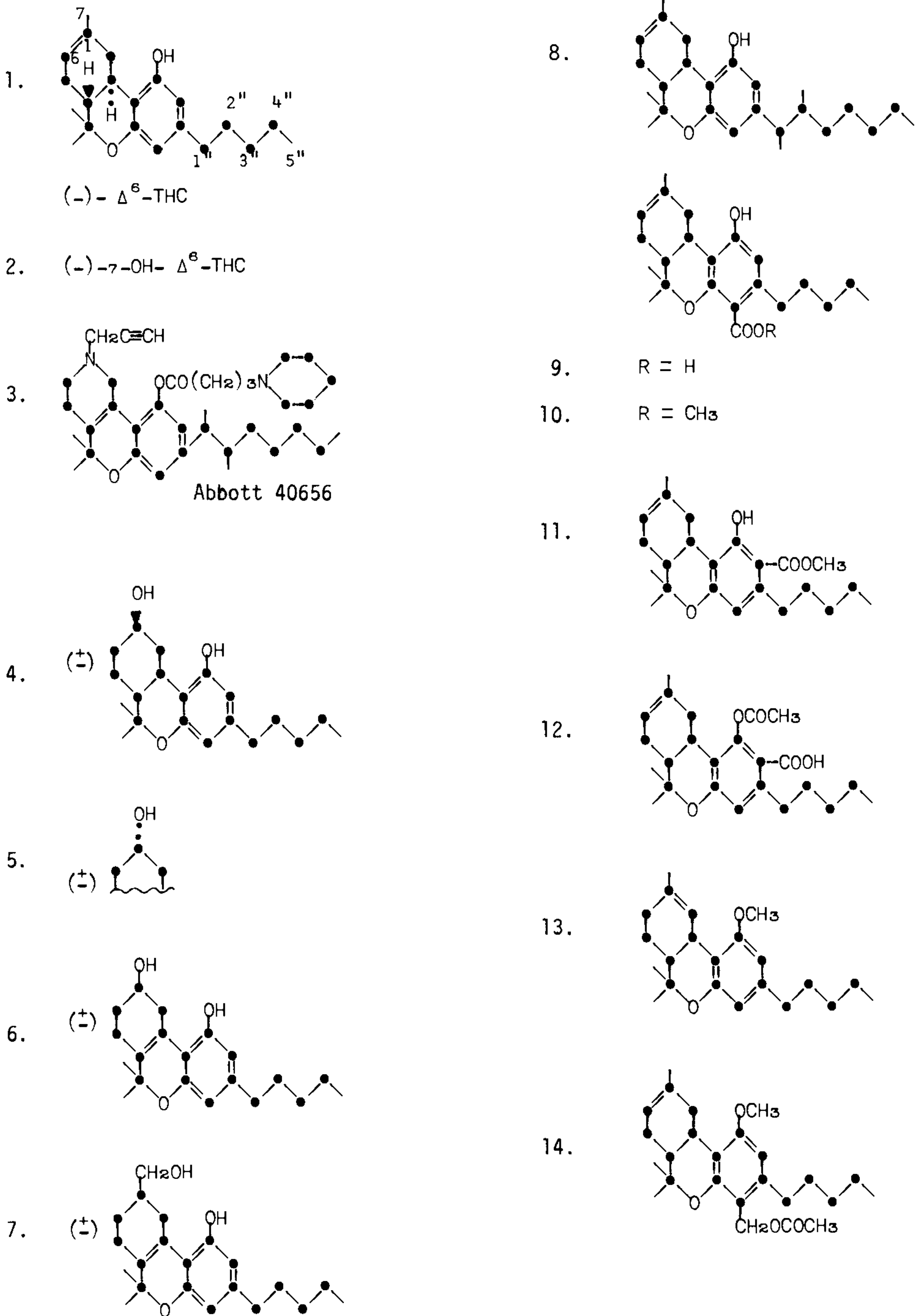


FIG. 2. Formulas of compound tested for inhibition of twitch response in Table 2.

with 7-hydroxy-Δ^6-THC (2) in the guinea pig ileum preparation. It is not known whether the ester linking of 3 was hydrolyzed before its activity was exerted. This is a prerequisite, for example, for esterified Δ^1-THC.

Compound 4 and 5 were racemates and lack the C-7 methyl group of THC. In compound 4 the hydroxyl group lies essentially in the plane of the cyclohexane ring (Wilson *et al.*, 1976) which as discussed by Mechoulam, McCallum and Burstein (1976) and elsewhere in this volume by Binder is necessary for psychotropic activity. In agreement with this, compound 4 was much more potent *in vitro* than its epimer 5. Compound 6, where the double bond is located in the 3, 4-position—which generally confers lower activity than the Δ^1- or Δ^6-isomers—showed low potency both *in vitro* and *in vivo*.

Compound 7 shows rather potent analgesic properties in the mouse hot plate test and similarly was a potent inhibitor of the twitch response.

Compound 8 is a long acting and potent psychotomimetic in man and was also effective in inhibiting the guinea-pig ileum twitch response. Therefore one might have expected a higher *in vitro* activity. However, since Δ^6-THC compared to 7-hydroxy-Δ^6-THC in the guinea pig ileum preparation shows a less potent and more gradual onset of action, it is possible that the lipophilic C_9-side chain decreases the penetration to the receptor.

Compounds 9 to 14 have all been tested in the rhesus monkey model and found to be inactive. When tested in the *in vitro* preparation in moderate concentrations they likewise lacked activity.

SUMMARY

We have found a striking relation between the psychotropic activity of various cannabinoids and their ability to inhibit the twitch response of the electrically stimulated guinea pig ileum. Further experiments with more purified receptor preparations are desirable to show whether the guinea pig ileum is a suitable organ to study the mechanism of action of cannabinoids.

ACKNOWLEDGEMENTS

The work in Sweden was supported by grants from the Medical Research Council, Magnus Bergvall's Stiftelse and from Harald and Greta Jeansson's Stiftelse. Dr. R. Wilson kindly supplied compounds 4–7.

REFERENCES

AGURELL, S., BINDER, M., FONSEKA, K., LINDGREN, J.-E., LEANDER, K., MARTIN, B., NILSSON, I. M., NORDQVIST, M., OHLSSON, A., WIDMAN, M., Cannabinoids: Metabolites hydroxylated in the pentyl sidechain. In Nahas, G. (Ed.) Marihuana: Chemistry, biochemistry and cellular effects, Springer Verlag, New York, pp. 141–157 (1976).

EDERY, H., GRUNFELD, Y., PORATH, G., BEN-ZVI, Z., SHANI, A., MECHOULAM, R., Structure-activity relationship in the THC-series. *Arzneimittelforsch.* **22**,1995–2003 (1972).

GILL, E. W., PATON, W. D. M., Pharmacological experiments *in vitro* on the active principles of cannabis. In Joyce, C. R. B., Curry, S. H. (Eds.) The botany and chemistry of cannabis. J. & A. Churchill, London, pp. 165–173 (1970).

LEANDER, K., DAHMÉN, J., AGURELL, S., OHLSSON, A., EDERY, H., PORATH, G., LEVY, S., MECHOULAM, R., Synthesis and psychotropic activity of side-chain hydroxylated Δ^6-THC metabolites. *Acta Pharm. Suec.*, (in press).

MECHOULAM, R., MCCALLUM, N. K. BURSTEIN, S., Recent advances in the chemistry and biochemistry of cannabis. *Chem. Rev.*, 75–112 (1976).

PATON, W. D. M., The response of the guinea-pig ileum to electrical stimulation by coaxial electrodes. *J. Physiol.* **127**,40–41P (1955).

PEREZ-REYES, M., TIMMONS, M. C., LIPTON, M. A., CHRISTENSEN, H. D., DAVIS, K. H., WALL, M. E., A comparison of the pharmacological activity of Δ^9-THC and its monohydroxylated metabolites in man. *Experientia* **29**,1009–1010 (1973).

ROSELL, S., AGURELL, S., MARTIN, B., Effects of cannabinoids on isolated smooth muscle preparations. In Nahas, G. (Ed.) Marihuana: Chemistry, biochemistry and cellular effects. Springer Verlag, New York, pp. 397–405 (1976).

WILSON, R. S., DEWEY, W. L., to be published.

WILSON, R. S., MAY, E. L., MARTIN, B. R., DEWEY, W. L., 9-nor-9-hydroxy-hexahydrocannabinols. Synthesis, behavioral and analgesic properties and comparison with the tetrahydrocannabinols. *J. Med. Chem.* **19**,1165–1167 (1976).

YISAK, W.-A., WIDMAN, M., LINDGREN, J.-E., AGURELL, S., Neutral *in vivo* metabolites of cannabinol isolated from rat faeces. *J. Pharm. Pharmacol.* **29**,487–490 (1977).

Δ^7-TETRAHYDROCANNABINOL, A NON-PSYCHOTROPIC CANNABINOID: STRUCTURE-ACTIVITY CONSIDERATIONS IN THE CANNABINOID SERIES

MICHAEL BINDER, HABIB EDERY* and GILA PORATH*

Institut für Physiologische Chemie, Ruhr-Universität Bochum, P.O. Box 102148, D-4630 Bochum 1, Germany.

also *Israel Institute for Biological Research, Sackler School of Medicine, Tel Aviv University, Ness-Ziona, Israel.*

Summary. An improved synthesis, purification and spectroscopic data of (3R, 4R)-$\Delta^{1(7)}$-tetrahydrocannabinol (Δ^7-THC), a double bond isomer of Δ^1-THC are described. The conformation of the isoprenoid ring of Δ^7-THC was determined by nmr, based on model considerations. The ring is locked in a half chair conformation, the exocyclic methylene carbon being located ca 100 pm perpendicular above the plane of the aromatic ring. When tested for cannabinoid-like activity in the Rhesus monkey, Δ^7-THC was found to be inactive in dosage up to 5 mg/kg i.v. Δ^7-THC neither antagonized nor synergized the behavioural changes elicited by the standard Δ^1-THC. Because of its close structural resemblance to the psychotropically active cannabinoids Δ^1- and Δ^6-THC, Δ^7-THC is suggested as a model compound to differentiate the general effects produced by these cannabinoids from their specific psychotropic effects. The influence of side chain hydroxyl groups on the psychotropic activity of cannabinoids, determined for 4″-hydroxy-Δ^1-THC, a microbial metabolite of Δ^1-THC is discussed in relation to a hypothetical THC receptor.

IN SPITE of the fact that the drug hashish, derived from the indian hemp, *Cannabis sativa*, has been used for several thousand years, very little is known about the intimate mechanism of the psychotropic activity of its main active constituent (3R, 4R)-Δ^1-tetrahydrocannabinol (Δ^1-THC). At present there is an increasing amount of information available about various pharmacological and biochemical effects of Δ^1-THC which may or may not be related to its psychotropic activity (for general information see Mechoulam, 1973; Nahas, 1976; Braude & Szara, 1976). In contrast to the hallucinogenic indolealkylamines (dimethyltryptamine, LSD-25, psilocybin) and the phenylisopropylamines (i.e. amphetamines), Δ^1-THC does not resemble any of the known neurotransmitters. Studies on the interaction of Δ^1-THC with release or uptake of neurotransmitters so far have led to no conclusive results (Bannerjee & others, 1975; Ho & Johnson, 1976; Domino, 1976). There are two major views concerning a possible mechanism of action. Δ^1-THC might interact with a special receptor (Edery & others, 1971; Mechoulam & others, 1976), a protein or lipoprotein located in the neurolemma, implicating thus the

speculative existence of a physiological THC agonist. Alternatively it might interact with the lipid phase of the membrane by changing its fluidity, as do the steroid anaesthetics (Lawrence & Gill, 1975) or by altering its composition (Burstein & Hunter, 1977; Greenberg & others, 1977). We are in favour of the receptor hypothesis (Binder, 1977), because there are a number of facts indicating a high selectivity and stereospecificity required for the action of Δ^1-THC. Structure-activity relationships in the THC series have been discussed in detail (Edery & others, 1971; Mechoulam & Edery, 1973a). We believe that many peripheral effects of the drug could be mediated by less specific interaction of apolar parts of the highly lipophilic molecule with apolar regions of various enzymes or lipid membrane constituents. This view is supported by the fact that the amounts of Δ^1-THC required for such actions usually exceed the *in vivo* concentrations of Δ^1-THC reached upon smoking or ingestion (200 nM) by a factor of 10^3. When looking for the mechanism of the psychotropic action of Δ^1-THC, the need for a model compound which allows us to differentiate between the specific psychotropic and other effects became apparent. Usually the behaviourally inactive cannabinoids cannabidiol (CBD) and cannabinol (CBN) are used for this purpose (Lawrence & Gill, 1975; Gill, 1977) as in studies on liposomes and membrane models. Both compounds are models of limited value, their stereochemistry being markedly different from that of Δ^1-THC as well as from its also psychotropically active isomer Δ^6-THC. Model considerations led us to the conclusion that either the enantiomeric (3S, 4S)-Δ^1-THC, which is difficult to obtain in an optically pure form, or another double bond isomer, (3R, 4R)-Δ^7-THC might be the desired model compound. Δ^7-THC, which was first synthesized in racemic form by Fahrenholtz & others (1967) who also casually referred to its inactivity in monkeys though neither doses nor other details were given (Mechoulam & Edery, 1973b) and by Nilsson & others (1971), closely resembles Δ^1- and Δ^6-THC, the double bond having been shifted to an exocyclic position. The conformation of Δ^7-THC was analyzed by nmr and compared to the known conformations of the THC's, CBN (Archer & others, 1970) and CBD. The compound is very close to Δ^1-THC concerning its stereochemistry and its physicochemical properties and thus may be expected to give the same unspecific interactions as Δ^1-THC while simultaneously not fitting on a hypothetical THC receptor. An improved synthesis, purification, spectroscopic properties and tests in the Rhesus monkey of Δ^7-THC are now described.

The influence of hydroxyl groups introduced into the side chain of psychotropic cannabinoids is discussed in relation to the receptor concept, based on data on the Δ^6-THC series (Agurell & others, 1976) and on a microbial transformation product of Δ^1-THC, 4″-hydroxy-Δ^1-THC (Binder, 1976) which also has been tested in the Rhesus monkey.

MATERIALS AND METHODS

SYNTHESIS

Δ^6-THC. Δ^6-THC was synthesized according to Petrzilka & others (1969) from olivetol (Fluka) and (+)-trans-p-mentha-2,8-dien-1-ol (Firmenich).

Δ^7-THC. The synthesis described by Nilsson & others (1971) was slightly modified. A solution of Δ^6-THC (469 mg) in 200 ml propan-2-ol and 2 ml xylene was irradiated with a medium pressure Hg immersion lamp (125 W). The reaction was monitored every 15 min by gas liquid chromatography (g.l.c.). After two hours the starting material had been

completely replaced by a product peak of slightly lower retention time (70% area of starting material) and two minor impurities of higher retention time. The solvent was evaporated in vacuo. The residue (524 mg) was chromatographed on 35 g silica gel (Merck) and eluted with petrol ether 40°–60° (PE)/ether 99 : 1 (fract. 1–10, 35 ml each), 97 : 3 (fract. 11–30) and 95 : 5 (fract. 31–40). Fractions 19–29 contained Δ^7-THC (272 mg = 58%) which, according to thin layer chromatography (t.l.c.) on silica gel in several solvent systems did not show impurities. T.l.c. on silica gel plates impregnated with dimethylformamide (DMF), however, revealed the presence of a less polar impurity (ca. 15%) which could not be removed by column chromatography on silica gel or Florisil. The g.l.c. system originally used (SE-30) gave identical retention times for both compounds; on OV-17 a slight separation was achieved. The impurity was removed by repeated chromatography on Sephadex LH-20 (Pharmacia) (column 1 m × 1 cm) in $CHCl_3$/PE/ethanol 10 : 10 : 1. In a typical run (181 mg Δ^7-THC) 50 fract. (10 ml) were collected at a rate of 0.5 ml/min. Fract. 22–25 contained Δ^7-THC plus impurity (86 mg), fract. 26–28 contained pure Δ^7-THC (92 mg) which on t.l.c. (DMF system) and g.l.c. (OV-17) was 99.8% pure. The compound, a colourless oil of high viscosity was stored as a 1% solution in ethanol in the dark at −20°.

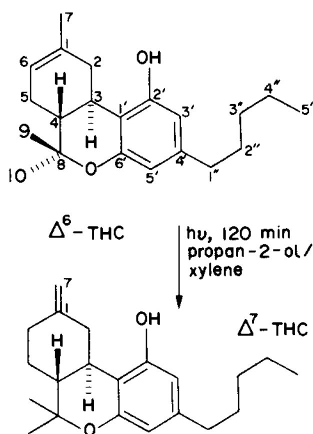


FIG. 1. Synthesis of Δ^7-THC.

GAS LIQUID CHROMATOGRAPHY

G.l.c. was carried out on a Hewlett-Packard 5711 gas chromatograph (FID). Two systems were used: (a) column 1.80 m × 2.5 mm, 3% SE-30 on Gas Chrom Q, 30 ml N_2/min; (b) same column, 3% OV-17 on Gas Chrom Q, 30 ml N_2/min, 210° and 250° respectively.

THIN-LAYER CHROMATOGRAPHY

T.l.c. was performed on precoated plates (0.25 mm, silica gel, Merck) in (a) $CHCl_3$, (b) PE/ether 95 : 5 3 × and (c) PE/ether 8 : 2.

DMF system: plates were impregnated with DMF vapor for 45 min at 60° after application of the samples and developed three times in PE. The cannabinoid spots were visualized by spraying with a 0.1% solution of Fast Blue B Salt (Merck) in 0.1 N NaOH.

NMR AND MASS SPECTRA (MS)

Nmr spectra and decoupling experiments were recorded in $CDCl_3$ on a Bruker HSX-90 spectrometer with $Si(CH_3)_4$ as internal standard. The ms was determined on a Varian MAT CH-5 instrument and processed by a Varian SS-100 data system.

TESTS FOR CANNABINOID ACTIVITY

Five male Rhesus monkeys (3.5–4.5 kg body weight) were used for evaluating the psychotropic activity of Δ^7-THC. One week in advance the animals were checked for their ability to react to cannabinoids (Grunfeld & Edery, 1969) by injecting the standard Δ^1-THC. Due to their water insolubility both cannabinoids were dissolved in propylene glycol. The volume injected (antecubital vein) was always 0.05 ml/kg. The observation period extended from half an hour before to six hours after administration. When the same animal was used twice, at least one week was allowed to elapse between the experiments.

RESULTS AND DISCUSSION

SPECTROSCOPIC DATA OF Δ^7-THC

Mass spectrum. The ms of Δ^7-THC resembles those of Δ^1- and Δ^6-THC, differing only in the relative intensity of the peaks. The molecular ion at m/e 314 (100%) constitutes the base peak. Further peaks at m/e 299 (73, M^+-CH_3), 297 (17, M^+-OH), 271 (40, M^+-C_3H_7), 258 (43, M^+-C_4H_8), 257 (43, M^+-C_4H_9), 246 (68, M^+-68, retro-Diels-Alder cleavage), 243 (13, M^+-C_4H_8-CH_3), 231 (85, M^+-68-CH_3) and 193 (32, M^+-121). For a detailed discussion of the fragments see Budzikiewicz & others (1965).

Nmr spectrum. Signals at δ0.88 (t, 3, H-5″), 1.16 (s, 3, H-10), 1.51 (s, 3, H-9), 1.8 (m, 1, H-2β), 2.42 (t, 2, H-1″, J = 8 Hz), 2.50 (dt, 1, H-3, $J_{3,2\beta} = J_{3,4} = 10.5$ Hz, $J_{3,2} = 4$ Hz), 3.73 (d, 1, H-2, $J_{gem} = -13$ Hz), 4.74 (m, 2, H-7), 4.97 (s, 1, OH), 6.04 (d, 1, H-5′, J = 1.5 Hz), 6.25 (d, 1, H-3′).

CONFORMATION OF Δ^7-THC

According to model considerations (Dreiding models) based on the work of Archer & others (1970), Δ^7-THC can exist in two conformations which are shown in Fig. 3. The isoprenoid ring can assume either a slightly distorted chair conformation (IIb) where the exocyclic methylene carbon C-7 is located ca. 100 pm above the plane of the aromatic ring or a twist conformation (IIa) where C-7 is located 330 pm above this plane. For comparison, drawings of Δ^1- and Δ^6-THC are given in Fig. 4. In both compounds the distance of C-7 above the aromatic ring is 200 and 220 pm respectively. This seems to be a critical distance required for psychotropic activity. To substantiate our postulate that CBD and

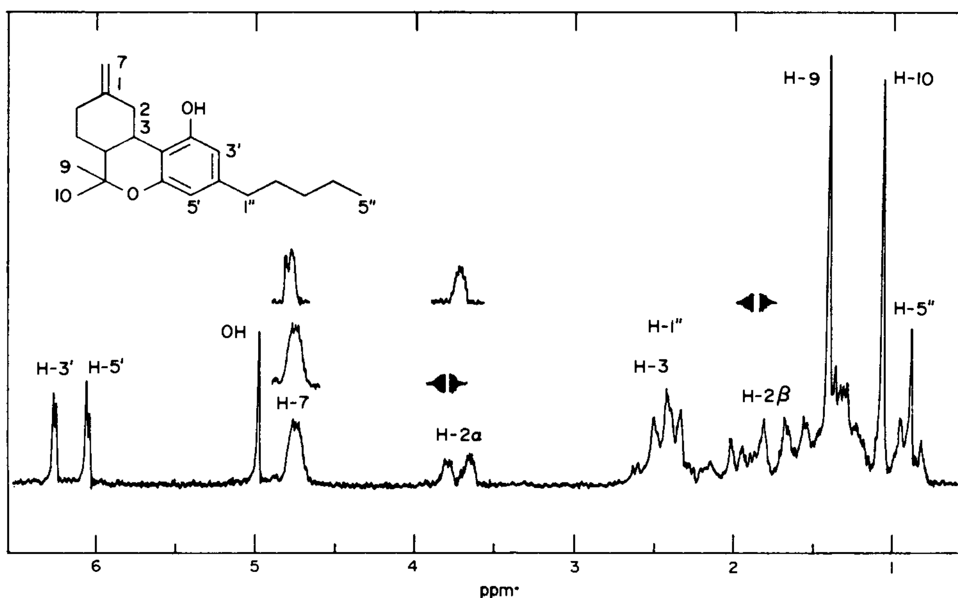


FIG. 2. 90 MHz nmr spectrum of Δ^7-THC in $CDCl_3$.

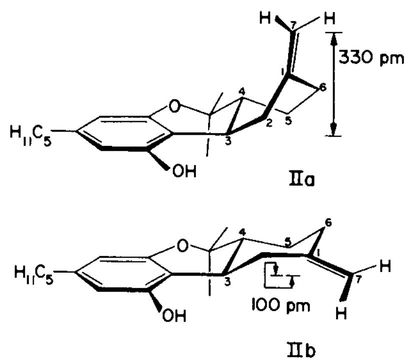


FIG. 3. Conformations of Δ^7-THC.

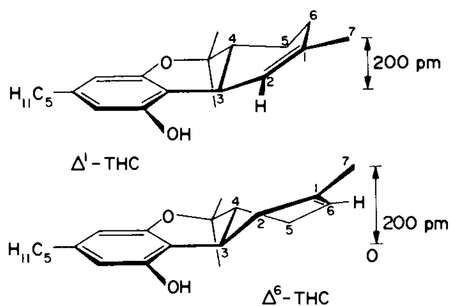


FIG. 4. Conformations of Δ^1- and Δ^6-THC.

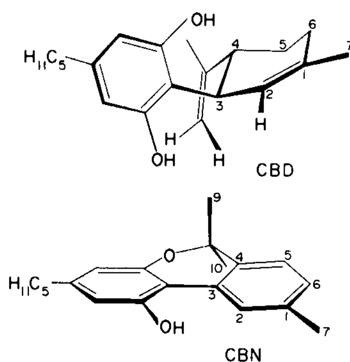
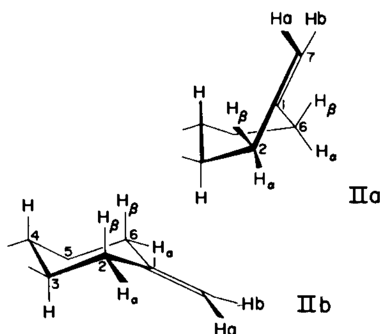


FIG. 5. Conformations of CBD and CBN.

CBN are not suited for comparison with Δ^1- and Δ^6-THC, steric drawings of both compounds are provided in Fig. 5. In CBD the isoprenoid ring retains the conformation realized in Δ^1-THC but the aromatic ring is twisted out of plane due to steric interactions with H-2 and the bulky substituent at C-4. CBN is fully aromatic and hence almost planar. The conformations of Δ^1-, Δ^6-THC and CBN have been confirmed by calculations (Archer & others, 1970). The conformation of CBD has been deduced from low temperature nmr spectra and theoretical calculations (Weiner & Meyer, 1976).

By means of nmr decoupling experiments it was possible to discriminate between the two conformations of Δ^7-THC derived from the Dreiding models. The conformations of the isoprenoid ring of Δ^7-THC, the dihedral angles (θ) between the protons in 2-, 6- and 7-position and the predicted coupling constants are given in Fig. 6.

In the case of the chair conformation IIb the protons 2α and 6α are located in the plane of the double bond and the 7-protons while the protons 2β and 6β are roughly perpendicu-



II b	$\theta°$	J(Hz)	II a	$\theta°$	J(Hz)
H-2β, Ha	105	2	H-2β, Ha	15	0
H-2α, Ha	15	0	H-2α, Ha	105	2
H-6β, Hb	110	2	H-6β, Hb	0	0
H-6α, Hb	10	0	H-6α, Hb	120	2

FIG. 6. Conformations of the isoprenoid ring of Δ^7-THC, dihedral angles and predicted coupling constants.

lar to this plane. The opposite is the case with the twist conformation IIa, where the protons 2β and 6β are located in the plane of the double bond and H-2α and H-6α assume a perpendicular position. The broadening of the signal of the 7-protons centered at δ4.74 should be due to an allylic coupling with the protons in the 2- and 6-positions. According to Bhacca & Williams (1966) the contribution of protons to allylic coupling depends strongly on the dihedral angle between these protons and the plane of the double bond; it is minimal when $\theta = 0°$ (0 Hz) and maximal when $\theta = 90°$ (2–3 Hz). Therefore proton 2β and 6β should be responsible for the broadening of the signal at δ4.74 when the chair conformation IIb is realized and H-2α and H-6α when the twist conformation IIa is realized.

In both conformations the proton 2α is located in the plane of the aromatic ring and therefore shifted to lower field at δ3.73. Irradiation on H-2α led to changes at δ2.60 and 1.85, the signals of H-3 and H-2β respectively. This was confirmed by irradiation at δ2.60 (H-3) which led to a sharp doublet (J_{gem} = 13 Hz) at δ3.73 and irradiation at δ1.85 (H-2β) leading to a singlet at δ3.73. Irradiation at δ4.74 (H-7) led to no change in the signal of H-2α but a slight disturbance was visible around δ1.8. The control experiment, irradiation at H-2α and H-2β gave clear results: no change in the signal of H-7 was obtained upon decoupling H-2α but a distinct change was obtained upon decoupling H-2β. Therefore we concluded that H-2β is the proton contributing to the allylic coupling and not H-2α, i.e. the chair conformation IIb of Δ^7-THC is realized. Concerning the distance of C-7 above the aromatic plane, this result places Δ^7-THC between Δ^1-THC and CBN. The physicochemical properties of Δ^7-THC, as evident from its behaviour on g.l.c., t.l.c. and column chromatography, however, differ little from those of Δ^1- and Δ^6-THC.

EVALUATION OF Δ^7-THC IN THE RHESUS MONKEY

Behavioural and somatic changes culminating in the typical crouched posture (" thinker position ") were observed after injection of 0.25 mg/kg Δ^1-THC. Contrastingly, the normal behavioural pattern of the monkey remained unaltered after administration of 1 or 5 mg/kg Δ^7-THC. The potential antagonistic or synergistic activity of Δ^7-THC towards the psychoactive Δ^1-THC was examined as follows: (a) animals were first administered with 0.25 mg/kg Δ^1-THC and when the full cannabinoid-induced syndrome had developed, 5 mg/kg Δ^7-THC was injected; (b) monkeys were injected with 5 mg/kg Δ^7-THC 30 min before administration of Δ^1-THC. The time interval for pretreatment was selected for theoretical reasons in the absence of pharmacokinetic data on Δ^7-THC. In no case did the administration of Δ^7-THC either prevent the appearance or modify the intensity and duration of the symptoms induced by Δ^1-THC.

The reason for the failure could be that the dose used (limited by the supply) was not sufficient to counteract the potent Δ^1-THC. Likewise it might be possible, though less probable, that when given in advance, Δ^7-THC could have been rapidly excreted thus reducing its concentration levels and becoming insufficient to prevent the binding of Δ^1-THC to its potential receptor sites.

On the molecular level the psychopharmacological inactivity of Δ^7-THC raises the question whether this may be due to its inadequate fit or no fit at all to a hypothetical THC receptor or to the fact, that Δ^7-THC can not be metabolized by microsomal enzymes in the same way as Δ^1- and Δ^6-THC (and probably as the less active synthetic cannabinoids

Δ^3-THC and (1R)-hexahydrocannabinol). Here the primary metabolic step is a hydroxylation at C-7 leading to the psychotropic metabolites 7-hydroxy-Δ^1- and 7-hydroxy-Δ^6-THC. Formation of this metabolite would be blocked in Δ^7-THC. Metabolism of Δ^7-THC could proceed in a different way, formation of a 1,7-epoxide (a 1,2-epoxide has been isolated after incubation of Δ^1-THC with a squirrel monkey tissue preparation by Gurny & others, 1972) with subsequent hydrolysis to a 1,7-diol. When 7-hydroxy-Δ^1-THC was first isolated by Nilsson & others (1970) and Wall & others (1970), Mechoulam (1970) argued that the psychotropic activity of Δ^1-THC might be due exclusively to its metabolite 7-hydroxy-Δ^1-THC, a view that has been disputed by Gill & others (1973) based upon experiments with Δ^1-THC and 7-hydroxy-Δ^1-THC in mice. Investigations on the metabolism of Δ^7-THC are in progress.

THE THC RECEPTOR CONCEPT

There are several arguments in favour of the receptor hypothesis: (a) (3R, 4R)-Δ^1-THC is psychotropic, the enantiomer (3S, 4S)-Δ^1-THC is considerably less active. In mice, this difference in activity has been demonstrated to be due neither to the distribution nor to the metabolism of the two enantiomers (Jones & others, 1974). (b) In the Δ^6-THC series derivatives hydroxylated in the side chain have been synthesized and tested in the Rhesus monkey (Agurell, Binder & others, 1976; Leander, Agurell & others, 1978). There is a distinct relationship between the position of the hydroxyl group in the side chain and the psychotropic activity of the compounds.

We have tested 4″-hydroxy-Δ^1-THC, a metabolite formed by microbial transformation of Δ^1-THC (Binder, 1976) that was first observed by Widman & others (1975) as a perfused lung metabolite of Δ^1-THC. In the Rhesus monkey the compound is roughly as active as Δ^1-THC. It seems reasonable that on the binding site of the THC receptor a functional

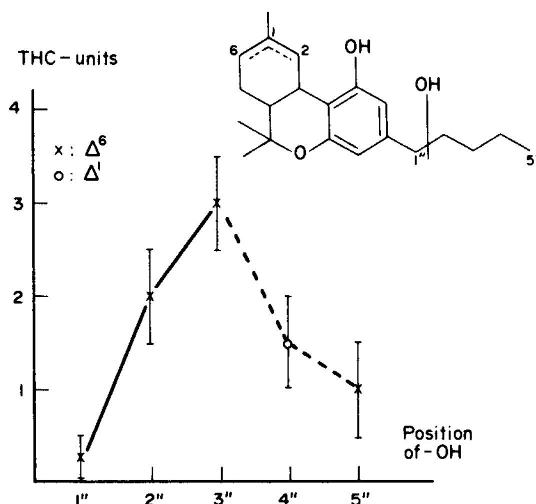


FIG. 7. Activity of Δ^6- and Δ^1-THC derivatives in the Rhesus monkey as a function of hydroxyl substituents in the side chain. All values refer to Δ^6-THC. The unit is defined as the reciprocal of the quotient of the amounts of derivative and Δ^6-THC leading to comparable behavioural changes.

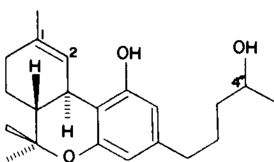


FIG. 8. Structure of 4″-hydroxy-Δ^1-THC.

group is located which could form a hydrogen bond to an HO-group in the side chain of the cannabinoid. Optimal formation of this hydrogen bond occurs when the HO-group is located in 3″-position. Due to the flexibility of the side chain it can be formed also with hydroxyl groups in 2″- and 4″-position but not when the HO-group is located in 1″-position, which abolishes the activity or in 5″-position, where it does not lead to a change in activity compared to the parent compound. Our results on 4″-hydroxy-Δ^1-THC fit into this scheme. The formation of the hydrogen bond increases the affinity of the cannabinoid to the binding site of the receptor and hence the activity of the cannabinoid.

TABLE 1. ACTIVITY OF 4″-HYDROXY-Δ^1-THC IN THE RHESUS MONKEY. RESULTS REFER TO Δ^6-THC: – NO CHANGE, + + AND + + + CHANGES EQUIVALENT TO 0.5–0.9 AND 1.0–2.0 MG/KG Δ^6-THC RESPECTIVELY (MECHOULAM & EDERY, 1973C).

dose mg/kg	activity
0.05	–
0.10	–
0.25	+ +
0.50	+ + +

Cannabinoids with lengthened and branched side chains have been tested for their psychotropic activity. Again we would argue that the effect of branched side chains, consisting rather in the prolongation of the cannabinoid action would be less specific than the observed effect of HO-groups which rather intensifies the action of the respective compound but does not lead to a prolongation of the effect.

Finally, based on the present data, we would like to postulate, that Δ^7-THC might represent a valuable tool to differentiate general effects (i.e. mediated by unspecific apolar interactions) of psychoactive cannabinoids from their specific psychotropic effects (i.e. effects mediated by combining with specific receptors) and we want to suggest that biochemical and pharmacological effects of Δ^1-THC, before being related to the psychotropic action of the drug should be checked against Δ^7-THC. It can, however, not be postulated that our data relate exclusively to a THC receptor responsible for the mediation of the psychotropic effects. Differences in stereochemistry, as has been pointed out by Paton (1975), could lead to specific actions on the level of the lipid phase of the membrane.

The main tool for cannabinoid receptor research, a selective antagonist of Δ^1-THC still remains to be discovered. The hypothesis of a THC receptor is supported by the high stereospecificity required for action and by the structure activity relationship in the side chain hydroxyl derivatives of Δ^6-THC. A similar relationship, demonstrated so far only for 4″-hydroxy-Δ^1-THC is to be expected for the corresponding derivatives of Δ^1-THC.

ACKNOWLEDGEMENT

The authors are indebted to Dr. D. Müller, Dept. of Mass Spectroscopy, Ruhr-University Bochum, for running the ms of Δ^7-THC and to Ms. Hermsdorf and Dr. W. Dietrich, Dept. of NMR Spectroscopy, Ruhr-University Bochum, for recording the nmr spectra.

REFERENCES

AGURELL, S., BINDER, M., FONSEKA, K., LINDGREN, J.-E., LEANDER, K., MARTIN, B., NILSSON, I. M., NORDQVIST, M., OHLSSON, A. and WIDMAN, M., In: Marihuana, Chemistry, Biochemistry and Cellular Effects, pp. 141-157, Editor: Nahas, G. G., Springer-Verlag, New York, 1976.

ARCHER, R. A., BOYD, D. B., DEMARCO, P. V., TYMINSKI, I. J. and ALLINGER, N. L., *J. Amer. Chem. Soc.*, **92**,5200-5206 (1970).

BANNERJEE, S. P., SNYDER, S. and MECHOULAM, R. (1975). *J. Pharmac. Exp. Ther.*, **194**,74-81.

BHACCA, H. S. and WILLIAMS, D. H., Applications of nmr Spectroscopy in Organic Chemistry, p. 108. Holden-Day, Inc., San Francisco, 1969.

BINDER, M. (1976). *Helv. chim. acta*, **59**,1674-1684.

BINDER, M. (1977). Paper presented at the Conference on Psychotropic Drug Action with Special Reference to Cannabinoids, Arad, Israel.

BRAUDE, M. C. and SZARA, S., Pharmacology of Marihuana, Raven Press, New York, 1966.

BUDZIKIEWICZ, H., ALPIN, R. T., LIGHTNER, D. A., DJERASSI, C., MECHOULAM, R. and GAONI, Y., *Tetrahedron*, **21**,1881-1888 (1965).

BURSTEIN, S. and HUNTER, S. A., *Biochem. Pharmacol.* (in press).

DOMINO, E. F., In: Marihuana, Chemistry, Biochemistry and Cellular Effects, pp. 407-413, Editor: Nahas, G. G., Springer-Verlag, New York, 1976.

EDERY, H., GRUNFELD, Y., BEN-ZVI, Z. and MECHOULAM, R., *Ann. N.Y. Acad. Sci.*, **191**,40-50 (1971).

FAHRENHOLTZ, K. E., LURIE, M. and KIERSTEAD, R. W., *J. Amer. Chem. Soc.*, **89**,5934-5941 (1967).

GILL, E. W., Paper presented at the Conference on Psychotropic Drug Action with Special Reference to Cannabinoids, Arad, Israel, 1977.

GILL, E. W., JONES, G. and LAWRENCE, D. K., *Biochem. Pharmacol.*, **22**,175-184 (1973).

GREENBERG, J. H., SAUNDERS, M. E. and MELLORS, A., *Science*, **197**,475-477 (1977).

GRUNFELD, Y. and EDERY, H., *Psychopharmacologia*, **14**,200-210 (1969).

GURNY, O., MAYNARD, D. E., PITCHER, R. G., and KIERSTEAD, R. W., *J. Amer. Chem. Soc.*, **94**,7928-7929 (1972).

HO, B. T. and JOHNSON, K. M., In: Marihuana, Chemistry, Biochemistry and Cellular Effects, pp. 367-382, Editor: Nahas, G. G., Springer-Verlag, New York, 1976.

JONES, G., PERTWEE, R. G., GILL, E. W., PATON, W. D. M., NILSSON, I. M., WIDMAN, M. and AGURELL, S., *Biochem. Pharmacol.*, **23**,439-446 (1974).

LAWRENCE, D. K. and GILL, E. W., *Molecular Pharmacology*, **11**,595-602 (1975).

LEANDER, K., AGURELL, S., LEVY, S., MECHOULAM, R., EDERY, H. and PORATH, G. Submitted for publication 1978.

MECHOULAM, R., *Science*, **168**,1159-1166 (1970).

MECHOULAM, R., Marijuana, Academic Press, New York, 1973.

MECHOULAM, R. and EDERY, H., In: Marijuana, pp. 101-136, Editor: Mechoulam, R., Academic Press, New York, 1973a.

MECHOULAM, R. and EDERY, H. Ibid., p. 119, 1973b.

MECHOULAM, R. and EDERY, H. Ibid., p. 124, 1973c.

MECHOULAM, R., MCCALLUM, N., LEVY, S. and LANDER, N., In: Marihuana, Chemistry, Biochemistry and Cellular Effects, pp. 3-13, Editor: Nahas, G. G., Springer-Verlag, New York, 1976.

NAHAS, G. G., Marihuana, Chemistry, Biochemistry and Cellular Effects, Springer-Verlag, New York, 1976.

NILSSON, I. M., AGURELL, S., NILSSON, J. L. G., OHLSSON, A., SANDBERG, F. and WAHLQVIST, M., *Science*, **168**,1228-1229 (1970).

NILSSON, J. L. G., NILSSON, I. M., AGURELL, S., ÅKERMARK, B. and LAGERLUND, I., *Acta Chem. Scand.*, **25**,768-769 (1971).

PATON, W. D. M., *Ann. Rev. Pharmacol.*, **15**,191-220 (1975).

PETRZILKA, T., HAEFLIGER, W. and SIKEMEIER, C., *Helv. Chim. acta*, **52**,1102-1134 (1969).

WALL, M. E., BRINE, D. R., BRINE, G. A., PITT, C. G., FREUDENTHAL, R. I. and CHRISTENSEN, H. D., *J. Amer. Chem. Soc.*, **92**,3466-3468 (1970).

WEINER, B. Z. and MEYER, A. Y., In: Recent Advances in the Chemistry and Biochemistry of Cannabis, By: Mechoulam, R., McCallum, N. K. and Burstein, S., *Chemical Reviews*, **76**,75-112 (1976).

WIDMAN, M., NORDQVIST, M., DOLLERY, C. T. and BRIANT, R. H., *J. Pharm. Pharmacol.*, **27**,842-848 (1975).

THE RADIOIMMUNOASSAY OF CANNABINOIDS; ITS CLINICAL, PHARMACOLOGICAL AND FORENSIC APPLICATIONS

VINCENT MARKS and J. D. TEALE

Department of Biochemistry (Division of Clinical Biochemistry), University of Surrey, and Department of Clinical Biochemistry, St. Luke's Hospital, Guildford.

Summary. A radioimmunoassay for cannabinoids in blood and urine using an antiserum raised in sheep and [^{3}H]-THC as label has been developed and used routinely in our laboratory during the past four years. It has proved itself useful clinically in the elucidation of obscure psychiatric disorders due to cannabis intoxication, in the study of cannabis bioavailability, the epidemiology of illicit cannabis use and its association with serious motor accidents.

THE detection and practicable measurement of cannabinoids in biological fluids still presents many difficulties despite enormous expenditure by various government and other agencies and efforts by research workers throughout the world. Radioimmunoassay (RIA) is the most promising method currently available. This is a sensitive and specific analytical procedure which has been successfully applied to the detection and measurement of a wide range of drugs(5, 7) including cannabinoids(6, 11, 12). In this paper we describe briefly the applications of an assay for cannabinoids in blood and urine developed in our laboratory over the past five years, which has now found widespread application in clinical and forensic laboratories, both in the U.K. and abroad, thus qualifying it for consideration as a "mature" assay. Other authors have reported in varying detail their experience with similar assays(1, 2, 4) though none, as far as we know, has yet been used in clinical studies.

ANTIBODY PRODUCTION

Antibodies to tetrahydrocannabinol (THC) were produced by immunising rabbits and sheep with conjugates of THC and bovine serum albumin (BSA). In preliminary experiments THC was covalently linked to BSA by one, four, six and ten carbon chains between its hydroxyl group and the lysine residues of BSA. The one carbon link was formed by direct reaction of the chlorocarbonate derivative of THC with BSA. The four carbon link was made by refluxing THC with succinic anhydride (C4) to form a hemisuccinate which

was then attached to BSA using carbodiimide or isobutyl chloroformate. Six and ten carbon links were prepared by making the hemiadipate (C_6) and hemisebacate (C_{10}) derivatives of THC using carbonyldiimidazole and effecting their attachment to BSA by means of carbodiimide or isobutyl chloroformate[5].

In other experiments BSA was linked to THC through a position other than its hydroxyl group. Diazotized *p*-aminobenzoic acid was attached to THC at the *para* position to the hydroxyl group. The carboxylic acid derivative, so formed, was attached to BSA either by carbodiimide or isobutyl chloroformate. The Mannich reaction, involving formaldehyde, was also used to form a link between BSA and the position *para* to the hydroxyl group of the THC molecule. In each conjugate preparation the average number of haptens conjugated per molecule of BSA was determined by including a small amount of tritiated THC in the reaction and determining the specific activity of the conjugate.

The most immunogenic conjugates were those containing four or six carbon links. Changing the point of attachment of THC from the hydroxyl group to the *para* position resulted in loss of immunogenicity. There was no definite correlation between the number of haptens in each conjugate and antibody production; in general however, the most immunogenic conjugates were those containing between 20 and 40 THC residues per BSA molecule which is in agreement with observations made with other haptens[8]. Because, in the preliminary experiments, sheep were more responsive than rabbits to the immunogens, they were used in all of our subsequent work.

Five mg of the THC-hemisuccinate-BSA conjugate dissolved in 1.5 ml sterile water, was emulsified with 3 ml of a modified Freund's complete adjuvant[5] and injected into the leg muscles of ten sheep. Three booster doses of 2.5 mg THC—hemisuccinate—BSA were given at monthly intervals for three months and then irregularly. The animals were bled from time to time and the serum tested for its capacity to bind to [^{3}H]-THC displaceably. Each bleed was coded separately and treated as unique.

No antibodies capable of binding [^{3}H]-THC appeared until 6–9 months after primary immunisation in any of the sheep. Of the 10 sheep originally immunised four produced potentially useable antibodies. One animal, S133Y, was selected for further study and all subsequent development, clinical and experimental work was carried out with an antiserum coded 133Y/25/4 obtained from this animal. Later bleeds have been characterised, used and distributed to other laboratories. Reimmunisation is normally carried out when the antiserum titre has fallen to a low level as we have found from experience with THC, and other haptens, that this is the most effective way of maintaining good antibody production suitable for RIA. (Fig. 1). Sheep 133Y is, at this writing, still alive and well and producing high avidity antisera.

ASSAY DEVELOPMENT

During the preliminary experiments we used an iodinated conjugate of THC hemisuccinate and a synthetic polymer containing Glutamic acid, Lysine, Alanine and Tyrosine (GLAT) as label. This bound avidly to the serum but was not displaceable nor prevented from binding to the antibody by native THC. We have had similar experiences with other haptens labelled with iodine[9].

Subsequently a high specific activity [^{3}H]-THC label (26 μCi/μg) became available from the Radiochemical Centre, Amersham and proved extremely useful. Consequently, we

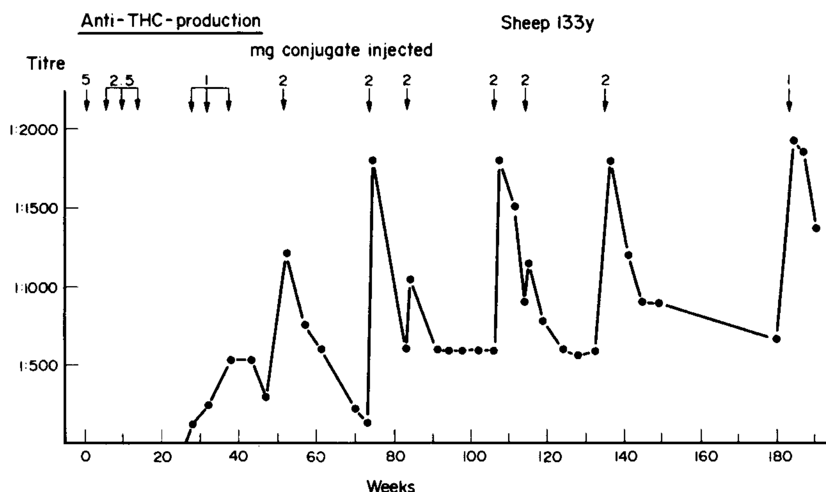


FIG. 1. Antibody titre (final dilution) of anti-THC antiserum raised in sheep 133Y in response to priming and boosting immunisations with THC-BSA conjugate during the first four years of its life.

have not tried to prepare any more iodinated THC labels though experience with other drugs since this work began leaves us in little doubt that such labels could be made and would be useful. They would have many advantages over the [^{3}H]-THC label we are currently using including increased sensitivity and ease of performance.

Because of interference in the assay by high concentrations of protein, especially lipoproteins, we used ethanol and later methanol, in the ratio of 2 parts to 1 part of plasma to effect protein precipitation and solubilisation of cannabinoids. The ethanol extract could be added in small amounts directly to the assay tube without serious interference with the antibody-antigen reaction. Even this slight interference was avoided, however, by substituting methanol for ethanol as precipitant. Urine could be added directly to the assay tube in amounts up to 100 μl without any interference. Triton X 405 was used to keep THC in solution during the assay and to prevent it binding to the walls of the assay tube which must be made of glass and not plastic. Separation of bound from free label was achieved by the use of dextran-coated charcoal[(12)].

The assay currently in use in our laboratory[(12)] has been described in detail and will only briefly be alluded to here. It is sensitive to 50 pg of THC per assay tube but this could almost certainly be improved 5–10 fold by using a high specific activity [^{125}I] label. It is possible using semi-automated equipment for one person to carry out between 50 and 100 assays in an afternoon.

Antisera raised in S133Y are specific for the closed three-ring cannabinoid nucleus group of compounds reacting, to a varying extent, with a large number of substances having this general configuration—but with no others. (Table 1). Group specificity confers many advantages on this antiserum compared with one capable of reacting only with THC—were such a one available—the most important being that it enables an assay using the antiserum to be employed to detect cannabis products in blood and urine.

Most of the circulating cannabinoids, and virtually all of those in the urine, are hydroxylated or acidic derivatives of the cannabinoid nucleus even when pure THC, which is seldom the case, has been ingested, inhaled or injected. Absolute specificity—or as near

TABLE 1. SIMILARITY BETWEEN CROSS-REACTIVITIES OF TWO ANTISERA RAISED IN DIFFERENT SPECIES AGAINST DIFFERENT THC-CONJUGATES. ANTISERUM 133Y/30/9 WAS PRODUCED IN GUILDFORD; R41/11 WAS GENEROUSLY SUPPLIED BY PROFESSOR M. CAIS FROM JERUSALEM.

Antisera cross-reactivities		
Antiserum	133Y/30/9	R41/11
Species	Sheep	Rabbit
Immunogen	(Ptn)NH—CO—$(CH_2)_2$—C(=O)—O; CH_3; O; C_5H_{11}	NH (Ptn); O=C; OH; O; C_5H_{11}
Final titre	1 : 2400	1 : 18000
Cannabinoid	% cross-reactivity	
Δ^9-THC	100	100
11-hydroxy-Δ^9-THC	160	100
8,11-dihydroxy-Δ^9-THC	150	200
11-carbomethoxy-Δ^8-THC	46	54
11-carboxy-Δ^8-THC methyl ether	1.4	6.4
Cannabinol	66	40
11-hydroxy-cannabinol	6.3	3.2
Cannabidiol	3.2	2.0

absolute specificity as is possible with any analytical technique, including GC—MS—can be achieved, when necessary, by combining a reverse-phase high pressure liquid chromatography (HPLC) separation system with the RIA immunodetection system. This combination brings together the exquisite sensitivity of RIA and its immunospecificity with the separative advantages of HPLC. We have used such a system, developed in conjunction with our colleagues at the Home Office, Aldermaston, to follow the absorption of THC and its elimination from the body in volunteers and to demonstrate the presence of circulating THC in the blood of victims of motor car and other severe accidents[14].

APPLICATIONS

For most practical purposes straight RIA of urine or an unpurified methanol extract of plasma provides all the information required and has proved particularly useful in epidemiological and bioavailability studies and in forensic investigations.

In a study involving the analysis of 1002 urine specimens from various sources, 82 random specimens from hospital in-patients were all negative for cannabinoids (Fig. 2). Approximately 30% of the specimens collected from subjects attending two Government drug addiction centres, and 55% from several small privately run addiction clinics were positive for cannabinoids which gives some measure of the frequency of cannabis use by the attendees within the past three days. Three out of 172 samples submitted from recently

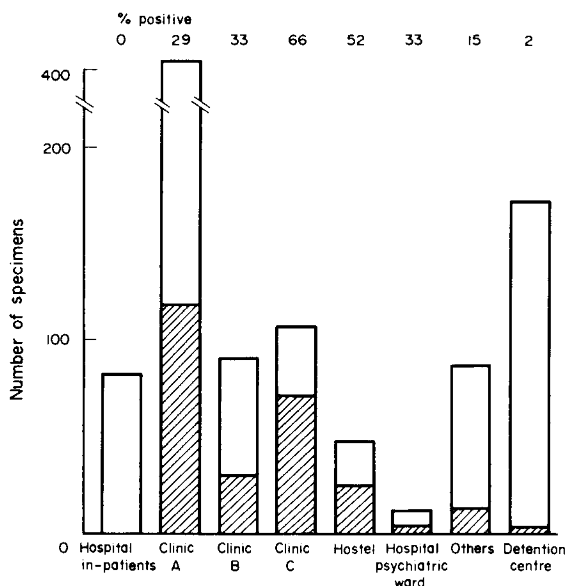


FIG. 2. Results of analysis for cannabinoids in 1002 urine specimens received in a hospital laboratory providing a diagnostic service for general hospital, psychiatric hospital and clinic patients. Clinics A and B are government clinics, clinic C a privately run drug clinic not prescribing prohibited drugs.

admitted inmates of a detention centre were positive. Each of the subjects subsequently admitted to having smoked cannabis in the 48 hours prior to arrival in the detention centre.

Clinically, the assay, which is well suited to routine application in a hospital laboratory, and is freely available in the U.K. through the Supraregional Assay Service, has been used to ascertain the cause of coma as the result of cannabis use which had not previously been suspected[3] and to eliminate, or confirm, cannabis as the cause of strange behaviour leading to psychiatric referral in a large number of cases.

Recognition that cannabinoids in general, and THC in particular, have pharmacological properties of potential therapeutic value has rekindled interest in the bioavailability and pharmacokinetics of these compounds. We have been concerned with several clinical trials, all of which are still incomplete, into the rates of absorption, disposition and effectiveness of cannabinoids as an adjunct to the treatment of neoplastic disease with cytotoxic drugs. It is clear from the results obtained, so far, that there are wide variations in the rate of absorption of THC from the gut between individuals and that the clinical effectiveness of the drug varies widely but it is too early to say whether these two phenomena are related.

The cannabis assay was first used in 1975 to ascertain the causative/contributory role of cannabis in a fatal motor car accident[13]. Subsequently a pilot study, a preliminary report of which has already been published[14] revealed a disturbingly high incidence (6%) of cannabis use amongst 128 victims of fatal road traffic accidents (Fig. 3). Although lack of control over selection of the material submitted for analysis precluded the drawing of statistically valid conclusions, the study confirmed the practicability of an investigation into the possible causative/contributory role of cannabis in road traffic accidents based on sound epidemiological principles and employing blood or urine as analyte.

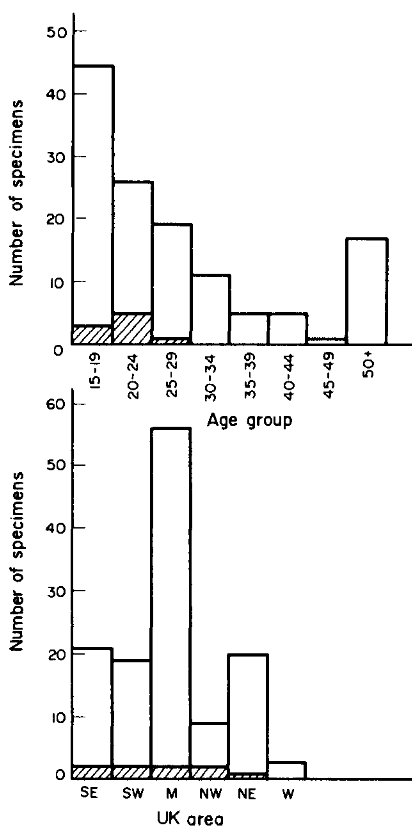


FIG. 3. Results of the analysis for cannabinoids of 128 blood specimens received from the driver victims of fatal motor accidents between July 1976 and April 1978.
The upper panel shows results grouped according to age of subjects; the lower according to the part of the country from which the specimens were received. Positive results are shown shaded.

It is pertinent here to discuss briefly the question of what substance or substances should be measured and in what fluid, in the unlikely event that cannabis use becomes socially and legally acceptable and its involvement in road traffic accidents is established. The use of alcohol as a model for cannabis is clearly inappropriate for a number of reasons. First, alcohol is completely and rapidly metabolised to CO_2 and water and it is only alcohol and not its metabolites that circulate in the blood. Secondly, alcohol is rapidly and evenly distributed throughout the body water and does not accumulate in body tissues. Hence the concentration of alcohol in urine water is, for all practical purposes, identical with that in plasma or tissue water. In the case of the cannabinoids, however, the metabolites may be equally or even more pharmacologically potent than the parent compound (THC) which is rarely taken alone except experimentally. There is therefore, no particular virtue in confining one's measurements to THC, as some authorities have suggested. Because cannabinoids persist in the body for days and sometimes longer after their administration, they are regularly detectable in the urine long after their most obvious pharmacological effects have dissipated. Thirdly, whilst a reasonably good correlation between alcohol concentration in blood and impairment of performance can be demonstrated, at least

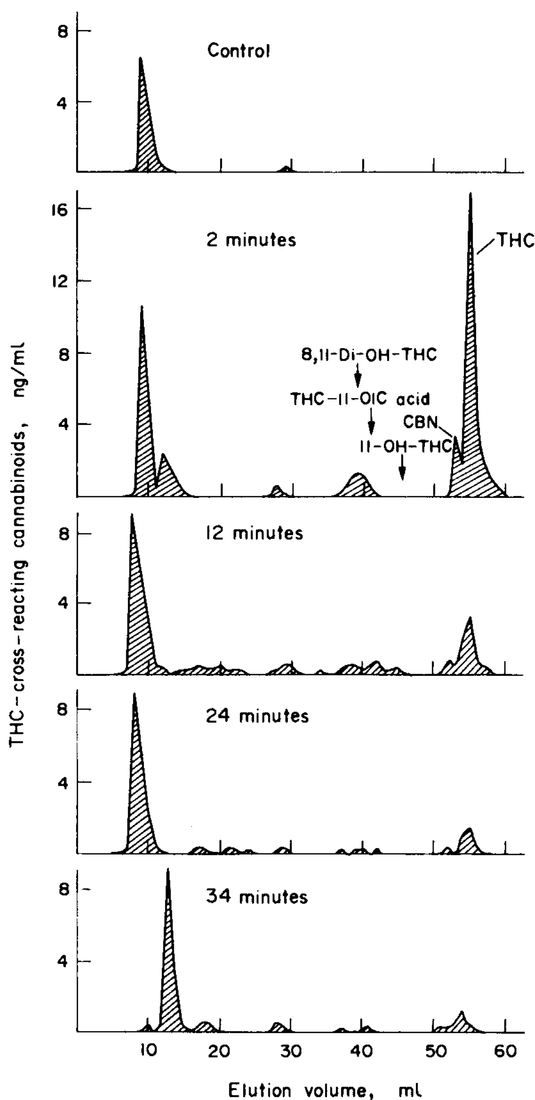


FIG. 4. Radioimmunochromatograms of THC and its metabolites—in a non-naive volunteer subject—at intervals before and following the smoking of a cigarette impregnated with 10 mg THC (Study carried out by P. L. Williams and A. C. Moffat).

amongst non-habituated subjects, no such correlation has been established for cannabis. It is indeed unlikely that such a correlation would exist in the ordinary course of events since most cannabis preparations contain a number of different pharmacologically active ingredients.

It is certain that there is not, and never will be, an " ideal " immunoassay for cannabis. Probably the best assay for most purposes would be one that measured THC, its metabolites and closely related analogues as though they were all the same since it is only when substances being measured and the standards behave identically, that truly accurate analytical results can be obtained. None of the antisera used in the radio and other types of

immunoassay described to date, have these desirable properties. Indeed, some authors have gone to great lengths to try and obtain antisera "specific" for THC without success. Since any cross-reactivity with metabolites and analogues, no matter how slight, inevitably produces "inaccurate" results. Investigators should beware of claims by authors that one assay is more specific or accurate than another. Nevertheless, it is possible, should it be necessary and providing a preliminary separation of HPLC is carried out, to measure virtually any specific or previously designated cannabinoid or cannabinoid metabolite capable of cross-reacting with the antiserum (Fig. 4) both accurately and precisely so long as a suitable standard is available.

Clearly much remains to be done to determine which cannabinoid or metabolite correlates best with pharmacological effects. In the meantime RIA provides the only practicable method of detecting and measuring cannabinoids in blood, albeit only semiquantitatively, in a variety of situations where this information is required. Other techniques have been described for detecting and possibly measuring cannabinoids and their metabolites in urine but none, apart from an enzyme-immunoassay[10], which is still in its developmental phase, is anywhere near as sensitive, specific or practicable as RIA.

REFERENCES

1. Chase, A. R., Kelley, P. R., Taunton-Rigby, A., Jones, R. T. and Harwood, T., Quantitation of Cannabinoids in biological fluids by radioimmunoassay. In "Cannabinoid assays in Humans" ed. R. E. Willette. NIDA Research Monograph No. 7, p. 1, Washington, 1976.
2. Cook, C. E., Hawes, M. L., Amerson, E. W., Pitt, C. G. and Williams, D., Radioimmunoassay of Δ^9-Tetrahydrocannabinol. In "Cannabinoid assays in Humans" ed. R. E. Willette. NIDA Research Monograph No. 7, p. 15, Washington, 1976.
3. Garrett, C. P. O., Braithwaite, R. A. and Teale, J. D.: An unusual case of tetrahydrocannabinol intoxication confirmed by radioimmunoassay. *British Medical Journal*, **2**,166 (1977).
4. Gross, S. J. and Soares, J. R., Separate Radioimmune Measurements of Body Fluid Δ^9-THC and 11-Nor-9-Carboxy-9-THC. In "Cannabinoid assays in humans" ed. R. E. Willette. NIDA Research Monograph No. 7, p. 10, Washington, 1976.
5. Marks, V., Morris, B. A. and Teale, J. D., Pharmacology. *British Medical Bulletin*, **30**,80 (1974).
6. Marks, V., Teale, J. D. and Fry, D., Detection of cannabis products by radioimmunoassay: studies in drug dependent subjects. *British Medical Journal*, **3**,348 (1975).
7. Mould, G. P., Aherne, G. W., Morris, B. A., Teale, J. D. and Marks, V., Radioimmunoassay of Drugs and its Clinical Application. *European Journal of Drug Metabolism and Pharmacokinetics*, **4**,171 (1977).
8. Robinson, J. D., Morris, B. A., Piall, E. M., Aherne, G. W. and Marks, V.: The Use of Rats in the Screening of Drug-Protein Conjugates for Immunoreactivity. In "Radioimmunoassay in Clinical Biochemistry" ed. C. A. Pasternak, p. 101, London, 1975.
9. Robinson, J. D., Aherne, G. W., Teale, J. D., Morris, B. A. and Marks, V., Problems Encountered in the Use of Radioiodination Tags in Radioimmunoassays for Drugs. In "Radioimmunoassay in Clinical Biochemistry" ed. C. A. Pasternak, p. 113, London, 1975.
10. Rodgers, R., Crowl, C. P., Eimstad, W. M., Hu, M. W., Kam, J. K., Ronald, R. C., Rowley, G. L. and Ullman, E. F., Homogeneous Enzyme Immunoassay for Cannabinoids in Urine. *Clinical Chemistry*, **24**,95 (1978).
11. Teale, J. D., Forman, E. J., King, L. J. and Marks, V., Radioimmunoassay of Cannabinoids in Blood and Urine. *The Lancet*, **2**,553 (1974).
12. Teale, J. D., Forman, E. J., King, L. J., Piall, E. M. and Marks, V., The development of a radioimmunoassay for cannabinoids in blood and urine. *Journal of Pharmacy and Pharmacology*, **27**,465 (1975).
13. Teale, D. J. and Marks, V., A Fatal Motor-Car Accident and Cannabis Use, Investigation by Radioimmunoassay. *The Lancet*, **1**,884 (1976).
14. Teale, J. D., Clough, J. M., King, L. J., Marks, V., Williams, P. L. and Moffat, A. C., The Incidence of Cannabinoids in Fatally Injured Drivers: An Investigation by Radioimmunoassay and High Pressure Liquid Chromatography. *Journal of the Forensic Science Society*, **17**,177 (1977).

DETERMINATION OF CANNABINOIDS IN URINE BY EMIT® HOMOGENEOUS ENZYME IMMUNOASSAY

KENNETH E. RUBENSTEIN*

Summary. Two THC immunogens were prepared by conjugating Δ^8- or Δ^9-THC to either bovine-γ-globulin or bovine serum albumin. Antibodies were raised to these in sheep. A series of THC-mitochondrial malate dehydrogenase conjugates were prepared and assayed for activity. This declined sharply with increasing substitution up to 5 THC residues (to 20%) and then more slowly to 8% with 12.2 residues. Maximum inhibition by the antibody (51%) occurred with enzyme containing about 4.2 residues. Assay calibrators were prepared from THC-9-acid. The assays were performed on 50 μl urine samples using a 60 sec. reaction time and spectrophotometric recording. The method was most sensitive to THC-9-acid and 11-hydroxy-Δ^9-THC with a detection range of 0.5–10 μg/l. Negligible cross reactivity was found with a large number of other drugs. The method was used to detect THC and CBN in urines from several volunteers who had received the drugs orally; CBD gave negative results as its concentration was below the 15 μg/l value chosen as the cut-off point to avoid false positives. Urinary cannabinoids were also measured for several days in four subjects who had received a single marihuana cigarette.

INTRODUCTION

THE generality of the technique employed in EMIT® chemistries has been demonstrated with the introduction of immunoassays for many compounds including opiates, thyroxine, antiepileptic drugs, cardioactive drugs and antiasthmatics. Based upon the observation that certain enzymes, when covalently bound to haptens, could be inhibited by antibodies directed against the haptens, EMIT assays employing the enzymes lysozyme, malate dehydrogenase (MDH) and glucose-6-phosphate dehydrogenase have been developed (Schneider *et al.*, 1973; Ullman *et al.*, 1975; Chang *et al.*, 1975).

Further application of the technique, using MDH conjugated with a derivative of Δ^9-tetrahydrocannabinol (Δ^9-THC), has demonstrated that the procedure is equally useful in the determination of cannabinoids in urine. Pig heart mitochondrial malate dehydrogenase (MDH) was chosen for development of a THC assay because of its high stability, its availability and its ease of detection by a simple highly sensitive assay. As little as 10^{-11} molar enzyme can be detected in a one minute spectrophotometric measurement.

* The data reported herein has been described in: Rodgers *et al.*, 1978 and Rowley *et al.*, 1976. Tables and graphs have been reproduced with the permission of the authors and *Clinical Chemistry*.

Similarly, the mechanism of antibody inhibition of morphine conjugates of MDH has also been studied and an immunoassay for morphine has been developed which permits detection of as little as 2×10^{-9} molar morphine in the assay mixture (Rowley *et al.*, 1975).

PREPARATION OF THE COMPONENTS

Antibodies to two immunogens were used in these studies. The first, prepared from the THC analog, IIIa (Fig. 1), was synthesized by treating $\Delta^{9,(11)}$-THC (I) with one equivalent of ozone at low temperature to yield an ozonide which was reduced with zinc in acetic acid to yield ketone II in 55% yield. Compound II was treated with 1-^{14}C-O-carboxymethylhydroxylamine in anhydrous refluxing methanol to yield the desired acid, IIIa, in 61% yield.

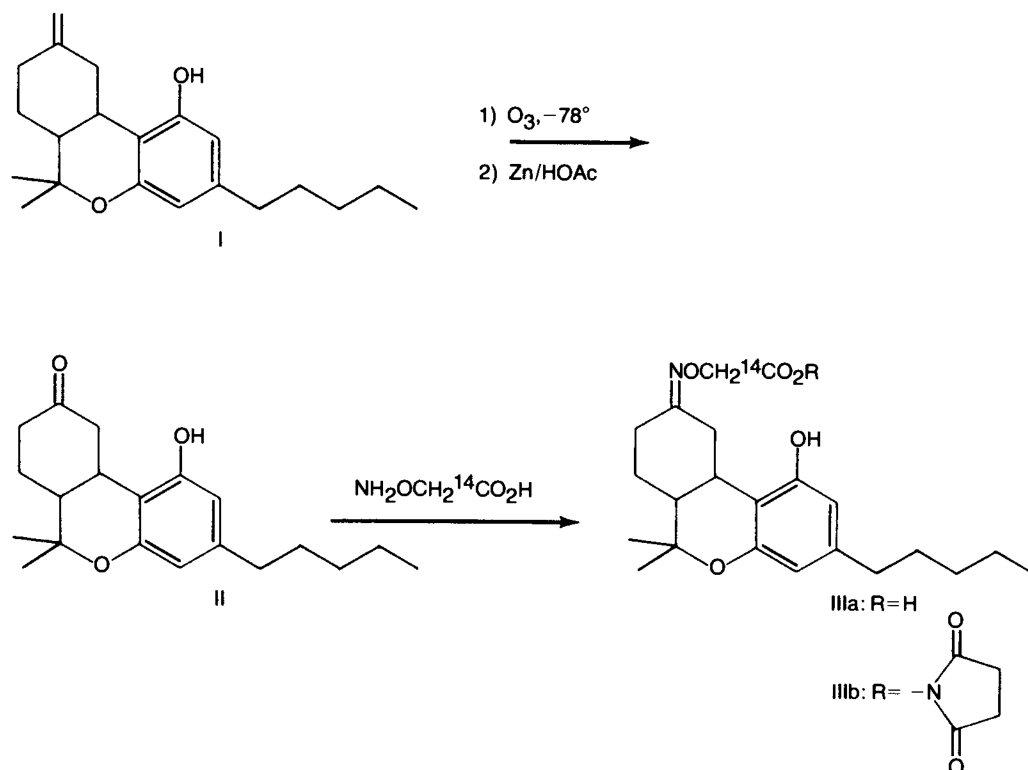


FIG. 1.

The THC antigen was prepared by conjugating the carboxymethyloxime (IIIa) to bovine γ-globulin via its N-hydroxysuccinimide ester, (IIIb) (Fig. 1). The ester (IIIb) was prepared by condensation with N-hydroxysuccinimide using the condensing reagent, N-dimethylaminopropyl-N'-ethyl carbodiimide hydrochloride in anhydrous dimethylformamide. Subsequently, the dimethylformamide solution of "active ester" was added to a buffered solution of the protein containing 30% dimethylformamide cosolvent at pH 8.5. The protein was exhaustively dialyzed to remove noncovalently bound THC residues. The

number of bound residues was determined by scintillation counting of the radiolabeled products to yield conjugates containing 21 and 32 THC residues per protein molecule in two separate preparations. Antibodies were obtained from sheep by immunizing with the conjugates (Rowley *et al.*, 1976).

THC-MDH conjugates were prepared by conjugating MDH with the NHS ester (IIIb) under conditions identical to those above. A series of THC-MDH conjugates were thus prepared by adding increasing amounts of IIIb to constant amounts of MDH in separate reaction vessels. Noncovalently bound THC residues were removed either by exhaustive dialysis or by gel chromatography on Sephadex G-25. The number of bound radiolabeled THC residues and the residual enzyme activity of each conjugate was determined. As can be seen in Fig. 2, enzyme activity of the conjugates decreased sharply with increasing substitution up to about 5 residues bound (20% activity). Substitution beyond 5 residues provided a conjugate (12.2 residues) with only a moderate further loss of activity (8% activity). Addition of excess THC antibody to the conjugates reduced their enzymatic activity. The maximum inhibition for each conjugate was only slightly affected by the species of animal used to produce the antibody.

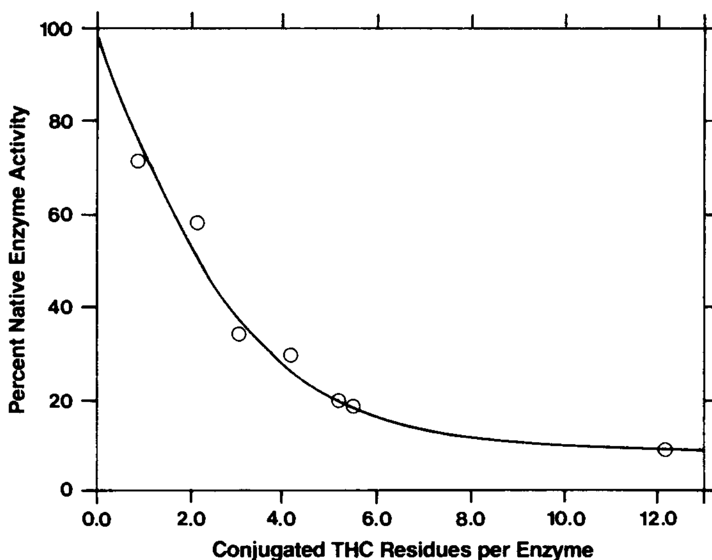


FIG. 2.

Maximal inhibition was, however, directly dependent on the number of bound THC residues. Inhibition increased sharply with the number of THC groups on the enzyme and reached a maximum when at about 4.2 residues (51% inhibition) (Fig. 3). Upon substituting the enzyme more heavily (12.2 residues) the antibody induced inhibition decreased to 35%.

Initial attempts to prepare urine calibration material with Δ^9-THC were unsuccessful, because this compound quickly adsorbs onto the walls of either glass or plastic containers (Rodgers *et al.*, 1978). For example, a urine pool containing an added 25 μg of Δ^9-THC per liter lost about 80% of the drug when stored in glass at 4°C for 24 hours. Similar behavior was previously reported by Garrett and Hunt (1974), and for this reason, the more soluble

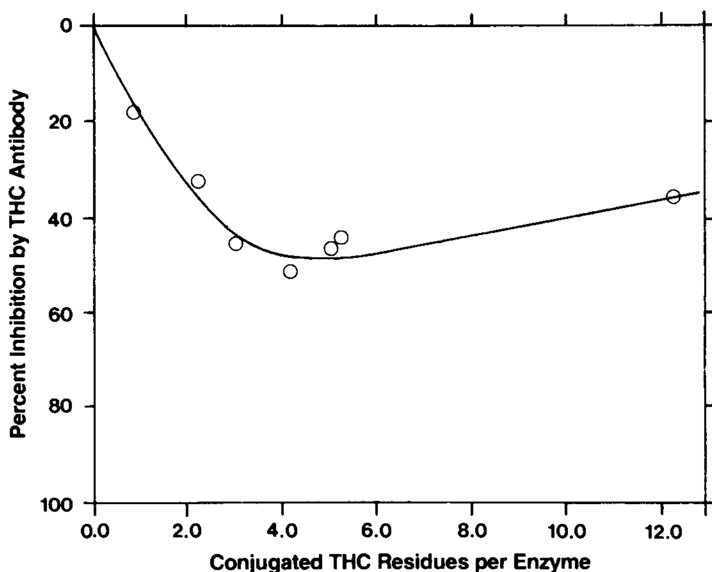


FIG. 3.

THC-9-acid (Fig. 4) was used to prepare the assay calibrators. This material cross reacts well with the antibodies (Rowley *et al.*, 1976) and is a major urinary metabolite of Δ^9-THC (Lemberger *et al.*, 1971).

The second immunogen, prepared from compound V, (Fig. 4) was conjugated to bovine serum albumin by reductive amination with sodium cyanoborohydride. Sheep were immunized every two weeks with an emulsion containg the conjugate and the animals were subsequently bled every two months (Rodgers *et al.*, 1978).

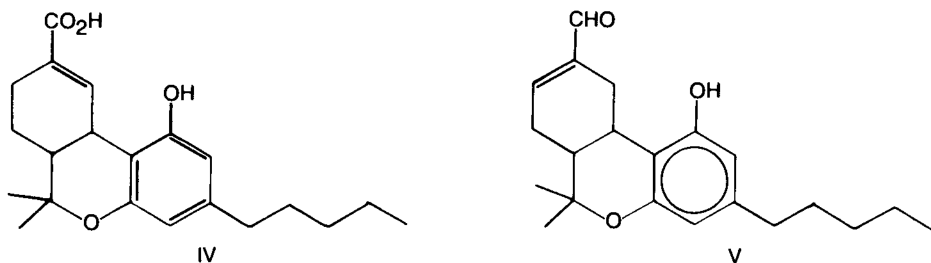


FIG. 4.

ASSAY PROTOCOL

Using the Syva® Model 1500 Pipetter-Diluter, 50 μl of the urine sample is delivered with 250 μl of assay buffer to a disposable glass test tube. Antibody reagent (50 μl) plus assay buffer (250 μl) and enzyme reagent (50 μl) plus assay buffer (250 μl) are then added to the sample mixture to give a total assay volume of 900 μl. The final reaction mixture is then vortex-mixed for 2–3 seconds and aspirated into the spectrophotometer flow-cell

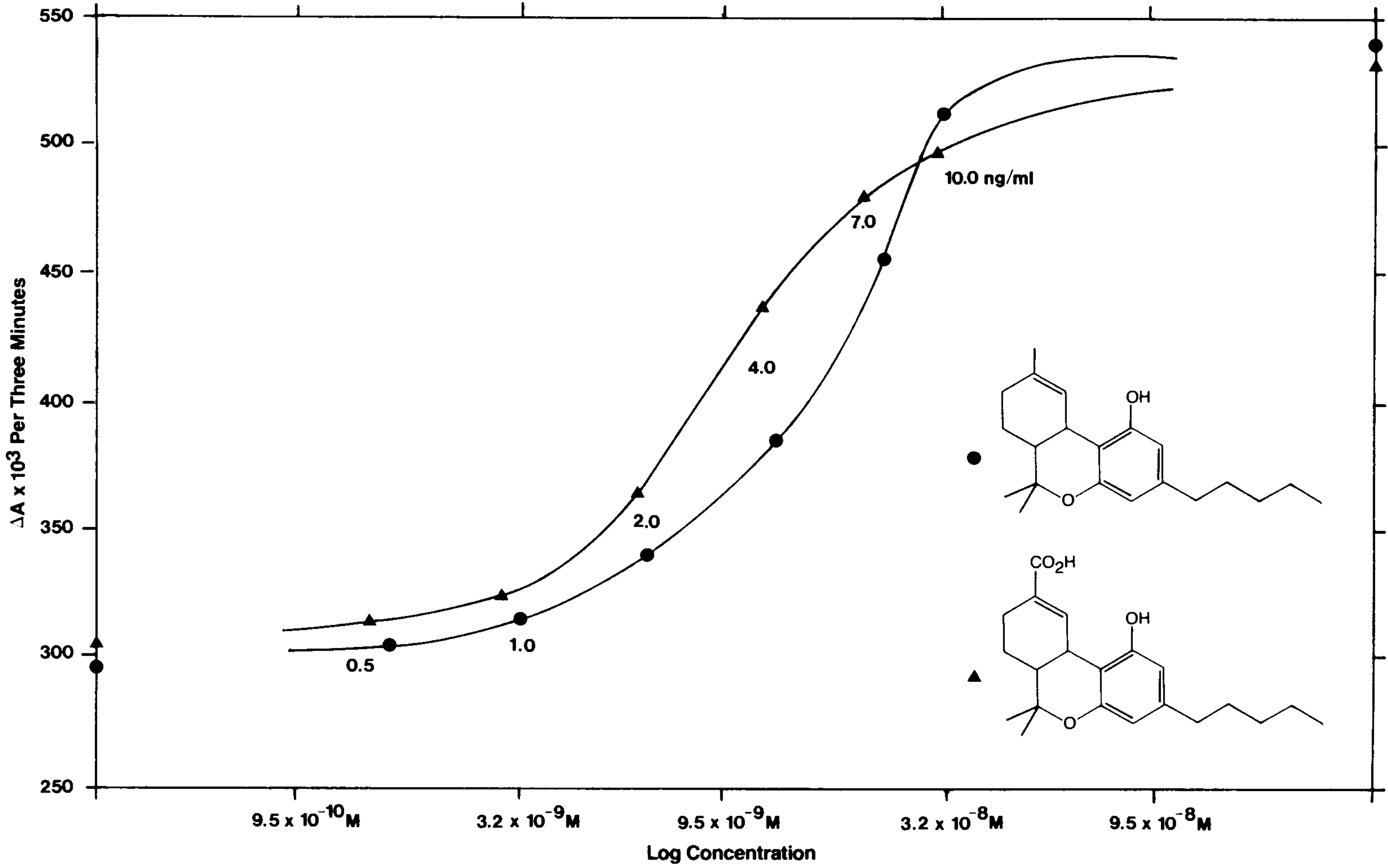


FIG. 5.

(Gilford Stasar III). Data handling is accomplished using the Syva® Model 2400 Timer-Printer which is directly interfaced to the spectrophotometer. Activated upon aspiration of the reaction mixture, the Timer-Printer controls the 13 second thermal equilibration delay, prints the initial reading and 60 seconds later prints the difference between the initial and final readings. The amount of drug in the sample is then determined by using a standard curve, which is prepared by plotting calibrator readings *vs.* concentrations, as a reference (Fig. 5).

SENSITIVITY/CROSSREACTIVITY

The antibodies used in this assay are specifically intended to permit detection of various Δ^9-THC congeners and metabolites. Using antibody derived from V, the assay is most sensitive to THC-9-acid and to 11-hydroxy-Δ^9-THC; the response to cannabinol and to Δ^9-THC itself is about 30% less. By contrast, cannabidiol is much less cross reactive, 47-fold as much being required to give the same response as THC-9-acid.

Drug-response curves with antibody derived from IV were obtained for both THC and 11-nor-Δ^9-THC-9-oic acid. Enzyme rate measurements were made over 3 minute periods

TABLE 1. CROSSREACTIVITY OF THE EMIT ASSAY FOR CANNABINOIDS IN URINE: DRUGS AND THEIR METABOLITES TESTED

Crossreactivity of Compounds at Concentrations < 1000 mg/liter[a]		Crossreactivity of Compounds at Concentrations > 1000 mg/liter[a]	
17β-Estradiol	> 10	Methoxyphenamine	Barbital
Testosterone	> 10	Methylphenidate	Caffeine
Thyroxine	> 10	Nalorphine	Cocaine
Benzphetamine	> 100	Naloxone	Codeine
Butabarbital	> 100	Promethazine[c]	Cyclopentamine
Chlorpromazine	> 100	Phenmetrazine	Meperidine[d]
Cholesterol	> 100	Phenobarbital	Diphenhydramine
Cortisol	> 100	Phentermine	Ecgonine
Propoxyphene HCl[b]	> 100	Phenylpropanolamine	Ephedrine
Des-N-CH_3-diazepam	> 100	Probarbital	Glutethimide
Flurazepam	> 100	Propylhexedrine	Homatropine
Medazepam	> 100	Salicylic acid	5-(p-OH-phenyl)-5-phenytoin
Nylidrin	> 100	Secobarbital	Librium
Pentobarbital	> 100	Sulthiame	Lidocaine
Thorazine	> 100	Talbutal	Mephentermine
Dextromethorphan	1000	Thiopental	Mescaline
		Thiamytal	α-Oxymethadol
		Diazepam	Amphetamine
		Aprobarbital	Atropine
		Benzoyl ecgonine	Methadone
		Methadone metabolite	Methamphetamine
		Metharbital	

a. Concentration of compound in synthetic urine that gives an assay response equivalent to 15 μg of THC-9-acid per liter

b. "Darvon".

c. "Phenergan".

d. "Demerol".

(Fig. 5). The useable range of detection was the same for both compounds, 0.5–10 μg/l. However, response to IV in mid-range was about 1.5 times higher than response to THC.

To examine the crossreactivity of the assay to various natural hormones, drugs, and their metabolites, each compound in Table 1 was added to synthetic urine to give a concentration of either 1000 mg/liter or the highest concentration at which it was soluble. None of the compounds tested, using antibody derived from V, exhibited clinically significant crossreactivity including steroid hormones and cholesterol, which were tested at concentrations much higher than amounts normally present in urine.

SENSITIVITY AND PRECISION

To monitor for the presence or absence of cannabinoids in urine, it is necessary to select a specific minimum "cutoff" reading above which samples will be identified as positive. Data (Fig. 6) generated using antibody derived from V suggest that a 15 μg/liter cutoff provides a practical distinction between positive and negative samples without correction for endogenous enzyme activity. This cutoff is expected to produce $< 5\%$ false positives and to assure that $> 95\%$ of all samples containing 25 μg/liter will be correctly identified as positive. By using 25 μg/liter as a cutoff, false positives are virtually eliminated, while providing a $> 95\%$ probability of detection of samples containing at least 45 μg/liter (Rodgers *et al.*, 1978).

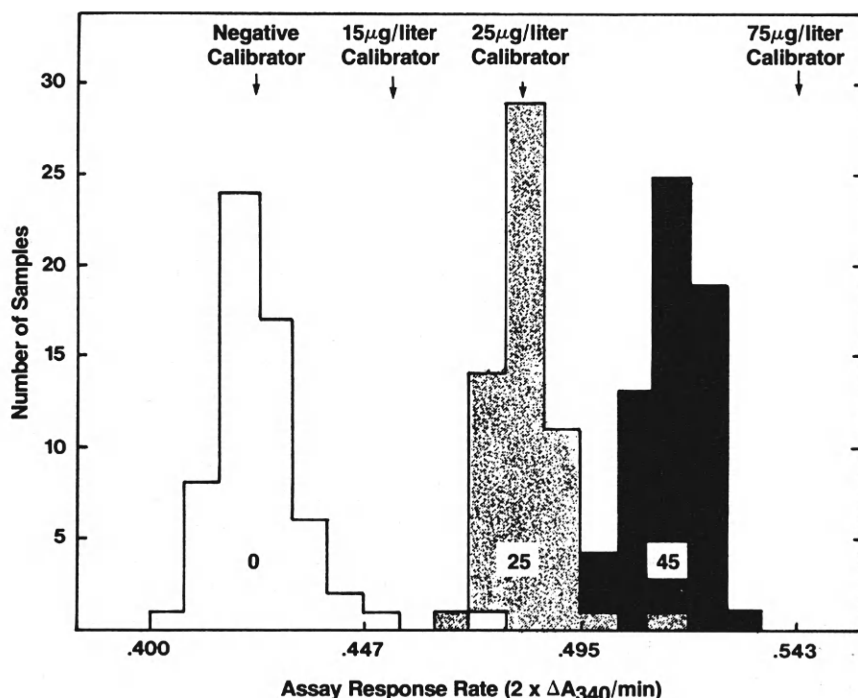


FIG. 6. Distribution of assay responses of 60 urine samples with no drug added, and with 25 and 45 μg of THC-9-acid added per liter.

Sample to sample and pooled sample precision data (antibody derived from V) are presented in Table 2. In the latter study aliquots of a pool were supplemented with 15, 25 and 75 μg of THC-9-acid per liter. Each aliquot was assayed 20 times by a single operator and each level exhibited high precision. By contrast the study in which individual urine samples were spiked with 25 and 45 μg/liter showed adequate but lower precision. This data, represented in histogram form (Fig. 6) suggests that variations in urine composition have a minor effect on the assay response.

TABLE 2. PRECISION OF THE EMIT® CANNABINOID ASSAY

	Number of determinations	Assayed mean (μg/liter)	Standard deviation (μg/liter)	Coefficient of variation (%)
Pooled samples:				
Containing THC-9-Acid				
15 μg/liter	20 replicates	15.1	0.60	4.0
25 μg/liter	20 replicates	25.3	0.75	3.0
75 μg/liter	20 replicates	74.9	1.48	2.0
Sample-to-sample precision:				
Individual specimens Containing THC-9-acid				
25 μg/liter	60 samples	25.6	4.3	16.8
45 μg/liter	60 samples	45.3	5.2	11.5

CLINICAL RESULTS

" Blind " clinical studies were done on urine samples provided by the Veterans Administration Hospital, Palo Alto, California. The analyst was provided only an identifying number with each sample and was unaware of the sample history (Rodgers *et al.*, 1978).

The subjects tested were administered, orally, 30-mg Δ^9-THC, 100 mg of cannabinol, or 100 mg of cannabidiol. Urine samples were obtained before administration of the dose and at various intervals afterward. The results (antibody from V) are presented in Table 3. Urine from all of the subjects who received Δ^9-THC or cannabinol was strongly positive for one or two days after the dose, but urine samples taken after the oral dose of cannabidiol tested negative (< 15 μg of THC-9-acid equivalents per liter) by our assay, a result that was to be expected because 470 μg of cannabidiol per liter is required to give the same response as is obtained with 15 μg of THC-9-acid per liter. One subject (VA-I) showed the presence of cannabinoids before dosing with Δ^9-THC and cannabidiol. This subject reported frequent use of cannabinoids before the experiment and appeared to be continually excreting metabolites. In addition to these positive samples, 12 negative urine samples were included which were correctly identified as negative.

Because the volume of urine produced changes during the day, the assay results are also reported as micrograms of THC-9-acid equivalents per gram of creatinine. The adjusted

TABLE 3. URINARY EXCRETION OF ORALLY ADMINISTERED CANNABINOIDS

Subject	Compound administered	Dose	Time interval: Before dose (hours)	Time interval: After dose hours	Cannabinoid concentration determined by EMIT® assay μg/liter	Cannabinoid concentration as a function of creatinine level[a] $\mu g/g$
VA-I[b]	Δ^9-THC	30 mg	(24)		(216)	(108)
				2	132	825
				6	918	1187
				12	883	460
				24	341	376
VA-II	Δ^9-THC	30 mg	(12)		(negative)[c]	(negative)[c]
				6	561	637
				24	561	339
				48	363	534
VA-I[b]	Cannabidiol	100 mg	(12)		(132)	(160)
				2	63	175
				48	118	92
VA-III	Cannabidiol	100 mg	(12)		(negative)	(negative)
				2	negative	negative
				6	negative	negative
				48	negative	negative
VA-III	Cannabinol	100 mg		2	128	662
				6	624	996
				12	715	715

a. Because the volume of urine produced is variable, results are reported as micrograms of THC-9-acid equivalents per gram of creatinine.
b. This subject was a frequent user of cannabinoids before the experiment.
c. Throughout this table, "negative" means the measured concentration was < 15 μg of THC-9-acid equivalents per liter.

a show a maximum for metabolites excreted at about 6 hours after the dose, followed a very slow decline in values for THC-9-acid equivalents.

n a second clinical study (Fig. 7), urine samples were collected from reliable volunteers various times after each had smoked a single marihuana cigarette of unknown origin and nabinoid content. Each specimen was subsequently assayed by the EMIT cannabinoid ay. Subject A, a moderately frequent user of cannabinoids, showed a higher cannabin- l excretion before smoking than the peak values of the other three subjects. Subjects B l C reached higher peak cannabinoid values than did D and also peaked earlier (2 hours 6 hours) after smoking. These differences probably represent differences in absorption ong the various individuals as well as differences in potency of the marijuana cigarettes. e values were not corrected for changes in urinary volume.

Further, results with clinical specimens indicate that peak excretion of cannabinoids urs from 2–6 hours after exposure, while cannabinoid excretion can remain high for as g as 24 hours. Frequent users (several exposures per week) have basal values for tabolites in their urine that exceed the peak values attained by relatively infrequent rs.

These data, and the fact that the period of intoxication lasts only from 1 to 4 hours ton *et al.*, 1973) indicate that the EMIT enzyme immunoassay for Δ^9-THC and its tabolites in urine is suited for general screening as an indicator of cannabinoid use.

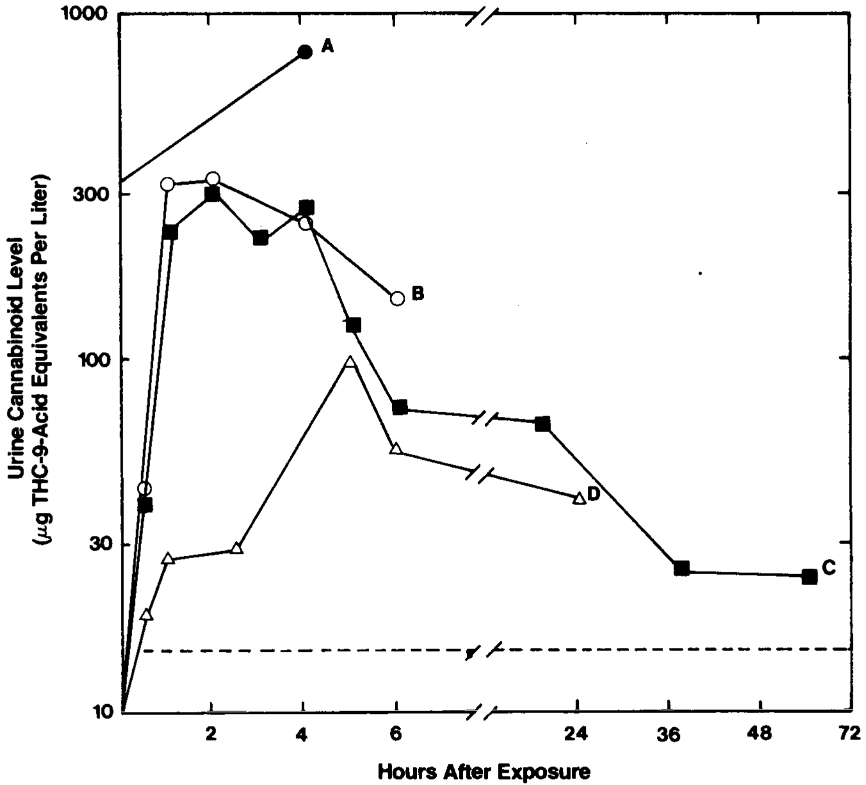


FIG. 7. Urinary cannabinoid concentrations at various times after smoking a single marihuana cigarette. The dashed line represents the cutoff above which samples are considered positive (15 μg of THC-9-acid equivalents per liter).

ACKNOWLEDGEMENTS

The author thanks Dr. L. E. Hollister and S. Kanter (Veterans Administration Hospital, Palo Alto) for providing urine samples used in clinical investigations, and Ms. W. L. Thole for editorial assistance. Financial support for a portion of this study was provided by the National Institute on Drug Abuse.

REFERENCES

CHANG, J. J., CROWL, C. P., and SCHNEIDER, R. S., Homogeneous enzyme immunoassay for digoxin, *Clin. Chem.* **21**,967 (1975).

GARRETT, E. R., and HUNT, C. A., Physicochemical properties, solubility, and protein binding of Δ^9-tetrahydrocannabinol, *J. Pharm. Sci.* **63**,1056 (1974).

LEMBERGER, L., AXELROD, J., and KOPIN, I. J., Metabolism and disposition of tetrahydrocannabinols in naive subjects and chronic marijuana users. *Ann. N.Y. Acad. Sci.* **191**,142 (1971).

PATON, W. D. M., and PERTWEE, R. G., The actions of cannabis in man. In "Marijuana: Chemistry, Pharmacology, Metabolism and Clinical Effects," R. Mechoulam, Ed., Academic Press, New York, 1973.

RODGERS, R., CROWL, C., EIMSTAD, W., HU, M. W., KAM, J. K., RONALD, R. C., ROWLEY, G. L., and ULLMAN, E. F., Homogeneous enzyme immunoassay for cannabinoids in urine. *Clin. Chem.* **24**,95–100 (1978).

LEY, G. L., ARMSTRONG, T. A., CROWL, C. P., *et al.*, Determination of THC and its metabolites by EMIT® homogeneous enzyme immunoassay: A summary report. In "Cannabinoid Assays in Humans," NIDA Research Monograph 7, R. E. Willette, Ed., p 28, 1976.
LEY, G. L., RUBENSTEIN, K. E., HUISJEN, J., and ULLMAN, E. F., Mechanism by which antibodies inhibit hapten-malate dehydrogenase conjugate. An enzyme immunoassay for morphine. *J. Biol. Chem.* **250**,3759 (1975).
NEIDER, R. S., LINQUIST, P., WONG, E. T., RUBENSTEIN, K. E. and ULLMAN, E. F., Homogeneous enzyme immunoassay for opiates in urine. *Clin. Chem.* **19**,821 (1973).
MAN, E. F., BLAKEMORE, J., LEUTE, R. K., EIMSTADT, W., and JARLITSCH, A., Homogeneous enzyme immunoassay for thyroxine. *Clin. Chem.* **21**,1011 (1975).

IN VITRO METABOLISM OF TETRAHYDROCANNABINOL BY RHESUS MONKEY LIVER AND HUMAN LIVER

M. WIDMAN and M. HALLDIN

Department of Pharmacognosy, Faculty of Pharmacy, BMC, Box 579, S-751 23 Uppsala, Sweden

and

B. MARTIN

Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

TETRAHYDROCANNABINOL (THC) and related cannabinoids are known to be extensively metabolized by liver microsomal enzymes in different species[1]. In addition to hydroxylation allylic to the double bond, oxidation at various carbons of the side-chain seems to be a general metabolic process for the cannabinoids in most animals. More importantly, the metabolites of Δ^6-THC that are hydroxylated in the side chain have been shown to be behaviourally active in rhesus monkeys[2,3]. Previously Wall and Brine[4] have reported the rhesus monkey to metabolize Δ^1-THC to a number of dihydroxy metabolites in addition to 7-, 6α-, and 6β-hydroxy metabolites. No monohydroxy side-chain metabolites were found. We have now looked more closely into the relative proportions of these side-chain hydroxy metabolites formed by rhesus monkey liver to see if their presence ought to be considered for the overall psychoactive effect of THC.

Regarding the metabolic fate of THC in humans Wall *et al.*[5] have reported 1-nor-Δ^1-THC-1-carboxylic acid to be one of the predominant metabolites in plasma after either intravenous administration or smoking. 7-Hydroxy-Δ^1-THC was found in amounts of only one-twentieth that of the Δ^1-THC. Other minor metabolites found were 6β-hydroxy-, 6α-hydroxy- and 6,7-dihydroxy-Δ^1-THC. To obtain evidence for the presence of side-chain hydroxy metabolites of THC in humans we have now carried out an *in vitro* study using human liver.

Δ^1-THC was used in the human study while Δ^6-THC was used for the rhesus monkey study. Otherwise the two experiments were carried out under similar conditions. The rhesus monkey liver (130 g) was homogenised with 1.15% KCl and the homogenate was centrifuged at 10000 g for 10 min. The 10000 g supernatant was added to phosphate buffer containing appropriate cofactors[6,7]. Finally 30 mg of Δ^6-THC-4″, 5″-^{3}H (0.11 mCi/mmole) was added in an emulsion of Tween 80 and the mixture was incubated

at 37° for 45 min. The human liver (100 g) obtained from a black male (brain-dead donor) 20 min. after death was treated as the monkey liver and incubated with 10 mg of Δ^1-THC-1″, 2″-^{3}H (0.25 mCi/mmole) at 37° for 45 min. The incubation mixtures were extracted with petroleum ether and diethyl ether as described earlier[6, 7]. The diethyl ether extracts containing the metabolites were separated and purified on Florisil and Sephadex LH-20 columns[6, 7]. A final separation and purification step was done by thin-layer chromatography[6, 7] prior to the identification of the metabolites by gas chromatography—mass spectrometry and comparison to reference compounds[8, 9].

The rhesus monkey metabolized Δ^6-THC to various monohydroxy metabolites, as can be seen in Table 1. 7-Hydroxy-Δ^6-THC was the most abundant metabolite formed. Furthermore, all the side-chain hydroxy metabolites except 5″-hydroxy-Δ^6-THC were isolated. 4″-Hydroxy-Δ^6-THC was present in about one-third and 3″-hydroxy-Δ^6-THC in about one-sixth of that of 7-hydroxy-Δ^6-THC. The less psychoactive metabolites 1″-hydroxy- and 2″-hydroxy-Δ^6-THC made up for about one-twentieth of that of 7-OH-Δ^6-THC, respectively. In addition to these metabolites 5-hydroxy-Δ^6-THC was isolated as a mixture of both the α- and the β-isomer.

The human liver on the other hand showed a much more simple metabolic pattern. Surprisingly no side-chain hydroxy metabolites were found (Table 1). Far the most abundant metabolite was 7-hydroxy-Δ^1-THC. About 70% of converted material was due to this metabolite. Other metabolites found were 6β-hydroxy-Δ^1-THC which was present in an amount of about one-fifteenth of that of 7-hydroxy-Δ^1-THC while 6α-hydroxy-Δ^1-THC was present only in trace amounts.

TABLE 1. RELATIVE PROPORTIONS OF IN VITRO METABOLITES[a]

	Human liver Δ^1-THC		Monkey liver Δ^6-THC
7-OH	+ + +		+ + +
6α-OH	tr.	5α-OH	+ +
6β-OH	+	5β-OH	
1″-OH	–		+
2″-OH	–		+
3″-OH	–		+ +
4″-OH	–		+ +
5″-OH	–		–

a. +, + +, + + + indicate minor to major metabolite; tr. indicates trace; – indicates "not found".

CONCLUSION

The results of this study shows that side-chain hydroxylation of THC does not seem to be a general pathway of biotransformation in man while it has to be considered in the rhesus monkey. Thus, there seems to be a species difference between man and animals. However, one has to remember that this study has been carried out with only one human liver and of course the metabolic pattern might differ slightly from one person to another.

ACKNOWLEDGEMENTS

We thank Dr. S. Agurell and Dr. L. Harris for advice and support, Dr. J.-E. Lindren for recording the mass spectra, Ms. J. Montgomery and Ms. D. Rosin for skilled technical assistance. This work was supported by the Swedish Medical Research Council and the National Institute on Drug Abuse, Grant No. DA-00490.

REFERENCES

1. MECHOULAM, R., MCCALLUM, N. K., and BURSTEIN, S., Recent advances in the chemistry and biochemistry of cannabis. *Chem. Rev.* **76**,75 (1976).
2. AGURELL, S., BINDER, M., FONSEKA, K., LINDREN, J.-E., LEANDER, K., MARTIN, B., NILSSON, I. M., NORDQVIST, M., OHLSSON, A., and WIDMAN, M., Cannabinoids: Metabolites hydroxylated in the pentyl side chain. In: "Marihuana: Chemistry, Biochemistry and Cellular Effects." Ed. G. G. Nahas, Springer-Verlag, New York, p. 141, 1976.
3. LEANDER, K., DAHMÉN, J., AGURELL, S., OHLSSON, A., EDERY, H., PORATH, G., LEVY, S., and MECHOULAM, R., Synthesis and psychotropic activity of side-chain hydroxylated Δ^6-THC metabolites. Submitted for publication.
4. WALL, M. E., and BRINE, D. R., Identification of cannabinoids and metabolites in biological materials by combined gas-liquid chromatography—mass spectrometry. In: "Marihuana: Chemistry, Biochemistry, and Cellular Effects," Ed. G. G. Nahas, Springer-Verlag, New York, p. 51, 1976.
5. WALL, M. E., BRINE, D. R., and PEREZ-REYES, M., Metabolism of cannabinoids in man. In: "The Pharmacology of Marihuana." Ed. M. C. Braude and S. Szara, Raven Press New York, p. 93, 1976.
6. WIDMAN, M., DAHMÉN, J., LEANDER, K., and PETERSSON, K., *In vitro* metabolism of cannabinol in rat and rabbit liver. Syntheses of 2″-, 3″- and 5″-hydroxycannabinol. *Acta Pharm. Suec.* **12**,385 (1975).
7. MARTIN, B., NORDQVIST, M., AGURELL, S., LINDGREN, J.-E., LEANDER, K., and BINDER, M., Identification of monohydroxylated metabolites of cannabidiol formed by rat liver. *J. Pharm. Pharmacol.* **28**,275 (1976).
8. BINDER, M., AGURELL, S., LEANDER, K., and LINDGREN, J.-E., Zur identification potentieller Metabolite von Cannabisinhaltstoffen: Kernresonanz- und massen spektroskopische Untersuchungen an seitenketten-hydroxylierten Cannabinoiden. *Helv. Chim. Acta* **57**,1626 (1974).
9. FONSEKA, K., WIDMAN, M., and AGURELL, S., Chromatographic separation of cannabinoids and their mono-oxygenated derivatives. *J. Chromatogr.* **120**,343 (1976).

PHARMACOKINETICS AND DISPOSITION OF Δ^9-TETRAHYDROCANNABINOL AND ITS METABOLITES

EDWARD R. GARRETT

The Beehive, College of Pharmacy, Box J-4, J. Hillis Miller Health Center, University of Florida, Gainesville, Florida 32610.

Summary. The time course of intravenously administered Δ^9-tetrahydrocannabinol (Δ^9-THC) in various species can be fitted by a sum of up to five exponentials, indicative of variable rates of permeation into and return from multiple body compartments. No dose dependent pharmacokinetics has been observed. The drug is rapidly and completely metabolized, concomitantly with its rapid distribution into, and sequestration by, tissues. Both bound and unbound Δ^9-THC in the plasma are effectively cleared by the liver with a 50% hepatic efficiency referenced to *total* drug in plasma. The overall volume of distribution referenced to plasma-unbound drug is 50–100 × 10^3 liters. The terminal slow decay of Δ^9-THC in the body with apparent half lives of up to 7 days is due to its slow release from these tissues. This terminal phase could not be significantly affected by any enzymic induction. Such a phenomenon implies a high accumulation in body tissues; the amount of Δ^9-THC in the body would be 10-fold greater than from a single dose after 27 days of repetitive daily dosing. The slow elimination of metabolites is a consequence of the slow rate-determining return of Δ^9-THC from deep compartments to the plasma. Intravenously administered metabolites are rapidly eliminated. In general by 5 days, 15–17% of the dose appears as metabolites in the urine and 40–45% is biliary excreted to appear in the feces. The studies in bile cannulated dogs show a 15% enterohepatic recirculation of biliary excreted metabolites. Available data is consistent with a similar pharmacokinetic pattern for the primary metabolite 11-hydroxy Δ^9-tetrahydrocannabinol. The high non-saturable protein binding (97%) of Δ^9-THC can be used to solubilize this highly insoluble material (0.77 mg/l in 0.15 *M* NaCl at 23°). Δ^9-THC is rapidly degraded in acidic solutions and the kinetics have been completely determined.

THE pharmacokinetics and disposition of Δ^9-tetrahydrocannabinol (Δ^9-THC), the active constituent of marijuana, and its formed metabolites have been clarified in recent years[6, 11, 12, 14, 17, 21]. The high initial plasma level of Δ^9-THC or Δ^8-THC, observed on initial administration, rapidly decreased to a small fraction of its value which then persisted with slow diminution to reflect an extremely long terminal half-life (Figs. 1–5).

The Δ^9-THC apparent volume of distribution of the central compartment referenced to total drug concentrations in plasma (1.31 ± 0.07 liters in the dog[6]) is close to the volume of plasma in the body. This is a simple consequence of the compound's high plasma protein binding, largely lipoprotein[20], of 97%[4, 23]. Similar protein binding was shown for 11-OH-Δ^9-THC, except that the binding was largely to albumin[22, 23].

The precipitous drop in plasma level after bolus i.v. administration occurs immediately (Fig. 5). The drug is concomitantly and quickly metabolized and distributed into

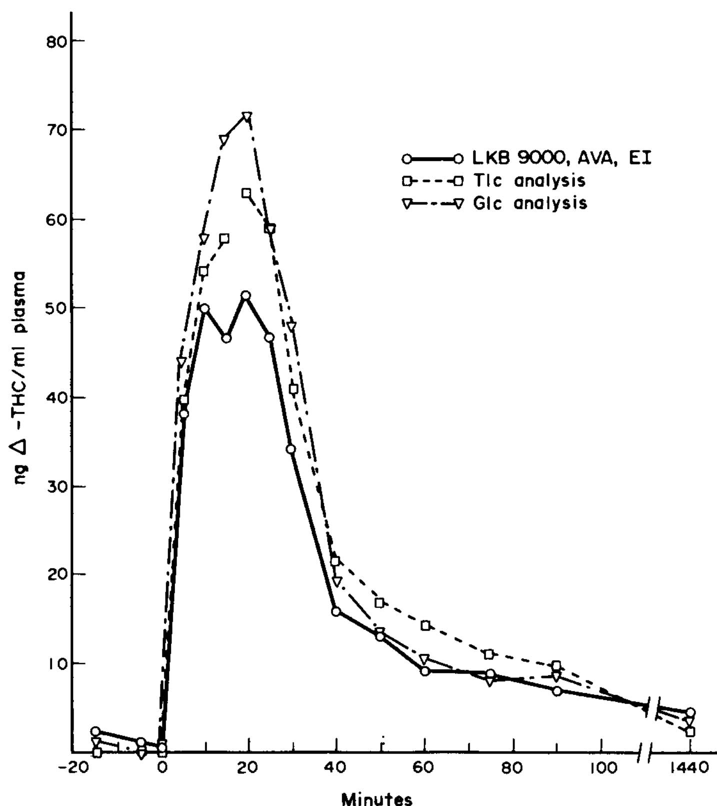


FIG. 1. Averaged plasma levels of Δ^9-THC after the intravenous infusion of 5 mg Δ^9-THC to 4 subjects as determined by mass spectrometry (LKB mass spectrometer), analysis of TLC-separated material, and GLC-electron capture analysis of the pentafluoropropionate ester derivative. (Fig. 10 of Wall *et al.*, NIDA Res. Monograph Series[21] and reproduced with the permission of the publisher, National Institute on Drug Abuse, Rockville, MD 20857.)

tissues[6, 18], and is concentrated in the lung, liver, myocardium, adrenals, spleen[11] and later in fat[9] after distribution (Fig. 6). Less than 1% as THC gets into the brain which apparently has no metabolizing action[2, 11]. The drug appearance in mouse brain is delayed (*ca.* 15 min), indicative of a slower penetration into this tissue[8]. The psychoactive effects reasonably parallel this presumed time course of drug appearance in the brain[8, 16] (Fig. 7).

Subsequently, the rate of loss of Δ^9-THC slows measurably (Figs. 1–5) and a sum of up to five exponentials (Fig. 5), the number dependent on the sensitivity of the plasma assays, are necessary to characterize the plasma level-time curve[6]. This is indicative of a minimum of 5 composite body compartments, into which the drug has variable rates of permeation. This is consistent with the known distribution patterns of lipophilic Δ^9-THC into non-vascularized tissues such as fat[9]; its high sequestration in certain organs such as liver, lung etc. and its extremely high binding and adhesion properties[4, 20, 22, 23].

Ultimately, a terminal phase for Δ^9-THC elimination is observed from plasma levels whose half life can range up to 7 days[6]. Total metabolite presence in the plasma reaches an early maximum, consistent with the premise of rapid metabolism (Fig. 8). A major

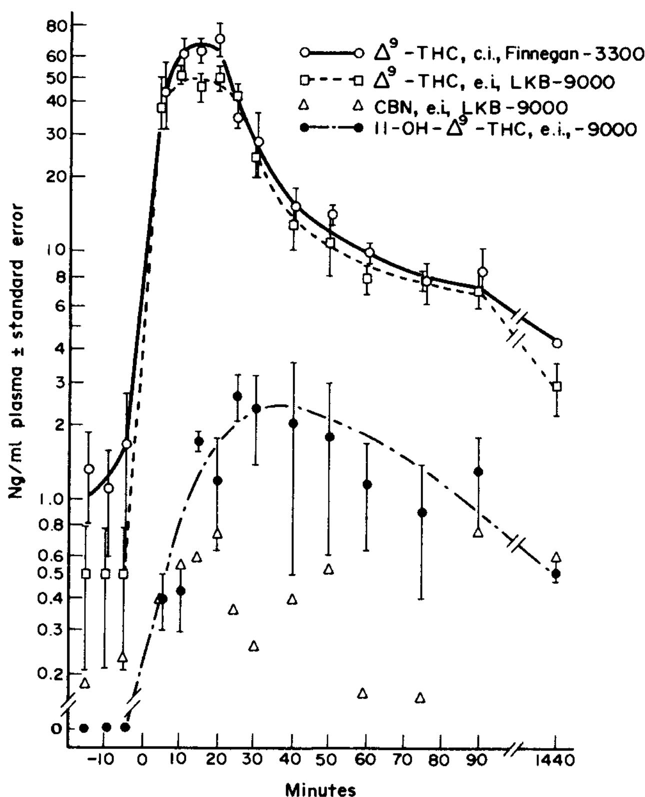


FIG. 2. Averaged plasma levels of Δ^9-THC, 11-hydroxy-Δ^9-THC and cannabinol (CBN) after the intravenous infusion of Δ^9-THC as determined by mass spectrometry (Fig. 9 of Wall *et al.*, NIDA Res. Monograph Series[21] and reproduced with the permission of the publisher, National Institute on Drug Abuse, Rockville, MD 20857.)

metabolite, 11-hydroxy Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC) is rapidly formed and peaks early (Fig. 2). Its pharmacokinetics appears to be very similar to that of Δ^9-THC (Fig. 9). Concomitantly, but more slowly than this metabolite, there are formed more polar, but relatively neutral metabolites (which could include ring and side chain hydroxylated derivatives of both Δ^9-THC and 11-OH Δ^9-THC), weak acids (such as the 11-oic acid derivatives) and their strongly acid conjugates; glucuronides and sulfates (Fig. 10). A nonpolar acyloxy-11-hydroxy has been observed[10] and is consistent with the pharmacokinetic analysis of metabolites that led to the conclusion of tissue accumulation of less than 10% of the administered THC as very slowly eliminated non-polar metabolites[6].

For all practical purposes, the Δ^9-THC is completely metabolized[6, 11]. The polyhydroxylated compounds, the 11-oic acids and their conjugates are biliary and renally excreted[6, 11]. The biliary excreted acids and hydroxylated compounds and their conjugates are enterohepatically recirculated[6].

The only study[21] of concomitant 11-OH-Δ^9 and Δ^9-THC plasma levels in man (Fig. 2) was entirely consistent with the predicted[6] time course of the plasma levels of the former on the premise that its metabolic clearance and apparent volume of distribution was the

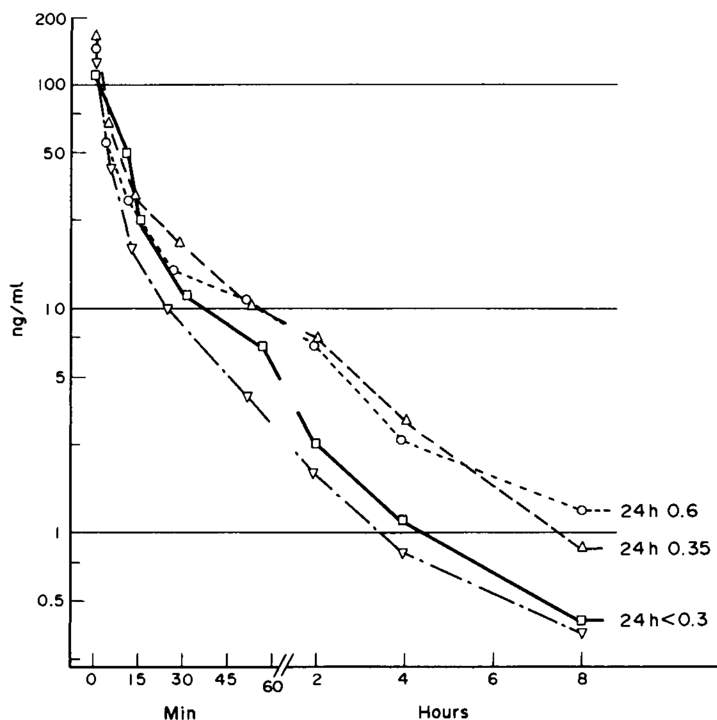


FIG. 3. Plasma levels of Δ^8-THC in man after smoking 8.3 mg Δ^8-THC in a cigarette as determined by mass fragmentography. (Fig. 8 of Ohlsson *et al.*, NIDA Res. Monograph Series[17] and reproduced with the permission of the publisher, National Institute on Drug Abuse, Rockville, MD 20857.)

same as Δ^9-THC (Fig. 11). The predicted maximum plasma level of 11-OH-Δ^9-THC was 4% of the maximum values of intravenously administered Δ^9-THC which was close to that observed by Wall *et al.*[21] (See Fig. 2).

Complete pharmacokinetic studies as a function of Δ^9-THC i.v. doses with concomitant monitoring of plasma, biliary, urine and fecal levels of drug and metabolites have only been conducted in the dog[6].

These studies were conducted over a large range of intravenous Δ^9-THC doses (0.1–2.0 mg/kg) and showed that plots of plasma-levels (Fig. 5) and urinary and fecal amounts (Fig. 12) of Δ^9-THC and its total metabolites against time are superimposable, indicative of no dose dependencies and no saturable metabolisms, and thus imply that enzymic induction is highly improbable. The terminal phase for the loss of total radioactivity in the plasma (the sum of the derived metabolites of Δ^9-THC) on administration of labeled Δ^9-THC was consistent with a single exponential decay with a 171 hr (7.1 days) half life (Fig. 13). This decay, monitored after the 7000 min time when Δ^9-THC assay sensitivity was too low for evaluation, can be considered to reflect the Δ^9-THC plasma level. At 7000 min, 30% of the drug dose was still in the body.

If it were the slow release of sequestered, bound or distributed metabolites that was responsible for this terminal phase, direct administration of metabolites would show the same pharmacokinetic pattern. However, such a study demonstrated that 91% of intravenously administered biliary metabolites appeared in both bile and urine within

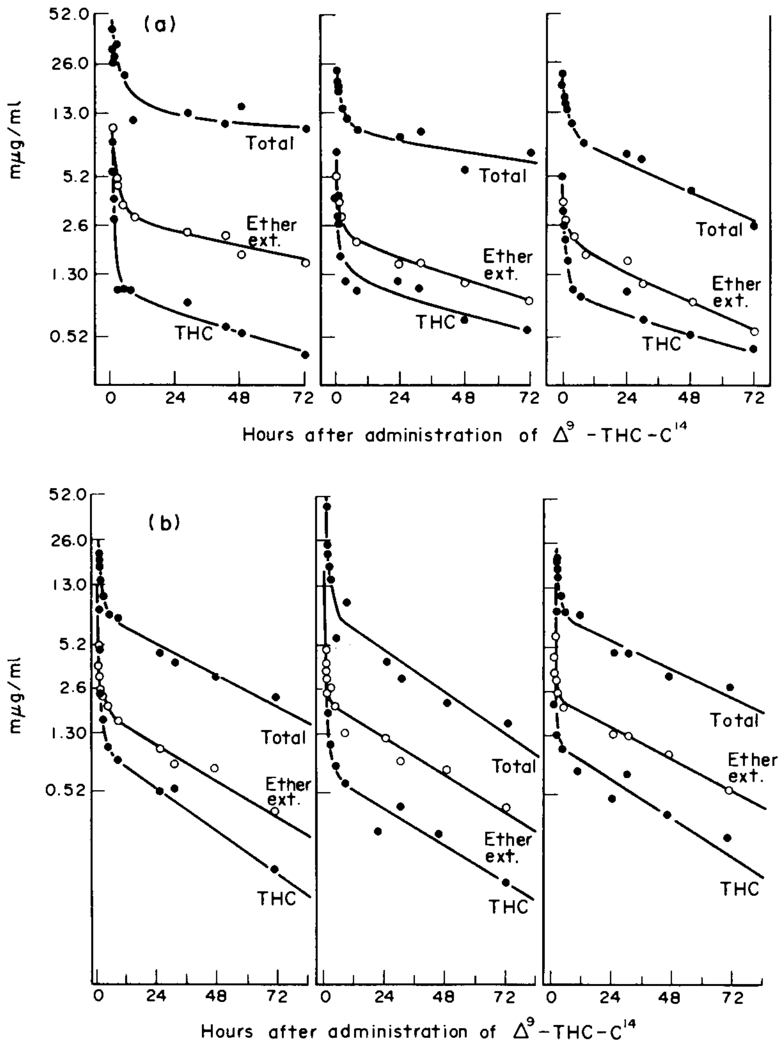


FIG. 4. Plasma levels of Δ^9-THC, total radioactivity, and ether-extractable radioactivity in three marijuana-naive subjects (A) and three chronic marijuana users (B) after i.v. injection of ^{14}C-Δ^9-THC. Values on the ordinate represent Δ^9-THC equivalents calculated from the specific activity of the administered drug (Fig. 1 of Lemberger *et al.*, *Annals N.Y. Acad. Sci.*[14] and reproduced with the permission of the publisher.)

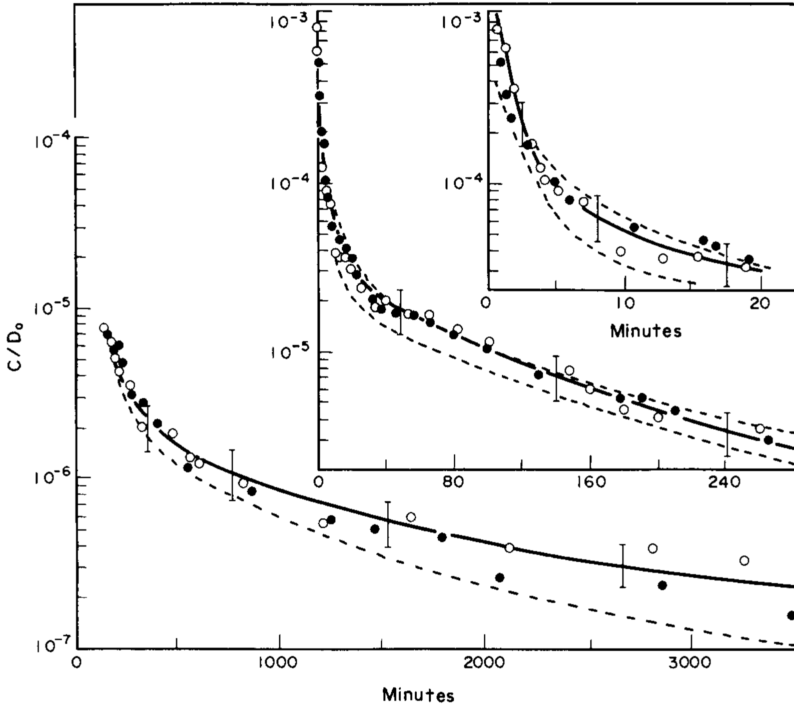


FIG. 5. Semilogarithmic plots of the plasma Δ^9-tetrahydrocannabinol levels as C/D_0, the ratio of concentration per ml of plasma (C) to the dose (D_0), against time for the 0.5-(○) and 2.0-(●) mg/kg i.v. doses of a dog. The solid curve give the calculated values of C/D_0, $\hat{Y}$, for the best fit to a sum of five exponentials for the 0.5 mg/kg data, and the vertical bars are the range for ± 2 SD of the weighted residuals. The dashed lines represent the range for the maximum and minimum of all $\hat{Y}$ values calculated. The concentrations of Δ^9-tetrahydrocannabinol in plasma corresponding to the fraction $C/D_0 = 10^{-4}$ are 635 (○) and 2800 (●) ng/ml. (Fig. 9 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.) Note the superimposability of 0.5 and 2.0 mg/kg data which indicates no dose dependency in the dog.

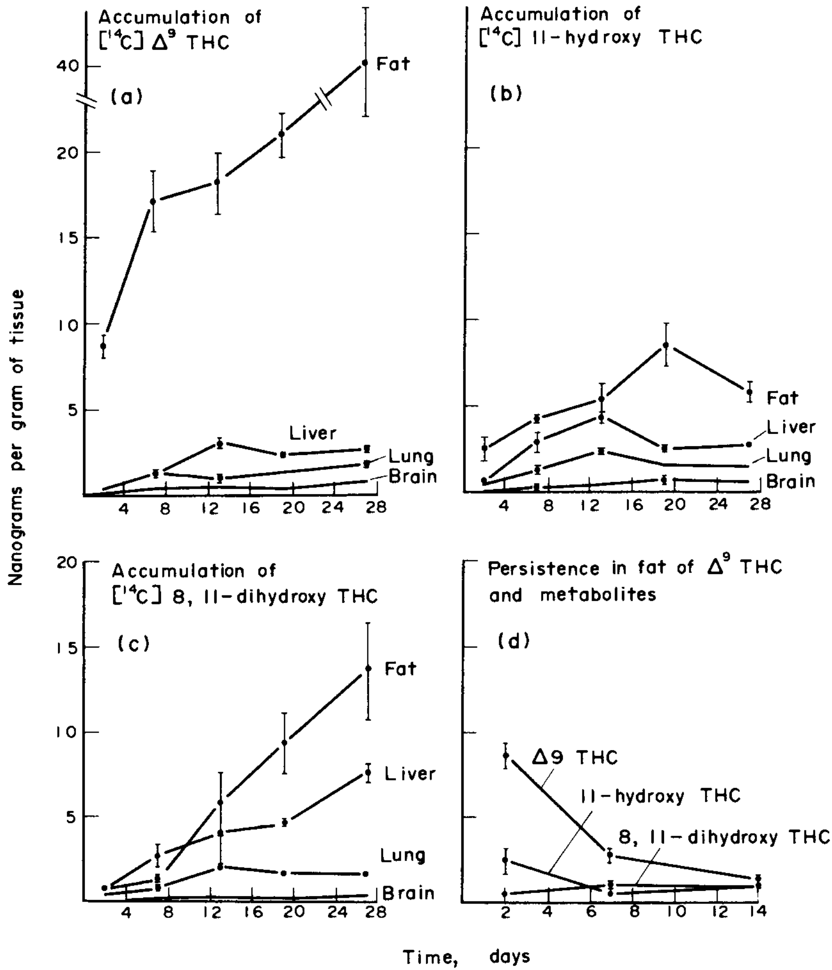


FIG. 6. The distribution of Δ^9-THC, 11-hydroxy-THC, and 8,11-dihydroxy-THC in rat tissues after repeated subcutaneous doses of [^{14}C]Δ^9-THC. (A to C)—The [^{14}C]Δ^9-THC was given every other day for the stated number of days. (D)—A single dose of [^{14}C]Δ^9-THC was given, and tissues were examined at the times indicated. Results are expressed as mean $\pm$ standard error of the mean for four animals at each time point. (Fig. 1 of Kreuz and Axelrod, Science[9] and reproduced with the permission of the copyright owner. *Copyright 1973 by the American Association for the Advancement of Science*).

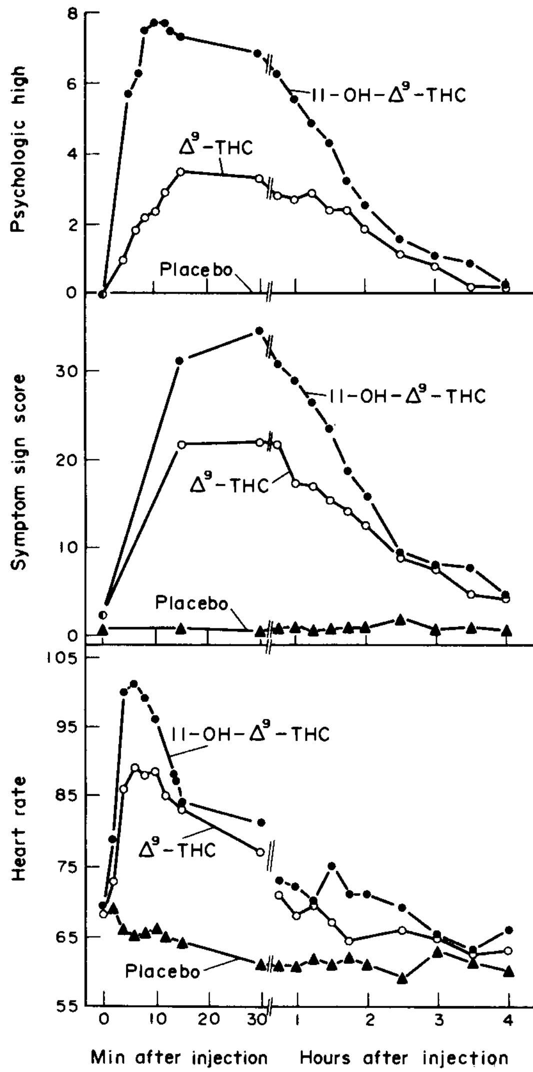


FIG. 7. Comparison of the effects of the intravenous administration of alcoholic vehicle, Δ^9-THC, and 11-OH-Δ^9-THC on psychologic high, symptom sign score, and heart rate. Each point represents the mean value obtained from six subjects to whom the drugs were administered in a double-blind, crossover fashion. (Fig. 3 of Lemberger *et al.*, J. Clin. Inv.[16] and reproduced with the permission of the copyright owner.)

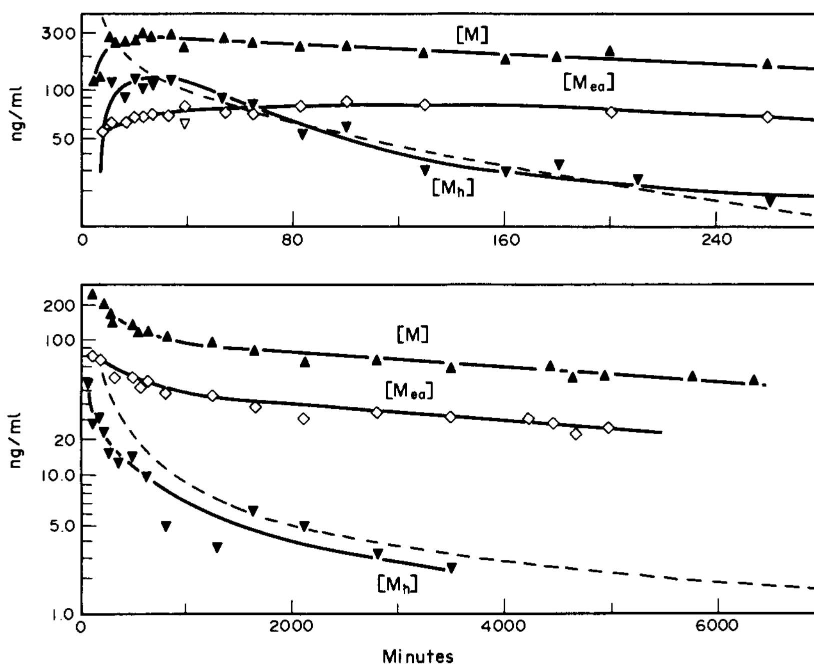


FIG. 8. Semilogarithmic plots of Δ^9-tetrahydrocannabinol metabolites in plasma against time for the 0.5 mg/kg i.v. dose of Δ^9-tetrahydrocannabinol in the dog. The concentration of total metabolites in plasma [M] (▲), are as nanograms per milliliter equivalents of Δ^9-tetrahydrocannabinol. The concentration of heptane-extractable metabolites (at pH 10.5) in plasma, $[M_h]$ (▼), are as nanograms per milliliter equivalents of Δ^9-tetrahydrocannabinol. The concentration of ethyl acetate-extractable metabolites (at pH 2.5; 5 ml of solvent/ml of plasma) in plasma, $[M_{ea}]$ (◇), are as nanograms per milliliter equivalents. The best fit values for C/D_0, $\hat{Y}$ are given as concentration of Δ^9-tetrahydrocannabinol per milliliter of plasma in the dashed curve. (Fig. 12 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

500 min of administration, with 96% eliminated by 1500 min (Fig. 14); the terminal half-life of administered radiolabeled metabolites was 230 min[6]. These facts support the premise that the rate determining step for metabolite elimination of Δ^9-THC administration is not the elimination of such metabolites but the slow return of Δ^9-THC from its sequestration and binding in "deep" compartments to its site of rapid metabolism in the circulating system (Fig. 15).

The logical consequence of these assertions is that the composition of metabolites would remain constant and in pseudo steady state equilibrium. This was confirmed by the constancy of physicochemical properties (various solvent extractions at selected pH values) of biliary (Fig. 16) and urinary (Fig. 17) metabolites with time subsequent to 100–200 min. Additional confirmation was obtained from the constancies of ratios of plasma levels of Δ^9-THC to total metabolites after 3000 min[6, 12].

Δ^9-THC was cleared from the body solely by metabolism and was 124 ± 22.7 ml/min (D_0/AUC) referenced to total plasma concentration for all studies in the dog[6] where hepatic plasma flows are in the range 237–476 ml/min, approximately 2–3 times the metabolic clearance of both bound and unbound drug in the plasma. Since the Δ^9-THC is 97% bound to plasma proteins[4], the hepatic efficiency of unbound drug can be calculated as an

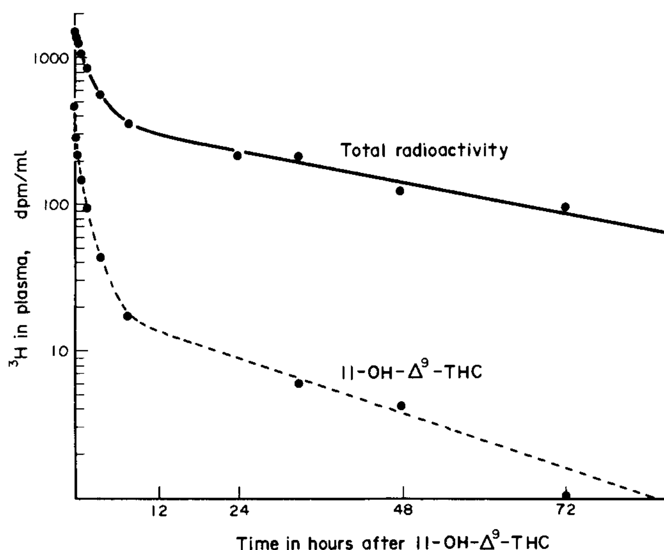


FIG. 9. Concentration of 11-OH-Δ^9 THC and total radioactivity in the plasma after intravenous injection of tritiated 11-OH-Δ^9THC. Unchanged 11-OH-Δ^9THC was measured by extracting the plasma (two times) with four volumes of a mixture of heptane and toluene (1 : 1). The recovery from plasma of authentic 11-OH-Δ^9THC under similar conditions is greater than 90 $\pm$ 5 percent. Radioactivity was assayed by means of liquid-scintillation spectrometry. (Fig. 2 of Lemberger *et al.*, *Science*[15] and reproduced with the permission of the copyright owner. *Copyright 1972 by the American Association for the Advancement of Science.*)

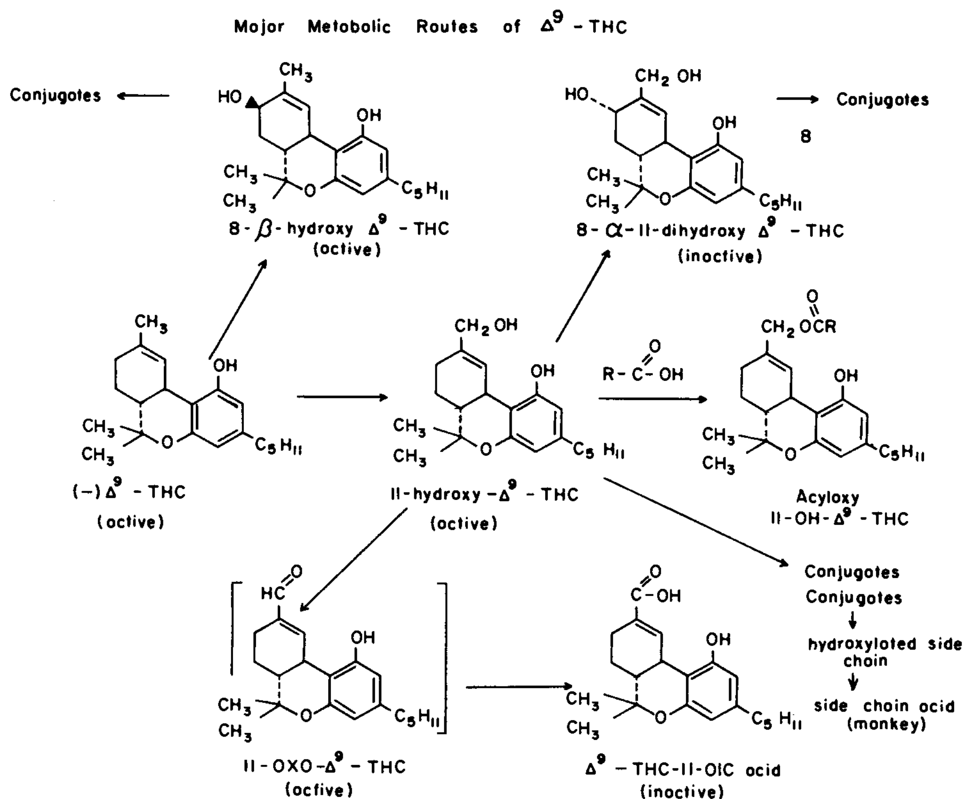


FIG. 10. Probable major metabolic paths of Δ^9-THC.

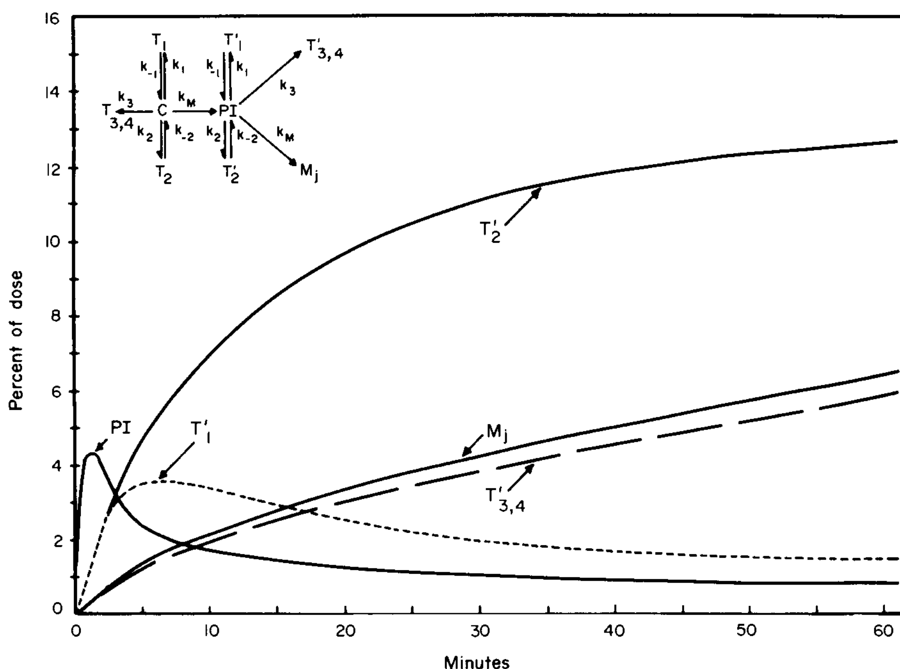


FIG. 11. Predicted time course of a Δ^9-THC active metabolite, PI, such as 11-OH-Δ^9-tetrahydrocannabinol, as generated by an analog computer for the specified scheme. PI is considered the primary intermediate in Δ^9-THC metabolism after intravenous administration of this drug to the dog. The generated sum of subsequent metabolites M_j, and tissue levels, T_1', T_2' and $T_3' + T_4'$ are also given on the presumptions that 11-Δ^9-OH-THC's metabolism and tissue distribution were the same as that experimentally determined for Δ^9-THC in the same animal (Fig. 18 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

astronomical 4132 ± 753 ml/min. This can only be rationalized by an apparent substantial fraction of the bound drug being cleared in a single pass through the liver. The transit time of plasma in the liver is undoubtedly sufficient for a series of reequilibrations between bound and unbound drug with only the former being actually metabolized. The simultaneous appearance in the plasma of non polar and acidic polar metabolites (Figs. 2, 4, 8) must be due to sequential metabolic processes, such as Δ^9-THC → 11-OH-THC → polar metabolites, occurring during a single pass through the liver. The rapidly appearing peak (25–30 min) for metabolites in plasma is consistent with the estimated metabolic half lives of 6.9 min (4.8–10.9 min)[6]. Since Δ^9-THC at the level of analytical sensitivity persisted in the plasma for 7000 min, non-metabolized drug must distribute rapidly into retaining tissues concomitantly with its rapid clearance from the central compartment and it can be calculated that 16–29% as Δ^9-THC is still in the tissue at 100 hrs with 11–20% of the dose in the body as metabolites.

These arguments are contradictory to statements[11, 13, 14] that induced metabolism significantly lessens the terminal half-life of Δ^9-THC in chronic users over those of naive individuals (Fig. 4). If these claimed apparent differences in apparent half-lives truly exist, they could be best explained as resulting in differences of the rates of return to plasma of the rate determining release of drug from deep compartments. Also, extended studies of

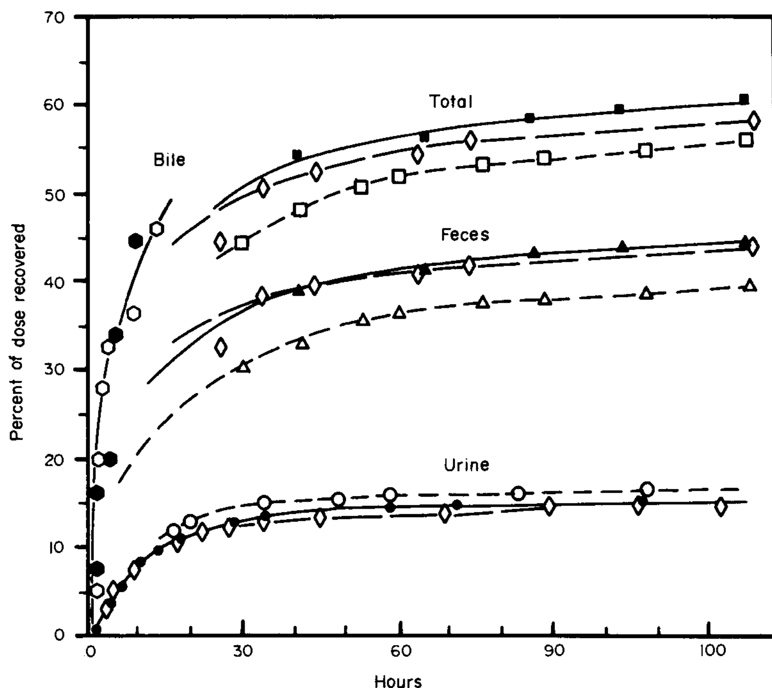


FIG. 12. Linear plots of the percent of dose recovered in urine and feces against time for the two intravenous doses in Dogs A and B. Key: Dog B urine, 0.5 (●) and 2.0 (○) mg/kg; Dog B feces, 0.5 (△) and 2.0 (▲) mg/kg; Dog B total (urine plus feces), 0.5 (□) and 2.0 (■) mg/kg; Dog A urine, feces, and total, 0.1 mg/kg dose (◇). The percent of dose excreted in the sampled bile of Dog A, corrected for the "dead" time of the cannula, is given as ○ for the 0.1 mg/kg dose and as ⬢ for the 2.0 mg/kg dose. The percent of dose was calculated from [Δt × bile flow rate (mg/min) × 100]/dose, where Δt is the time of collection. (Fig. 5 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.) Note the superimposability of the 0.5 and 2.0 mg/kg data which indicates no dose dependency in the dog.

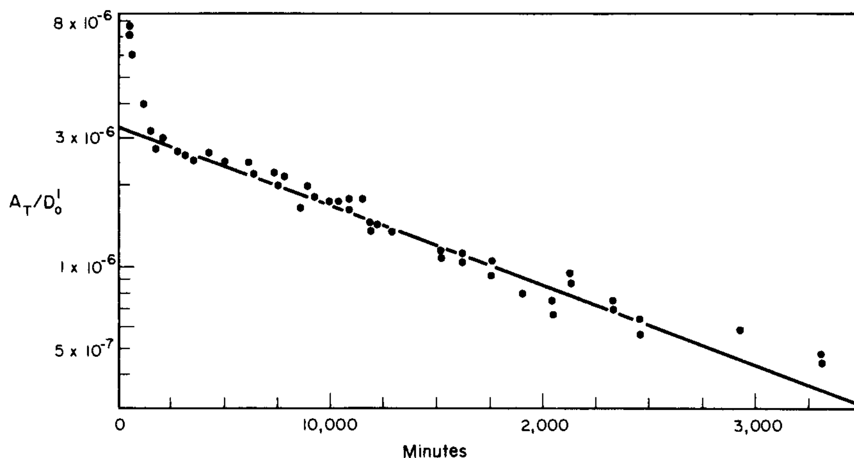


FIG. 13. Semilogarithmic plot of the fraction A_T/D_0' of the total radioactive dose, D_0', per milliliter of plasma against an extended time of study for the 2.0 mg/kg dose of Δ^9-tetrahydrocannabinol in the dog. The apparent rate constant, k_5, obtained by least-squares regression was 6.74×10^{-5} min^{-1} ($t_{1/2} = 7.1$ days). (Fig. 4 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

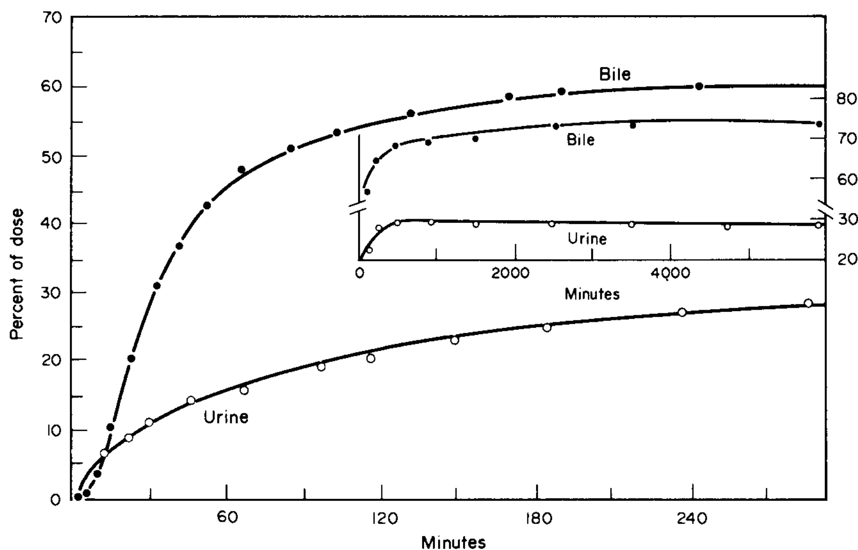


FIG. 14. Linear plots of the percent of the radioactivity recovered in bile (●) and urine (○) against time for the intravenous administration of biliary Δ^9-tetrahydrocannabinol metabolites to Dog C. (Fig. 8 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

Δ^9-THC and metabolites distribution in various animal species have failed to show significant differences[11].

The observed pharmacokinetics are consistent with the observations[9] that drug accumulates in body fat after repetitive administration. Overall values of distribution in the dog are 1500–3000 l referenced to total drug in plasma for 12–17 kg dogs. The wide distribution is clearly shown by the fact that the volume would be 50–100 × 10^3 liters if referenced to unbound concentrations of drug in the plasma! This implies significant differences in the disposition and pharmacodynamics between lean and obese individuals. On the basis of these pharmacokinetic studies in dogs, significant accumulation of drug can be predicted[6] on chronic administration; chronic daily administration would achieve a steady state only after 30 days. The amount of Δ^9-THC in the body would increase 5-fold by 10 days and 10-fold by 27 days over that of the single dose. Thus a pharmacodynamic response could increase with chronic dosing or be maintained for weeks after the cessation of dosing if tolerance did not develop[6].

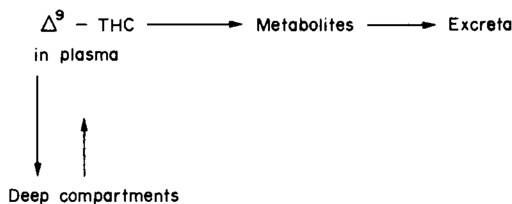


FIG. 15. Symbolic representation of simultaneous rapid metabolism and distribution of Δ^9-THC in the body where ultimately the final rate of excretion of drug and/or metabolites in the body depends on the slow return of unmetabolized Δ^9-THC from deep compartments where it is tightly bound and/or sequestered.

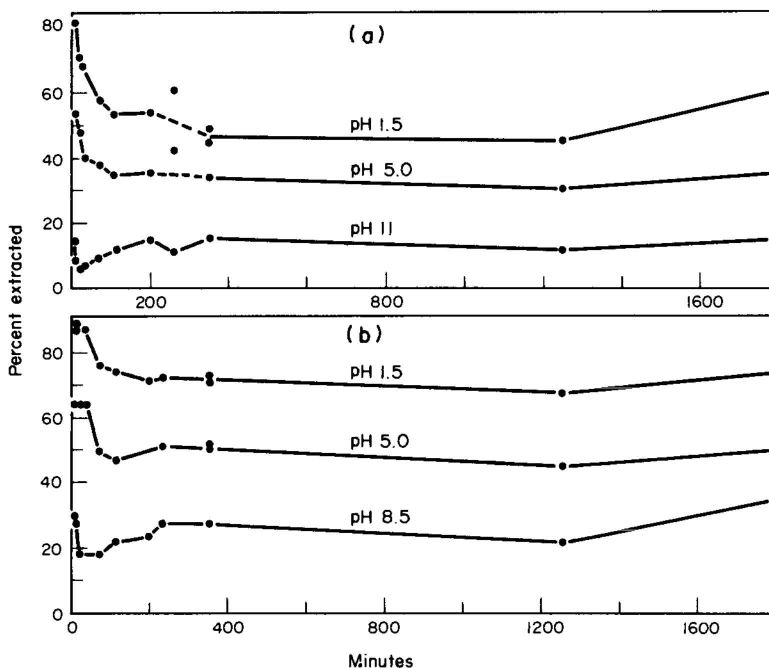


FIG. 16. Percent of biliary metabolites in a given sample that were extracted into benzene (a) or ethyl acetate (b) at selected pH values plotted against time after the administration of 0.5 mg of Δ^9-tetrahydrocannabinol/kg i.v. to a dog. (Fig. 10 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

The percent of an i.v. administered dose in the urine and feces of the normal dog over 5 days was 15–17% and 40–45% respectively[6]. These disposition ratios were similar in the rat, monkey and man[11], but reversed in the rabbit[1]. As explained above, the elimination of the residual must depend on the slow release of sequestered Δ^9-THC from deep tissues. No radioactivity was found in feces on administration of labeled Δ^9-THC to the bile-cannulated dog when all bile was collected[6]. A 10–15% excess recovered in the bile in such a dog compared to feces in the normal dog can be attributed to the enterohepatic recirculation of 15% of the biliary excreted metabolites, probably largely due to the reabsorption of gastrointestinally hydrolyzed biliary secreted conjugates. This excess in the bile compared to that in the feces is clearly shown in Fig. 12. Plasma levels of total radioactivity after 300 min were significantly less for the bile-cannulated dog with total bile collection than for the normal dog[6]. The lack of potential enterohepatic recirculation of metabolites in the former readily explains this difference.

Δ^9-THC has a very limited solubility in 0.15 *M* NaCl, 0.77 mg/liter at 23° and self-emulsifies[4]. It is best administered intravenously, dispersed in the plasma of the subject since the high 97% protein binding performs its own solubilization. Equilibration in a pharmaceutical formulation has been effected with an albumin solution and other macromolecules[11, 19]. Although the RBC/plasma water partition coefficient reflects the adhesive properties of this drug with a value of 12.5, the actual amounts in the RBC's of blood are small due to the highly competitive protein binding with the drug in the plasma-water[4, 6].

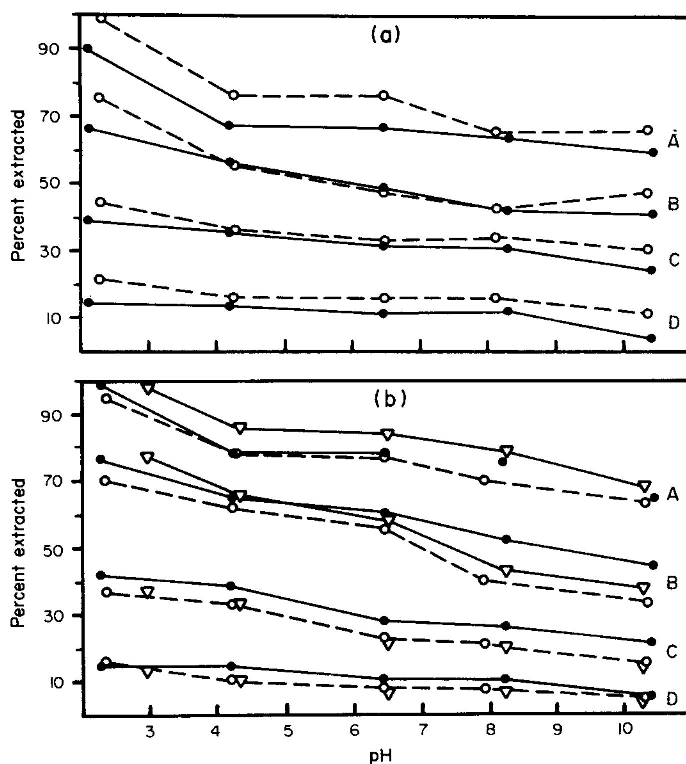


FIG. 17. Plots of the percent of urinary metabolites extracted into various solvents as a function of pH. Aliquots of the urine from the 2.0 mg Δ^9-tetrahydrocannabinol/kg study in a dog were adjusted to the indicated pH and extracted with tetrahydrofuran (A), ethyl acetate (B), heptane containing 1% butanol (C), or heptane containing 1.5% isopentyl alcohol (D). Urine collection intervals in (a) were 50–73 (○) and 133–195 (●) min; in (b), they were 325–381 (●), 646–855 (○), and 1244–1474 (▽) min. (Fig. 11 of Garrett and Hunt, *J. Pharm. Sci.*[6] and reproduced with the permission of the copyright owner.)

Variable results are obtained on i.p. or subcutaneous administration with incomplete absorption due to the difficult solubilization. The efficiency but variability of the pulmonary route which bypasses the liver is well known[11].

The high hepatic clearance of Δ^9-THC *1* readily explains the low oral bioavailability of this compound. This is further compounded by its high susceptibility to strong acid degradation[5, 7] which splits the ether linkage of the molecule to cannabidiol *10*, 6, 12-dihydro-6-hydroxycannabidiol *8* and possibly isocannabidiol *9* (Fig. 18). An equilibration is effected among these compounds in the strongly acid solutions necessary for ether solvolysis. Concomitantly, and also under the milder acidic conditions of pH greater than 3, Δ^9-THC *1* undergoes acid catalyzed hydration of its endocyclic bond to 9-hydroxyhexahydrocannabinol *4* which, in strong acid solution, can give analogs of compounds with the hydrolyzed ether linkages described above. Concomitantly, and also under milder acidic condition, Δ^9-THC is isomerized practically irreversibly to Δ^8-THC *2* which can undergo the same hydrolytic transformations of Δ^9-THC. A final equilibrium can be established among all these compounds except for Δ^9-THC. Thus if these products and their derived metabolites are found in the body it is possible that they may be simple

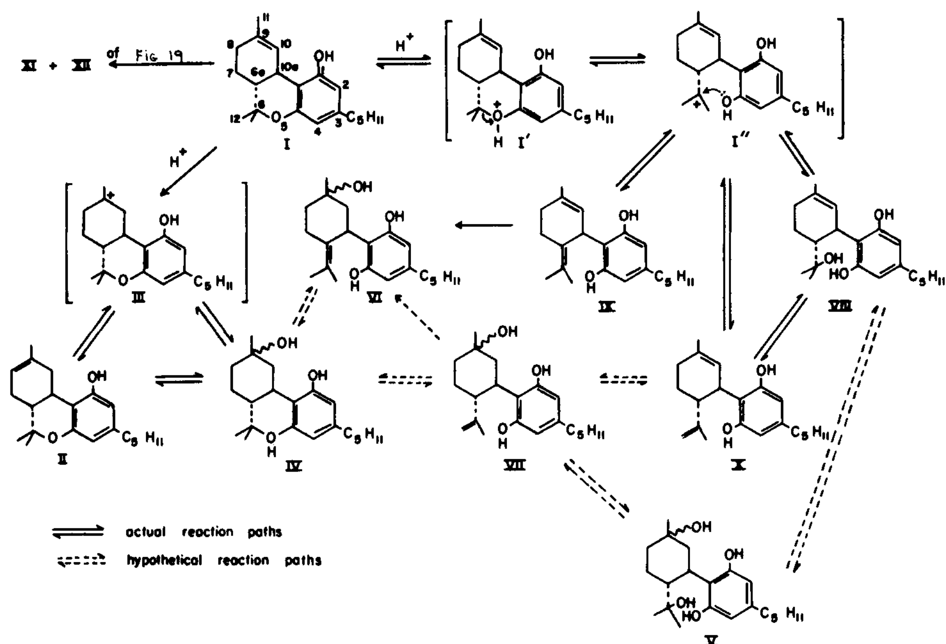


FIG. 18. Solvolytic transformations of Δ^9-THC.

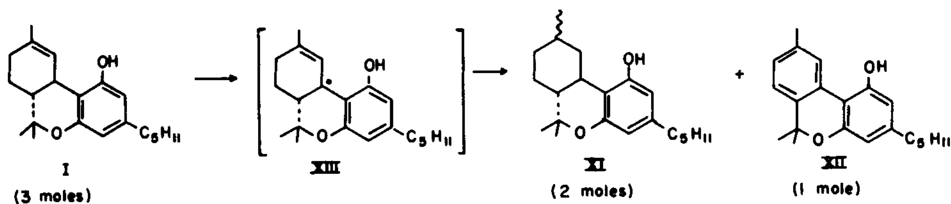


FIG. 19. The catalyzed redox transformation of Δ^9-THC.

chemical consequences of the route of administration or the methods of procedure in the treatment of the biological fluids. Also, the appearance of small amounts of cannabinol and hexahydrocannabinol in the body can be readily ascribed to such technological factors. Mildly acidic treatment in the presence of free radical activations, such as by chloroform or silicic acid, promotes the disproportionation of Δ^9-THC to these compounds (Fig. 19).

ACKNOWLEDGEMENTS

The work reported herein performed by Edward R. Garrett and colleagues was supported by Grant DA-00743 from the *National Institute on Drug Abuse, Rockville, MD 20857.*

REFERENCES

1. AGURELL, S., NILSON, I. M., OHLSSON, A., and SANDBERG, F., On the metabolism of tritium-labeled Δ^1-THC in the rabbit. *Biochem. Pharmacol.* **19**,1333 (1970).
2. CHRISTENSEN, H. D., FREUDENTHAL, R. I., GIDLEY, J. T., ROSENFELD, R., BOEGLI, G., TESTING, L., BRINE, D. R., PITT, C. G. and WALL, M. E. Activity of Δ^8- and Δ^9-tetrahydrocannabinol and related compounds in the mouse. *Science*, **171**,165 (1971).
3. DEWEY, W. L., MARTIN, B. R., BECKNER, J. S., and HARRIS, L. S. A comparison of the subcellular distribution of cannabinoids in the brains of tolerant and nontolerant dogs, rats and mice after injecting radiolabelled Δ^9-tetrahydrocannabinol. *Marijuana: Chemistry, Biochemistry and Cellular Effects* (G. G. Nahas, ed.) Springer-Verlag, New York, p. 349, 1976.
4. GARRETT, E. R. and HUNT, C. A. Physicochemical properties, solubility and protein binding of Δ^9-tetrahydrocannabinol. *J. Pharm. Sci.* **63**,1056 (1974).
5. GARRETT, E. R. and TSAU, J., Stability of tetrahydrocannabinols I. *J. Pharm. Sci.* **63**,1563 (1974)
6. GARRETT, E. R. and HUNT, C. A., Pharmacokinetics of Δ^9-tetrahydrocannabinol in dogs. *J. Pharm. Sci.* **66**,395 (1977).
7. GARRETT, E. R., GOUYETTE, A. J. and ROSEBOOM, H., Stability of tetrahydrocannabinols II. *J. Pharm. Sci.* **67**,27 (1978).
8. GILL, W. E. and JONES, G., Brain levels of Δ^1-tetrahydrocannabinol and its metabolites in mice. *Biochem. Pharmacol.* **21**,2237 (1972).
9. KREUZ, D. S. and AXELROD, J., Delta-9-tetrahydrocannabinol: Localization in body fat. *Science* **179**,391 (1973).
10. LEIGHTY, E. G., FENTIMAN, A. F. and FOLTZ, R. L., Long-retained metabolites of Δ^8- and Δ^9-tetrahydrocannabinols identified as novel fatty acid conjugates, *Research Communications in Chemical Pathology and Pharmacology* **14**,13 (1976).
11. LEMBERGER, L., The metabolism of tetrahydrocannabinols. *Adv. Pharmacol. Chemother.* **10**,221 (1972).
12. LEMBERGER, L., SILBERSTEIN, S. D., AXELROD, J. and KOPIN, I. J., Marijuana: studies on the disposition and metabolism of delta-9-tetrahydrocannabinol in man. *Science* **170**,1320 (1970).
13. LEMBERGER, L., TAMARKIN, N. R., AXELROD, J. and KOPIN, I. J., Delta-9-tetrahydrocannabinol: metabolism and disposition in long-term marijuana smokers. *Science*, **173**,72 (1971).
14. LEMBERGER, L., AXELROD, J. and KOPIN, I. J., Metabolism and disposition of tetrahydrocannabinols in naive subjects and chronic marijuana users. *Ann. N.Y. Acad. Sci.* **191**,142 (1971).
15. LEMBERGER, L., CRABTREE, R. E. and ROWE, H. M., 11-Hydroxy-Δ^9-tetrahydrocannabinol: pharmacology, disposition and metabolism of a major metabolite of marijuana in man. *Science* **177**,62 (1972).
16. LEMBERGER, L., MARTZ, R., RODDA, B., FORNEY, R. and ROWE, H., Comparative pharmacology of Δ^9-THC and its 11-OH-Δ^9-THC metabolite. *J. Clin. Inv.* **52**,2411 (1973).
17. OHLSSON, A., LINDGREN, J.-E., LEANDER, K. and AGURELL, S., Detection and quantification of tetrahydrocannabinol in blood and plasma. *Cannabinoid Assays in Humans.* NIDA Research Monograph Series, No. 7. U.S. Dept. Health, Education and Welfare, Rockville, MD 20852, pp. 48–63 (1976).
18. RYRFELDT, A., RAMSAY, C. H., NILSSON, I. M., WIDMAN, M. and AGURELL, S., Whole-body autoradiography of Δ^1-tetrahydrocannabinol and $\Delta^{1(6)}$-tetrahydrocannabinol in mouse. *Acta Pharm. Suecica*, **10**,13 (1973).
19. SOFIA, R. D., KUBENA, R. K. and BARRY, H., III, Comparison of four vehicles for intraperitoneal administration of Δ^1-tetrahydrocannabinol. *J. Pharm. Pharmac.* **23**,889 (1971).
20. WAHLQUIST, M., NILSSON, I. M., SANDBERG, F. and AGURELL, S., Binding of Δ^1-tetrahydrocannabinol to human plasma proteins. *Biochem. Pharmacol.* **19**,2579 (1970).
21. WALL, M. E., HARVEY, T. M., BURSEY, J. T., BRINE, D. R. and ROSENTHAL, D., Analytical methods for the determination of cannabinoids in biological media. *Cannabinoid Assays in Humans.* NIDA Research Monograph Series, No. 7, U.S. Dept. Health, Education and Welfare, Rockville, MD 20856, pp. 107–117 (1976).
22. WIDMAN, M., NILSON, I. M., NILSSON, J. L. G., AGURELL, S., BORG, H. and GRANSTAND, B., Plasma protein binding of 7-hydroxy Δ^1-tetrahydrocannabinol: An active Δ^1-tetrahydrocannabinol metabolite. *J. Pharm. Pharmac.* **25**,453 (1973).
23. WIDMAN, M., AGURELL, S., EHRNEBO, M. and JONES, G., Binding of (+)- and (−)-Δ^1-tetrahydrocannabinols and (−)-7-hydroxy-Δ^1-tetrahydrocannabinol to blood cells and plasma proteins in man. *J. Pharm. Pharmac.* **26**,914 (1974).

SUMMARY OF SESSION 1

D. J. HARVEY and R. BOURDON

THE first session was concerned with the metabolism and pharmacokinetics of the cannabinoids, the measurement of cannabinoids in body fluids and tissues, and structure-activity relations particularly of the metabolites. The high potency of Δ^1-THC and its rapid plasma clearance necessitate the use of highly sensitive methods for quantitation and the most important of these were reviewed briefly. Several specific assays were described in detail. TLC methods were reported to be sensitive and accurate but not always applicable to studies in humans when for example, radiolabelled cannabinoids are used. GLC assays can achieve high sensitivity (2 ng/ml) but require extensive purification of the sample. Radioimmunoassay offers the widest scope for rapid results, is sensitive to the low ng/ml range but is not selective as there is extensive cross reactivity between, for example, Δ^1-THC, other cannabinoids and several metabolites. This can, however, be advantageous in some clinical and forensic studies where metabolite concentrations are considerable. Where specificity is required, the cannabinoids can be separated by HPLC. A related EMIT assay based on Δ^1-THC antibody inhibition of a THC-mitochondrial malate dehydrogenase complex was described; this was rapid but showed the same limited specificity as the conventional radioimmunoassay. A lower concentration limit of 15 ng/ml was imposed to avoid false positive results. GC—MS methods were reported to be the most specific and sensitive for individual cannabinoids. Although detection limits in the range 0.1–2 ng/ml of plasma are common, extensive sample purification using such techniques as Sephadex LH-20 chromatography limit the number of samples that can be handled. Chemical derivatization is also necessary for high sensitivity with trimethylsilyl derivatives giving the best overall results. Specific assays were presented for Δ^1-THC, CBN, 7-hydroxy-Δ^1-THC and Δ^1-THC-7-oic acid using deuterated internal standards and 3″-hydroxy-Δ^1-THC using the 7-hydroxy metabolite on a standard. A GC—MS method using an 8-channel peak selector was also described for the semi-quantitative measurement of most of the major metabolites of Δ^1-THC present in mouse liver.

Various routes of administration are commonly used for Δ^1-THC but it has been shown that these can lead to widely varying plasma concentrations. This was particularly evident in a study where plasma levels were measured in the same subject after intravenous, oral or pulmonary (smoking) administration. Intravenous infusion gives the most reproducible results for acute studies; pulmonary, subcutaneous, and intraperitoneal routes lead to more variable plasma concentrations because of factors such as difficult solubilization and different rates of absorption. The oral route gives the least predictable plasma levels with

maximum concentrations considerably lower than those achieved by the other routes. Instability of Δ^1-THC at low pH, variable intestinal absorption, and extensive first pass metabolism by the liver seem to be the main factors affecting the ultimate plasma levels. Typical maximum plasma levels in man after an intravenous infusion of 4–5 mg of Δ^1-THC are in the range 50–200 ng/ml. Smoking gives somewhat lower values of around 50–70 ng/ml after 2–3 minutes for an equivalent dose, whereas after an oral dose of 20 mg, peak plasma levels of only 8–10 ng/ml have been recorded and these occur from 0.5 to 1.5 hours after administration. Plasma protein binding is extensive, only about 3% of the circulating Δ^1-THC is in the free state, the remaining 97% is bound to lipoprotein. 7-hydroxy-Δ^1-THC binds extensively to albumin.

Plasma levels drop rapidly after the initial peak, for example in man a peak concentration of 70 ng/ml measured 3 min after inhaling marihuana smoke had dropped to 5 ng/ml at 1 hour and 1 ng/ml at 4 hours. To fit the plasma level-time curves up to five exponentials are needed suggesting at least five body compartments. The terminal elimination phase has a half life of up to seven days. The 7-hydroxy metabolite shows similar kinetics but its peak plasma concentration is only about 4% of that found for the parent drug. Pharmacokinetic data are lacking for most other metabolites but it appears that Δ^1-THC-7-oic acid is eliminated more slowly than the 7-hydroxy metabolite. The observed pharmacokinetics are consistent with rapid uptake of Δ^1-THC into tissues followed by a slow release. Lung, myocardium, adrenal gland and spleen seem to be the main initial sites for accumulation, fat is also important, but the drug accumulates more slowly, probably because of poor vasculature. Only about 1% reaches the brain but it has been suggested that higher concentrations of the 7-hydroxy metabolite may be reached although the brain itself does not have the ability to metabolise the drug.

Metabolism of Δ^1-THC by the liver is rapid and complete with several metabolic steps apparently occurring during the first pass. The liver seems to have a high capacity for metabolising both free and protein bound drug. In dogs, the metabolism shows no dose dependent effects over the range 0.1 to 2.0 mg/kg, no saturable metabolic routes and no enzymic inhibition. In addition, the ratio of metabolites shows little variation with time, consistent with the slow release of Δ^1-THC from tissues. The metabolites are excreted rapidly in faeces and urine, but the extensive retention of unmetabolised drug by the tissues results in slow overall elimination. For example, after five days the dog excretes only about 40–45% of an intravenous dose in the faeces and a further 15–17% in the urine. Similar patterns have been observed in the rat, monkey and man but in the rabbit, the urinary and faecal ratios are reversed. About 10% of the biliary metabolites are enterohepatically recirculated. Only a small percentage of the metabolites appear to be retained by the tissues, for example, in the dog although 16–29% of the administered dose is retained in the tissues after 100 hours only 11–20% of this has been attributed to metabolites.

Δ^1-THC is metabolised mainly by allylic hydroxylation to 7-hydroxy-Δ^1-THC which is subsequently oxidised to Δ^1-THC-7-oic acid. Allylic hydroxylation also occurs in the 6-position and aliphatic hydroxylation in the side-chain can be extensive. All five side-chain carbons can be hydroxylated but the preferred positions show considerable species variation. Although the 3″- and 4″-positions are favoured, the guinea-pig produces relatively large amounts of the 1″-hydroxy metabolite and the mouse hydroxylates extensively at position 2″. Possibly the most characteristic feature of Δ^1-THC metabolism is the very large number of metabolites that are produced. Many of these are substituted with two or three hydroxy, ketone or acid groups and to date some 75 such compounds have been

observed in mammals and microorganisms but not all have yet been reported. Oxidative degradation of the side-chain (β-oxidation) is extensive in species such as the guinea-pig but appears to be less so in mouse and rat. Highly substituted or degraded metabolites have not been observed in Rhesus monkeys or humans and, in addition, the human liver *in vitro* apparently does not produce side-chain hydroxy metabolites; the 7-hydroxy metabolite accounts for most of the metabolic fraction. Other metabolic routes include reduction and epoxidation of the double bond, the latter route being particularly significant for Δ^7-THC where allylic 7-hydroxylation is blocked. Conjugation has been observed with glucuronic acid and a number of aliphatic acids. CBD and CBN are metabolised in a similar way, and when all three major cannabinoids are administered together, as for example in cannabis tincture, the metabolic profiles of each cannabinoid appear to remain unchanged. The effect of deuterum labelling on metabolic profiles was briefly reported, 2″-hydroxylation is inhibited in the mouse by deuterium substitution as predicted but paradoxically, deuterium substitution at the major site of biotransformation, the 7-position, appeared to increase the metabolic rate.

Several of the monohydroxy metabolites show pharmacological activity, sometimes as great as that of Δ^1-THC itself. The 7- and 3″-hydroxy metabolites are particularly active. Whether these contribute significantly to the pharmacological activity of Δ^1-THC is still debatable but in view of the low plasma concentration of the 7-hydroxy metabolite, most of the activity is probably due to Δ^1-THC itself. However, as noted above, the relative concentrations in brain could well be different. In man, at least, it was suggested that Δ^1-THC and possibly its 7-hydroxy metabolite are the only compounds contributing significantly to the overall activity. The pharmacological activity of the side-chain hydroxylated metabolites was estimated by their ability to reduce the response of the electrically stimulated guinea-pig ileum; this effect was shown to correlate well with psychotropic activity. A number of other cannabinoids were tested using this preparation and several cannabinoid methyl ethers and carboxylic acid derivatives were found to be inactive.

The question of whether the action of THC is produced by a relatively non-specific interaction with membranes or a specific interaction with a receptor, or both has still not been answered. Δ^7-THC, a pharmacologically inactive analogue of Δ^1- and Δ^6-THC was proposed on a suitable control compound for differentiating between these modes of action as it is sterically similar to the other THC's. It was argued that because certain metabolites, particularly the side-chain alcohols, were active, with a clear activity maximum with 3″-hydroxy-Δ^1-THC, that a receptor must be involved. However as membrane interactions can also show similar specificity the existence of a receptor must still remain not proved.

RÉSUMÉ

I—DOSAGE

Dans les liquides biologiques, les concentrations atteintes par les divers cannabinoïdes sont généralement de l'ordre de quelques nanomoles. C'est pourquoi le dosage de chacun d'eux s'effectue préférentiellement par couplage CPG-SM: les résultats obtenus ainsi par diverses équipes sont remarquablement concordants.

Lors d'un screening systématique, on peut faire appel aux méthodes radioimmunologiques, bien que, dans le plasma comme dans les urines, les divers cannabinoïdes interfèrent avec le THC (vis à vis de son anticorps.).

Cette méthode a été utilisée avec succès en Angleterre au cours d'études de biodisponibilité, ou pour détecter les emplois illicites, notamment chez les conducteurs impliqués dans des accidents de la circulation.

Enfin les méthodes immunoenzymologiques en phase homogène type EMIT prennent une importance grandissante.

Il demeure qu'une méthode simple et rapide permettant de doser le THC dans les liquides biologiques reste à établir: tache difficile en raison de la disparition rapide du THC plasmatique et de son absence dans l'urine.

II—PHARMACOCINÉTIQUE

Quelques points doivent être soulignés:

—la cinétique du Δ^9 THC qui ne dépend pas de la dose administrée est caractérisée par la décroissance multiexponentielle de la concentration plasmatique: cette évolution est liée au fait que les vitesses de pénétration et de sortie relatives à chacun des nombreux compartiments diffèrent entre elles.

—le volume de distribution du Δ^9 THC est particulièrement élevé et voisin de 50×10^3 litres.

—sa demi vie apparente dans les tissus est voisine de 7 jours. En conséquence on observe une importante accumulation. Ainsi après 27 jours d'administration, les liquides biologiques contiennent 10 fois plus de cannabinoïdes qu'après administration d'une seule dose.

—le Δ^9 THC est complètement métabolisé après fixation par le foie. Au cinquième jour, les métabolites éliminés par l'urine représentent 15 % de la dose administrée; 40 à 45 % de celle-ci d'abord excrétés par la bile sont finalement éliminés par les feces. Il existe de plus un cycle entérohépatique portant sur 15 % de l'excrétion biliaire.

—la biodisponibilité du THC est très différente selon la voie d'administration:

la moitié du THC introduit dans une cigarette atteint le sang circulant lors de la combustion; ainsi, sous cette forme, 5 mg de THC conduisent à une concentration plasmatique maximale de quelque 100 nanogrammes/ml à la cinquième minute.

par contre après ingestion d'une dose de 20 mg, la concentration plasmatique maximale n'est que de 10 nanogrammes/ml à la première heure.

Sous forme de fumée, la biodisponibilité du THC est ainsi plus de 10 fois supérieure à celle de l'administration per os.

A. Isolated Cell Preparations

INHIBITORY EFFECTS OF DELTA-9-TETRAHYDROCANNABINOL AND OTHER PSYCHOTROPIC DRUGS ON CULTURED LYMPHOCYTES

JACQUES BANCHEREAU, BERNARD DESOIZE, COLETTE LÉGER and GABRIEL NAHAS

Department of Anesthesiology, College of Physicians and Surgeons, New York, N.Y.

and

I.N.S.E.R.M., Laboratoire de Pharmacologie et de Toxicologie Cellulaires, Hôpital F. Widal, Paris

Summary. Delta-9-tetrahydrocannabinol (THC) depresses *in vitro* phytohemagglutinin (PHA) induced lymphocyte transformation as measured by ^{3}H-thymidine and ^{3}H-uridine uptake. Cells are harvested after 3 days of culture when there is maximum thymidine uptake and when there is still enough glucose in the medium to meet energy requirements. Care is exerted to disperse the THC-ethanol solution uniformly in the culture, and tritiated THC is used to verify the drug's presence in the medium. Ethanol, in concentrations used in these experiments, does not alter thymidine uptake. The respective concentration of protein (serum) and cells in the culture medium does influence the extent of THC inhibition. With 20% serum in the medium, a concentration of 1.6×10^{-4} M THC is required to inhibit by 50% the thymidine uptake (IC 50) occurring in a parallel control culture; with 5% serum in the medium the IC 50 is 3.5×10^{-5} M.

Other commonly used lipophilic psychotropic drugs such as diazepam, imipramine, diphenylhydantoin, chlordiazepoxide exert a similar inhibition on thymidine uptake of cultured lymphocytes; haloperidol (IC 50 = 1×10^{-5} M) has a higher cytotoxicity than THC; phenobarbital and meprobamate are less toxic (IC 50 = 10^{-3} M).

THE interference of cannabinoids with immunological mechanisms has been the subject of numerous studies (Table 1). Some authors reported a decrease in T cell mediated immunity and B cell numeration in street marihuana smokers (Cushman and Khurana, 1975; Cushman and Khurana, 1977; Nahas *et al.*, 1974). Others (Silverstein and Lessin, 1974; White *et al.*, 1975; Lau *et al.*, 1975; Rachelefski *et al.*, 1977) did not duplicate these results. However, studies in animals have substantiated a depressive effect of THC and marihuana on their immune response (Armand *et al.*, 1974; Daul and Heath, 1976; Levy *et al.*, 1975; Rosenkrantz *et al.*, 1976). Furthermore, studies describing the inhibitory effects of cannabinoids on leucocyte motility (Schwartzfarb *et al.*, 1974) or on pulmonary macrophages (Gaul and Mellors, 1975; Mann *et al.*, 1971) have not been challenged.

TABLE 1. CANNABINOID EFFECTS ON THE CELLULAR-MEDIATED AND HUMORAL IMMUNE RESPONSES

Cannabinoid	Dose (mg/kg)	Route	Species	Cell type and source	Mitogen or antigen
					Route
Cellular-mediated immune response					
Marihuana	4 × /wk[a]	Lungs	Man	Lymphocyte, blood	*In vitro*
	4 × /wk	Lungs	Man	Lymphocyte, blood	*In vitro*
	3 × /wk	Lungs	Man	Lymphocyte, blood	*In vitro*
	3 × /wk	Lungs	Man		intraderm.
	1 × /wk	Lungs	Man	Macrophage, alveoli	None
	1 × /wk	Lungs	Man	Lymphocyte, blood	*In vitro*
	> 6 × /wk	Lungs	Man	Lymphocyte, blood	*In vitro*
	15 × /wk	Lungs	Monkeys	Lymphocyte, blood	*In vitro*
Marihuana ext.	2 μg/ml	*In vitro*	Man	Leukocyte, blood	None
Δ^9-THC	2–22 μM	*In vitro*	Man	Lymphocyte, blood	*In vitro*
		In vitro	Rat	Lymphocyte, spleen	*In vitro*
	9×10^{-7} M	*In vitro*	Man	Lymphocyte, blood	*In vitro*
	2×10^{-4} M	*In vitro*	Man	Lymphocyte, blood	*In vitro*
	210 × 14 days	Oral	Man	Lymphocyte, blood	*In vitro*
	1.6×10^{-5} M	*In vitro*	Mouse[g]	Lymphocyte, spleen	*In vitro*
	50–200	Oral	Mouse	Lymphocyte, spleen	*In vitro*
	50–200	Oral	Mouse		Skin
	0.3–1.3	IP	Rat	Macrophage, perit.	Intraderm.
	10.4×10^{-4}	*In vitro*	Man	Lymphocyte, blood	*In vitro*
Humoral immune response					
Marihuana	3 × /wk	Lungs	Man	Lymphocyte, blood	*In vitro*
Δ^9-THC	140	IP	Mouse	Lymphocyte, spleen	*In vitro*
	25–200	Oral	Mouse	Lymphocyte, spleen	IP
	1–10	Oral	Rat	Lymphocyte, spleen	*In vitro*
Marihuana	15 × /wk	Lungs	Monkey	Blood	IP
	> 6 × /wk	Lungs	Man	Blood	*In vitro*

a. Time since last use of marihuana variable.
b. SR BC = sheep red blood cells; PHA = phytohemagglutinin; MLC = mixed lymphocyte culture (allogeneic cells); 2,4-DNCB = 2,4-dinitrochlorobenzene.
c. Leucocytes from both marihuana smokers and nonsmokers responded similarly.
d. Inhibition also induced by cannabinol and cannabidiol.
e. A decreased incorporation of ^{3}H-leucine and ^{3}H-uridine was also seen.

TABLE 1—*Continued*

Mitogen or antigen Type	Methodology	Finding	Reference
SR BC[b]	T-cell rosette	Inhibition	Cushman and Khurana, 1977; 1975
PHA; MLC[b]	^{3}H-thymidine incorp.	Inhibition	Nahas *et al.*, 1974
PHA	^{3}H-thymidine incorp.	No change	White *et al.*, 1975
2,4-DNCB[b]	Skin test	No change	Silverstein and Lessin, 1974
None	Morphology	Change	Mann *et al.*, 1971
SRBC; PHA	T cell rosette, % lymphocyte stimulation	Inhibition[j]	Petersen *et al.*, 1976
PHA, MLC	^{3}H-Thymidine incorp.	No change	Rachelefski *et al.*, 1976
Con A		Inhibition	Daul and Heath, 1976
None	Migration	Inhibition[c]	Schwartzfarb *et al.*, 1974
PHA	^{3}H-thymidine incorp.	Inhibition	Armand *et al.*, 1974
PHA	^{3}H-thymidine incorp.	Inhibition	Armand *et al.*, 1974
SR BC	T-cell rosette	Inhibition[d]	Cushman and Khurana, 1975
PHA; MLC	^{3}H-thymidine incorp.[e]	Inhibition[f]	Desoize *et al.*, 1975
PHA	^{3}H-thymidine incorp.	No change	Lau *et al.*, 1975
Δ^9-THC	^{3}H-thymidine incorp.	Stimulation	Nahas *et al.*, 1973
PHA	^{3}H-thymidine incorp.	Inhibition	Levy *et al.*, 1975
Allograft	Graft-survival	Increased	Levy *et al.*, 1975
Adjuvant	Migrat. inhib. factor	Suppressed	Gaul and Mellors, 1975
PHA	^{3}H-thymidine incorp.	No change	Rachelefski and Opelz, 1977
SR BC-hemolysin[h]	B-cell rosette	No change	Cushman and Khurana, 1977
Pokeweed	^{3}H-thymidine incorp.	No change	White *et al.*, 1975
SR BC	Plaque formation	Inhibition	Lefkowitz and Yang, 1975
Lipopoly B[i]	^{3}H-thymidine incorp.	Inhibition	Levy *et al.*, 1975
SR BC	Plaque formation Serum antibodies	Inhibition	Rosenkrantz *et al.*, 1976
	Immunoglobulins	Decreased	Daul and Heath, 1976
	Immunoglobulins A, M, D Immunoglobulin E	No change Increased	Rachelefski *et al.*, 1976

f. Inhibition also obtained with various cannabinoids and 11-OH metabolites.
g. Sensitized to Δ^9-THC.
h. Trypsinized sheep red blood cells coated with antisheep hemolysin for detection of B-cell C_3 receptor.
i. E coli lipopolysaccharide B.
j. Effects of marihuana smoking on T cells during a 24 hour period after smoking cells are inhibited in the first 6 hours.

This table is a revision of the one compiled by H. Rosenkrantz in *Marihuana: Chemistry, Biochemistry and cellular effects*, p. 443, G. G. Nahas, W. D. M. Paton and J. E. Idänpään-Heikkilä, eds. Springer-Verlag. New York, 1976.

Others investigators (Levy *et al.*, 1975; Petersen *et al.*, 1976) have substantiated the *in vitro* depressive effects of cannabinoids on the thymidine incorporation in cultured lymphocytes first reported by Nahas *et al.* (1974). An inhibitory action of cannabinoids on cell proliferation and on synthesis of macromolecules in eucaryote cells was also observed by several investigators in normal as well as abnormal cell lines (Blevins and Regan, 1976; Huot, 1976; Carchman *et al.*, 1976; Bram and Brachet, 1976) with concentrations of 10^{-6} to 10^{-4} M. However, one report by Rachelefski and Opeltz (1977) claims that "delta-9-THC in concentrations of 0.6×10^{-4} to 10.6×10^{-4} M has no effect on DNA synthesis of resting normal human lymphocytes or on their responses to PHA or to allogeneic cells".

In the present paper additional studies describing the inhibitory effects of THC on thymidine and uridine incorporation in cultured lymphocytes are reported; special emphasis is placed on the techniques used in these experiments, since differences in the techniques might account for differences in results. Furthermore, other liposoluble psychotropic drugs known to act on the double lipid layer of the plasma membrane are studied on the same model: imipramine, diazepam, chlordiazepoxide, haloperidol, phenobarbital, diphenylhydantoin and meprobamate.

MATERIALS AND METHODS

CULTURE PREPARATION

The microculture technique used is that of Hartzman *et al.* (1971). Venous blood from healthy male donors is collected under heparin (from beef intestinal mucosa, Upjohn Co., 50 IU/ml). Mononucleated cells are collected according to the technique of Boyum (1968) by density gradient centrifugation with Ficoll-Isopaque. The cells are washed 3 times and suspended in RPMI 1640 with penicillin and streptomycin. Contamination with polymorphonuclear cells is less than 5%. Except for a few experiments, the cell concentration was 10^6 cells per ml. Cell suspension is transferred with an automatic Hamilton syringe into the wells of disposable culture plates. Each well is filled with 0.2 ml of suspension (which corresponds to 2×10^5 cells per well) and 0.05 ml of pooled human serum, or with 0.05 ml of a dilution of this serum with RPMI. Phytohemagglutinin (PHA) is added to a final concentration of 4 μg/ml (except when mentioned). The cells are incubated at 37°C for a few hours to 7 days in an incubator saturated with water vapor containing 5% CO_2. Cellular division is evaluated by measuring ^{3}H-thymidine uptake (2 Ci/mM, New England Nuclear). ^{3}H-thymidine is added 2 to 6 hours before harvesting. Uridine (25 Ci/mM, New England Nuclear) is added at the onset of the culture.

HARVESTING AND MEASUREMENT OF RADIOACTIVITY

Cell death is determined by the cells' inability to exclude trypan blue, or with a phase contrast microscope. When a higher ratio than usual of cellular death is found in the control cultures, results are discarded. Microscopic examination also allows for a morphological examination of lymphoblastic transformation. Cells are harvested with a Multiple Automatic Samples Harvester (MASH) described by Hartzman *et al.* (1971). Filters are dried and transferred into vials for scintillation counting. Cultures are generally harvested

at the 72nd hour after onset, unless otherwise specified. Thirty experiments were performed with lymphocyte samples from 9 healthy male donors.

Uptake of radioactivity in cells is measured after washing with isotonic saline. Incorporation of radioactivity in DNA and RNA is measured after washing twice with 5% trichloracetic acid and methanol.

Thymidine uptake varies considerably in lymphocytes sampled at different times from the same donor, and from one donor to the next (from 10,000 to 70,000 cpm). For this reason, and in order to express results obtained in a uniform and comparative fashion, the uptake of the precursor (cpm) into the test culture was compared with its uptake into a parallel control culture. Results were then expressed in terms of "percentage of uptake" into the parallel control cultures. Dose-response curves were performed and the concentration of drug required to inhibit 50% of uptake of precursor was determined (IC 50).

PREPARATION OF THC AND OTHER PSYCHOTROPIC DRUGS

THC is available in ethanol solution, containing 20 mg/ml (NIDA). This ethanol solution is first evaporated and serum added according to the technique of Perez-Reyez *et al.* (1972). Final concentrations of THC vary from 10^{-5} to 4.7×10^{-4} M.

The following drugs dissolved in alcohol (same final concentration as for THC) were also tested: imipramine, diazepam, chlordiazepoxide, haloperidol, phenobarbital, diphenylhyandtoin, meprobamate.

INTERACTION OF THC WITH PHA

Several experiments were performed. In one, THC was added to the medium 30 minutes before, at the same time, or 30 minutes after the addition of PHA to the culture. In another, THC was incubated with PHA for two hours at 37°C before being added to the culture. In a third, cultures were incubated with increasing concentrations of PHA in the absence of or in the presence of THC.

FATE OF THC IN CULTURE

In some experiments, tracer amounts of tritiated THC (100 mCi/mg) were added to the culture at the onset and after 72 hours of culture, and radioactivity measured both times.

Cold THC was also extracted from the culture medium, shortly after and following 72 hours of culture. Chromatographs were made with the original THC alcoholic solution and THC extracted at the onset and after 72 hours.

INTERACTIONS OF THC WITH SERUM OR CELLS

The effect of an increasing number of cells on thymidine uptake was studied in the presence of the same THC concentration; the effect of varying serum concentration from 0 to 20% in the culture medium on thymidine was also investigated. Different fractions of the serum were tested in the cultures to find out which one interacts with the drug.

TABLE 2a. INHIBITORY EFFECT OF DELTA 9 THC ON THYMIDINE UPTAKE BY CULTURED LYMPHOCYTES STIMULATED WITH PHA. SERUM CONCENTRATION IN CULTURE IS 20%. EACH EXPERIMENT PERFORMED IN QUADRUPLICATE. RESULTS REPRESENT THE MEAN ± STANDARD ERROR.

		Control	THC Concentration					"ID 50"
			4.7×10^{-5} M	1.17×10^{-4} M	1.76×10^{-4} M	2.35×10^{-4} M	4.70×10^{-4} M	
Exp 1	cpm	26555 ± 1805	27174 ± 461	21919 ± 566		2781 ± 122	259 ± 46	1.62×10^{-4} M
	(%)*		102.3%	82.5%		2.9%	0.9%	
Exp 2	cpm	14089 ± 743	21938 ± 1319	14691 ± 1053		2712 ± 525	318 ± 65	1.90×10^{-4} M
	(%)*		155.7%	104.2%		19.2%	2.3%	
Exp 3	cpm	24922 ± 1230	25606 ± 2320	21577 ± 772	8285 ± 492	2113 ± 27	368 ± 88	1.55×10^{-4} M
	(%)*		102.7%	86.5%	33.25%	8.4%	1.4%	
Exp 4	cpm	51417 ± 2561	43017 ± 3681	29439 ± 581	559 ± 142	285 ± 60	272 ± 70	1.25×10^{-4} M
	(%)*		83.6%	57.2%	1.0%	0.5%	0.5%	
Mean			111.0% ± 15.5	82.6% ± 9.7	17.7% ± 16.1	7.7% ± 4.1	1.3% ± 0.3	$1.58 \pm 0.13 \times 10^{-4}$ M

* of parallel control culture

TABLE 2b. INHIBITORY EFFECT OF DELTA 9 THC ON URIDINE UPTAKE BY CULTURED LYMPHOCYTES STIMULATED WITH PHA. SERUM CONCENTRATION IN CULTURE IS 20%. EACH EXPERIMENT PERFORMED IN QUADRUPLICATE. RESULTS REPRESENT THE MEAN ± STANDARD ERROR.

		Control	THC Concentration					"ID 50"
			4.7×10^{-5} M	1.17×10^{-4} M	1.76×10^{-4} M	2.35×10^{-4} M	4.70×10^{-4} M	
Exp 1	cpm	22550 ± 320	26849 ± 324	24074 ± 505		1703 ± 234	158 ± 7	1.80×10^{-4} M
	(%)*		119%	106.7%		7.5%	0.7%	
Exp 2	cpm	23116 ± 2389	26450 ± 797	15675 ± 2357	9082 ± 2513	940 ± 38		1.55×10^{-4} M
	(%)*		114.4%	67.8%	39.2%	4%		
Exp 3	cpm	27627 ± 881	20766 ± 1960	20653 ± 1265	490 ± 36	418 ± 32	496 ± 51	1.35×10^{-4} M
	(%)*		75.7%	75.3%	1.8%	1.5%	1.8%	
Mean			103% ± 13.7	83.2% ± 11.9	20.5% ± 18.7	4.3% ± 1.7	1.2% ± 0.5	$1.56 \pm 0.13 \times 10^{-4}$ M

* of parallel control culture

MEASUREMENTS OF GLUCOSE, LACTATE AND PROTEIN IN THE CULTURE MEDIUM

At different time intervals, 0.1 ml of the microculture supernatant is removed for measurement of glucose and lactate. Cells are resuspended in the remaining medium, washed three times with phosphate buffered saline dissolved in NaOH, and protein concentration is evaluated according to the Lowry technique. Glucose concentration is measured with the Orthotoluidin technique. Lactate concentration is evaluated with the LDH technique (Boehringer Mannheim Kit). All of these experiments are performed in triplicate or quadruplicate.

RESULTS

UPTAKE AND INCORPORATION OF PRECURSORS

Inhibition of cellular uptake of thymidine (Table 2a) and uridine (Table 2b) is similar to the inhibition of their incorporation in the macromolecules DNA and RNA.

FATE OF THC IN THE CULTURE

After adding ^{3}H-THC to the culture, radioactivity measured at onset and after 72 hours of culture is the same. Chromatographs of the original THC solution and of THC extracted at onset of culture and 72 hours after were compared. A red coloration characteristic of delta-9-THC was present with the three samples. The Rf were identical for the three samples, with each of three different solvents: hexane-petrol ether (Rf = 0.47), Chloroform (Rf = 0.61), dimethylformamide (Rf = 0.83).

INTERACTIONS THC-ETHANOL

Ethanol inhibits blastogenesis at a concentration greater than 1% (Table 3). In all experiments, final concentration of ethanol was never higher than 0.5%, a dose that does not inhibit cell growth (Koch and Koch, 1974).

TABLE 3. EFFECTS OF ETHANOL ON PHA INDUCED LYMPHOCYTE TRANSFORMATION AS MEASURED BY H^3 THYMIDINE UPTAKE AFTER 3 DAYS OF CULTURE. EACH VALUE REPRESENTS THE MEAN ± S.E. OF FOUR EXPERIMENTS.

Ethanol Concentration (%)	(Moles)	^{3}H-thymidine uptake (% of control cultures)
0.02	4.3×10^{-3}	108.8 ± 4.2
0.1	2.2×10^{-2}	101.6 ± 2.3
0.2	4.3×10^{-1}	102.6 ± 2.1
0.5	1.1×10^{-1}	110.2 ± 5.8
1.0	2.2×10^{-1}	93.7 ± 5.2
1.5	3.3×10^{-1}	58.8 ± 3.6
2.0	4.3×10^{-1}	35.6 ± 2.8
5.0	1.1	18.6 ± 2.2

TABLE 4. THC-ETHANOL INTERACTION ON PHA INDUCED LYMPHOCYTE TRANSFORMATION AS MEASURED BY H^3-THYMIDINE UPTAKE AFTER 3 DAYS OF CULTURE.

THC ($\times 10^{-4}$ M)	Ethanol ($\times 10^{-2}$ M)	^{3}H-Thymidine uptake	
		With ethanol (Mean of 11 exp. ± S.E.)	Without ethanol (Mean of 3 exp. ± S.E.)
1.6	4.3	59.9 ± 4.3	57.3 ± 3.7
2.2	7.6	26.9 ± 4.1	27.2 ± 2.9
2.5	8.7	13.3 ± 4.8	15.4 ± 3.1

When ethanol was evaporated from the THC solution, and the drug diluted with serum, the effect of THC on thymidine uptake was comparable (Table 4).

INTERACTION THC-PHA

The same degree of inhibition is observed when THC is added to the medium 30 minutes before, at the same time, or 30 minutes after the addition of PHA. Preincubation of THC

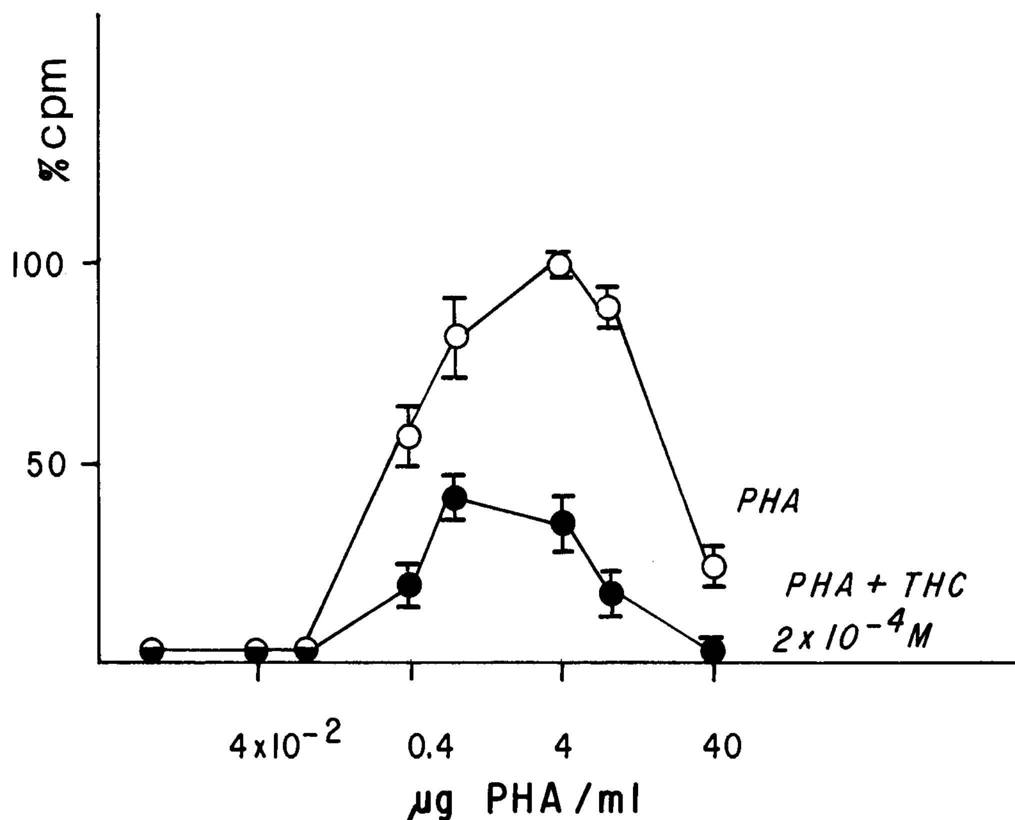


FIG. 1. ^{3}H-thymidine incorporation in lymphocytes incubated with increasing concentrations of PHA, with or without THC (2×10^{-4} M). Each point is the mean of 4 different experiments, ±S.E.

with PHA during 2 hours at 37°C does not alter the inhibitory effect of THC on cells.

The dose-response curve relating PHA concentration to thymidine uptake is illustrated in Fig. 1. Lymphocyte transformation is a function of PHA concentration up to 4 μg/ml of mitogen. With higher doses, PHA becomes toxic to the cells. The stimulatory effect of the mitogen is offset by its toxic effect. The dose response curve relating the combined effects of PHA and THC (2×10^{-4} M) on thymidine uptake follows a similar pattern with a maximal response for dosages of PHA ranging from 1 to 4 μg/ml. The latter dose was selected in the present experiments.

INTERACTIONS THC-SERUM AND THC-CELLS
(Figure 2, Tables 5 and 6)

^{3}H-thymidine uptake is negligible in the presence of THC (1.6×10^{-5} M) when serum is not present in the culture medium (Fig. 2). As serum concentration increases from 0 to 1%, the rate of blastogenesis increases. With serum concentrations exceeding 10% the rate of thymidine uptake slows down and reaches an asymptote with 20% serum concentration. This concentration was used in the experiments where effects of THC on uridine and thymidine incorporation were studied.

Of all the different fractions of serum tested, only native α-1-lipoprotein interacted with THC and antagonized its inhibitory effect on thymidine uptake (Table 5).

The inhibition of thymidine by THC (1.76×10^{-4} M) is a function of cell concentration (Table 6). The greater the number of cells, the lower the toxicity of the drug.

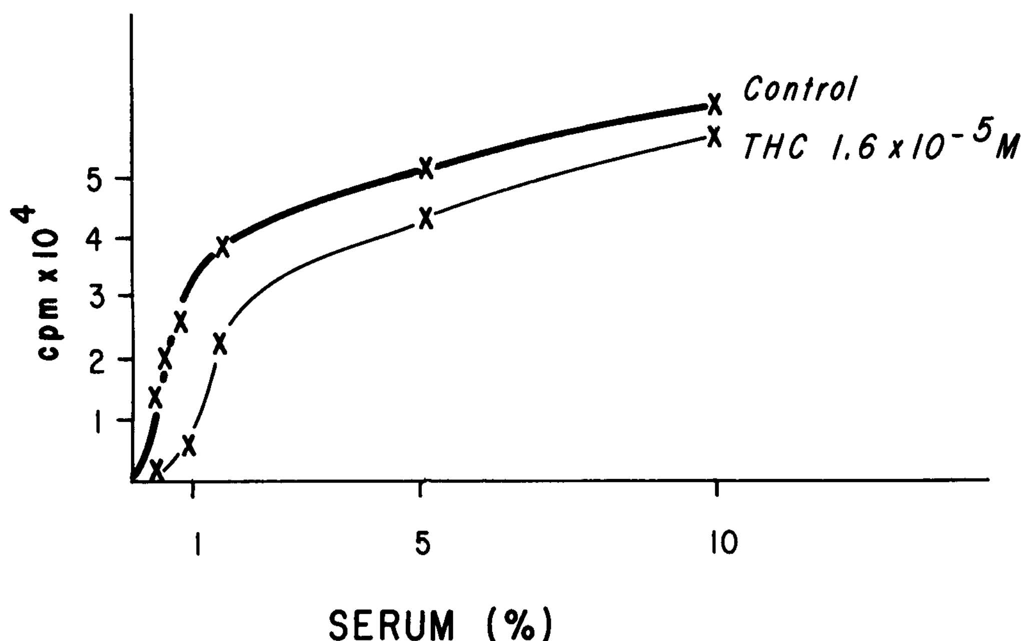


FIG. 2. THC effect on ^{3}H-thymidine incorporation in lymphocytes incubated in a medium that contains an increasing concentration of serum. Each point is the mean of triplicate cultures.

TABLE 5. EFFECT OF SERUM CONCENTRATION AND OF ITS DIFFERENT FRACTIONS ON THC TOXICITY

Fractions	^{3}H-Thymidine Incorporation (% of control cultures) THC Concentrations (× 10^{-5} M) 6.4	9.5
Serum 20%	98 ± 1	88 ± 6
Serum 10%	75 ± 5	52 ± 8
Serum 10% + Serum-albumin	84 ± 1	50 ± 5
Serum 10% + Cohn Fraction	45 ± 6	51 ± 3
Serum 10% + Phytic Extract	70 ± 5	40 ± 6
Serum 10% + Delipided α-1-lipoproteins	71 ± 6	49 ± 2
Serum 10% + α-1-lipoproteins	92 ± 2	84 ± 12

TABLE 6. EFFECTS OF CELL CONCENTRATION ON PHA INDUCED LYMPHOCYTE TRANSFORMATION AS MEASURED BY H^3 THYMIDINE UPTAKE AFTER 3 DAYS OF CULTURE. EACH VALUE REPRESENTS THE MEAN ± S.E. OF FOUR EXPERIMENTS.

Number of cells in the medium (in millions)		0.4	0.8	1.6	2.4
	Controls	18673 ± 872	38195 ± 2170	44991 ± 2232	35763 ± 1868
Exp I	THC 1.76 × 10^{-4} M	7381 ± 489	18254 ± 572	25010 ± 465	26580 ± 482
	Percentage	39.5%	47.7%	55.5%	74.3%
	Controls	31480 ± 1031	65839 ± 4887	59303 ± 5103	31287 ± 519
Exp II	THC 1.76 × 10^{-4} M	11340 ± 316	26642 ± 1725	28092.9 ± 1915	30390 ± 2279
	Percentage	36%	40.5%	47.4%	97.1%

METABOLISM OF LYMPHOCYTES IN CULTURE FOR SEVEN DAYS (Figures 3 and 4).

During the first three days, ^{3}H-thymidine incorporation and cellular protein concentration increase rapidly in control cultures (Fig. 3). At the 72nd hour, maximum ^{3}H-thymidine incorporation is observed. After this time, incorporation falls and reaches zero after 7 days. Cellular protein concentration begins to decrease after the fourth day. In cultures incubated with THC, the rate of growth is slower and decreases only after the fifth or sixth day.

In the control cultures, glucose consumption is very high (Fig. 4). After the fourth day, only a tenth of the initial glucose concentration remains in the medium, and a few hours later, the last molecules of glucose have been consumed. For each molecule of glucose consumed, under the present experimental conditions, 1.6 molecules of lactate are excreted, a ratio similar to that found with other cells (Ekinjian *et al.*, 1975). At the fourth day, this lactate excretion increases up to a plateau that corresponds to the exhaustion of glucose.

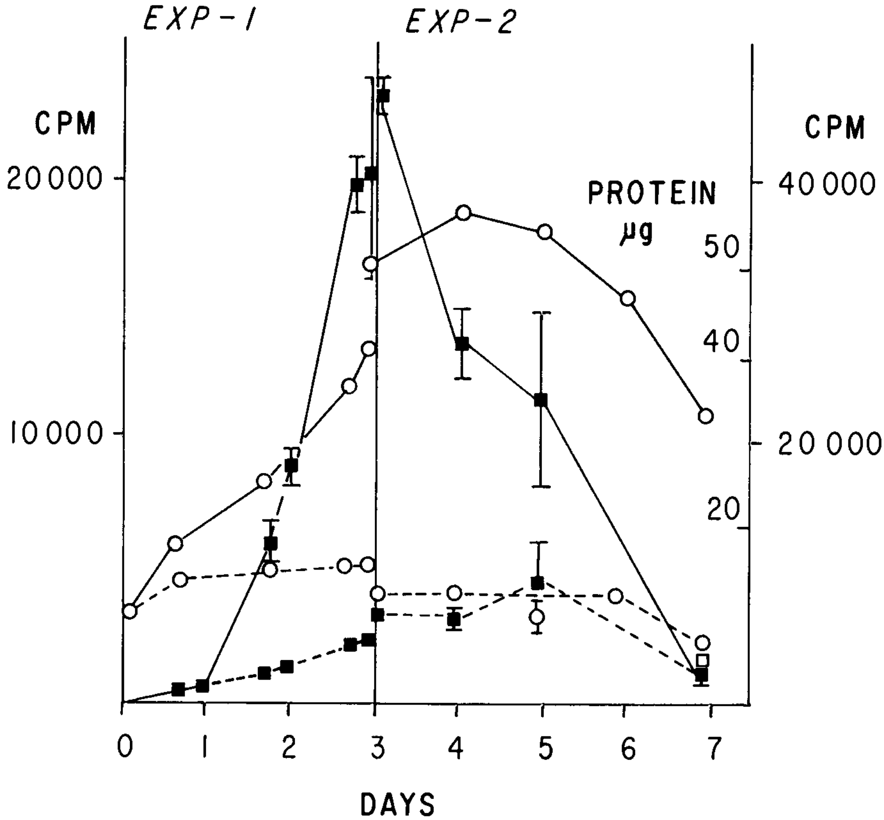


FIG. 3. ^{3}H-thymidine incorporation, after a 2 hour (experiment 1) or a 6 hour label (experiment 2); cellular protein concentration per culture, during a 7 day culture. Each point is the mean of the triplicate cultures, ± S.E. when S.E. is larger than the point.
■ ^{3}H-thymidine incorporation
○ Cellular Proteins
—— Control Cultures
--- Experimental cultures (2.0 × 10^{-4} M THC)

INHIBITION OF PRECURSOR UPTAKE BY THC AND OTHER PSYCHOTROPIC DRUGS

With a concentration of 20% serum in the culture medium, the inhibitory effect of THC on thymidine uptake is first observed at a concentration of 1.17 × 10^{-4} M (Table 2a). Inhibition is total with dosages of 2.35 to 4.7 × 10^{-4} M. The calculated dose that corresponds to a 50% decrease of thymidine uptake (IC 50) is 1.58 × 10^{-4} M (±0.13 × 10^{-4}). Inhibition of uridine uptake is observed with the same concentrations of THC (Table 2b).

With a 5% serum concentration in the medium, the dose of THC required to inhibit thymidine incorporation is smaller. In this case, IC 50 is 3.5 × 10^{-5} M (Fig. 5a and 5b). Comparable IC 50 are observed for imipramine (IC 50 = 4 × 10^{-5} M), and for diazepam (IC 50 = 7 × 10^{-5} M). Haloperidol is more toxic than THC with an IC 50 = 10^{-5} M.

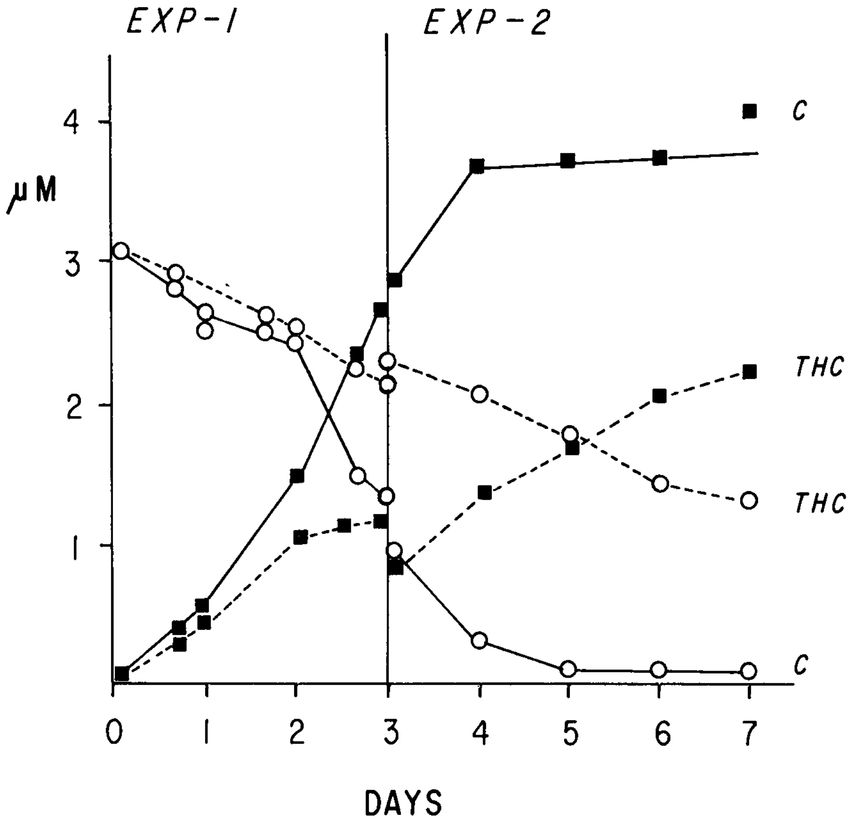


FIG. 4. Glucose and lactate concentration in the culture medium from the 1st to the 3rd day (experiment 1), and from the 3rd to the 7th day of culture (experiment 2). Each point is the mean of the triplicate cultures. S.E. is smaller than the point surface.

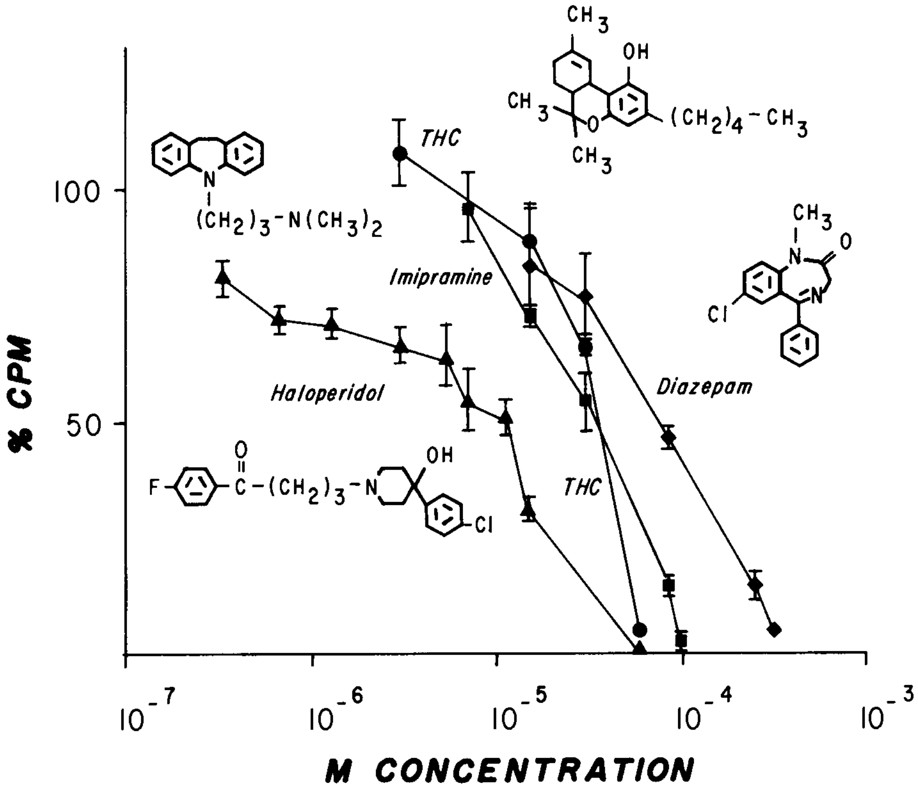
○ Glucose concentration in the medium
■ Lactate concentration in the medium
—— Control cultures
--- Experimental cultures

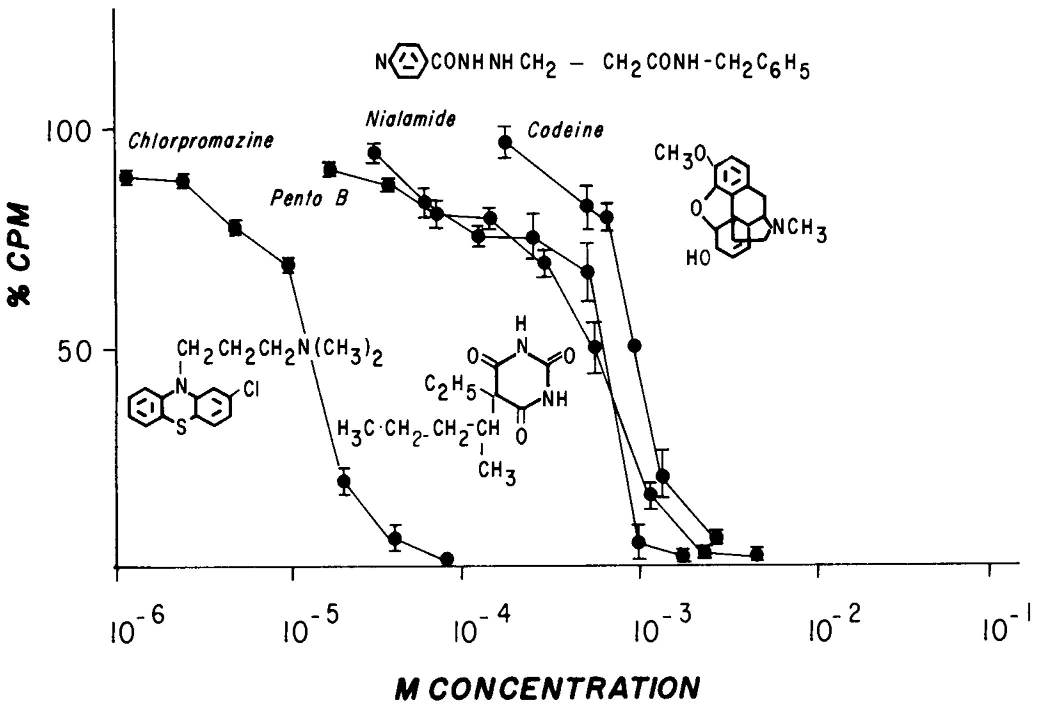
The other drugs that were studied exhibit less cellular toxicity than THC. Dihydantoin with an IC 50 = 1.1×10^{-4} M, chlordiazepoxide with an IC = 1.3×10^{-4} M, phenobarbital with an IC 50 = 1.3×10^{-3} M, and meprobamate with an IC 50 = 1.7×10^{-3} M.

DISCUSSION

Present results confirm those reported by Nahas *et al.*, 1974, Armand *et al.*, 1974, Desoize *et al.*, 1975, Desoize and Nahas, 1975, Nahas *et al.*, 1977, who described the inhibitory effects of THC 10^{-5} to 10^{-4} M on thymidine uptake by lymphocytes stim-

FIG. 5*a* and 5*b*. Inhibitory effects of Δ9-THC, Haloperidol, imipramine, diazepam, chlordiazepoxide, dihydantoine, phenobarbital and meprobamate on PHA induced lymphocyte transformation (5% serum in culture medium) as measured by ^{3}H thymidine uptake after 3 days of culture. Each point represents the mean ± S.E. of 4 different experiments. Inhibition of thymidine uptake was calculated in reference to the control cultures.


THC
Imipramine
Haloperidol
Diazepam
THC
% CPM
100
50
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
M CONCENTRATION


Chlorpromazine
Nialamide
Codeine
Pento B
% CPM
100
50
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
10⁻¹
M CONCENTRATION

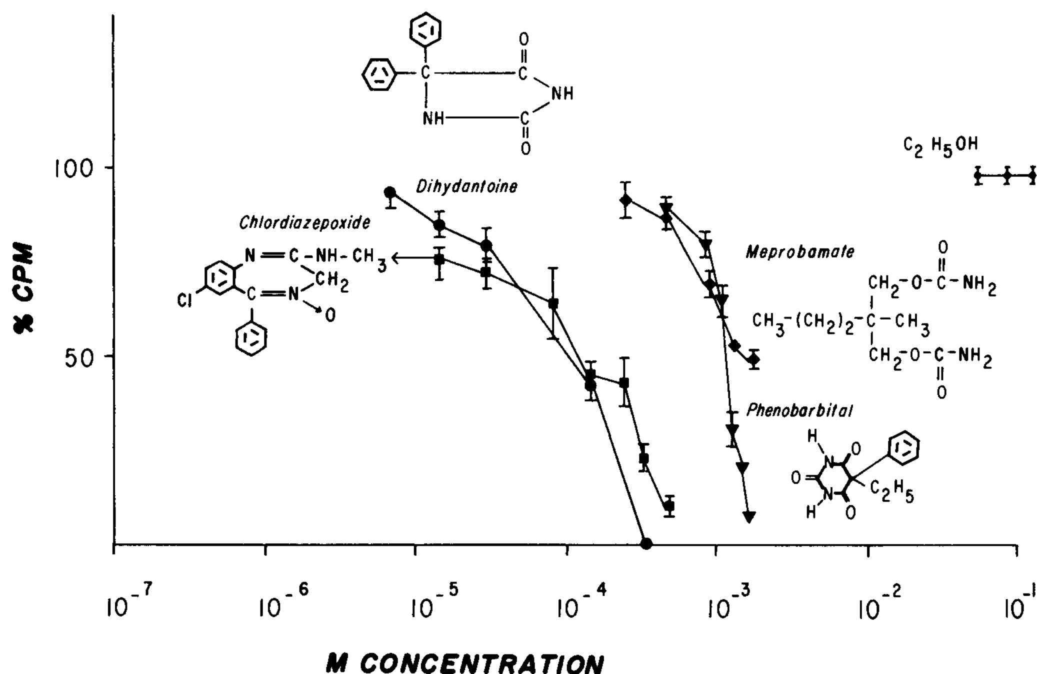


FIG. 5c.

ulated with mitogens. These experiments indicate that lymphocytes in culture, like all other eucaryotic cells which have been studied (Blevins and Regan, 1976; Zimmerman and McClean, 1973; Bram and Brachet, 1976; Carchman *et al.*, 1976) display an inhibition of growth when exposed to THC.

This study confirms that the toxicity of THC is related to the concentration of serum protein in the medium; the higher the concentration, the lower the toxicity; the drug being bound to the lipid fraction of the lipoproteins as noted by Capelle *et al.* (1976): with 20% serum in the medium, IC 50 of THC is 1.58×10^{-4} M; with 5% serum in the medium, IC 50 of THC is 3.5×10^{-5} M. Also, toxicity of any given amount of THC is related to the concentration of cells in the culture; the greater the number of cells, the smaller the amount of THC available to each cell.

Other commonly used psychotropic fat soluble drugs also exert an inhibitory effect on DNA metabolism. The inhibition induced by imipramine and diazepam occurs with concentrations comparable to those of THC. These experiments appear to show that the ability of psychotropic drugs to decrease blastogenesis of cultured lymphocytes correlates to some extent with their therapeutic efficacy: haloperidol inhibits with $10^{-6} - 10^{-5}$ M concentrations, meprobamate with 10^{-3} M concentrations.

The inhibitory effect of these drugs on cellular metabolism may be explained by their lipophilic nature: anesthetics, tranquilizers, and THC dissolve in the double lipid layer of the plasma membrane (Paton and Pertwee, 1972; Seeman, 1972) and alter its characteristics (Chari-Bitron, 1971).

However, our observations on the inhibitory effect on thymidine incorporation (IC 50 = 10^{-4} to 10^{-5} M depending upon serum concentration in medium) are in conflict with those reported by Rachelfski and Opelz (1977). These authors state "THC in concen-

tration of 10^{-3} M does not affect DNA synthesis of resting normal lymphocytes or their response to PHA or allogenic cells". They used different techniques in their studies.

In the first place, they did not verify the presence of THC in the culture medium. In the present studies, evidence that this drug was effectively present in the culture medium is provided by the use of labeled THC. Radioactivity in the medium was similar at the start and the end of the culture, furthermore, THC was not catabolised. In the experiments of Rachelfski and Opelz, it is possible that when the alcohol solution was added to the culture, the induced precipitate of protein might have absorbed most of the THC and prevented a homogeneous distribution of the drug in the culture. Their results might have been affected by the high final concentration of ethanol added at the same time as THC. They did not check the presence of THC in the culture medium.

In our experiments, after 3 days of incubation, at the time of harvesting, glucose concentration in the medium is sufficient to supply the energy requirement of the control culture. However, at the fourth day only 1/10 of the original glucose concentration is left. We noted that Rachelfski and Opelz harvested their cultures after $4\frac{1}{2}$ days of incubation. At that time, glucose concentration might have been insufficient to meet entirely the needs of the control cultures, while still adequate for those of the THC treated cells which were growing more slowly. When cultures are treated with THC, there is less glucose consumption because the drug slows down metabolism leaving enough glucose to supply energy throughout the period of culture.

Rachelfski and Opelz do not specify the protein concentration of the serum utilized in their cultures, which is as we have shown an important variable to control.

Any or all of these factors might account for the discrepancies between these results and those reported by Rachelfski and Opelz. The results of the experiments described in this paper indicate that the toxic effect of THC cannot be differentiated by present technique from that of other fat soluble psychotropic drugs.

REFERENCES

ARMAND, J. P., HSU, J. T. and NAHAS, G. G., Inhibition of blastogenesis of T-lymphocytes by delta-9-THC. *Fed. Proc.* **33**,539 (1974).

BLEVINS, R. D. and REGAN, J. D., Delta-9-THC: Effects on macromolecular synthesis in human and other mammalian cells, in Nahas, G. G., Paton, W. D. M. and Idänpään-Heikkilä, J. E., Editors, "Marihuana: Chemistry, Biochemistry and Cellular Effects," Springer-Verlag, New York, pp. 213–222, 1976.

BOYUM, A., Isolation of leucocytes from human blood, *Scan. J. Clin. Lab. Invest.* **21** (suppl. 97), 31 (1968).

CHARI-BITRON, A., Stabilisation of rat erythrocyte membrane by delta-1-THC, *Life Sci.* **10**,1273 (1971).

BRAM, S. and BRACHET, P., Inhibition of proliferation and differentiation of Dictyostelium Discoideum Amoebae by tetrahydrocannabinol and cannabinol, in: Nahas, G. G., Paton, W. D. M. and Idänppään-Heikkilä, J. E., Editors, "Marihuana: Chemistry, Biochemistry and Cellular Effects," Springer-Verlag, New York, p. 207-211, 1976.

CAPELLE, N., TREICH, I., HOELLINGER, H. et SCHERMANN, J. M., Liaison du delta-9-THC aux protéines sériques humaines. *J. Eur. Tox.*, **9**,5–9 (1976).

CARCHMAN, R. A., WARNER, W., WHITE, A. C. and HARRIS, L. S., Cannabinoids and Neoplastic growth, in: Nahas, G. G., Paton, W. D. M. and Idänppään-Heikkilä, J. E., Editors, "Marihuana: Chemistry, Biochemistry and Cellular Effects." Springer-Verlag, New York, pp. 329–345, 1976.

CUSHMAN, P. and KHURANA, R., Effects of marihuana smoking and tetrahydrocannabinol on T-cell rosettes, *Fed. Proc.* **34**,783 (1975).

CUSHMAN, P. and KHURANA, R., A controlled cycle of THC smoking: T and B cell rosette formation, *Life Sci.* **20**,971 (1977).

DAUL, C. B. and HEATH, R. G., The effect of chronic marijuana usage on the immunological status of rhesus monkeys, *Life Sci.* **17**,875 (1976).

DESOIZE, B., HSU, J., NAHAS, G., and MORISHIMA, A., Inhibition of human lymphocyte transformation in vitro by natural cannabinoids and olivetol, *Fed. Proc.* **34**,783 (1975).

DESOIZE, B. and NAHAS, G., Effets inhibiteurs du delta-9-THC sur la synthèse des protèines et des acides nuclèiques, *C. R. Acad. Sci. Paris*, **281**,475 (1975).

EKINDJIAN, G. C., TAILLEMITE, C., AGNERAY, J. et JARDILLIER, J. C., Etude du metabolisme glucidique du fibroblaste en culture. *C. R. Acad. Sci. Paris*, **280**,1421 (1975).

GAUL, C. C. and MELLORS, A., 1975. Delta-9-tetrahydrocannabinol and decreased macrophage migration inhibition activity, *Res. Commun. Chem. Pathol. Pharmacol.* **10**,559 (1975).

HARTZMAN, R. J., SEGALL, M., BACH, M. L. and BACH, F. M., Histocompatibility matching. Miniaturization of the MLC test, *Transplantation*, **2**,268 (1971).

HUOT, J., Cellular alterations induced in vitro by delta-1-tetrahydrocannabinol: Effects on cell proliferation, nucleic acids, plasma cell membrane ATPase, and adenyl cyclase. In: Nahas, G. G., Paton, W. D. M. and Idänppään-Heikkilä, J. E., Editors, "Marihuana: Chemistry, Biochemistry and Cellular Effects." Springer-Verlag, New York, pp. 313–327, 1976.

KOCH, F. and KOCH, G., Reversible inhibition of macromolecular synthesis on HeLa cells by ethanol, *Res. Com. Chem. Pathol. Pharmacol.*, **9**,291 (1974).

LAU, R. J., LERNER, C. B., TUBERGEN, D. G., BENOWITZ, N., DOMINO, E. F. and JONES, R. T., Non-inhibition of phytohemagglutinin (PHA) induced lymphocyte transformation in humans by delta-9-tetrahydrocannabinol, *Fed. Proc.* **34**,783 (1975).

LEFKOWITZ, S. S. and YANG, C., Drug induced immunosuppression of the plaque forming cell response, 75th Meeting, *Am. Soc. Microbiol.* p. 81 (1975).

LEVY, J. A., MUNSON, A. E., HARRIS, L. S. and DEWEY, W. L., Effects of delta-9-tetrahydrocannabinol on the immune response of mice, *Fed. Proc.* **34**,782 (1975).

MANN, P. E. G., COHEN, A. B., FINLEY, T. N. and LADMAN, A. J., Alveolar macrophages. Structural and functional differences between non-smokers and smokers of marihuana and tobacco, *Lab. Invest.*, **25**,111 (1971).

NAHAS, G. G., SUCIU-FOCA, N., ARMAND, J. P. and MORISHIMA, A., Inhibition of cellular mediated immunity in marihuana smokers, *Science*, **183**,419 (1974).

NAHAS, G. G., ZAGURY, D., SCHWARTZ, I. W. and NAGEL, M. D., Evidence for the possible immunogenicity of delta-9-tetrahydrocannabinol in rodents. *Nature*, **243**,407 (1973).

NAHAS, G. G., MORISHIMA, A. and DESOIZE, B., Effects of cannabinoids on macromolecular synthesis and replication of cultured lymphocytes. *Fed. Proc.*, **36**,1748 (1977).

PATON, W. D. M. and PERTWEE, R. G., The general pharmacology of cannabinoids, in Paton W. D. M. and Crown, J., Editors, "Cannabis and its derivatives," Oxford University Press, pp. 50–75, 1972.

PEREZ-REYEZ, M., TIMMONS, M. C. and LIPTON, A., Intravenous injection in man of delta-9-tetrahydrocannabinol and 11-delta-9-THC, *Science*, **117**,633 (1972).

PETERSEN, B. H., GRAHAM, J. and LEMBERGER, L., Marijuana, THC and cell function, *Life Sci.* **19**,395 (1976).

RACHELEFSKI, G. S. and OPELZ, G., Normal lymphocyte function in the presence of delta-9-THC, *Clin. Pharmacol. and Therapeu.*, **21**(1),44 (1977).

RACHELEFSKI, G. S., OPELZ, G., MICKEY, M. R., LESSIN, P., KINCHI, M., SILVESTEIN, M. J. and STIEHM, E. R., Intact humoral and cell mediated immunity on chronic marijuana smoking, *J. Allergy Clin. Immunol.*, **58**,483 (1976).

ROSENKRANTZ, H., MILLER, A. J. and ESBER, H. J., Delta-9-tetrahydrocannabinol suppression of the primary immune response in rats, *J. Toxicol. Environ. Health*, **1**,119 (1976).

SCHWARTZFARB, L., NEEDLE, M. and CHAVEZ-CHASE, M., Dose related inhibition of leucocyte migration by marihuana and delta-9-THC *in vitro*, *J. Clin. Pharmacol.*, **14**,35 (1974).

SEEMAN, P., The membrane actions of anesthesics and tranquilizers, *Pharmacol. Rev.*, **24**,583 (1972).

SILVESTEIN, M. J. and LESSIN, P. J., Normal skin test responses in chronic marihuana users, *Science*, **186**,740 (1974).

WHITE, S. C., BRIN, S. C. and JANICKI, B. W., Mitogen-induced blastogenic responses of lymphocytes from marihuana smokers, *Science*, **188**,71 (1975).

ZIMMERMAN, A. M. and MCCLEAN, D. K., Action of narcotic and hallucinogenic agents on the cell cycle, in Zimmerman, A. M., Padilla, G. M. and Cameron, I. L., Editors, "Drugs and the Cell Cycle," Academic Press, Inc., New York, pp. 67–94, 1973.

INHIBITION BY THC OF THYMIDINE TRANSPORT: A PLASMA MEMBRANE EFFECT

BERNARD DESOIZE, COLETTE LÉGER, JEAN-CLAUDE JARDILLIER and GABRIEL NAHAS

Laboratoire de Biochimie, Faculté de Pharmacie, 51096 REIMS CEDEX.

Laboratoire de Pharmacologie et de Toxicologie Cellulaires.
200, rue du Faubourg Saint-Denis, 75475 PARIS CEDEX 10

and

Department of Anesthesiology, College of Physicians and Surgeons,
Columbia University. NEW YORK, N.Y.

Summary. 10^{-6} to 10^{-4} M of delta-9-tetrahydrocannabinol (THC) or of other cannabinoids, which all have in common the C ring of olivetol, inhibit in cultured lymphocytes incorporation of ^3H thymidine. The inhibitory effect of olivetol derivatives is related to their octanol-water partition coefficient (liposolubility). Within 15 minutes of incubation, THC inhibits precursor pool formation and macromolecular incorporation of thymidine, uridine and leucine. THC inhibits also ^{14}C aminoisobutyric acid uptake into the cell, but does not alter the cellular "leakage" of this amino acid analogue. Using isotopic dilution technique with L1210 murine lymphoma cells and human lymphocytes, it was observed that THC decreases ^3H thymidine uptake within fifteen seconds after addition of the drug to the culture. Experiments performed at 0°C indicate that THC has no action on thymidine binding to the carrier. All of these observations suggest that THC in micromolar concentration inhibits DNA synthesis through a "non-specific" alteration of membrane configuration. This effect, due to the liposolubility of the drug, could induce conformational changes of membrane bound transport systems which would inhibit their function.

THE inhibitory effects of delta-9-tetrahydrocannabinol and other cannabinoids, in 10^{-6} to 10^{-4} M concentration, on cell division and macromolecular synthesis of cultured lymphocytes[1, 2, 3] and other eucaryocytic cells[4, 5, 6, 7, 8] has been reported by a number of investigators. It was suggested[9] that this effect was related primarily to the liposolubility of these compounds in the double lipid layer of the plasma membrane[10]. Others reported that THC exerted its inhibitory effect on the mitochondrion[11]. However, the 10^{-3} M concentrations of THC used in these latter experiments are lethal to the whole cell culture. Furthermore, the present authors have observed that THC has no action on cellular glycolysis and does not alter ATP concentration[12].

The purpose of the present study was to further investigate the possible mediation of the inhibitory effect of THC on macromolecular synthesis through a non-specific action of the

drug on the plasma membrane of the cell. In this investigation, the relationship between the octanol-water partition of olivetol derivatives and inhibition of thymidine incorporation was studied first. The effect of THC on cellular pool formation of precursors and on their macromolecular incorporation was investigated next. Finally, the kinetics of precursor transport system were measured, using the isotopic dilution technique with 15 to 60 second pulse exposure.

METHODS

MATERIALS

Methyl-^{3}H-Thymidine (2Ci/mMole and 47.5 Ci/mMole), 5-^{3}H-Uridine (52 Ci/mMole), 4,5-^{3}H-Leucine (50 Ci/mMole), 1-^{14}C-Aminoisobutyric acid (AIBA, 10 mCi/mMole) are obtained from Amersham. THC was kindly provided by NIDA (National Institute on Drug Abuse). The drug solubilized in alcohol is mixed with the culture medium and added to the cultures so as to reach a final concentration of 10^{-5} to 1.5×10^{-4} M. Similar amounts of alcohol vehicle are added to the control cultures. Purified phytohemaglutinin (PHA) is obtained from Burroughs Wellcome Company. Media, biological extracts and salt solutions are obtained from Grand Island Biological Company (GIBCO), New York. Olivetol and its derivatives are obtained from Aldrich Chemicals.

CULTURE OF HUMAN LYMPHOCYTES

The technique used is that of Hartzman *et al.*[13]. Venous blood is collected under heparin (50 IU/ml) from healthy male donors. Mononucleated cells are separated according to the technique of Boyum[14] by density gradient centrifugation with Ficoll-Isopaque. After three washes the cells are resuspended at a concentration of 0.8×10^6 cells per ml in RPMI 1640 supplemented with antibiotics, glutamine, 20% of pooled human serum and 4 μg/ml of purified PHA. Cells are cultured under a volume of 0.25 ml, 1.25 ml of 5 ml at 37°C, in a 5% CO_2 atmosphere saturated with water vapor.

CULTURE OF L1210 CELLS (MURINE LYMPHOMA)

The cells are cultured at a concentration of 0.2×10^6 cells per ml in Dulbeco's modified Eagle's medium (GIBCO) supplemented with antibiotics, glutamine and 10% of foetal calf serum (GIBCO), under a volume of 30 ml in Roux' flasks (Corning) at 37°C in a 5% CO_2 atmosphere saturated with water vapor.

LIPID SOLUBILITY OF OLIVETOL ANALOGUES AND INHIBITION OF THYMIDINE UPTAKE

Human lymphocytes are cultured with increasing concentrations of diphenol derivatives during three days. ^{3}H-Thymidine (0.5 μCi) is added at the 66th hour. The cells in triplicate

culture are harvested at the 72nd hour with a Multiple Automatic Sample Harvester (MASH, Biological Associates). Viability of the cells is verified at the end of the culture with trypan Blue or phase contrast microscopy. Results are discarded when a higher than usual ratio (7.5%) of cellular death is found. Cellular radioactivity is measured by scintillation counting. As thymidine uptake varies considerably in different lymphocyte populations, uptake of the precursor into the test cultures is compared with its uptake into parallel control cultures. Results are then expressed in terms of percentage of precursor uptake in the control culture. The concentration of drug which inhibits 50% uptake of the precursor (I.C. 50) is obtained from the dose response curves. The I.C. 50 of these drugs is correlated with their octanol water partition coefficient on log/log coordinates. Partition coefficient is measured as follows: the compounds are dissolved in octanol (0.5 mg/ml). Octanol and buffer are mixed three times an hour during a 30 hour incubation. The drug concentration in octanol and in buffer are measured by UV spectrophotometry.

MEASUREMENTS OF THE CELLULAR POOL AND OF MACROMOLECULAR INCORPORATION OF ^{3}H-THYMIDINE, URIDINE, LEUCINE AND OF ^{14}C-AMINOISOBUTYRIC ACID (AIBA) UPTAKE

0.8×10^6 human lymphocytes are cultured in a volume of 1 ml of medium. At the 64th hour, 2 μCi of one of the labeled precursors, thymidine, uridine and leucine, or of ^{14}C-aminoisobutyric acid (AIBA), are added to the culture with different concentration of THC dissolved in 0.05 ml of human pooled serum. After 15 min. the cells are washed three times by centrifugation in a refrigerated centrifuge ($400 \times g \times 10$ min.). The cell pellet is then resuspended in 5% ice cooled trichloracetic acid, and incubated at 37°C for one hour. Radioactivity is evaluated in the supernatant which contains the cellular pool (acid soluble) and in the precipitate which contains the macromolecules (acid insoluble).

MEASUREMENT OF ^{14}C-AIBA TRANSPORT AND LEAKAGE

0.8×10^6 human lymphocytes are cultured in a 1 ml volume. At the 64th hour the cells are centrifuged and resuspended for a second 2 hour incubation in 0.18 ml Phosphate Buffered Saline (PBS, pH = 7.4) and 0.02 ml of RPMI containing 0.4 μCi of ^{14}C-AIBA. After 2 hours, the cells are centrifuged again and resuspended in a radioactive free medium. Following different time intervals up to 45 min., the cells are centrifuged a third time ($150 \times g \times 10$ min.) and the radioactivity is evaluated in the supernatant and in the cells, after resuspension of the pellet in 1 ml of medium and 0.25 ml of serum. Quenching is similar in the supernatant and in the pellet. In other experiments, the cultures, after the second centrifugation, are resuspended for the third incubation in 1 ml of RPMI and 0.25 ml of serum containing different concentrations of THC (1 to 25×10^{-4} M) and reincubated at 37°C during 30 min. in a 5% CO_2 atmosphere saturated in humidity.

MEASUREMENT OF ^{3}H-THYMIDINE UPTAKE AFTER 15 TO 30 SECOND PULSE EXPOSURES

The isotopic dilution technique described by Strauss[15] was adapted for the present experiments. L1210 cells (murine lymphoma) are centrifuged (150 × g × 10 min.) and resuspended (15×10^6 cells per ml) in PBS supplemented with 1 g/l of glucose (PBSG); 0.1 ml of this suspension is mixed in a hemolysis tube at time "0" with 0.07 ml of PBSG containing 0.5 μCi of tritiated thymidine, uridine or leucine, 0.1 ml of PBSG containing cold thymidine and 0.03 ml of fetal calf serum. The cells are incubated with increasing concentration of cold thymidine (3.5×10^{-8} to 10^{-2} M) but a constant concentration of ^{3}H-thymidine (0.5 μCi per tube). THC in 10^{-5} to $2.5\ 10^{-4}$ M concentration is added to the test cultures.

When human lymphocytes, stimulated with PHA, are used after 3 days of culture, the number of cells cannot be accurately measured at the time of the experiment because of the cellular aggregates. In this instance, lymphocytes are counted at the onset of culture, and diluted to 8×10^5 cells/ml; after 3 days of culture we assume that there are 1×10^6 cells/ml[16]. The cell suspension is shaken in a water bath at 37°C during incubations varying from 7 to 60 sec. A 0.2 ml volume of the suspension is filtered through a fiberglass filter, and washed with isotonic ice cold mannitol (0.3 M). The time interval between the moment the cells are added to the medium prewarmed at 37°C, and the moment the cold mannitol is poured on the cells may be as short as 7 sec. The filter is placed in a counting vial, the cells are dissolved with 0.5 ml of 1 N NaOH by warming one hour at 37°C. The solution is neutralized with 0.05 ml of HNO_3 (10 N) and Scintillation fluid (Instagel, Packard) then added. The quenching is constant in all experiments. The radioactivity harvested in the filter decreases to a plateau (background radioactivity) with increasing concentration of cold thymidine. Transport is calculated by subtracting background radioactivity retained on the filter. Other experiments are performed in similar fashion after incubating cells at 0°C instead of 37°C.

RESULTS

INHIBITORY EFFECTS OF OLIVETOL DERIVATIVES ON THYMIDINE UPTAKE (Fig. 1 and 2)

The I.C. 50 of these compounds is significantly correlated with their octanol-water partition coefficient ($r = 0.9467$, $p < 0.001$). The ortho position of the hydroxyl group as well as the length of the alkyl chain increase the cytotoxicity of the molecule: the greater its liposolubility, the greater its toxicity.

EFFECTS OF THC ON PRECURSOR UPTAKE AND INCORPORATION AFTER 15 MINUTE INCUBATION (Table 1)

After 15 minutes THC inhibits uptake of precursors into the cellular pool and their incorporation into the macromolecules: THC 2.5×10^{-4} M inhibits by 50 to 60% cellular

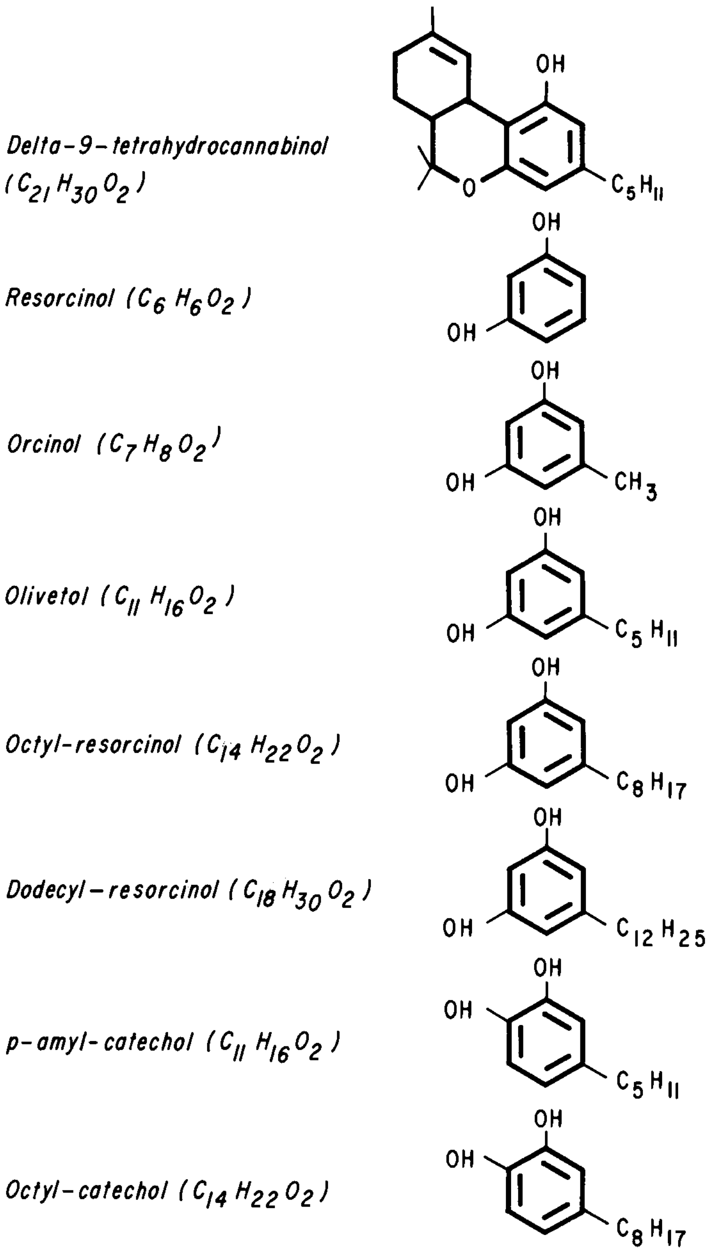


FIG. 1. Structure of delta-9-THC, olivetol and olivetol analogues.

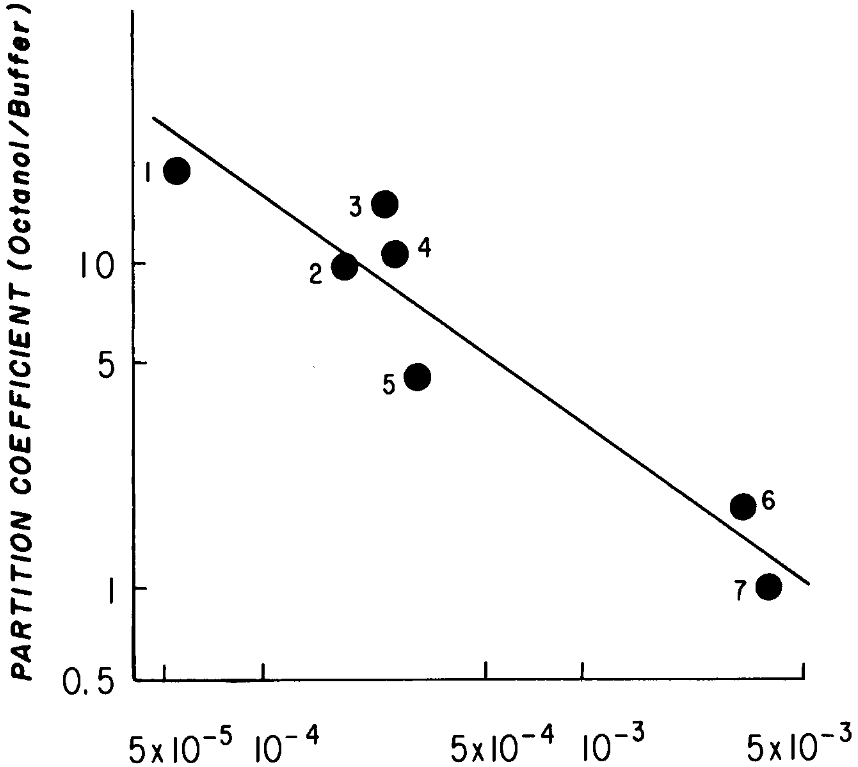


FIG. 2. The relationship between the molar concentration of olivetol and its analogues required to inhibit by 50% thymidine incorporation (I.C. 50) and their octanol water partition coefficient (liposolubility). (1) Octyl-catechol. (2) Dodecyl-resorcinol. (3) Octyl-resorcinol. (4) Amyl-catechol. (5) Olivetol. (6) Resorcinol. (7) Orcinol.

TABLE 1. INHIBITION OF CELLULAR UPTAKE AND OF MACROMOLECULAR INCORPORATION OF LABELED PRECURSORS AND OF ^{14}C-AIBA BY DELTA-9-TETRAHYDROCANNABINOL (THC, 2.5 × 10^{-4} M). THE DRUG IS INCUBATED 15 MINUTES WITH PHA STIMULATED CULTURED HUMAN LYMPHOCYTES. THE SERUM CONCENTRATION OF THE MEDIUM IS 25%. RESULTS ARE EXPRESSED IN TERMS OF PERCENTAGE OF PRECURSOR UPTAKE IN A PARALLEL CULTURE. EACH FIGURE REPRESENTS 2 TO 5 EXPERIMENTS.

	% Inhibition by THC (2.5 × 10^{-4} M)	
	Uptake into Cellular Pool	Incorporation into Macromolecules
^{3}H-thymidine	50.5% ± 1	59.0% ± 1
^{3}H-uridine	60.2% ± 3	64.6% ± 4
^{3}H-leucine	9.5% ± 1	28.0% ± 1
^{14}C-AIBA	50.0% ± 3	—

uptake and macromolecular incorporation of ^{3}H-thymidine and ^{3}H-uridine. The incorporation of ^{3}H-leucine, into protein, however, is less inhibited (10%) than its cellular uptake (28%). ^{14}C-AIBA cellular uptake is inhibited by THC in a manner comparable to the inhibitory effect of this drug on thymidine and uridine.

EFFECT OF THC ON ^{14}C-AMINOISOBUTYRIC ACID (AIBA) TRANSPORT (Tables 2 and 3)

Appearance of radioactivity in the supernatant following centrifugation is an indication of the plasma membrane reverse transport or "leakage" of ^{14}C-AIBA. In the first experiment (Table 2) after the cells have been resuspended, the cellular leakage of ^{14}C-AIBA is proportional to the time of incubation; the concentration of label in the supernatant is already measurable after 10 minutes of centrifugation (corresponding to time "zero" in this experiment) which is required to separate cells from supernatant. In the second experiment (Table 3) when THC, 1 to 2.5 × 10^{-4} M was incubated with the cells for 30 min., there was no change in the cellular "leakage of AIBA".

TABLE 2. MEASUREMENTS OF PLASMA MEMBRANE LEAKAGE OF ^{14}C-AIBA FROM HUMAN LYMPHOCYTES. THE CELLS ARE INCUBATED FOR 2 HOURS WITH ^{14}C-AIBA, CENTRIFUGED AND RESUSPENDED IN A NEW MEDIUM. RADIOACTIVITY IS EVALUATED IN THE SUPERNATANT AND IN THE CELLS.

Second Incubation time* (min.)	% of ^{14}C-AIBA in supernatant	
	#1	#2
0	—	44
15	32	54
30	42	65
45	50	66

* Not including the 10 min. centrifugation required to separate the cells from the supernatant.

TABLE 3. EFFECT OF THC ON PLASMA MEMBRANE "LEAKAGE" OF ^{14}C-AIBA FROM HUMAN LYMPHOCYTES. THE CELLS ARE INCUBATED FOR 2 HOURS WITH ^{14}C-AIBA CENTRIFUGED AND RESUSPENDED FOR 30 MINUTES IN A NEW MEDIUM CONTAINING VARYING CONCENTRATIONS OF THC.

THC concentration (× 10^{-4} M)	% ^{14}C-AIBA in supernatant
0	55.0 ± 0.3
0.95	53.0 ± 1.9
1.6	55.0 ± 1.2
2.5	53.0 ± 1.3

EFFECT OF THC ON ^{3}H-THYMIDINE UPTAKE AFTER 15 TO 60 SECONDS (Figs. 3–8)

Transport of the precursor is measured by subtracting the radioactivity retained by the filter after an incubation of the cells with a high concentration of thymidine, from the radioactivity retrieved after an incubation with a low concentration of the precursor (with a *constant* concentration of labeled precursor). As cold thymidine concentration increases, radioactivity harvested on the filter decreases to a plateau which measures background radioactivity (corresponding to mechanical trapping, adsorption and passive diffusion of the labeled molecule) (Fig. 3). In this system, thymidine transport is linear with time over

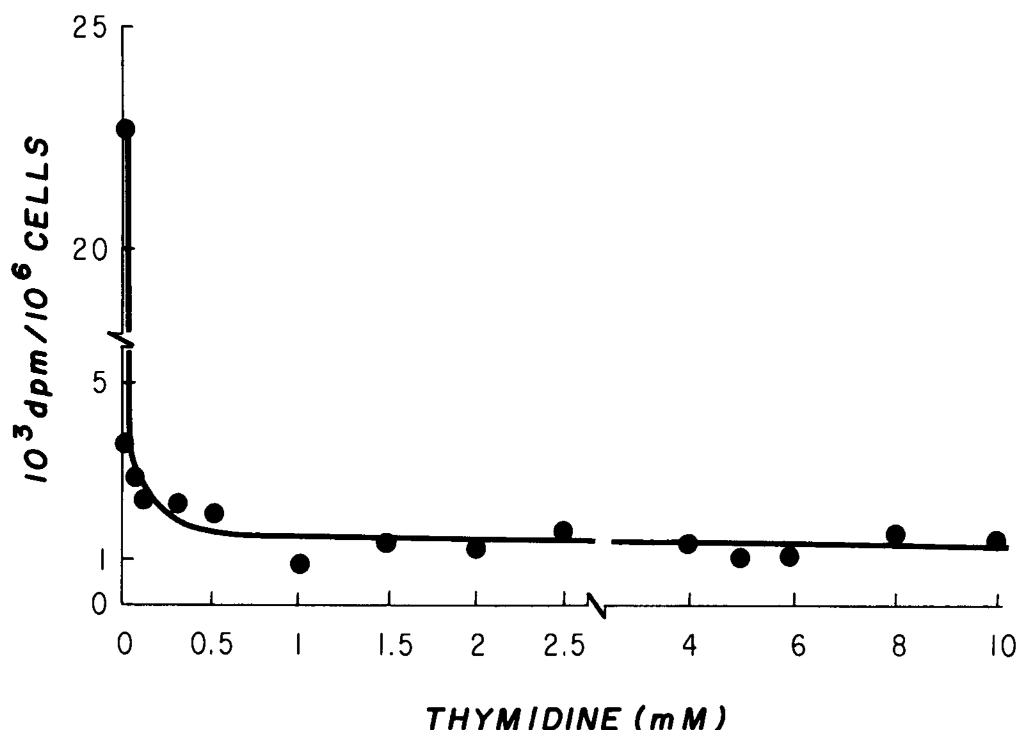


FIG. 3. Radioactivity harvested after 30 sec. incubation of L1210 cells in the presence of increasing doses of cold thymidine and the same amount of ^{3}H-thymidine (3.2×10^{-8} M).

60 seconds and extrapolates to zero, indicating unidirectional flow (Fig. 4). Inhibition of thymidine transport by THC is observed within 15 seconds after addition of the drug to L1210 lymphoma cells and is significant after 30 seconds. This inhibition is dose related (Fig. 5). After a 30 second pulse, thymidine uptake is linearly correlated to the logarithm of THC concentration ($p < 0.001$). The concentration of THC required to inhibit 50% uptake of thymidine (I.C. 50) in this model is 3.4×10^{-4} M.

In human PHA stimulated lymphocytes a significant inhibition by THC of thymidine transport is observed within 15 sec. (Fig. 6). When the cells are incubated at 0°C, addition of THC to the cultures does not change uptake of ^{3}H thymidine (Fig. 7).

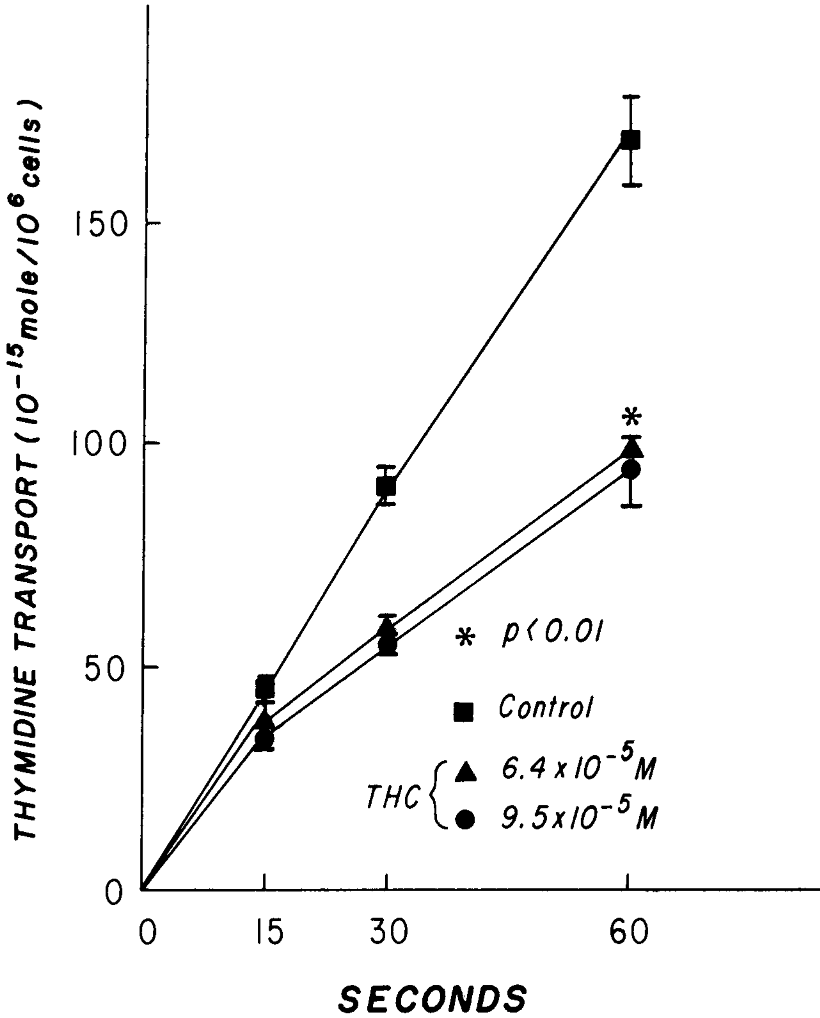


FIG. 4. Inhibition of thymidine transport by THC (6.4 and 9.5 × 10^{-4} M) in L1210 lymphocytes incubated at 37°C. Each point represents triplicate measurements.

Uptake of uridine and leucine are also significantly inhibited by THC; within 15 sec. for leucine and after 60 sec. for uridine. Uptake curves of these precursors measured during 60 sec. do not extrapolate through zero (Fig. 8).

DISCUSSION

All cannabinoids have in common the C ring of olivetol, and inhibit to the same extent macromolecular synthesis(9). Olivetol also exerts an inhibitory effect on thymidine uptake similar to that produced by THC(17). When the liposolubility of olivetol is increased through lengthening of its aliphatic chain or changing the position and number of hydroxyl groups, the cytotoxicity of the resulting compounds is increased. Inhibition of

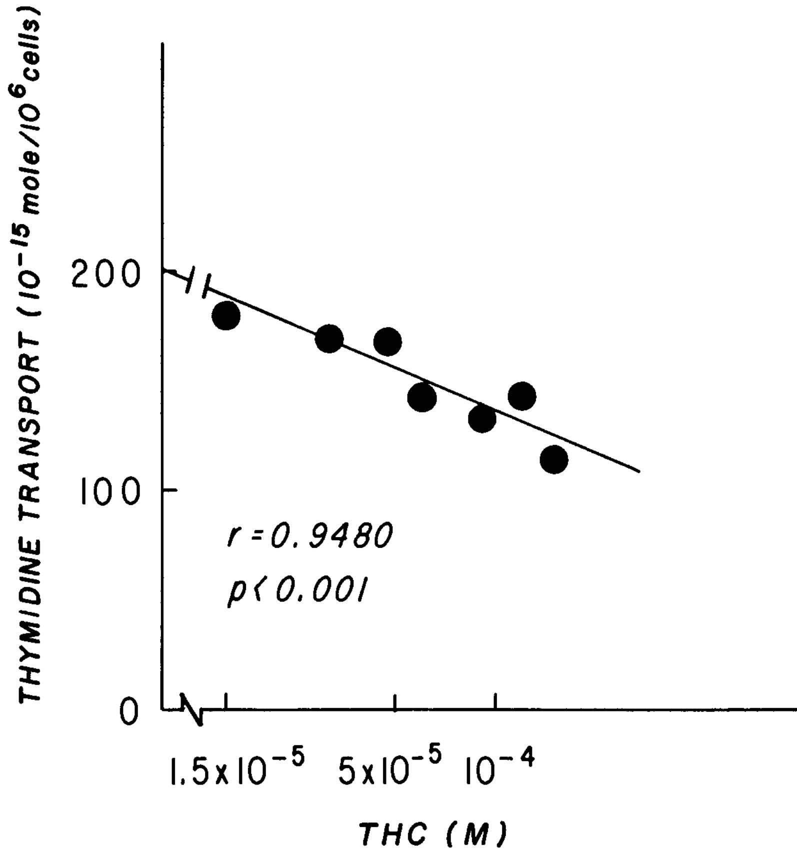


FIG. 5. Effects of increasing dose of THC on thymidine transport in L1210 cells after 30 seconds incubation.

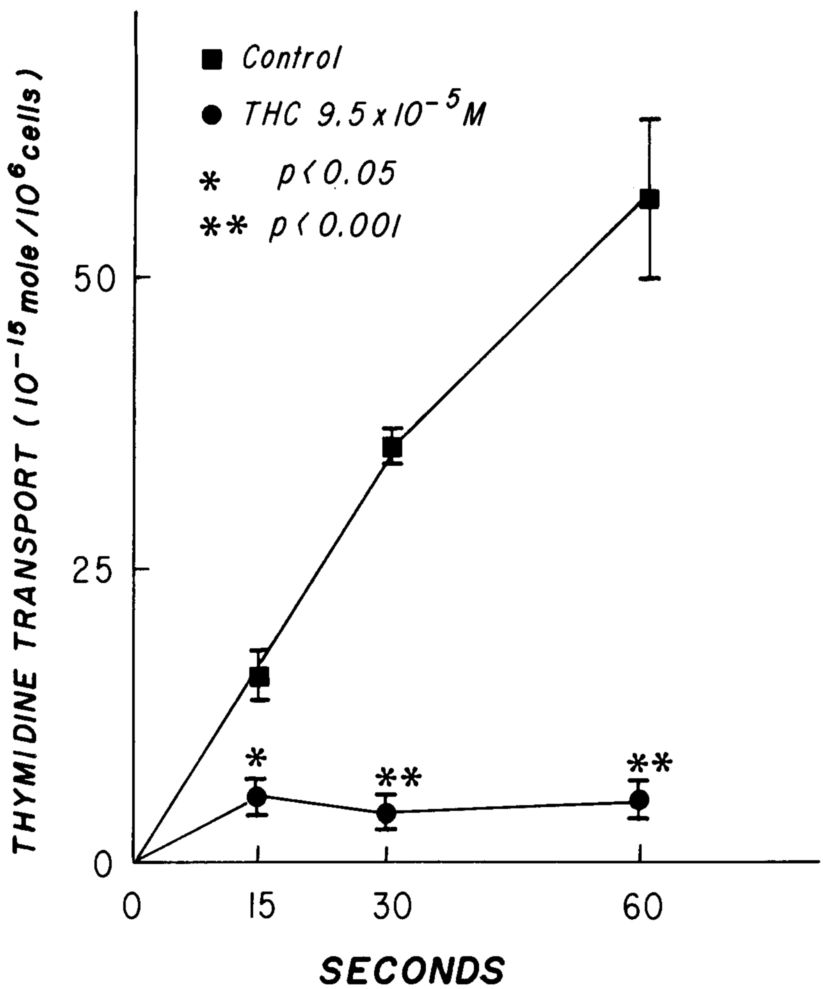


FIG. 6. Inhibition of thymidine transport by THC (9.5×10^{-5} M) in PHA stimulated human lymphocytes. Each point represents triplicate measurements.

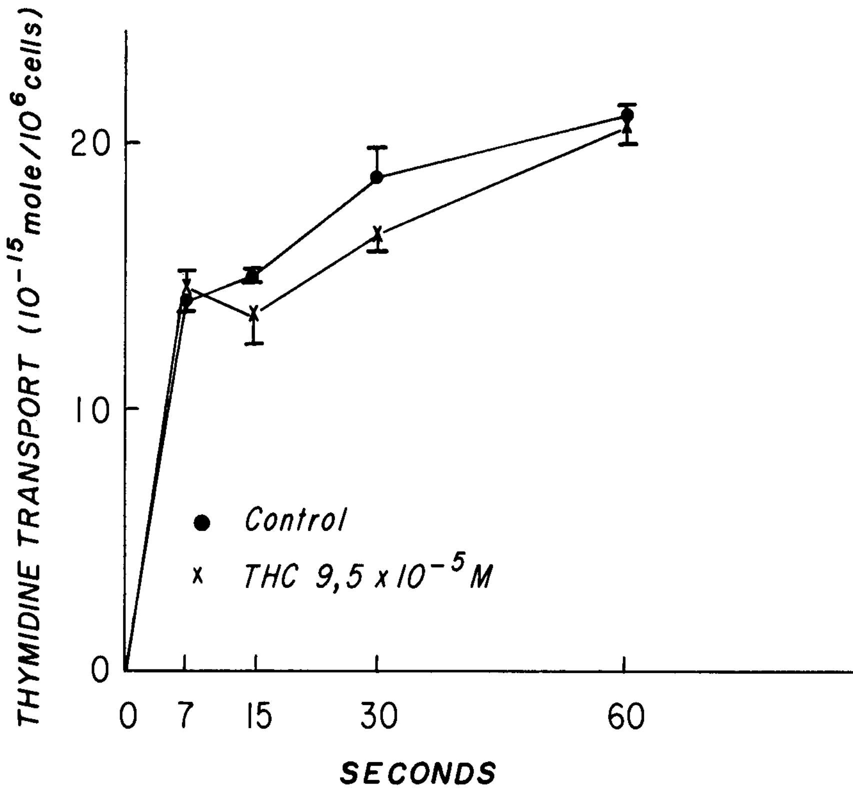


FIG. 7. Effect of THC on thymidine uptake of L1210 cells incubated at 0°C.

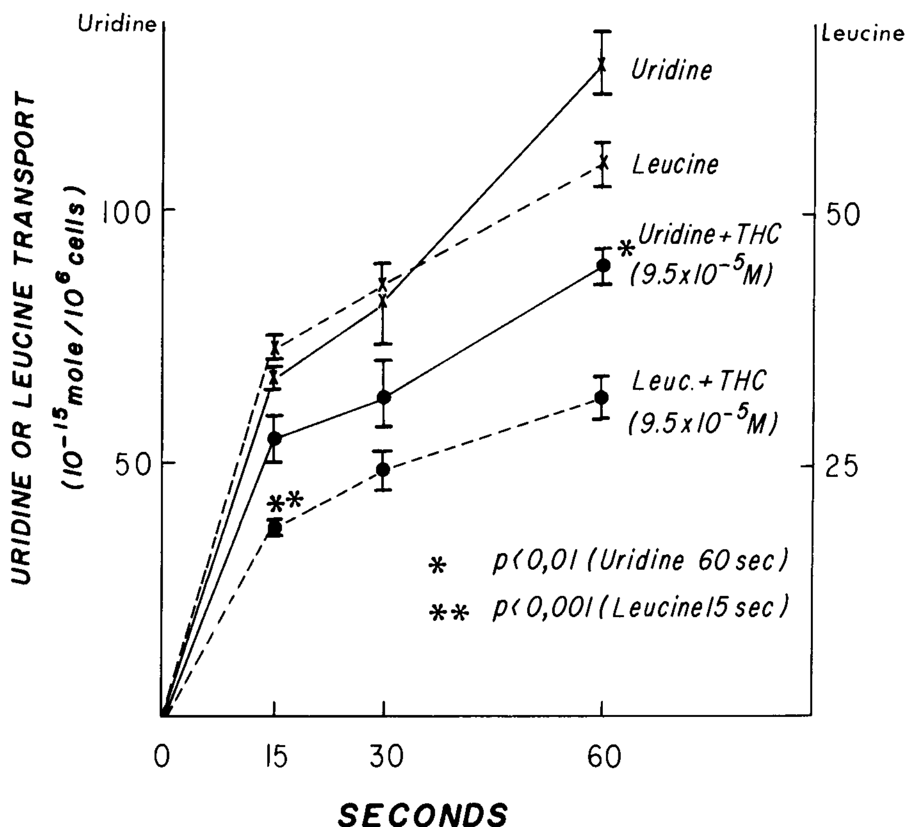


FIG. 8. Inhibition of uridine and leucine uptake by THC. Each point represents triplicate measurements.

thymidine uptake by these compounds is significantly correlated with their octanol water partition coefficient.

In the course of incubation periods with THC, which do not exceed 15 minutes, the amount of precursor uptake corresponds to the difference between the amount entering into the cell, and the amount leaving the cell. This latter is referred to as "plasma membrane leakage". Blevins and Regan[5] suggested that THC increases plasma leakage, thereby decreasing cellular pool formation of precursor. To investigate plasma leakage, ^{14}C-AIBA was used. This amino acid derivative is transported into the cell like other amino acids, but is not incorporated into the macromolecule. In the present experiments THC (1 to 2.5×10^{-4} M) does not influence aminoisobutyric acid cellular "leakage". The inhibition of precursor incorporation during 15 minutes incubation cannot be explained by an increase "leakage" of the membrane, as suggested by Blevins and Regan[5].

Our last series of experiments indicate that THC interferes with the membrane transport system of thymidine: The velocity of thymidine transport is constant during at least one minute. We have observed in other experiments that this transport rate remains constant for as long as 4 minutes. Within 15 seconds after addition of THC to the L1210 cells, a decrease in precursor transport is observable and within 30 seconds it is significant. This

inhibition is of a greater magnitude in normal human lymphocytes than in lymphoma murine cells.

The uptake curves of uridine and leucine, unlike that of thymidine, do not extrapolate through zero indicating that one is not measuring a simple, unidirectional transport. Nevertheless, THC produced a very rapid inhibition of the cellular uptake of these precursors.

At 0°C enzymatic activity is very slow, while the velocity of association of the substrate (thymidine) with the "enzyme" is little affected. During the first seven seconds of incubation, one may assume that thymidine uptake represents thymidine bound to the receptor sites of the carrier. By extrapolation we calculated that if 1×10^{-14} moles of thymidine were bound to a million cells (Fig. 7), there should be approximately 6,000 thymidine binding sites per cell. The very small increment of thymidine uptake during the remaining 53 seconds of incubation reflects the weak activity of the transport system. At 0°C: At that temperature THC has no measurable effect on thymidine uptake, an indication that the drug does not act on the binding of thymidine to the carrier but rather on the transport function of the carrier. This mechanism could account for the non competitive inhibitory effect of THC on thymidine uptake we reported earlier[(9)].

The precise molecular mechanism of action of the drug on the membrane cannot be determined by the present experiments. However, the concentration required to produce the observed inhibition indicates that the effect of THC on the membrane is "non-specific" and related to its liposolubility[(18)]. Similar inhibition of macromolecular synthesis in cultured lymphocytes has been observed with other psychotropic drugs in 10^{-6} to 10^{-3} M concentrations[(19)]. This inhibitory effect was correlated to the liposolubility of these drugs: the higher their octanol water partition coefficient, the greater their cytotoxicity[(20)].

Like other lipophiles and psychotropic drugs, THC expands erythrocyte membranes and increases their resistance to hemolysis [(21, 22)]. The concentration of THC required to decrease *in vitro* hemolysis by 50% (AH 50) is similar (10^{-5} M) to the concentration of this drug which will inhibit by 50% (I.C. 50) thymidine incorporation in cultured human lymphocytes[(19)].

Membrane expansion by THC could, as a result, produce conformational changes of the phospholipid and protein components and membrane bound carriers might be inhibited[(18, 23)]. An inhibition of the transport system of the precursors required for macromolecular synthesis would produce a decrease of their intracellular pool. As a secondary consequence, there would be a decrement in macromolecular synthesis by lack of precursors.

Such a nonspecific effect of THC is exerted with micromolar concentrations which might be reached in vivo only in heavy chronic consumption. By contrast, the acute psychotropic effects of this drug are exerted with nanomolar concentrations as a result of stereospecific interaction with receptor sites located in the central nervous system[(24)].

ACKNOWLEDGEMENTS

We wish to express our thanks to H. Hollinger who kindly prepared ^{3}H-THC, and Mrs M. Escaig who made the autoradiography and prepared the sample for the electron microscope.

REFERENCES

1. New, R. L., Powers, M., King, S. and Garner, L. I., *J. Clin. Pharmacol.* **10**,228 (1970).
2. Nahas, G. G., Suciu-Foca, N., Armand, J. P. and Morishima, A., *Science*, **183**,419 (1974).
3. Mellors, A., In: "Marihuana: Chemistry, Biochemistry and Cellular Effects," Eds. G. G. Nahas, W. D. M. Paton, J. E. Idanpaan-Heikkila, Springer-Verlag, New York, p. 265, 1976.
4. Zimmerman, A. and MacClean, D. K., In "Drugs and the Cell Cycle," Eds. A. M. Zimmerman, G. M. Padilla and I. L. Cameron, Academic Press, New York, p. 67, 1973.
5. Blevins, R. D. and Regan, J. D., In "Marihuana: Chemistry, Biochemistry and Cellular Effects," Eds. G. G. Nahas, W. D. M. Paton, J. Idanpaan-Heikkila, Springer-Verlag, New York, p. 213, 1976.
6. Bram, S. and Brachet, P., *Ibid.*, p. 207.
7. Huot, J., *Ibid.*, p. 313.
8. Carchman, R. A., Harris, L. S. and Munson, A. E., *Cancer Res.*, **36**,95–100 (1976).
9. Nahas, G. G., Morishima, A. and Desoize, B., *Fed. Proc.*, **36**,1748 (1977).
10. Paton, W. D. M., Pertwee, R. G. and Temple, D., In "Cannabis and its Derivatives," W. D. M. Paton and J. Crown, Eds., Oxford University Press, London, p. 50, 1972.
11. Birmingham, M. K. and Bartova, A., In "Marihuana: Chemistry, Biochemistry and Cellular Effects," Eds. G. G. Nahas, W. D. M. Paton, J. E. Idanpaan-Heikkila, Springer-Verlag, New York, p. 425, 1976.
12. Desoize, B. and Nahas, G. G., *C. R. Acad. Sci., Paris*, **281**,475 (1975).
13. Hartzman, R. J., Segall, M., Bach, M. L. and Bach, F. M., *Transplantation* **ii**,268 (1971).
14. Boyum, A., *Scand. J. Clin. Lab. Invest.* **21**,31 (1968).
15. Strauss, R. P., Sheehan, J. M. and Kashket, E. R., *J. Exp. Med.*, **144**,1009 (1976).
16. Iscove, N. N. and Melchers, F., *J. Exp. Med.*, **147**,973 (1978).
17. Desoize, B., Hsu, J., Nahas, G. G. and Morishima, A., *Fed. Proc.*, **34**,783, (1975).
18. Seeman, P., *Pharmacol. Rev.* **24**,583 (1972).
19. Desoize, B., Leger, C., Banchereau, J. and Nahas, G. G., *Fed. Proc.* **37**,739 (1978).
20. Nahas, G. G., Desoize, B. and Leger, C., *C. R. Acad. Sci., Paris*, In Press.
21. Chari-Bitron, A., In "Marihuana: Chemistry, Biochemistry and Cellular Effects," Eds. G. G. Nahas, W. D. M. Paton, J. E. Idanpaan-Heikkila, Springer-Verlag, New York, p. 273, 1976.
22. Sofia, R. D., Delgado, C. J. and Douglas, J. F., *European Journal of Pharmacology*, **27**,155–157 (1974).
23. Greenberg, J. H. and Mellors, A., *Biochem. Pharmacol.* **27**,329 (1978).
24. Seeman, P., *Biochem. Pharmacol.* **26**,1741 (1977).

EFFECT OF Δ^9-TETRAHYDROCANNABINOL IN NUCLEOSIDE AND AMINO ACID UPTAKE IN REUBER H-35 HEPATOMA CELLS

HELMUT JERING and EFRAÍN TORO-GOYCO

Department of Biochemistry and Nutrition
Medical Sciences Campus, University of Puerto Rico
San Juan, Puerto Rico 00936

Summary. DELTA-9-THC in 5×10^{-5} M concentration is a non-competitive inhibitor of thymidine uptake into Reuber hepatoma cells, but does not change uptake of uridine and leucine. Adenosine and guanosidine are affected by the drug in a similar fashion. Thymidine kinase activity is not influenced by THC. THC might inhibit DNA synthesis by limiting the supply of DTTP.

INTRODUCTION

AN ANTINEOPLASTIC effect has been attributed to Δ^9-tetrahydrocannabinol and to other cannabinoids[1, 2]. Such an effect could be attributed, in the absence of gross evidence of cytotoxicity to an impairment of macromolecular synthesis.

Studies conducted using different cell lines in tissue culture tend to indicate that this antineoplastic effect may be due to the inhibition of macromolecular synthesis (DNA, RNA and protein) by Δ^9THC[3–6]. The mechanism by which Δ^9THC reduces macromolecular synthesis is subject to speculation. Accepting as an established fact the dogma of molecular biology that the macromolecular processes of replication, transcription and translation are fundamental for protein biosynthesis and hence to eventual cellular replication and growth, one is tempted to suggest direct effects on any of these processes as possible sites of action of the drug. On the other hand, a decrease in the accessibility to the cells of the chemicals indispensable for these processes to occur (diminished availability of nucleotides and amino acids) by impairment of the transport processes at the cell membrane will yield analogous results.

The choice of a probable site of action to explain the diminution of macromolecular synthesis is further complicated by the fact that from available data existing in the literature it can be inferred that the various cell lines studied seem to react differently to the drug. For example, in Lewis lung tumor cells[3] Δ^9THC was reported to inhibit only thymidine incorporation; while in cultured lymphocytes both uptake and incorporation of

thymidine was repressed[5]. Since it was evident to us that much more additional work needs to be done on this subject we were stimulated to study the effect of Δ^9THC on nucleoside and amino acid uptake by another cell line. In this communication we present evidence indicating that Δ^9THC inhibits the uptake of various nucleosides by Reuber H-35 hepatoma cells. We further demonstrate that Δ^9THC reduces the level of intracellular thymidine nucleotides, without affecting the enzyme thymidine kinase, responsible for their intracellular synthesis.

MATERIALS AND METHODS

CELL CULTURE

H4-II-E-C3 hepatoma cells, derived from Reuber H-35 hepatoma cells[7] were a gift from McArdle Laboratory, Madison, Wisconsin. Cells were grown in 75 cm^2 Falcon tissue culture flasks in a humidified 5% CO_2, 95% air atmosphere at 37°C. The growth medium was Williams E medium supplemented with 10% heat inactivated fetal calf serum. Tissue culture reagents were purchased from Flow Laboratories, Rockville Maryland. No antibiotics were used. Cells were quantitated using a hemocytometer and cell viability was monitored by trypan blue dye exclusion (0.5% trypan blue).

MATERIALS

Methyl-^{3}H-deoxythymidine, 2,8-^{3}H-guanosine, 5-^{3}H-uridine were obtained from NEN, 5-^{3}H-L-proline and 4,5-^{3}H-L-leucine from Schwarz, Mann. Unlabeled nucleotides were purchased from Calbiochem. All other chemicals were reagent grade. Δ^9THC was kindly provided by L. S. Harris, Virginia Commonwealth University, Richmond, Virginia. This preparation was found to be homogeneous by thin layer chromatography[8]. Precoated silica gel plates from Kontes/Quantum and polyethyleneimine (PEI) cellulose plates from E.M. Laboratories were used for thin layer chromatography.

UPTAKE STUDIES

5×10^5 cells in 5 ml medium were seeded in 6 cm plastic dishes (Flow Laboratories) and 16–18 h were allowed for cell attachment. The cells were pre-incubated with different concentrations of Δ^9THC (10 μl in ethanol) as listed in the figure legends. Δ^9THC concentrations are always referred as the amount of Δ^9THC added to the culture dishes. No corrections were made for non-specific absorption to the plastic dishes and for serum binding. The same amount of ethanol was added to control cultures as to those treated with Δ^9THC. After a 15 min preincubation period radioactive nucleoside was added to a final concentration as indicated in the figure legends and the dishes were further incubated for varying intervals at 37°C. Uptake of radioactive label was terminated by aspirating the medium, rinsing three times with 3 ml ice-cold Hanks balanced salt solution (HBSS) and adding 0.5 ml of ice-cold 5% TCA. This manipulation took 15–20 sec. After 30 min at 4°C the TCA supernatant (TCA soluble fraction) was counted in Aquasol (NEN) with a

counting efficiency of 45%. The TCA precipitate, which firmly adhered to the dishes, was washed 3 times with 3 ml ice-cold 5% TCA, 2 times with absolute methanol, air-dried, solubilized with 0.5 ml of a 1 M methylbenzethonium hydroxide solution in methanol at 65°C for 15 min, transferred to scintillation vials with 3 ml of methanol and counted in PPO toluene (4 g PPO/l). Unless specified otherwise, all measurements were made in triplicate sister cultures and the averages were plotted. For measurements of amino acid uptake the medium was replaced with 2.5 ml of HBSS containing 10% fetal calf serum after the 16–18 h incubation period.

ANALYSIS OF NUCLEOTIDE POOLS

The TCA supernatant (0.5 ml) was washed three times with water-saturated ether. During this process no radioactivity was lost. A 100 μl aliquot of the ether-washed aqueous phase was spotted together with marker (thymidine, dTMP, dTDP, dTTP) on PEI cellulose thin layer sheets and developed in one dimension essentially as described by Randerath and Randerath[9] (3 min water, 3 min 0.3 M LiCl, 6 min 0.8 M LiCl, and up to 15 cm 1.4 M LiCl). After drying, carrier spots were marked under ultraviolet light, cut out and counted in PPO/toluene (4 g PPO/l) with a counting efficiency of 12%.

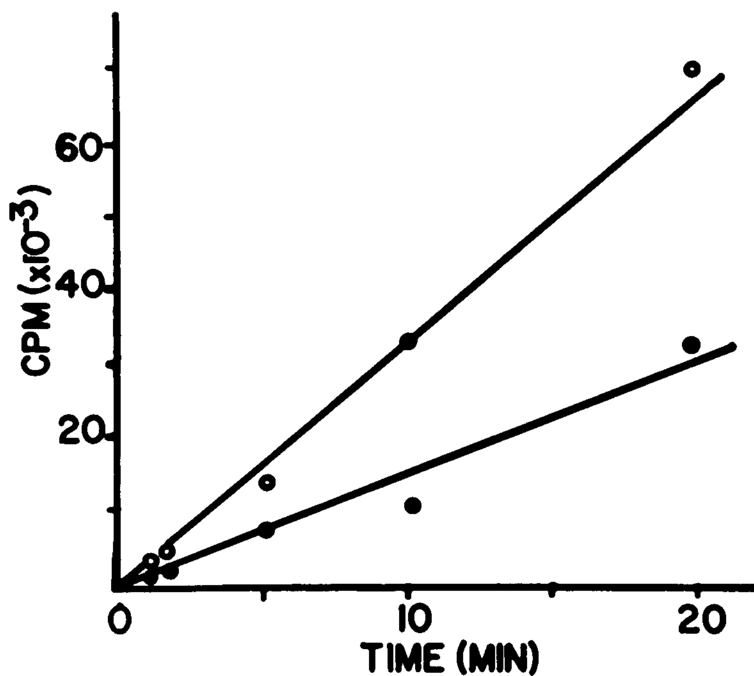
PREPARATION AND ASSAY OF THYMIDINE KINASE

Three 75 cm^2 flasks, each containing 5×10^6 cells were incubated with 5×10^{-5} M Δ^9THC, (dissolved in 75% dimethylsulfoxide (DMSO)/water) for 3 h. The final concentration of DMSO was 0.75%. Out of six control flasks, three were incubated with the drug vehicle alone. After trypsinisation the cells were washed with medium, followed by lysing buffer (10 mM Tris-HCl pH 7.6, 0.5 M KCl, 20 mM $MgCl_2$, 1 mM β-mercaptoethanol, 2×10^{-4} M adenosine triphosphate). The packed cells were suspended in four volumes of lysing buffer and lysed by quick freezing and thawing (4 times). The 100,000 g supernatant of the lysed cells was used as a source of thymidine kinase. The standard assay mixture (0.1 ml) contained 100 mM Tris-HCl pH 7.8, 3.3 mM β-mercaptoethanol, 10 mM ATP, 20 mM $MgCl_2$, 5×10^{-5} M thymidine (specific activity 0.5 Ci/mMole), and between 50 and 150 μg of protein. Under these conditions, enzyme activity was proportional to the amount of protein added and to the time of incubation (up to 25 min at 37°C). The amount of phosphorylated thymidine produced was measured as described by Breitman[10]. Protein was determined by the method of Lowry *et al.*[11].

RESULTS

UPTAKE STUDIES

The final ethanol concentration in the medium (0.2% or less) did not inhibit cell growth or nucleoside uptake. Radioactive thymidine was taken up at a constant rate into TCA insoluble material both in the presence or absence of Δ^9THC (Fig. 1a). In the TCA soluble



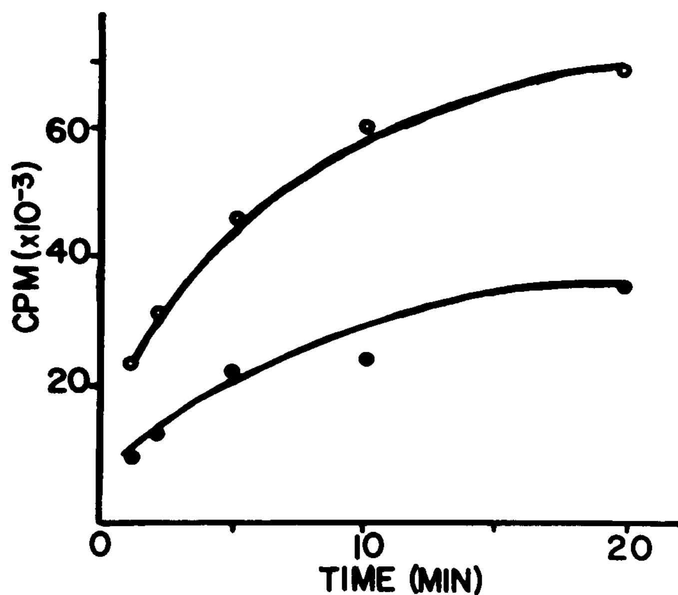


FIG. 1. Time course of thymidine uptake by Reuber H-35 hepatoma cells. Fig. 1a represents TCA-insoluble radioactivity, Fig. 1b TCA-soluble radioactivity. The final thymidine concentration was 1×10^{-6} M (2 μCi/ml). ○—○—○ no Δ^9THC present; ●—●—● in the presence of 7×10^{-5} M Δ^9THC. For experimental details see section on uptake studies.

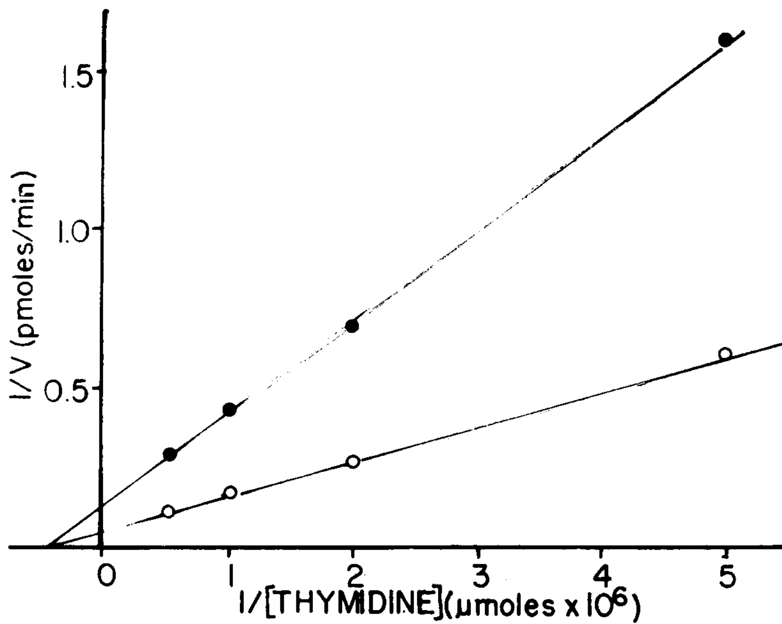


FIG. 2. Lineweaver-Burke plot showing non-competitive inhibition of thymidine uptake into TCA soluble material by 6 × 10^{-5} M Δ^9THC. The initial rates were estimated from duplicate 1 min values. The dishes were supplemented with 0.2, 0.5, 1 and 2 µM ^{3}H-thymidine (500 cpm/pmole). Uptake rates were calculated from the cpm per dish. ●—●—● with Δ^9THC, ○—○—○ without Δ^9THC.

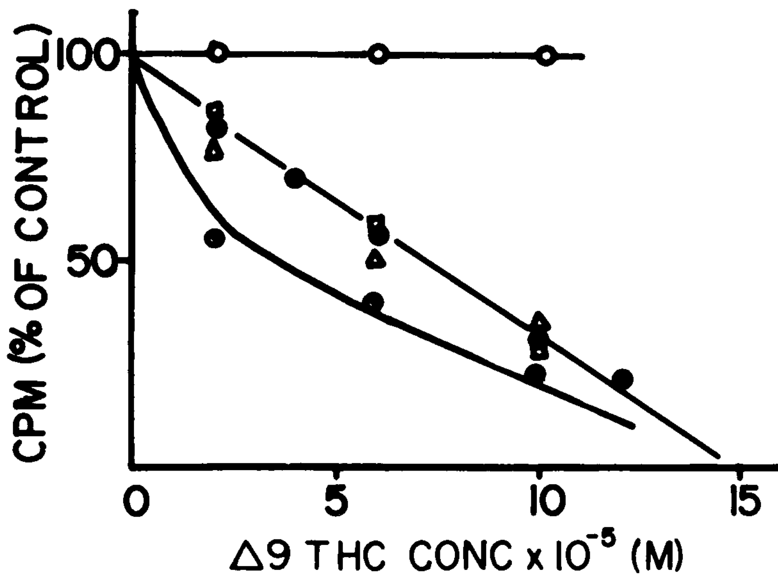


FIG. 3. Inhibition of nucleoside and amino acid uptake by different concentrations of Δ^9THC. The cells were pulse labeled with the radioactive precursor for 30 min, the reaction was stopped as described in the text and the TCA soluble radioactivity was measured. Results are expressed as % of control experiments, where the Δ^9THC concentration was zero. The TCA soluble radioactivity in cpm taken up by control cultures was: uridine 90,000; proline 29,000, leucine 10,200, thymidine 53,600, adenosine 752,000, cytidine 15,100, and guanosine 243,000. Symbols: —○—○— uridine (7.24 × 10^{-8} M, 2 µCi/ml), L-proline (1.18 × 10^{-6} M, 10 µCi/ml) and L-leucine (2 × 10^{-8} M, 4 µCi/ml). —●—●— thymidine (1 × 10^{-6} M, 2 µCi/ml), —□—□— adenosine (1 × 10^{-6} M, 2 µCi/ml), —△—△— guanosine (3.36 × 10^{-7} M, 2 µCi/ml), —⊘—⊘— cytidine (1 × 10^{-6} M, 1 µCi/ml). The concentrations are final concentrations.

fraction radioactivity accumulated and reached a plateau after 20 min. At this time apparently the intracellular nucleotide pools have equilibrated with exogenously added thymidine (Fig. 1b). Both uptake into TCA soluble material and incorporation were inhibited. A Lineweaver-Burke plot indicated that Δ^9THC is a non-competitive inhibitor of thymidine uptake (Fig. 2). The Km was estimated to be 2.2 μM which is slightly higher than the values of around 0.5 μM reported for Hela[12], 3T3[13] and Novikoff rat hepatoma cells[14], and 1 μM reported by Nahas *et al.*[5] for mitogen stimulated lymphocytes.

The effect of various Δ^9THC concentrations on the uptake of several nucleosides and amino acids is seen in Fig. 3. Even at high concentrations (10^{-4} M) Δ^9THC did not inhibit the uptake or uridine, L-proline, and L-leucine. Uptake of thymidine, adenosine, and guanosine was affected to the same extent by Δ^9THC with half maximal inhibition at 7×10^{-5} M Δ^9THC, whereas the inhibition of cytidine uptake was more pronounced (see Fig. 3).

ANALYSIS OF THYMIDINE NUCLEOTIDE POOLS

After pulse labeling with ^{3}H-thymidine the TCA soluble material was analyzed by thin layer chromatography. All radioactivity was recovered in the spots corresponding to thymidine, dTMP, dTDP and dTTP. No counts remained at the origin. Concentrations of 7×10^{-5} M Δ^9THC reduced the amount of label found in dTTP by about 50% (Fig. 4). The radioactivity contained in dTDP and dTMP was suppressed to the same extent.

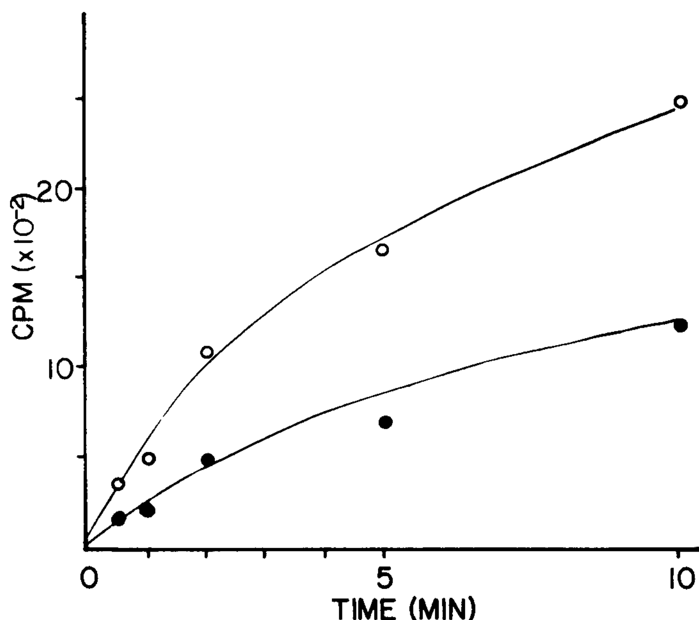


FIG. 4. Intracellular radioactive dTPP levels as a function of time in the absence and presence of Δ^9THC. After labeling the cells with 1×10^{-6} M thymidine (2 μCi/ml), the TCA soluble material was separated by thin layer chromatography and the radioactivity found in the spot corresponding to dTTP was plotted. O—O—O: dTTP in the absence of Δ^9THC; —●—●—: dTTP in the presence of 7×10^{-5} M Δ^9THC.

Intracellular free thymidine levels were not affected by Δ⁹THC. The small amounts of thymidine found (ca. 100 cpm) did not increase with increasing pulse-lengths (data not shown).

EFFECT OF Δ⁹THC ON THYMIDINE KINASE ACTIVITY

Lowered thymidine nucleotide pools might result from inhibition of thymidine kinase by Δ⁹THC. To confirm or discard this possibility the activity of thymidine kinase derived from Reuber hepatoma cells was measured. Neither Δ⁹THC nor the drug vehicle (75% DMSO) inhibited the enzyme, as seen in Table 1. The concentration of DMSO in the assay medium (7.5%) was 10 times higher than that of the preincubated cells. Preincubation of the cells for 3 h with 5×10^{-5} M Δ⁹THC prior to isolation of the enzyme resulted in no decrease of thymidine kinase activity.

TABLE 1. ACTIVITY OF THYMIDINE KINASE, EXPRESSED AS PMOLES PHOSPHORYLATED THYMIDINE PER MG PROTEIN AND PER 20 MIN INCUBATION AT 37°C. Δ⁹THC PRETREATED CELLS WERE PREINCUBATED FOR 3 H WITH 5×10^{-5} M Δ⁹THC PRIOR TO ENZYME ISOLATION, CONTROL CELLS RECEIVED ONLY SOLVENT (75% DMSO) AND NO ADDITION WAS MADE TO THE NAIVE CONTROL CELLS.

Thymidine kinase derived from	Δ⁹THC concentration 0	S[a]	3.2×10^{-4} M
Control cells	120	118	119
Naive control cells	119	119	120
Δ⁹THC pretreated cells	119	121	119

a. Assay mixture contained only solvent (10 μl 75% DMSO in a final volume of 0.1 ml).

DISCUSSION

In this kind of study cell viability is probably the single most important criterion in interpreting the data. Cannabinoids are known to be cytotoxic and it is important to determine whether the observed effects are due to cell death or just to impairment of a specific cellular process. The results presented here can be attributed with a reasonable degree of certainty, to impairment of a specific cellular process.

A second important consideration in this kind of study is the concentration of cannabinoids (in this case Δ⁹THC) used. Our results show that these concentrations were not toxic. However, how much of the total concentration of the drug is "free Δ⁹THC" is questionable. These cells cultures were grown and exposed to Δ⁹THC in the presence of serum. Proteins present in serum, especially the lipoproteins and albumin show a strong binding for Δ⁹THC and consequently we may expect a much lower effective concentration of Δ⁹THC in the medium. Nahas *et al.*[5] have demonstrated that the inhibition of thymidine incorporation by Δ⁹THC by lymphocytes depends on the amount of serum present in the

culture medium. Therefore it is likely that the actual free concentration of Δ^{9}THC interacting with the cells is lower than indicated in the text or figures. The finding that Δ^{9}THC did not inhibit uridine and leucine uptake (see Fig. 3) is in disagreement with previous reports. In studies performed with cultured lymphocytes, uridine and leucine uptake into TCA soluble material were inhibited to the same extent as thymidine uptake[5]. Obviously, the results obtained from experiments with one cell line cannot necessarily be applied to other cell lines. This view is supported by other data. End *et al.*[15] showed that Δ^{9}THC inhibited uptake of leucine and uridine in mouse neuroblastoma (NB2A) cells in tissue culture, whereas in rat glioma cells (C6) only leucine, but not uridine uptake was inhibited. In addition, Δ^{9}THC has been reported to have no effect on thymidine nucleotide pools in Lewis lung adenocarcinoma cells[3, 16].

Δ^{9}THC inhibited uptake of thymidine into thymidine nucleotides and TCA insoluble material to the same extent, suggesting a common underlying mechanism. As Δ^{9}THC decreased the amount of label found in dTTP, it is tempting to speculate that Δ^{9}THC might inhibit DNA synthesis (measured as thymidine incorporation) by limiting the supply of dTTP. This view is supported by the finding that in Hela S3 cells grown in thymidine free medium, exogenously added thymidine was incorporated into DNA almost immediately at full specific activity, blocking any further incorporation of de novo synthesized thymidine[17].

The inability to find any differences in the levels of intracellular pools of free thymidine in control and Δ^{9}THC treated cells can be explained by the loss of free intracellular thymidine by washing the cells to remove extracellular thymidine. Cass and Patterson[12] previously demonstrated that washing removes intracellular thymidine, but not thymidine nucleotides. It is a well known biochemical phenomenon that cells retain intracellular metabolites by phosphorylating them. Since cellular thymidine kinase was not affected by Δ^{9}THC, even when the cells had been exposed to Δ^{9}THC prior to isolation of the enzyme, we conclude that Δ^{9}THC interfered with thymidine uptake in some step before the formation of dTMP. A hypothesis presented here is that membrane associated proteins involved in thymidine transport are inhibited by Δ^{9}THC. It has been previously shown that Δ^{9}THC does strongly inhibit lysolecithin acyl transferase and (Na^+ + K^+) dependant ATPase, which are membrane associated enzymes[18, 19]. Further support for this hypothesis is obtained from the interpretation of the data presented in Fig. 2. The data, obtained using intact cells, may be ascribed to: (a) inhibition of an enzymatic process in the synthesis of nucleotides or (b) to the inhibition of a transport process at the membrane level, since both processes follow saturation kinetics. Having ruled out the first alternative by showing that thymidine kinase is not inhibited by Δ^{9}THC, the second alternative remains as a plausible explanation for the decreased uptake of thymidine.

Other investigators[20, 21] have already reported on the modifications occurring in the cell membrane structure and function in the presence of Δ^{9}THC. We must address ourselves to the effects of the drug on the membrane bound enzymes, especially those affecting ionic gradients and transport processes, such as the (Na^+ + K^+) ATPases. Work in our laboratory has shown that Δ^{9}THC affects the affinity of ATPases for Na^+[22]. Impairment of ionic gradients may have serious effects on cell function, especially macromolecular synthesis, without necessarily killing the cell. Disruptions in Na^+ transport lead to impaired nutrient transport and to unbalanced intracellular levels of K^+. It is a well established physiological fact that a smooth working, synchronous membrane must exist for the maintenance of the optimum intra and extracellular Na^+ and K^+ gradients. Alterations in

membrane function disrupt these gradients and other processes that eventually manifest themselves as impaired macromolecular synthesis. It has been conclusively shown that in order for protein synthesis and cell growth to proceed smoothly intracellular K^+ must remain within 60–80% of the control levels[23, 24]. A growing body of data point toward cannabinoids as causing alterations in membrane structure[20, 21], diminished membrane bound enzyme activity[18, 19] and diminished macromolecular synthesis[3–6].

ACKNOWLEDGEMENTS

This work was supported in part by Grant SP 30 CA 16598-02 from the National Cancer Institute and USPHS Grant No. DA-00490. We thank Professor L. S. Harris, Department of Pharmacology, Medical College of Virginia for his gift of the Δ^9THC, Mrs. Esther Torres for clerical help and Mr. Juan Caloca for the photographic work.

REFERENCES

1. HARRIS, L. S., MUNSON, A. E. and FRIEDMAN, M. A., *Pharmacologist* **16**,259 (1974).
2. MUNSON, A. E., HARRIS, L. S. and FRIEDMAN, M. A., *J. Natl. Cancer Inst.* **55**,597–602 (1975).
3. WHITE, A. C., MUNSON, J. A., MUNSON, A. E. and CARCHMAN, R. A., *J. Natl. Cancer Inst.* **56**,655–658 (1976).
4. CARCHMAN, R. A., HARRIS, L. S. and MUNSON, A. E., *Cancer Res.* **36**,95–100 (1976).
5. NAHAS, G. G., MORISHIMA, A. and DESOIZE, B., *Fed. Proc.* **36**,1748-1752 (1977).
6. DESOIZE, B. and NAHAS, G. G., *C. R. Acad. Sci.* **281**,475–478 (1975).
7. REUBER, M. D., *J. Nat. Cancer Inst.* **26**,891-899 (1961).
8. MARTIN, B. R., DEWEY, W. L., HARRIS, L. S. and BECKNER, J. S., *J. Pharmacol. Exp. Ther.* **196**,128–144 (1976).
9. RANDERATH, K. and RANDERATH, E., in "Methods in Enzymology," Vol. 12, 323–347, 1967.
10. BREITMAN, T., *Biochim. Biophys. Acta* **67**,153–155 (1963).
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., *J. Biol. Chem.* **193**,265–275 (1951).
12. CASS, C. E. and PATTERSON, A. R. P., *Exp. Cell Res.* **105**,427–435 (1977).
13. CUNNINGHAM, D. D. and REMO, R. A., *J. Biol. Chem.* **248**,6282–6283 (1973).
14. PLAGEMANN, P. G. W. and ERBE, J., *J. Cell. Physiol.* **83**,337–343 (1974).
15. END, D. W., THOURSEN, K., DEWEY, W. L. and CARCHMAN, R. A., *Mol. Pharmacol.* **13**,864–871 (1977).
16. CARCHMAN, R. A., WARNER, W., WHITE, A. C. and HARRIS, L. S., in "Marihuana: Chemistry, Biochemistry, and Cellular Effects," pp. 329–345; Nahas, G. G., ed., Springer-Verlag, New York, 1976.
17. KUEBBING, D. and WERNER, R., *Proc. Natl. Acad. Sci.* **72**,3333–3336 (1975).
18. GREENBERG, J. H., SAUNDERS, M. E. and MELLORS, A., *Science* **197**,475–477 (1977).
19. TORO-GOYCO, E., RODRÍGUEZ, M. B. and PRESTON, A. M., *Mol. Pharmacol.* **14**,130–137 (1978).
20. CHARI-BITRON, A., in "Marihuana: chemistry, biochemistry and cellular effects," 273–281 Nahas, G. G., ed., Springer-Verlag, New York, 1976.
21. LAURENT, B. and ROY, P. E., *Int. J. Clin. Pharmacol. Biopharm* **12**,261-266 (1975).
22. TORO-GOYCO, E., RODRÍGUEZ, M. B., PRESTON, A. M. and JERING, H. (these proceedings). 229–242.
23. LUBIN, M. *Federation Proc.* **23**,994–1001 (1964).
24. LEDBETTER, M. L. S. and LUBIN, M. *Exp. Cell Res.* 223-236 1977).

CANNABINOIDS: THE INFLUENCE ON CELL PROLIFERATION AND MACROMOLECULAR BIOSYNTHESIS

G. S. STEIN, M. J. MON, A. E. HAAS and R. L. JANSING

Department of Biochemistry and Molecular Biology, College of Medicine, The University of Florida, Gainesville, Florida 32610

and

J. L. STEIN

Department of Immunology and Medical Microbiology, College of Medicine, The University of Florida, Gainesville, Florida 32610

Summary. We have examined the influence of psychoactive (Δ^9-THC, Δ^8-THC, 11-OH-Δ^9-THC) and nonpsychoactive (cannabinol) cannabinoids on proliferation of human cells (HeLa S_3 cells grown in suspension culture) and on biochemical events associated with the proliferative process. The four cannabinoids studied brought about a dose-dependent inhibition in the rate of cell growth. Pulse-labeling studies indicated a cannabinoid-induced decrease in incorporation of ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine into DNA, RNA and protein, respectively, in intact cells and a comparable decrease in the acid-soluble intracellular precursor pools was observed. Results from in vitro nuclear and chromatin transcription experiments did not suggest a drug-induced change in RNA synthesis. It therefore appears that quantitative alterations in the synthesis and/or turnover of nucleic acids and proteins do not result from cannabinoid treatment of HeLa cells. However, other data suggest that cannabinoids affect the synthesis of specific macromolecules.

Since modifications in gene expression during the cell cycle are prerequisite for DNA replication and mitosis, the influence of cannabinoids on the structural and functional properties of the genome were examined. The four cannabinoids studied did not alter the staining pattern as observed on polyacrylamide gels of chromosomal proteins—histones and nonhistone chromosomal proteins—in HeLa cell chromatin. However, treatment of HeLa cells with psychoactive and nonpsychoactive cannabinoids resulted in changes in the incorporation of ^{3}H-leucine into histone fractions and into specific molecular weight classes of nonhistone chromosomal proteins; acetylation and phosphorylation of chromosomal proteins were also altered.

INTRODUCTION

ELUCIDATING the influence of cannabinoids on cell proliferation and on macromolecular biosynthetic events required for cell division is important from several standpoints. Because cannabinoids are drugs of abuse, it would be instructive to determine their effects on cell proliferation, which is associated with a broad spectrum of processes including wound healing, tissue regeneration, erythropoiesis, the immunological response and replacement of epithelial cells which line the gastrointestinal tract. Since cannabinoids have recently

been shown to be effective clinically as anti-emetics in conjunction with cancer chemotherapy, it is necessary to determine whether cannabinoids and chemotherapeutic agents function in a synergistic or antagonistic manner with respect to their influence on cell proliferation. Other clinical applications which further necessitate evaluation of possible cannabinoid-induced aberrations in the proliferative process include treatment of glaucoma.

In this chapter we will first consider the influence of two naturally occurring psychoactive cannabinoids (Δ^9-tetrahydrocannabinol (Δ^9-THC), and Δ^8-tetrahydrocannabinol (Δ^8-THC)), a psychoactive cannabinoid metabolite (11-OH-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC)) and a nonpsychoactive cannabinoid (cannabinol (CBN)) on cell division in exponentially growing human cervical carcinoma cells (HeLa cells) maintained in suspension culture. Then, since progression through the cell cycle requires a complex and interdependent series of biochemical events involving changes in gene expression, we will focus on cannabinoid-induced effects on DNA, RNA and protein metabolism as well as on cannabinoid-induced effects on the structural and functional properties of the genome.

Throughout the chapter we will attempt to emphasize experimental approaches we have taken and to evaluate their strengths and weaknesses with respect to the data presented. Additionally, we will try, where possible, to point out the directions that our work is presently taking.

I. INFLUENCE OF CANNABINOIDS ON CELL PROLIFERATION

Our initial approach to assessing the influence of cannabinoids on the ability of cells to proliferate was to examine the effect of systematically varied concentrations of psychoactive and nonpsychoactive cannabinoids on the growth kinetics of continuously dividing HeLa S_3 cells. At the start of each experiment the cell density was adjusted to 2.5×10^5 cells per milliliter to permit the potential for exponential growth. The cannabinoid was present in the culture medium throughout the experiment. The drug vehicle was 95% ethanol and the vehicle-treated culture contained the same concentration of ethanol (0.15%) as the drug-treated cultures. The growth rates were determined by measuring the increase in cell number at specific time intervals throughout the doubling time of the untreated control culture (approximately 24 hours). Cell viability (ascertained by trypan blue exclusion) was greater than 98% for the control, vehicle-treated and all cannabinoid-treated cultures at 10 minutes and 40 minutes after initiation of the growth experiments; thus it is unlikely that the cannabinoids and/or the drug vehicle produced toxic, nonspecific cell destruction that would result in incorrect initial "viable" cell number.

In general, micromolar concentrations of cannabinoids cause a dose-dependent depression in the exponential growth of HeLa cells. As shown in Fig. 1, Δ^9-THC at concentrations of 5 μM, 10 μM, 15 μM, 20 μM and 40 μM depressed the exponential growth of HeLa S_3 cells approximately 8%, 12%, 20%, 31% and 55%, respectively. A Δ^9-THC concentration of 0.5 μM exhibited no significant effect on growth when compared with the vehicle-treated cultures. Δ^8-THC-treated cultures (Fig. 2) exhibited growth depression of approximately 13%, 22% and 37% at respective Δ^8-THC concentrations of 1 μM, 5 μM and 10 μM. Figure 3 shows that 11-OH-Δ^9-THC at 5 μM, 10 μM and 15 μM concentrations exerted respective growth depressions of approximately 8%, 20%, and 32%.

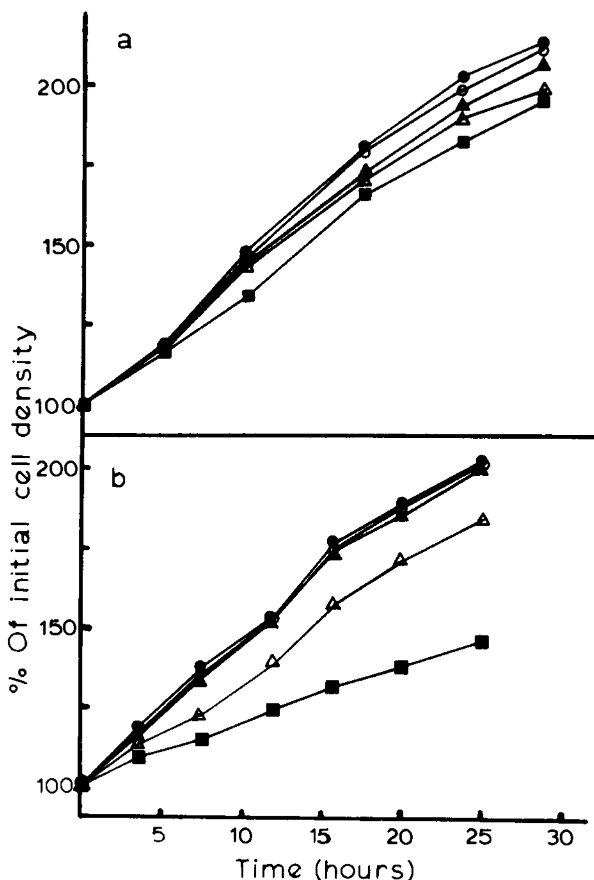


FIG. 1. Effects of varying concentrations of Δ^9-THC on exponentially growing HeLa S_3 cells in suspension cultures. The data are expressed as the percent of initial cell density as a function of time in the presence of: (a) control (●), vehicle (○), 5 μM Δ^9-THC (▲), 10 μM Δ^9-THC (△), 15 μM Δ^9-THC (■); and (b) control (●), vehicle (○), 0.5 μM Δ^9-THC (▲), 20 μM Δ^9-THC (△), 40 μM Δ^9-THC (■).

Cannabinol-treated cultures (Fig. 4) exhibited growth depressions of approximately 7%, 13% and 30% at respective cannabinoid concentrations of 5 μM, 10 μM and 15 μM. The vehicle-treated cultures (Figs. 1–4) displayed a very slight decrease in rate of growth compared with the untreated control cultures.

These growth kinetic studies serve as an indication of the relative influence of the four cannabinoids in terms of their overall effect on cell proliferation. These studies facilitate determining if there is a relationship between the psychoactive index of the compounds and their effect on cell growth. The results obtained would indicate that at approximately equal drug concentrations, the order of decreasing potency of the four cannabinoids studied is Δ^8-THC, 11-OH-Δ^9-THC, CBN, Δ^9-THC. This order of potency suggests that the degree of psychoactivity of the compounds is not directly related to their effects on cell proliferation, since the nonpsychoactive cannabinoid (CBN) falls in the midst of these psychoactive cannabinoids (Δ^8-THC, 11-OH-Δ^9-THC, Δ^9-THC) in terms of capacity to depress cell growth. It is important to note, as will be discussed later, that more Δ^8-THC is

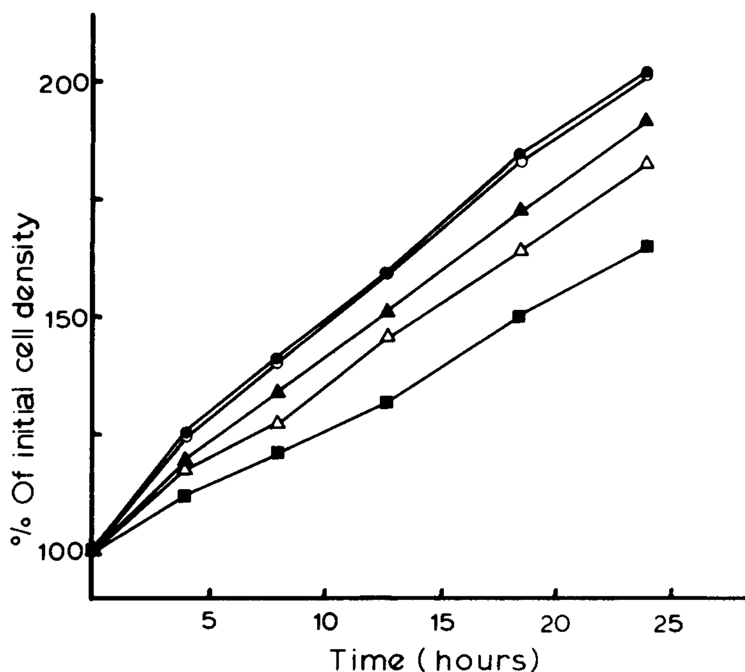


FIG. 2. Effects of varying concentrations of Δ^8-THC on exponentially growing HeLa S_3 cells in suspension cultures. The data are expressed as the percent of initial cell density as a function of time in a control (●) and in the presence of vehicle (○), 1 μM Δ^8-THC (▲), 5 μM Δ^8-THC (△), 10 μM Δ^8-THC (■).

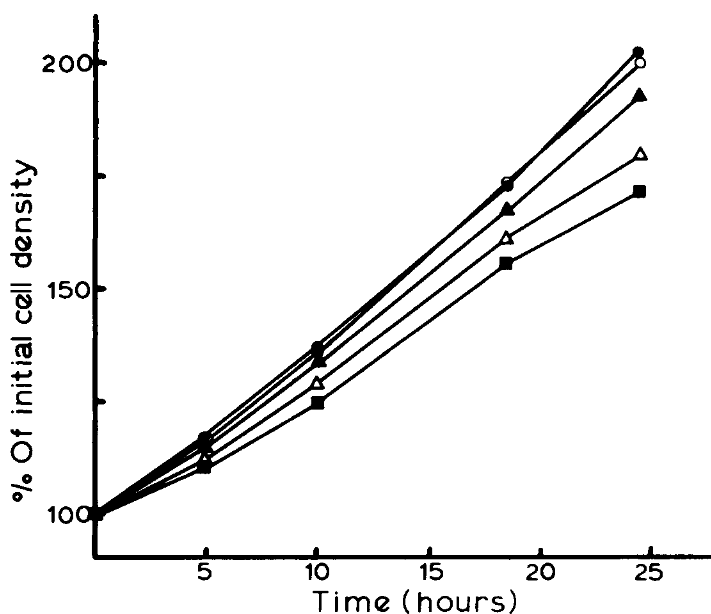


FIG. 3. Effects of varying concentrations of 11-OH-Δ^9-THC on exponentially growing HeLa S_3 cells in suspension cultures. The data are expressed as the percent of initial cell density as a function of time in a control (●) and in the presence of vehicle (○), 5 μM 11-OH-Δ^9-THC (▲), 10 μM 11-OH-Δ^9-THC (△), 15 μM 11-OH-Δ^9-THC (■).

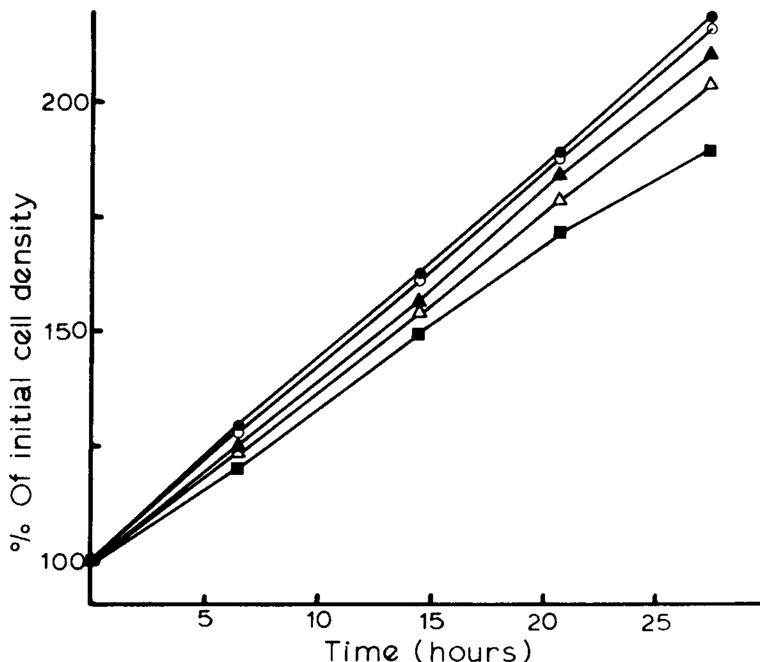


FIG. 4. Effects of varying concentrations of cannabinol (CBN) on exponentially growing HeLa S_3 cells in suspension cultures. The data are expressed as the percent of initial cell density as a function of time in a control (●) and in the presence of vehicle (○), 5 μM CBN (▲), 10 μM CBN (△), 15 μM CBN (■).

associated with the nucleus of the cell, and therefore its apparent higher potency may be due to a larger amount of the drug being present at a potentially active site.

The results from these growth kinetic studies were utilized to determine the drug concentrations employed in subsequent experiments. The concentrations chosen were those which exerted a 30–40% growth depression in continuously dividing HeLa S_3 cell cultures; 30 μM for Δ^9-THC, 10 μM for Δ^8-THC, 15 μM for 11-OH-Δ^9-THC and 15 μM for CBN.

Because most behavioral effects of cannabinoids have been shown to be reversible, we examined reversibility of cannabinoid-induced growth inhibition. HeLa S_3 cells were grown for 24 hours in the presence of 30 μM concentrations of Δ^9-THC, a drug concentration which exerts approximately 35% inhibition in the rate of cell growth, and then growth was observed for another 48 hours after release from Δ^9-THC treatment. During the first 24 hours, while in the presence of a 30 μM concentration of Δ^9-THC, the cells demonstrated a 35% decrease in growth compared with appropriate controls (Fig. 5a). After release from Δ^9-THC treatment, the cells demonstrated only a 21% decrease in growth within the next 24 hours (Fig. 5b), returning to their normal rate of cell proliferation during the following 24 hours (Fig. 5c). Thus the effect of Δ^9-THC on cell proliferation appears to be reversible within 48 hours after termination of the exposure of HeLa cells to the drug.

Two questions relevant to interpretation of these cell growth studies and other data to be described subsequently are: How much cannabinoid gets into or is associated with the cells? What is the subcellular distribution of the drug? Defining the drug level present in various intracellular compartments is important for evaluating the site of action and the

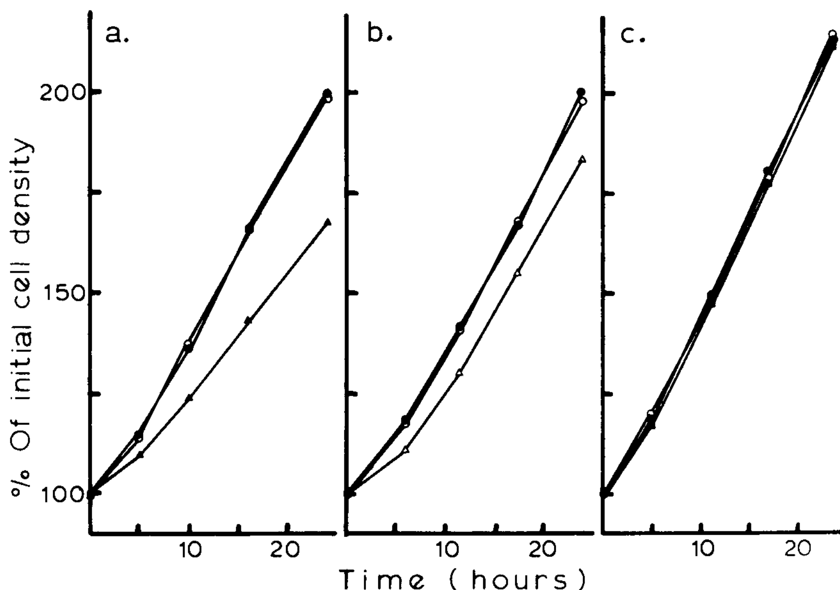


FIG. 5. Effect of removal of Δ^9-THC on exponentially growing HeLa S_3 cells previously exposed to 30 μM concentration of Δ^9-THC in suspension culture. (a) Initial 24-hour treatment of cells with 30 μM Δ^9-THC (▲). (b) Cell growth of pretreated cells (△) for the 24 hours following the first resuspension in Δ^9-THC-free medium. (c) Cell growth of pretreated cell (■) for the 24 hours following the second resuspension in Δ^9-THC-free medium. Untreated control (●) and vehicle-treated control (○) were also monitored for comparison.

mechanism of cannabinoids although caution must be exercised in assuming that the association of cannabinoids with particular intracellular organelles or macromolecular complexes reflects a functional interaction. Because cannabinoids exhibit a high affinity for nonspecific components of serum in the cell culture medium and for cell culture glassware, the concentration of cannabinoids in the growth medium may not be a reliable reflection of the amount of drug available to the cells. Knowing intracellular concentrations of cannabinoids is also essential for execution of experiments in which the drug is utilized in cell-free systems to insure that drug levels reflect conditions in intact cells.

To determine the amount of cannabinoids associated with the various cellular compartments of HeLa S_3 cells, the cells were incubated in the presence of ^{3}H-labeled cannabinoids for 30 minutes and the subcellular fractions were isolated by a detergent procedure routinely used in our laboratory[35]. To compensate for nonspecific sequestering of cannabinoids, all experiments were carried out in culture flasks of the same dimensions. The total recovery of the radiolabeled cannabinoids was greater than 86%. The percent of cannabinoids associated with the cells was measured to be from 6.3% to 10.00% (9.1 ± 0.2% for Δ^9-THC, 9.1 ± 0.3% for Δ^8-THC, 10.0 ± 0.17% for 11-OH-Δ^9-THC, 6.3 ± 0.1% for CBN) of that added to the culture medium. Of the drug entering the cell, 1.27% to 10.4% (1.27 ± 0.03% for Δ^9-THC, 10.4 ± 1.3% for Δ^8-THC, 1.06 ± 0.05% for 11-OH-Δ^9-THC, 1.65 ± 0.06% for CBN) was isolated in the "nuclear" fraction and 0.86% to 6.9% (0.96 ± 0.02% for Δ^9-THC, 6.9 ± 1.0% for Δ^8-THC, 0.86 ± 0.04% for 11-OH-Δ^9-THC and 1.39 ± 0.05% for CBN) was associated with chromatin (the isolated eukaryotic genome) (Table 1, data expressed as picograms of cannabinoid per 10^3 cells).

TABLE 1. THC (AT A CULTURE MEDIUM CONCENTRATION OF 30 μM) IN THE SUBCELLULAR FRACTIONS OF HELA S_3 CELLS

Fraction	PG Δ^9-THC/10^3 cells	PG Δ^8-THC/10^3 cells	PG 11-OH-Δ^9-THC/10^3 cells	PG CBN/10^3 cells
Cellular	1436 ± 26	1558 ± 56	1724 ± 21	1306 ± 14
Cytoplasmic	1418 ± 25	1394 ± 36	1708 ± 19	1285 ± 14
Nuclear	18 ± 0.5	162 ± 20	18 ± 0.9	22 ± 0.8
Nucleoplasmic	4 ± 0.2	56 ± 5	3 ± 0.2	3 ± 0.2
Chromatin	14 ± 0.3	107 ± 16	15 ± 0.7	18 ± 0.7

Exponentially growing HeLa S_3 cells in suspension cultures were exposed for 30 minutes at 37°C to 30 μM concentration each of ^{3}H-Δ^9-THC, ^{3}H-Δ^8-THC, ^{3}H-11-OH-Δ^9-THC, or ^{3}H-CBN, all with specific activities of 20 μCi/mg. Results were calculated as picograms (PG) of cannabinoid from the amount of ^{3}H-cannabinoid as determined from internal standards appropriate for each fraction isolated and were expressed as mean $\pm$ average deviation of three separate determinations.

Eleven-OH-Δ^9-THC exhibited the largest amount associated with the "cellular" fraction while Δ^8-THC exhibited the largest amounts associated with the "nuclear" and "chromatin" fractions. It is interesting to note that Δ^8-THC also displayed the most pronounced effect of all four cannabinoids in terms of depression of cell growth rates. Therefore, as previously mentioned, this apparent higher potency of Δ^8-THC may be related to the larger quantity of the drug associated with the cell's nucleus.

At a first glance our results appear to be inconsistent with those previously obtained from subcellular fractionation procedures in which crude nuclear preparations were made from mouse brain[10]. Dewey and coworkers reported that crude mouse brain nuclei contained 20–25% of the total intracellular radiolabeled Δ^9-THC, a higher percentage of the cellular cannabinoid than we find associated with the HeLa cell "nuclear" fraction. To resolve the apparent differences in the representation of ^{3}H-Δ^9-THC in nuclei from mouse brain and HeLa cells, we prepared a crude nuclear fraction from ^{3}H-Δ^9-THC-treated HeLa cells by the sucrose procedure of DeRobertis *et al.*[8]. In the HeLa nuclei prepared by the sucrose method, 86% of the radiolabeled cannabinoid was associated with the "cytoplasmic" fraction and 14% with the "nuclear" fraction. The level of Δ^9-THC in the sucrose-prepared HeLa nuclei compares quite favorably with that in mouse brain nuclei isolated by the same procedure[10]. Since the HeLa nuclei prepared by the sucrose method are associated with significant amounts of cytoplasmic material (as observed by phase contrast microscopy) and the HeLa nuclei prepared by our detergent procedures are not, it seems reasonable to conclude that the elevated levels of Δ^9-THC found in sucrose-prepared nuclei from HeLa or mouse brain cells are most likely attributable to drug present in cytoplasmic contaminants. However, the possibility that bona fide nuclear material is lost during preparation of nuclei with detergents should not be dismissed.

II. INFLUENCE OF CANNABINOIDS ON NUCLEIC ACID AND PROTEIN SYNTHESIS

Cell proliferation involves a complex and interdependent series of biochemical events requiring differential gene expression, and such modifications in gene expression, including RNA and protein synthesis, are prerequisite for DNA replication and mitotic division. We

therefore examined whether treatment of human cells with cannabinoids results in perturbations of macromolecular biosynthetic events essential for cell proliferation. We assayed the influence of cannabinoids (at concentrations which affect the rate of cell proliferation) on the biosynthesis of protein, DNA and RNA in continuously dividing HeLa S_3 cells by pulse labeling the cells with ^{3}H-leucine, ^{3}H-thymidine or ^{3}H-uridine, respectively, and determining the incorporation of these radioactive protein and nucleic acid precursors into acid-insoluble material. The effect of cannabinoids on cellular uptake of the radiolabeled acid-soluble precursors was also monitored since a decrease of radiolabeled precursors in the acid-soluble pool would limit the incorporation into the acid-insoluble fractions; thus any fluctuations in the precursor uptake would yield misleading data as to the actual direct effect of cannabinoids on macromolecular synthesis.

At the onset of these studies, the effect of the drug vehicle (95% ethanol) on the parameters assayed was ascertained. Exponentially growing HeLa S_3 cells were exposed for 1 hour to 24 mM, 48 mM, 72 mM and 96 mM concentrations of ethanol. The cells were then pulse labeled for 15 minutes with ^{3}H-leucine, ^{3}H-thymidine and ^{3}H-uridine and the incorporation of radioactivity into the acid-insoluble and acid-soluble fractions was determined (Fig. 6). After 1 hour of exposure the vehicle-treated cells displayed a marked, dose-dependent decrease in labeled precursor incorporation into DNA and RNA. The vehicle effect on the incorporation of ^{3}H-leucine into protein was insignificant within experimental error. The vehicle displayed no effect on the size of the intracellular radiolabeled pool and thus appeared to have no effect on the cellular uptake of the radiolabeled acid-soluble precursors.

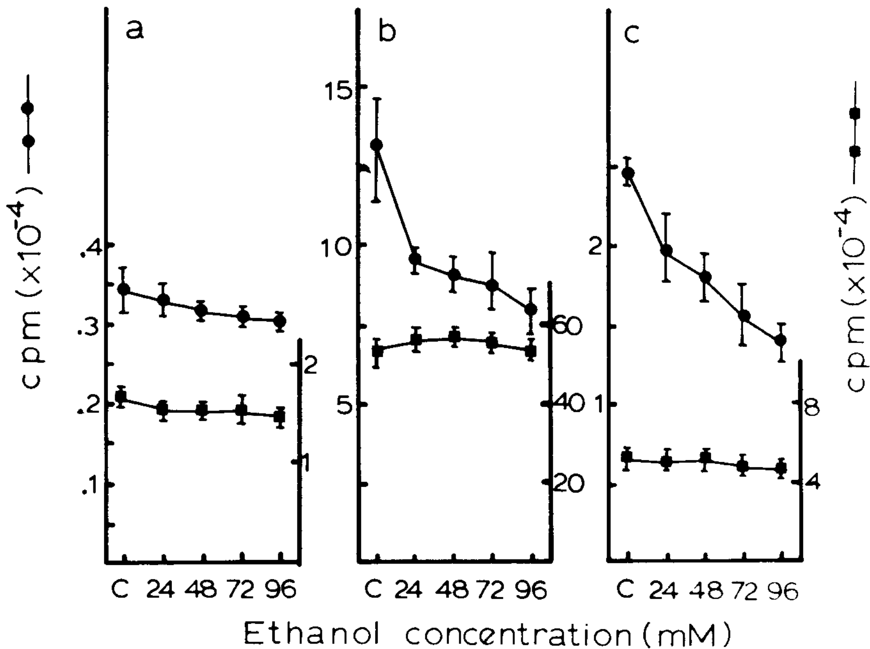


FIG. 6. Effects of drug vehicle (ethanol) on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (■) fractions was determined for cells pretreated for 10 hours with 24, 48, 72, and 96 mM concentrations of drug vehicle (ethanol). C = untreated control.

To determine whether the vehicle effect was temporary, the same pulse-labeling studies with ^{3}H-leucine, ^{3}H-thymidine and ^{3}H-uridine were carried out with HeLa S_3 cells treated with 0.15% ethanol for 1, 3, 6 and 9 hours prior to pulse labeling with the appropriate radioactive precursors (Fig. 7). Again, no significant vehicle effect was observed on incorporation of ^{3}H-leucine into protein; but the initial vehicle depression on the incorporation of ^{3}H-thymidine and ^{3}H-uridine into DNA and RNA, respectively, was reversed and returned to normal by 9 hours after introduction of the vehicle into the culture medium. Therefore in both drug and vehicle studies to be described subsequently, cells were treated for at least 10 hours in order to avoid vehicle depression of radiolabeled precursor incorporation into DNA and RNA.

Figures 8–12 show the effects of varying concentrations of cannabinoids on the incorporation of radiolabeled precursors into total cellular protein, DNA and RNA (acid-insoluble radioactivity) and into the intracellular precursor pools (acid-soluble radioactivity). In general, the cannabinoids exerted a significant dose-dependent depression on the apparent rates of DNA and RNA synthesis in HeLa cells after 10 hours of exposure to cannabinoid concentrations of 5 μM or greater. The inhibitory effect of cannabinoid on the apparent rate of protein synthesis was not as marked and in the case of Δ^9-THC required a concentration of at least 30 μM before an effect was observed.

As shown in Figs. 8 and 9, a dose-dependent depression (17%–63% for DNA, 19%–55% for RNA) in the apparent rates of DNA and RNA synthesis was observed after 10 hours of exposure to 10–40 μM concentrations of Δ^9-THC. A depressant effect on the apparent rate of protein synthesis (23%–25%) was observed at 30 μM and 40 μM concentrations of

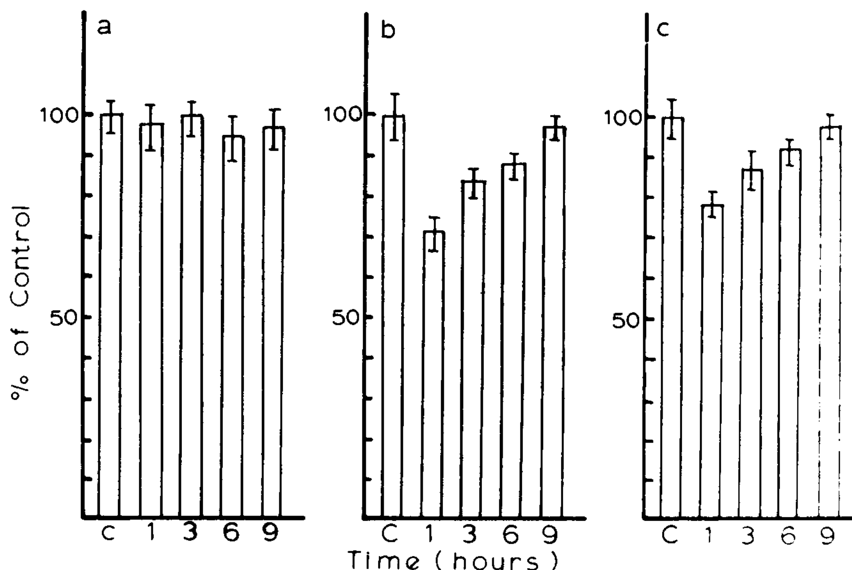


FIG. 7. Effects of drug vehicle (95% ethanol) on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble fractions was determined for cells pretreated with 71 μl of 95% ethanol/50 ml of culture media for 1, 3, 6, and 9 hours. Appropriate untreated controls were obtained for each radioactive precursor at each time interval. Results are plotted as percent incorporation of control for each radioactive precursor at 1, 3, 6, and 9 hours of preincubation with drug vehicle.

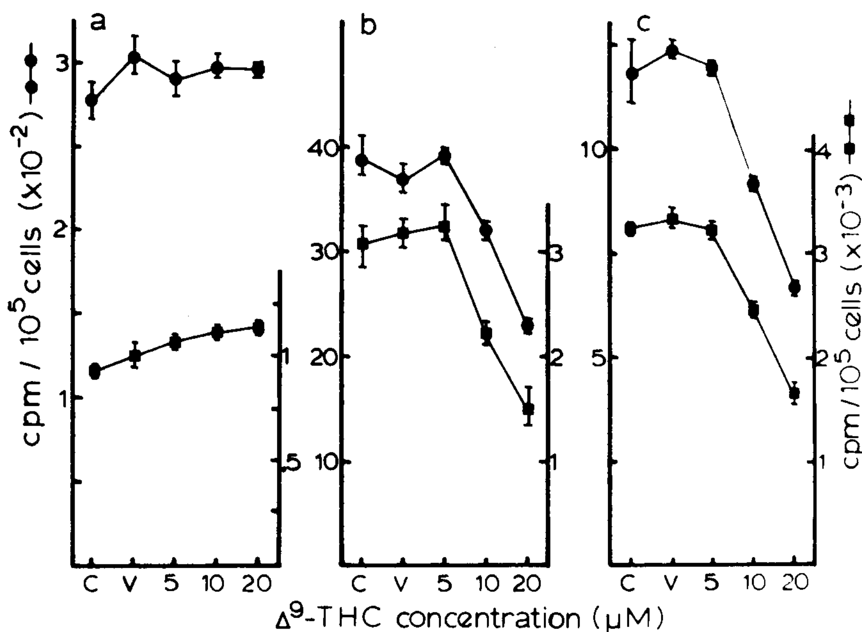


FIG. 8. Effect of Δ^9-THC on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension culture were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (■) fractions was determined for cells pretreated for 10 hours with 5 μM, 10 μM, and 20 μM concentrations of Δ^9-THC. C = control. V = vehicle-treated cells.

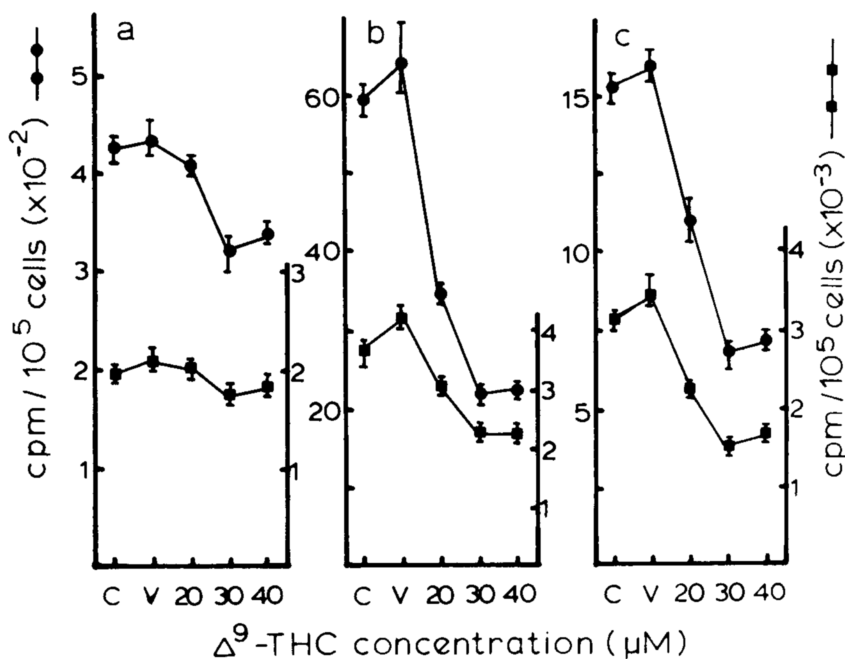


FIG. 9. Effect of Δ^9-THC on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (■) fractions was determined for cells pretreated for 10 hours with 20 μM, 30 μM, and 40 μM concentrations of Δ^9-THC. C = control. V = vehicle-treated cells.

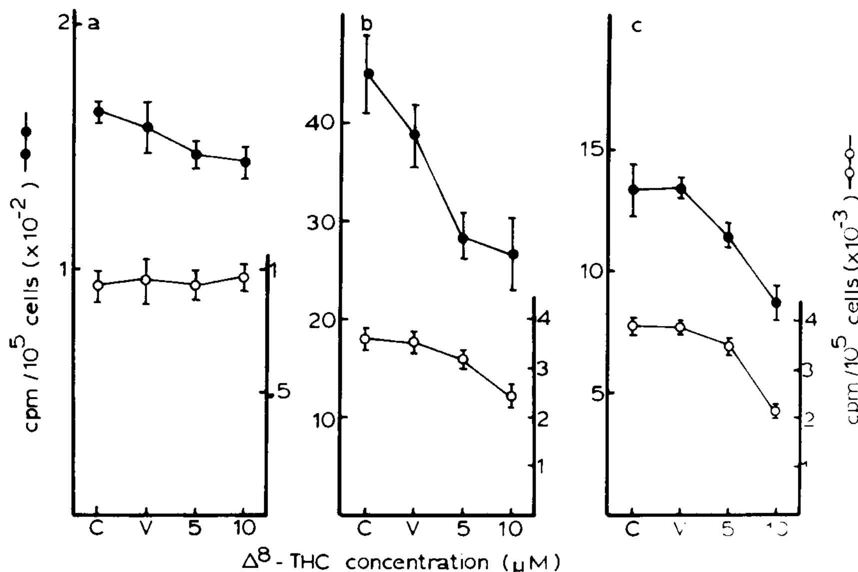


FIG. 10. Effect of Δ^8-THC on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (○) fractions was determined for cells pretreated for 10 hours with 5 μM and 10 μM concentrations of Δ^8-THC. C = control. V = vehicle-treated cells.

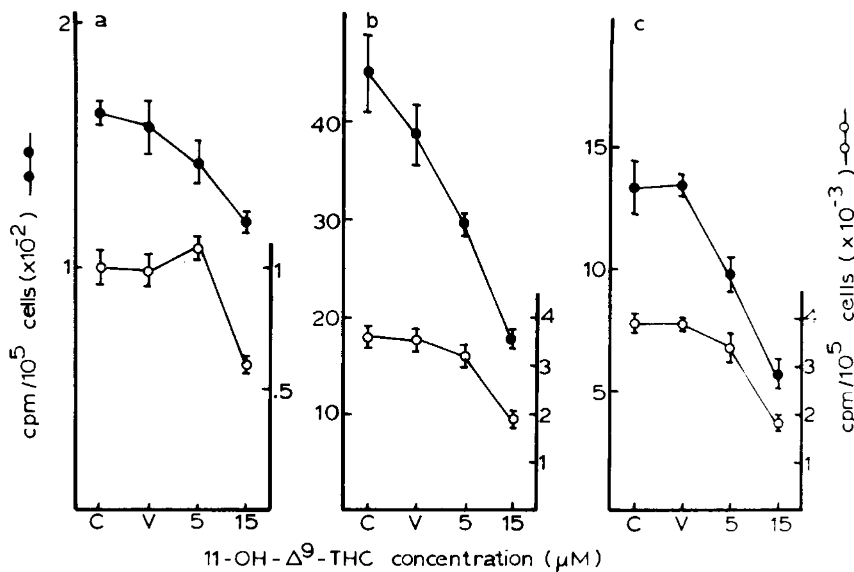


FIG. 11. Effect of 11-OH-Δ^9-THC on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (○) fractions was determined for cells pretreated for 10 hours with 5 μM and 15 μM concentrations of 11-OH-Δ^9-THC. C = control. V = vehicle-treated cells.

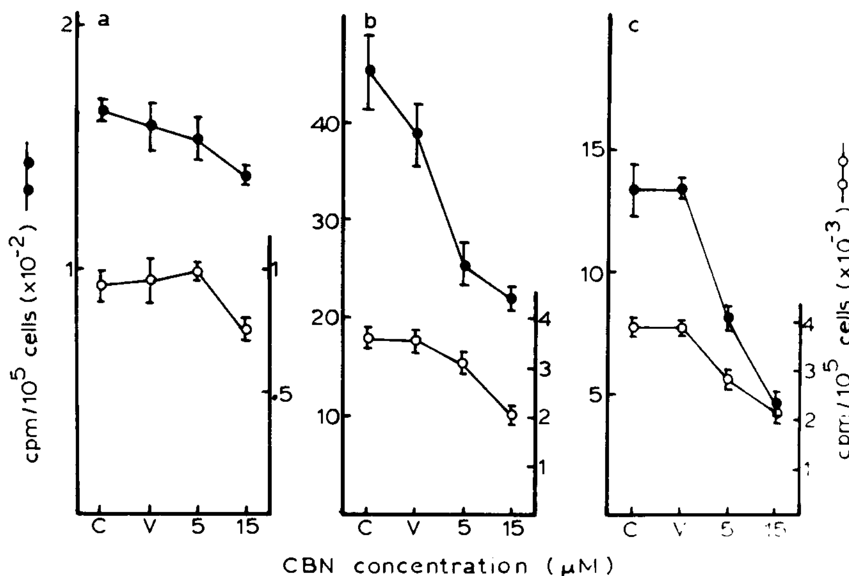


FIG. 12. Effect of cannabinol (CBN) on the incorporation of (a) ^{3}H-leucine, (b) ^{3}H-thymidine, and (c) ^{3}H-uridine into protein, DNA, and RNA respectively. Exponentially growing HeLa S_3 cells in suspension cultures were pulse-labeled for 15 minutes with the appropriate radioactive precursor. The incorporation of radioactivity into the acid-insoluble (●) and acid-soluble (○) fractions was determined for cells pretreated for 10 hours with 5 μM and 15 μM concentrations of CBN. C = control. V = vehicle-treated cells.

Δ^9-THC. Exposure to a 5 μM concentration of Δ^9-THC did not appear to affect the apparent rates of DNA or RNA synthesis, while exposure to 5 μM, 10 μM, or 20 μM concentrations of Δ^9-THC did not significantly affect the apparent rate of protein synthesis.

Figure 10 shows that Δ^8-THC depressed (35%–40% for DNA and 9%–30% for RNA) the apparent rates of DNA and RNA synthesis at 5 μM and 15 μM concentrations. Only a very slight inhibition (9%–12%) of the apparent rate of protein synthesis was observed at 5 μM and 10 μM concentrations of Δ^8-THC.

11-OH-Δ^9-THC (Fig. 11) caused decreases (34%–60% for DNA and 27%–59% for RNA) in the apparent rates of DNA and RNA synthesis at 5 μM and 15 μM concentrations. A 13%–27% depression in the apparent rate of protein synthesis was observed at 5 μM and 15 μM concentrations of 11-OH-Δ^9-THC.

Figure 12 shows that cannabinol exerted a depression (42%–51% for DNA and 40%–66% for RNA) on the apparent rates of DNA and RNA synthesis at 5 μM and 15 μM concentrations. The apparent rate of protein synthesis was depressed 12%–16% by 5 μM and 15 μM concentrations of cannabinol.

The data in Figs. 8–12 also clearly indicate that the observed decreases in the rates of incorporation of radiolabeled precursors into DNA, RNA and protein were accompanied by comparable decreases in the levels of radiolabeled precursors in the intracellular, acid-soluble precursor pools. This observation was true of all four cannabinoids studied. It is therefore important to consider the possibility that the observed cannabinoid-induced reduction of radiolabeled precursor incorporation into cellular macromolecules may reflect a decreased availability of intracellular radiolabeled precursor. Such cannabinoid-induced changes in nucleotide and amino acid precursor pools could be caused by a

decreased precursor uptake resulting from an inhibition in permeability or transport across the cellular membrane. Another possibility would be a cannabinoid-induced increase of the cell's endogenous precursor pools, thus impeding or slowing down exogenous precursor uptake.

To investigate further the cannabinoid effect on the intracellular precursor pools for RNA synthesis, we used two-dimensional thin layer chromatography to analyze both the size and the specific activity of the intracellular uridine precursor pools of cells treated with Δ^9-THC. Exponentially growing HeLa S_3 cells were maintained for 10 hours in the presence of a 30 μM concentration of Δ^9-THC and then pulse labeled for 15 minutes with ^{3}H-uridine. The acid-soluble intracellular material was isolated, extracted with anhydrous ether, and digested with *E. coli* alkaline phosphatase[4, 5, 12]; thus, the uridine pool measurements reflect the total uridine present as nucleoside and nucleotide precursors. The uridine pools were isolated by two-dimensional thin layer chromatography on MN300 cellulose[31]. The size of the uridine pool was measured spectrophotometrically and the radioactivity of ^{3}H-uridine was assayed in a liquid scintillation spectrometer. The specific activity of the uridine pool was calculated as a ratio of cpm ^{3}H-uridine/nmoles uridine. Figure 13 shows the results plotted as percent of the uridine pools of vehicle-

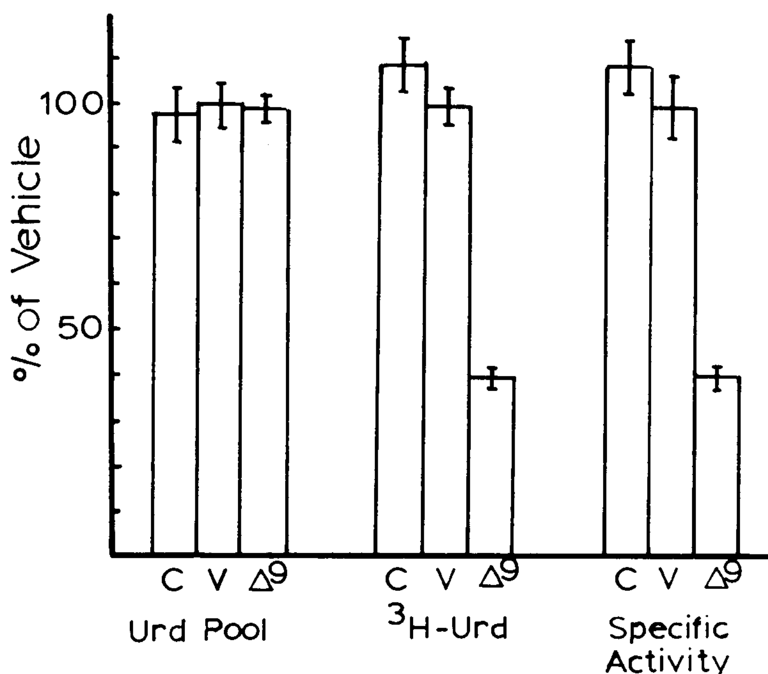


FIG. 13. Intracellular uridine pool, ^{3}H-uridine, and uridine pool specific activity of HeLa S_3 cells pulse-labeled with ^{3}H-uridine after 10 hours of exposure to 30 μM concentration of Δ^9-THC. Exponentially growing HeLa S_3 cells (30 ml) were grown for 10 hours in the presence of 30 μM concentration of Δ^9-THC and then pulse-labeled for 15 minutes at 37°C with 20 μCi of ^{3}H-uridine (23 Ci/mM). The acid-soluble intracellular pool was isolated and digested with *E. coli* alkaline phosphatase. The uridine pool was isolated by two-dimensional chromatography and measured spectrophotometrically (100% = 15 nM Urd). Radioactivity was measured in a scintillation counter (100% = 4723 cpm of ^{3}H-uridine). The specific activity was calculated as the ratio of cpm ^{3}H-uridine/mM uridine. Results are plotted as percent of vehicle and represent the mean $\pm$ AD of 3 separate samples. C = control. V = vehicle-treated control. Δ^9 = Δ^9-THC-treated sample.

treated cells (100% vehicle uridine pool = 15 nmoles of uridine; 100% vehicle ^{3}H-uridine = 4,723 cpm of ^{3}H-uridine). This procedure had a recovery of greater than 93% (with respect to uridine) as determined with known amounts of UTP, UMP, and uridine subjected to the same treatment as the actual cellular samples.

The size of the uridine pool of Δ^9-THC-treated cells was the same as that of untreated and vehicle-treated cells. However, the amount of ^{3}H-uridine taken up by the Δ^9-THC-treated cells was approximately 40% of that taken up by the untreated and vehicle-treated cells, and this decrease was also reflected in the specific activity of the drug-treated cells calculated from the former measurements of pool size and radioactivity. Thus, the observed decrease of precursor uptake by the cells can be attributed to a cannabinoid-induced decrease in permeability or transport across the cell membrane and not to a change in the size of the endogenous precursor pools of the cells.

To assess further the influence of cannabinoids on RNA synthesis and to circumvent the problem of cannabinoid-induced modifications in the acid-soluble, intracellular nucleotide precursor pool, we assayed RNA synthesis in two *in vitro* systems—in isolated nuclei and in chromatin. *In vitro* chromatin transcription was performed utilizing exogenously added *E. coli* RNA polymerase prepared according to the method of Berg *et al.*[1]. *E. coli* RNA polymerase has been successfully used for transcription of mRNA sequences coding for globin[7, 30], ovalbumin[45], and histones[14, 29, 41, 43]. Though it is probable that transcription with *E. coli* RNA polymerase is not identical to that with eukaryotic RNA polymerase, the *E. coli* polymerase has been found to transcribe certain specific genes (histone, globin and ovalbumin) with fidelity.

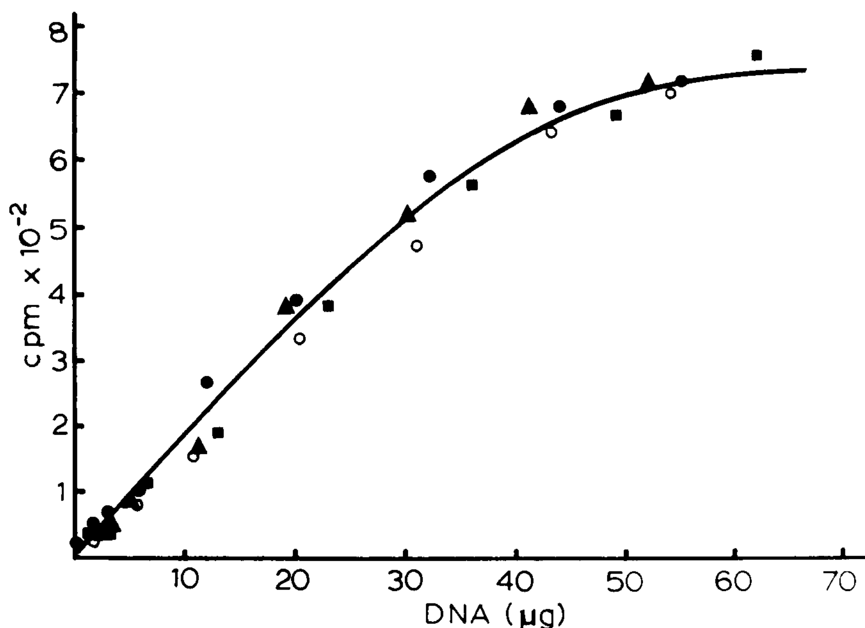


FIG. 14. Effects of Δ^9-THC and cannabinol (CBN) on the *in vitro* transcription of chromatin isolated from exponentially growing HeLa S_3 cells pretreated for 10 hours with 30 μM Δ^9-THC (▲) and 15 μM CBN (■) in suspension culture. Control (●) and vehicle-treated control (○) samples were also studied. *In vitro* transcription of isolated chromatin was carried out using *E. coli* RNA polymerase in the presence of ^{14}C-ATP. Results are plotted as cpm of incorporated ^{14}C-ATP vs μg of DNA in chromatin.

Exponentially growing HeLa S_3 cells were treated for 10 hours with a 30 μM concentration of Δ^9-THC or a 15 μM concentration of CBN. Untreated and vehicle-treated control cultures were also used for comparison. Chromatin was isolated as described previously[36] and then transcribed with *E. coli* RNA polymerase. Transcription was carried out with varying amounts of DNA as chromatin (ranging from 1.5 to 65 μg) for 10 minutes at 37°C in the presence of 0.4 μCi of ^{14}C-ATP as reported previously[39]. The results were plotted as cpm of ^{14}C-ATP incorporated into acid-insoluble material vs microgram of DNA in chromatin. Figure 14 shows no alteration in the template activity of chromatin isolated from cannabinoid-treated cells compared with that of chromatin from untreated and vehicle-treated cells. It is important to note that the total template activity of chromatin reflects the percent of the genome available for transcription and does not give qualitative information about possible changes in the expression of individual genes.

The possibility must be considered that in the process of isolating chromatin, the amount of bound cannabinoid may be diminished to such a small level that it cannot influence the transcription process. Therefore, the same transcription assays were carried out as described above but with a 30 μM concentration of Δ^9-THC or a 15 μM cannabinol concentration present in the transcription assay. As shown in Fig. 15, the presence of Δ^9-THC or cannabinol in the transcription assay itself did not appear to alter (compared with untreated and vehicle-treated controls) the template activity of chromatin isolated from cannabinoid-treated cells.

In the preceding transcription studies, an exogenous, prokaryotic RNA polymerase was used. It is possible that cannabinoids do not affect the transcriptional activity of the *E. coli* RNA polymerase but that they affect that of the eukaryotic RNA polymerase. *In vitro*

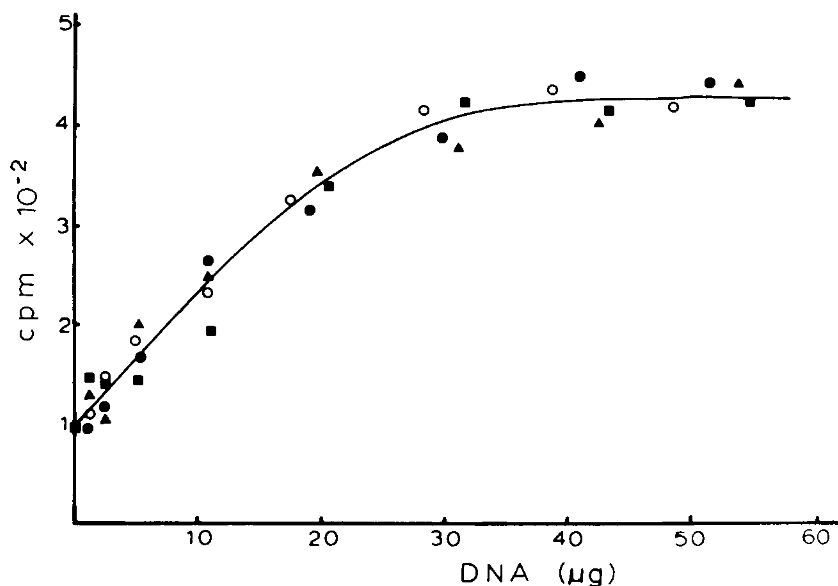


FIG. 15. Effects of Δ^9-THC and cannabinol (CBN) on the *in vitro* transcription of chromatin isolated from exponentially growing HeLa S_3 cells pretreated for 10 hours with 30 μM Δ^9-THC (▲) and 15 μM CBN (■) in suspension culture and transcribed *in vitro* with the same cannabinoid concentrations present in the transcription assay. *In vitro* transcription of isolated chromatin was carried out using *E. coli* RNA polymerase in the presence of ^{14}C-ATP. Results are plotted as cpm of incorporated ^{14}C-ATP vs μg of DNA in chromatin. Control (●) and vehicle-treated (○) samples were also studied.

transcription of nuclei has the advantage of using endogenous RNA polymerase in situ to ascertain the template activity of the genome. Nuclear isolation and *in vitro* transcription of nuclei were carried out by a modification of the procedure of Sarma *et al.*[34] as described by Detke *et al.*[9]. This procedure has been characterized in our laboratory and has been shown by Detke *et al.*[9]: (1) to give a 70%–80% yield of nuclei without intact cells observed in the nuclear suspension when examined by phase contrast microscopy; (2) to yield nuclei which exhibit linear incorporation of ^{3}H-UTP for 45–60 minutes; and (3) to yield nuclei which retain activity representative of all three classes of eukaryotic, DNA-dependent RNA polymerases.

In our experiments nuclei were isolated from exponentially growing HeLa S_3 cells exposed for 10 hours to the following cannabinoid concentrations: 30 μM Δ^9-THC, 10 μM Δ^8-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN. Transcription assays were carried out at 25°C in a total volume of 100 μl containing 25 μCi of ^{3}H-UTP and 1-5 × 10^7 nuclei/ml. The results were plotted as cpm of ^{3}H-UTP incorporated per nucleus vs time of incubation. Two types of experiments were performed: (1) transcription of nuclei without cannabinoid added to the transcription assay; and (2) transcription of nuclei with cannabinoid added to the transcription assay. The latter experiment was performed because it is possible that the cannabinoid could have been extracted from the nuclei during the isolation procedure. The cannabinoids were added to the nuclear transcription assay to achieve concentrations of 1.14 mM Δ^9-THC, 1.24 mM Δ^8-THC, 1.30 mM 11-OH-Δ^9-THC or 1.06 mM CBN. These concentrations were calculated from the previously obtained values of subcellular concentrations of cannabinoids. Figures 16–19 indicate that none of the cannabinoids tested caused any alterations (when compared to untreated and vehicle-treated controls) in the transcriptional activity of nuclei.

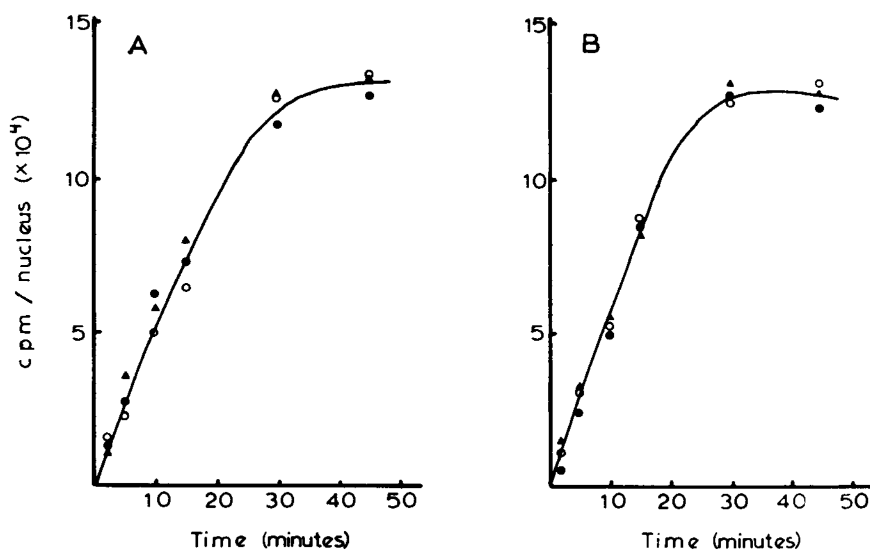


FIG. 16. Effect of Δ^9-THC on the *in vitro* transcription of nuclei isolated from HeLa S_3 cells exposed to 30 μM concentration of Δ^9-THC for 10 hours. Transcription assays were carried out at 25°C in a total volume of 100 μl containing 25 μCi of ^{3}H-UTP (in 0.05 mM UTP) and 1-5 × 10^7 nuclei/ml. Results are plotted as cpm of ^{3}H-UTP incorporated/nucleus vs time of incubation. (A) Nuclei transcribed without Δ^9-THC added to the transcription assay. (B) Nuclei transcribed with 1.14 mM concentration of Δ^9-THC added to the transcription assay. Control (●), vehicle-treated (○), and Δ^9-THC-treated (▲) samples were studied.

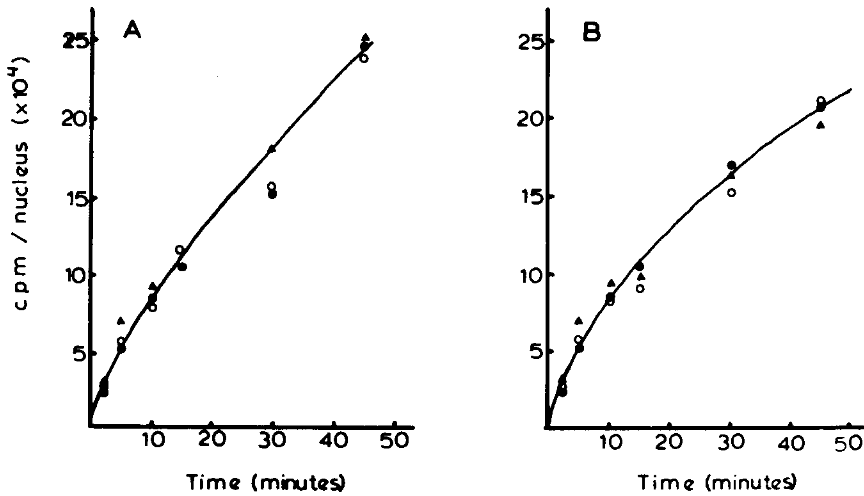


FIG. 17. Effect of Δ^8-THC on the *in vitro* transcription of nuclei isolated from HeLa S_3 cells exposed to 10 μM concentration of Δ^8-THC for 10 hours. Transcription assays were carried out at 25°C in a total volume of 100 μl containing 25 μCi of ^{3}H-UTP (in 0.05 mM UTP) and 1-5 $\times$ 10^7 nuclei/ml. Results are plotted as cpm of ^{3}H-UTP incorporated/nucleus vs time of incubation. (A) Nuclei transcribed without Δ^8-THC added to the transcription assay. (B) Nuclei transcribed with 1.24 mM concentration of Δ^8-THC added to the transcription assay. Control (●), vehicle-treated (○), and Δ^8-THC-treated (▲) samples were used.

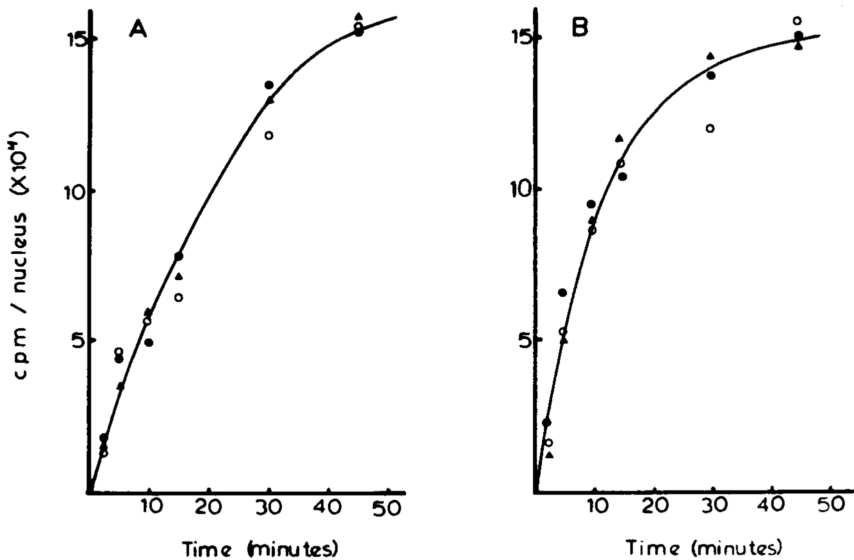


FIG. 18. Effect of 11-OH-Δ^9-THC on the *in vitro* transcription of nuclei isolated from HeLa S_3 cells exposed to 15 μM concentration of 11-OH-Δ^9-THC for 10 hours. Transcription assays were carried out at 25°C in a total volume of 100 μl containing 25 μCi of ^{3}H-UTP (in 0.05 mM UTP) and 1-5 $\times$ 10^7 nuclei/ml. Results are plotted as cpm of ^{3}H-UTP incorporated/nucleus vs time of incubation. (A) Nuclei transcribed without 11-OH-Δ^9-THC added to the transcription assay. (B) Nuclei transcribed with 1.30 mM concentrations of 11-OH-Δ^9-THC added to the transcription assay. Control (●), vehicle-treated (○), and 11-OH-Δ^9-THC-treated (▲) samples were used.

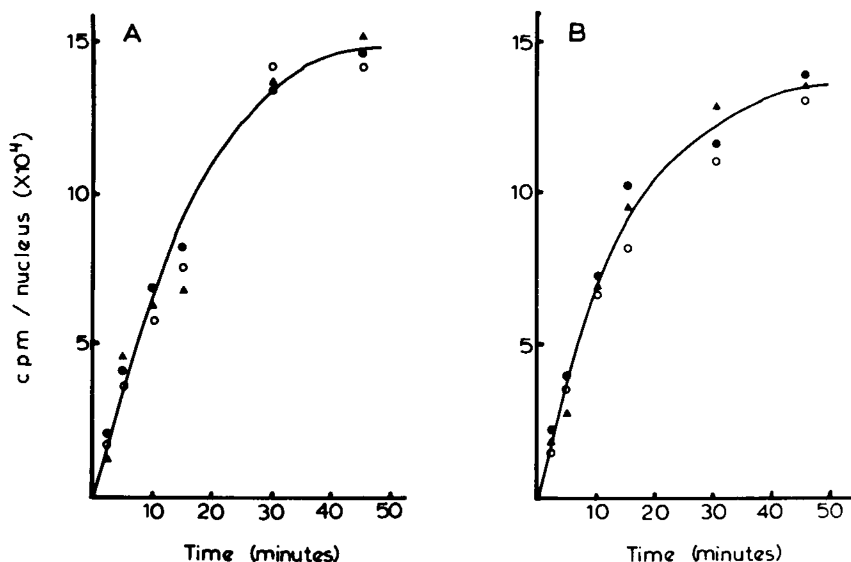


FIG. 19. Effect of CBN on the *in vitro* transcription of nuclei isolated from HeLa S_3 cells exposed to 15 μM concentration of CBN for 10 hours. Transcription assays were carried out at 25°C in a total volume of 100 μl containing 25 μCi of ^{3}H-UTP (in 0.05 mM UTP) and 1-5 × 10^7 nuclei/ml. Results are plotted as cpm of ^{3}H-UTP incorporated/nucleus vs time of incubation. (A) Nuclei transcribed without CBN added to the transcription assay. (B) Nuclei transcribed with 1.06 mM concentration of CBN added to the transcription assay. Control (●), vehicle-treated (○), and CBN-treated (▲) samples were used.

Taken together, results from our radiolabeled nucleic acid and protein precursor studies suggest that neither psychoactive nor nonpsychoactive cannabinoids bring about a quantitative change in RNA or protein synthesis. Those changes in the apparent rates of precursor incorporation observed in our laboratory and by others[2, 6, 21–27, 32, 46–48] can be explained by cannabinoid-induced modifications at the level of the cell membranes—a possibility which is consistent with reports contained in this volume from several laboratories dealing with cannabinoid-induced changes in nucleic acid precursor transport and in the activities of membrane-associated enzymes. However, in evaluating the influence of cannabinoids on macromolecular biosynthesis the key question is whether there is a drug-induced effect on expression of specific genes, i.e., changes in the transcription of defined mRNA sequences and/or in the translation of specific polypeptides. Using nucleic acid hybridization analysis with probes for specific genetic sequences and *in vitro* translation of isolated mRNAs from various intracellular compartments, we are presently examining cannabinoid-induced alterations in expression of defined genetic sequences at the transcriptional and at several post-transcriptional levels. Cannabinoid-induced variations in synthesis of specific molecular weight classes of chromosomal proteins will be described subsequently in this chapter.

III. INFLUENCE OF CANNABINOIDS ON THE GENOME

Because the biochemical events we have been considering reflect alterations in gene expression, it becomes important to determine if cannabinoids can influence the composi-

tion, architecture or functional interactions of macromolecules which comprise the genome. In eukaryotic cells the genome is a nucleoprotein complex, referred to as chromatin, consisting primarily of DNA and two classes of chromosomal proteins—histones and nonhistone chromosomal proteins. The histones have been shown to play a key role in the "packaging" of DNA into nucleosomes, structures which under the electron microscope resemble beads on a string. Several lines of evidence also suggest that histones may be responsible for the nonspecific repression of genetic sequences. While some of the complex and heterogeneous nonhistone chromosomal proteins are probably involved in genome structure and in a broad spectrum of enzymatic activities which occur at the level of the genome, other components of these proteins may determine the availability of genetic sequences for transcription. The structural and functional properties of chromatin have been extensively reviewed[3, 11, 37, 38, 40, 42]. We have approached the question of cannabinoid-induced effects on genome structure and function by examining drug-induced changes in the composition and metabolism of chromosomal proteins and by assessing the influence of cannabinoids on chromatin structure.

A. NONHISTONE CHROMOSOMAL PROTEINS

We have employed two methods for fractionating chromosomal proteins. Total chromosomal proteins were fractionated according to molecular weight by electrophoresis in the high resolution, one-dimensional, SDS-8.75% polyacrylamide gel system of

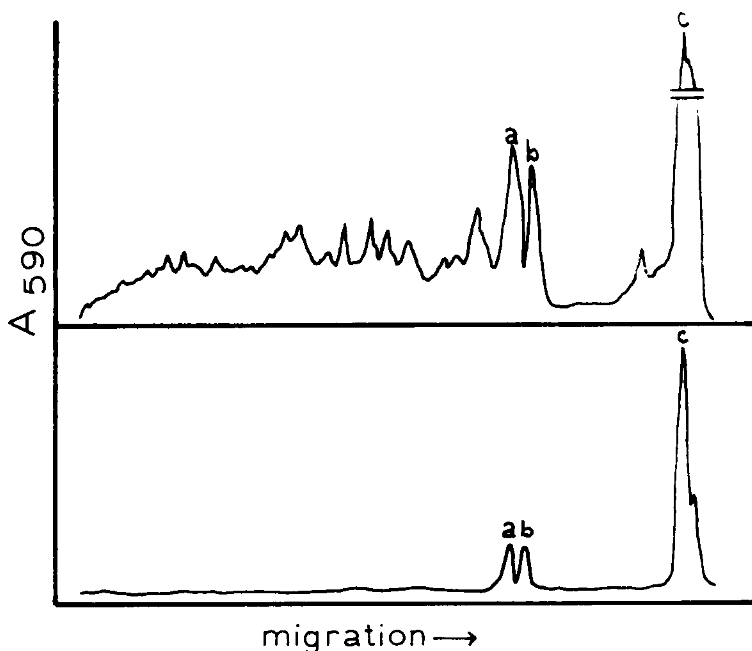


FIG. 20. Spectrophotometric profiles of total chromosomal proteins from HeLa S_3 cells and of calf thymus histones electrophoresed in 8.75% polyacrylamide gels. The upper profile shows the A_{590} of Coomassie Blue-stained total chromosomal proteins extracted from HeLa S_3 cells. The lower profile shows the A_{590} of Coomassie Blue-stained histones from calf thymus. Peaks labeled (a) and (b) correspond to histone H1. Peak labeled (c) contains the remaining four histones, H3, H2B, H2A, and H4.

Laemmli[20] using a Tris-glycine buffer. The relative mobilities of polypeptides in this gel system are directly proportional to the logarithms of their molecular weights. Figure 20 shows the spectrophotometric profiles of total chromosomal proteins from HeLa S_3 cells and of histones alone electrophoresed in Laemmli gels. As shown, the SDS-8.75% polyacrylamide Laemmli gel system fractionates nonhistone chromosomal proteins into a variety of bands ranging from approximately 200,000 to 17,000 in molecular weight, but histones are resolved into only three bands—two bands corresponding to H1 and one band (which moves with the solvent front) containing the four remaining histones (H3, H2B, H2A, H4). For this reason, histones were fractionated with respect to charge and molecular weight on 15% polyacrylamide-acetic acid-urea gels according to the method of Panyim and Chalkley[28]. Acetic acid-urea gels, 28 cm in length, can resolve each of the five histones.

To assess the effect of cannabinoids on chromosomal protein synthesis, the specific

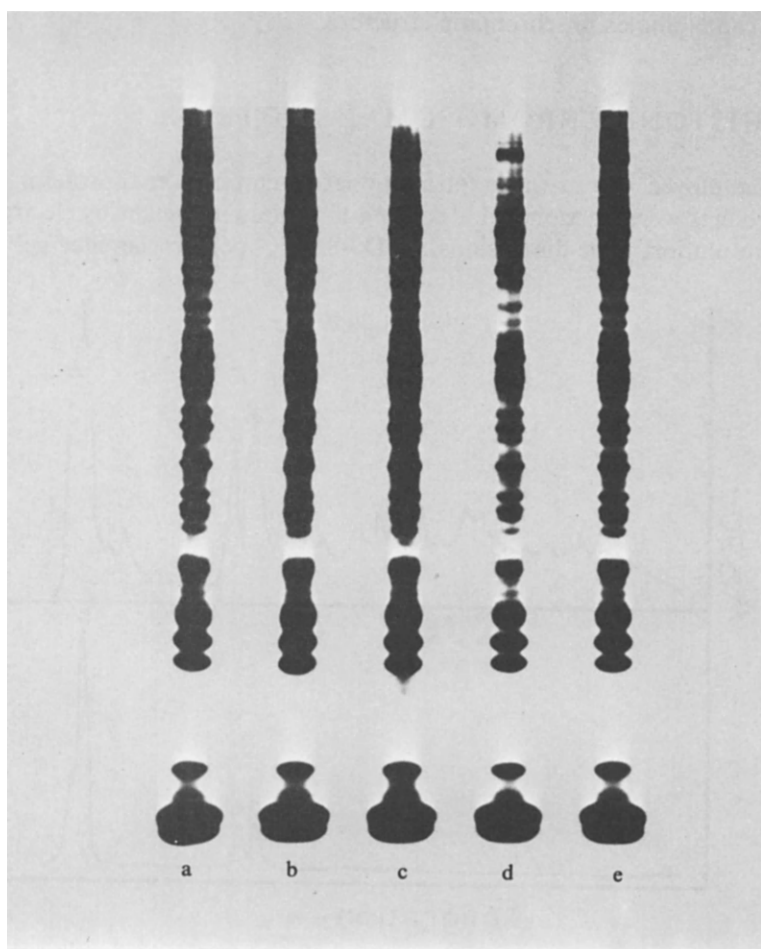


FIG. 21. Photograph of SDS-polyacrylamide slab gel of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hours to cannabinoid concentrations of 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), or 15 μM 11-OH-Δ^9-THC (e), and then pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes. Approximately 40 μg of protein were loaded for each sample. (a) = control. (b) = vehicle-treated control.

activities of these proteins were measured. HeLa S_3 cells were exposed for 10 hours to the following cannabinoid concentrations: 30 μM Δ^9-THC, 10 μM Δ^8-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN. We have found these concentrations to exert a 30%–40% depression in the proliferative capacity of HeLa S_3 cells. The cells were then resuspended in Earle's balanced salts solution and pulse labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes. Total chromosomal proteins were extracted from the drug-treated cells and from untreated and vehicle-treated cells and were fractionated in SDS-polyacrylamide slab gels as shown in Fig. 21. Figure 22 shows radioactivity and absorbance profiles of the stained gels. For study and comparison of specific activities, the total chromosomal proteins were divided into six molecular weight groups: (1) 200,000–150,000; (2) 150,000–100,000; (3) 100,000–70,000; (4) 70,000–50,000; (5) 50,000–35,000; and (6) 35,000–17,000. There are various peaks within each molecular weight group and each peak can contain many different proteins of similar molecular weight. Therefore, caution must be taken in the interpretation of any change observed, because it would reflect the net changes of many protein species within a group rather than alteration of individual proteins. Figure 23

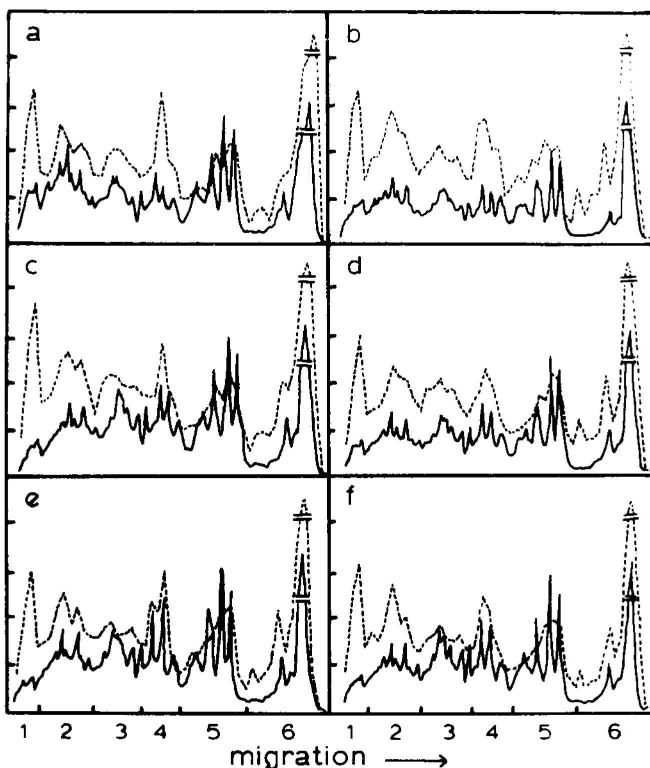


FIG. 22. Absorbance (A_{600} ——) and radioactivity (- - -) profiles of polyacrylamide gel electrophoresis of total chromosomal proteins extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes after treatment with cannabinoids for 10 hours. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). Control (a) and vehicle-treated (b) samples were also studied for comparison. The abscissa is divided into six molecular weight groups: (1) 200,000–150,000, (2) 150,000–100,000, (3) 100,000–70,000, (4) 70,000–50,000, (5) 50,000–35,000, (6) 35,000–17,000. Each mark on the ordinate represents 1% of total gel radioactivity and corresponds to 245 cpm in (a), 209 cpm in (b), 192 cpm in (c), 204 cpm in (d), 213 cpm in (e), and 213 cpm in (f). Approximately 40 μg of protein were loaded for each sample.

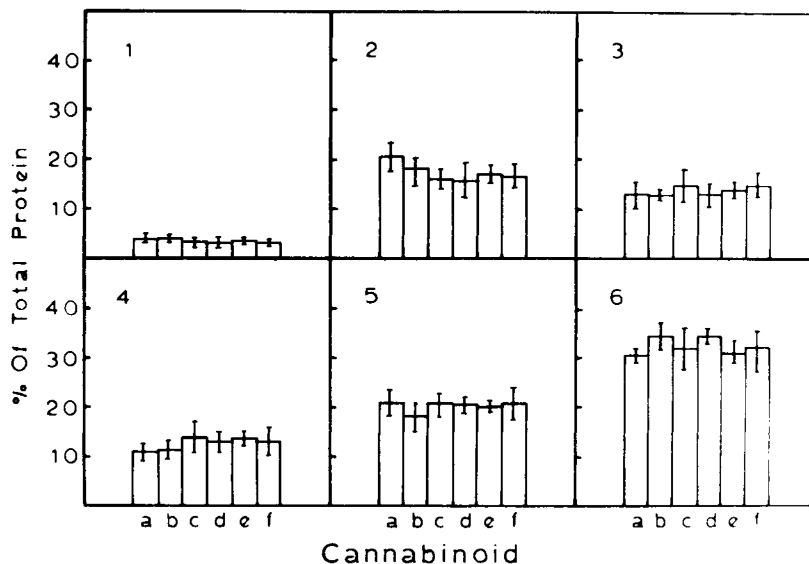


FIG. 23. Percent of total chromosomal proteins represented by different molecular weight groups of chromosomal proteins extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes after 10 hours of exposure to cannabinoids. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels and the relative amounts of protein were calculated from the A_{600} scans of the stained gels by integration of the absorbance profile area corresponding to each of six molecular weight groups: (1) 200,000–150,000, (2) 150,000–100,000, (3) 100,000–70,000, (4) 70,000–50,000, (5) 50,000–35,000, (6) 35,000–17,000. Results are plotted as the mean ±AD for two independent determinations. Each sample contained approximately 40 μg of protein.

shows the percent of total chromosomal proteins (calculated from the A_{600} profiles) represented by the different molecular weight groups extracted from each cannabinoid-treated sample and untreated and vehicle-treated controls. None of the four cannabinoids appeared to alter the relative composition of the six molecular weight groups of total chromosomal proteins. However, as shown in Fig. 24, the specific activities of these proteins exhibited varying fluctuations for the different molecular weight groups as expressed as percent of the specific activity of proteins extracted from vehicle-treated cells (72%–282% for Δ^8-THC, 75%–175% for Δ^9-THC, 62%–173% for 11-OH-Δ^9-THC, 55%–165% for CBN). The high molecular weight group 1 showed a marked increase in specific activity (165%–282% of vehicle) for all drug-treated samples, while the low molecular weight group 6 showed no significant changes. The intermediate molecular weight groups 2–5 displayed various fluctuations with a generalized decrease in specific activities. These results would suggest changes in the rate of synthesis or turnover of total chromosomal proteins as reflected by respective increases or decreases in specific activities. However, coupled with the previous observation of no change in the relative composition of the proteins, the data would suggest a change in turnover rather than a relative increased or decreased accumulation of any one particular molecular weight group of chromosomal proteins. Again, these results reflect the net observations for a series of molecular weight groups of proteins and do not exclude the possibility of variable changes for the individual proteins within each group.

The same experiment was carried out with cells pretreated with cannabinoids for 10

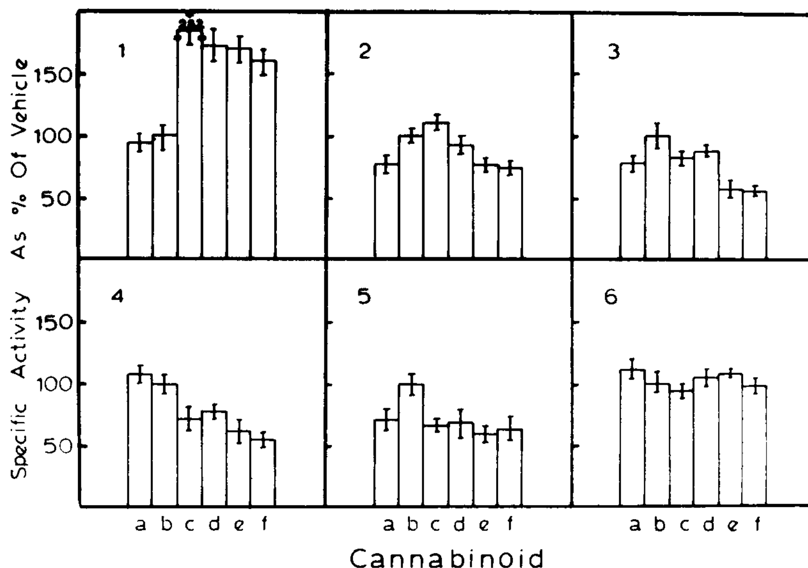


FIG. 24. Specific activities (as percent of vehicle-treated control) of different molecular weight groups of total chromosomal proteins extracted from HeLa S_3 cells pulse-labeled for 15 minutes with 15 μCi/ml of ^{3}H-leucine after 10 hours of exposure to cannabinoids. The following concentrations were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each of six molecular weight groups: (1) 200,000–150,000, (2) 150,000–100,000, (3) 100,000–70,000, (4) 70,000–50,000, (5) 50,000–35,000, (6) 35,000–17,000. Results are plotted as the mean $\pm$ AD for two independent determinations. Each sample contained approximately 40 μg of protein.

hours but with the pretreatment concentrations of cannabinoids in the pulse-labeling medium. As shown in Fig. 25, none of the cannabinoids appears to cause any significant changes in the relative composition of the six molecular weight groups of chromosomal proteins. Again, any changes observed in this experiment would represent net changes in molecular weight groups of protein rather than in individual proteins within each group. However, there were significant changes in specific activities and these changes differed somewhat from those previously obtained with cannabinoid-pretreated cells pulse-labeled in the absence of cannabinoids. These differences could be attributed to a loss of cannabinoids prior to pulse-labeling in the former experiment, or to an acute effect of the vehicle and/or drug (superimposed on ten hours of pretreatment) during the pulse-label in the latter procedure. Figure 26 shows that there was a generalized cannabinoid-induced decrease in the specific activities of all molecular weight groups when expressed as percent of the specific activity of the chromosomal proteins extracted from vehicle-treated cells. Only Δ^9-THC caused a significant increase in specific activity of the proteins of molecular weight groups 1 and 2 (165% and 125% of vehicle, respectively). Again, the interpretation of these results, as in the previous studies, would indicate a cannabinoid-induced change (generally a decrease) in the net turnover of the various molecular weight groups of chromosomal proteins. The wide variations observed in specific activities of the various molecular weight groups of chromosomal proteins treated with the same cannabinoids suggest that this effect was not due to a generalized cannabinoid-induced decrease in the amount of ^{3}H-leucine made available to the cell.

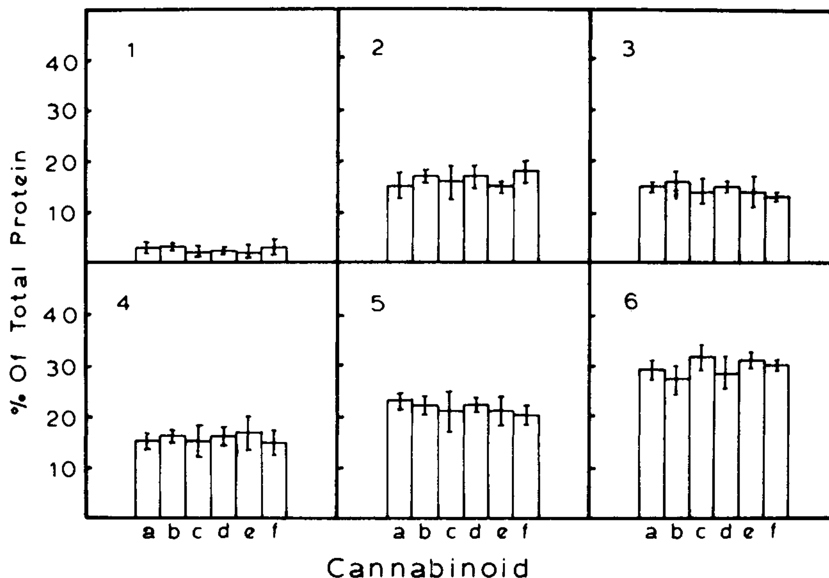


FIG. 25. Percent of total chromosomal proteins represented by different molecular weight groups of chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of ^{3}H-leucine for 15 minutes. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels and the relative amounts of protein were calculated from the A_{600} scans of the stained gels by integration of the absorbance profile area corresponding to each of six molecular weight groups: (1) 200,000–150,000, (2) 150,000–100,000, (3) 100,000–70,000, (4) 70,000–50,000, (5) 50,000–35,000, (6) 35,000–17,000. Results are plotted as the mean $\pm$AD of two independent determinations. Each sample contained approximately 80 μg of protein.

In interpreting experiments in which chromosomal proteins are pulse-labeled, such as those just described and to be discussed subsequently in this chapter, one must consider that we are dealing with cells that have been exposed to cannabinoids for 10 hours but have been pulse-labeled for only 15 minutes. Therefore when we speak of the effects of cannabinoids on the relative amounts of chromosomal proteins, we are referring to the entire time during which the cells were treated with the drug. However, when we talk about the incorporation of ^{3}H-leucine into chromosomal proteins, we are referring only to the effects observed during the 15 minutes of pulse-labeling. Thus, from the area of the optical density scans we can estimate the relative composition (not the absolute amounts) of the chromosomal proteins as affected by 10 hours of treatment with cannabinoids. The specific activity, on the other hand, gives the relationship between the newly synthesized chromosomal proteins (pulse-labeled for 15 minutes) and the chromosomal proteins already present (affected by 10 hours of cannabinoid treatment). Therefore, a decrease in specific activity could be related to various possible cannabinoid-induced effects among which are decreased synthesis of chromosomal proteins, increased breakdown of newly synthesized chromosomal proteins, and decreased transport into the nucleus of the cell. The converse of the above changes would apply to an increase in specific activity. These considerations hold for the ^{3}H-leucine pulse-labeling experiments of nonhistone chromosomal proteins just described and for the ^{3}H-leucine pulse-labeling experiments of histone proteins that follow.

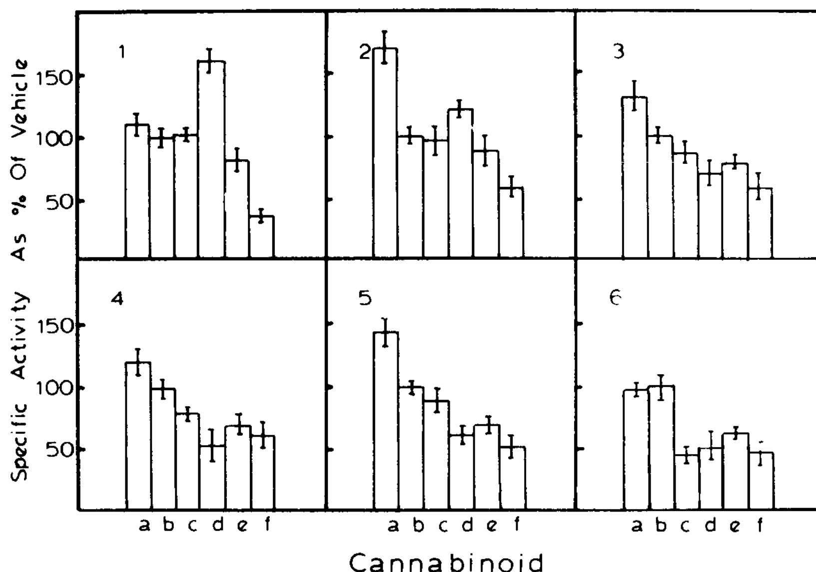


FIG. 26. Specific activities (as percent of vehicle-treated control) of different molecular weight groups of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of ^{3}H-leucine. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each of six molecular weight groups: (1) 200,000–150,000, (2) 150,000–100,000, (3) 100,000–70,000, (4) 70,000–50,000, (5) 50,000–35,000, (6) 35,000–17,000. Results are plotted as the mean $\pm$AD for two independent determinations. Each sample contained approximately 40 μg of protein.

B. HISTONES

The effect of cannabinoids on the composition and metabolism of histones was studied as follows. Exponentially growing HeLa S_3 cells were pretreated for 10 hours with cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN, concentrations which bring about approximately the same decrease in the proliferative capacity of HeLa S_3 cells. The cells were washed, resuspended in Earle's balanced salt solution and pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes. Histones were extracted from drug-treated cells and from untreated and vehicle-treated cells, and fractionated by electrophoresis in acetic acid-urea-15% polyacrylamide slab gels (Fig. 27). Figure 28 shows the radioactivity and absorbance profiles of the amido black-stained gels. The profiles of the histone gels were divided into five major histone classes: H1, H3, H2B, H2A, and H4. These profiles were utilized to calculate the percentages of total histone and the specific activity for each class of histones. Figure 29 shows the percent of total histone protein (calculated from absorbance profiles) represented by each of the five histones extracted from each cannabinoid-treated sample and untreated and vehicle-treated controls. None of the four cannabinoids appeared to cause significant changes in the relative compositions of the five histones. However, as shown in Fig. 30, specific

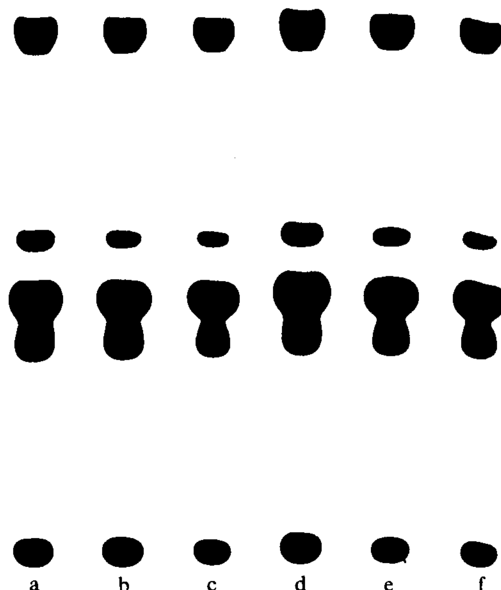


FIG. 27. Photograph of acetic acid-urea—15% polyacrylamide slab gel of histone proteins (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells exposed for 10 hours to cannabinoid concentrations of 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), or 15 μM CBN (f), and then pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes. Approximately 120 μg of protein was loaded for each sample. (a) = control. (b) = vehicle-treated control.

activities of the histones exhibited varying fluctuations for the different histone classes when expressed as percent of the specific activity of histones extracted from vehicle-treated cells (56%–105% for Δ^8-THC, 52%–83% for Δ^9-THC, 53%–94% for 11-OH-Δ^9-THC, 50%–107% for CBN). In general, there was a decrease in specific activity brought about by most of the cannabinoids. Histones H1, H2B and H2A exhibited the most marked decrease. As discussed previously, the lack of changes in the relative compositions of the histones, along with their decreases in specific activity, suggest a cannabinoid-induced change (generally a decrease) in the synthesis of histones.

Similar studies were carried out with cells pretreated with cannabinoids for 10 hours but with the pretreatment concentrations of cannabinoids present in the pulse-labeling medium. Figure 31 shows that cannabinoids do not appear to produce significant changes in the relative composition of the five histone classes. But, as was also observed in the "chronic cannabinoid treatment experiments" just described, the cannabinoids produced a generalized decrease in the specific activities of the different histone classes (Fig. 32) when expressed as percent of the specific activity of the corresponding histones extracted from vehicle-treated cells (50%–82% for Δ^8-THC, 61%–80% for Δ^9-THC, 65%–84% for 11-OH-Δ^9-THC, 75%–99% for CBN). Only 11-OH-Δ^9-THC exerted a significant specific activity increase in H1 (144% of vehicle). Thus, the results of this latter study also suggest a cannabinoid-induced change (generally a decrease) in the synthesis of histones.

In evaluating the histone pulse-labeling experiments (as was the case with the nonhistone chromosomal proteins), one must keep in mind the previously mentioned considerations with regard to the meaning of the data obtained from the optical density scans and the calculated specific activities. Additionally, one must consider the possibility of a cannabinoid-induced alteration in the binding of newly synthesized, radiolabeled histones

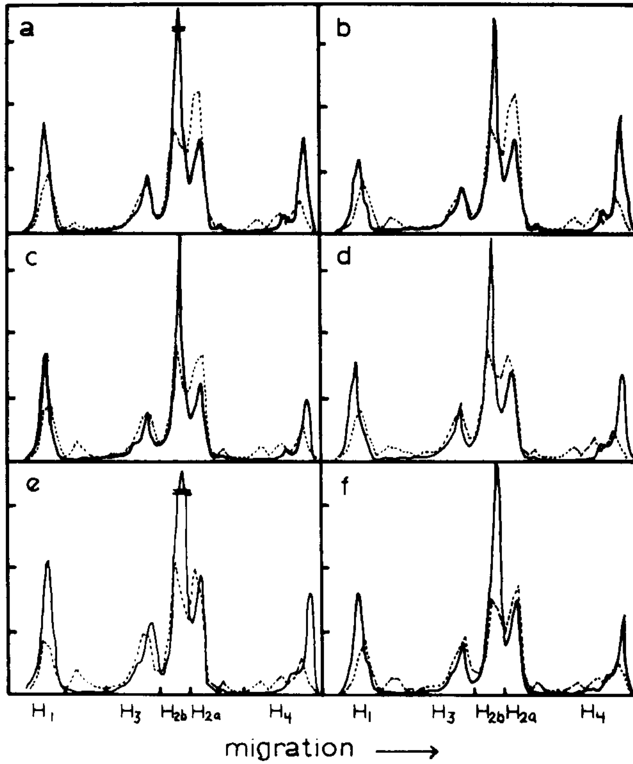


FIG. 28. Absorbance (A_{600} ——) and radioactivity (- - -) profiles of acetic acid-urea-15% polyacrylamide gel electrophoresis of histone proteins (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes after treatment with cannabinoids for 10 hours. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). Control (a) and vehicle-treated (b) samples were also studied for comparison. Each mark on the ordinate represents 5% of total gel radioactivity and corresponds to 996 cpm in (a), 1026 cpm in (b), 739 cpm in (c), 718 cpm in (d), 1038 cpm in (e), and 874 cpm in (f). Approximately 120 μg of protein were loaded for each sample.

to DNA and chromatin which would lead to differences in their extractibility and therefore to variations in specific activity. Such changes in the preferential release or restriction of histones may in part reflect subtle cannabinoid-induced variations in post-translational histone modifications.

Taken together, the composition and metabolism studies just described suggest that cannabinoids do not alter the relative composition of either histones or nonhistone chromosomal proteins associated with the genome. However, all four cannabinoids bring about varying fluctuations (mostly decreases) in the apparent rates of synthesis and/or turnover of both histones and nonhistone chromosomal proteins. Since chromosomal proteins have been implicated in the packaging, structure and functional integrity of the eukaryotic genome, alterations of this type can affect the structural and functional properties of the genome and thereby impair the normal patterns of gene expression essential, in a restricted sense, for regulation of cell proliferation and, in a general sense, for biological viability. Again, as with the cell growth studies, the psychoactive index of the cannabinoids does not appear to be directly related to their effects.

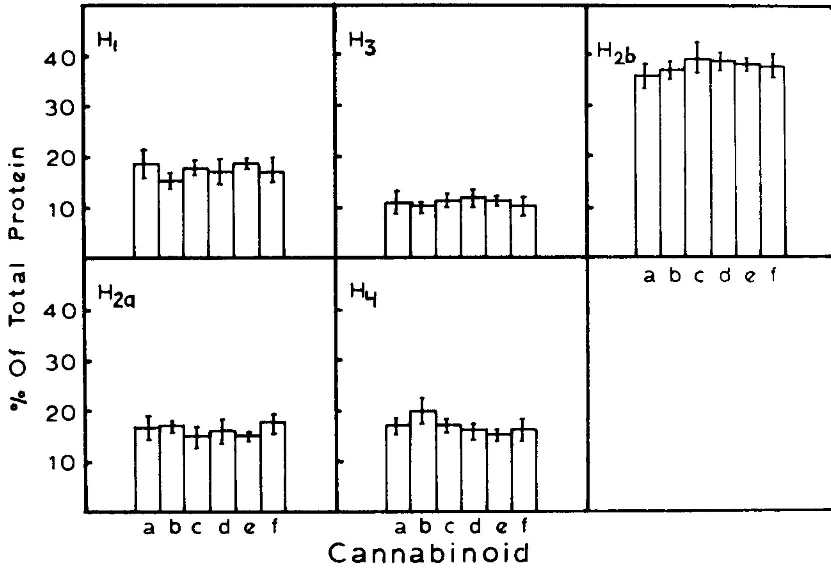


FIG. 29. Percent of total histone proteins represented by each histone (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells pulse-labeled with 15 μCi/ml of ^{3}H-leucine for 15 minutes after 10 hours of exposure to cannabinoids. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels and the relative amounts of protein were calculated from the A_{600} scans of the stained gels by integration of the absorbance profile area corresponding to each of the five histone peaks. Results are plotted as the mean ± AD of two independent determinations. Each sample contained approximately 120 μg of protein.

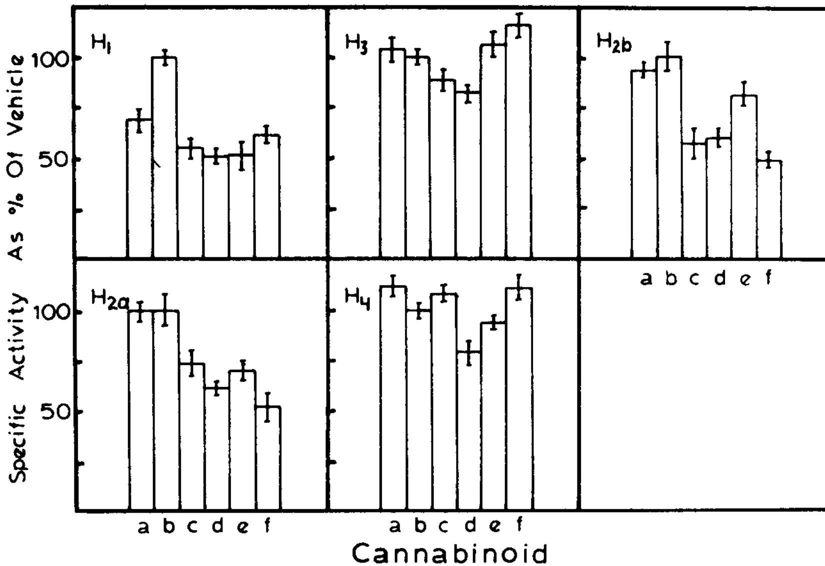


FIG. 30. Specific activities (as percent of vehicle-treated control) of histone proteins (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells pulse-labeled for 15 minutes with 15 μCi/ml ^{3}H-leucine after 10 hours of exposure to cannabinoids. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean ± AD of two independent determinations. Each sample contained approximately 120 μg of protein.

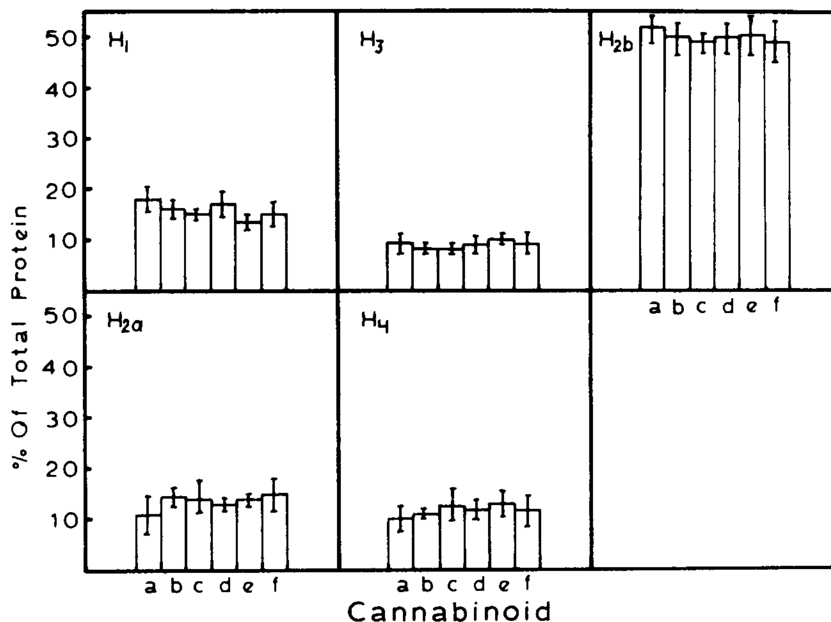


FIG. 31. Percent of total histone proteins represented by each histone (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of ^{3}H-leucine for 15 minutes. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels and the relative amounts of protein were calculated from the A_{600} scans of the stained gels by integration of the absorbance profile area corresponding to each of the five histone peaks. Results are plotted as the mean ± AD of two independent determinations. Each sample contained approximately 120 μg of protein.

C. POST-TRANSLATIONAL MODIFICATIONS OF CHROMOSOMAL PROTEINS

1. Acetylation

We have also examined the influence of cannabinoids on two post-translational modifications of chromosomal proteins—acetylation of histones and phosphorylation of histones and nonhistone chromosomal proteins. These reversible, enzyme-catalyzed modifications of chromosomal proteins have been implicated in mediating chromosomal protein-DNA as well as chromosomal protein-chromosomal protein interactions which may in part be responsible for determining the availability of genetic sequences for transcription. Hence, chromosomal protein acetylation and phosphorylation may play a central role in structural as well as functional properties of the genome (reviewed in References 13 and 33).

To study the effect of cannabinoids on histone acetylation, exponentially growing HeLa S_3 cells were pretreated for 10 hours with cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN. The cells were washed, resuspended in Earle's balanced salt solution and then pulse-labeled with 30 μCi/ml of

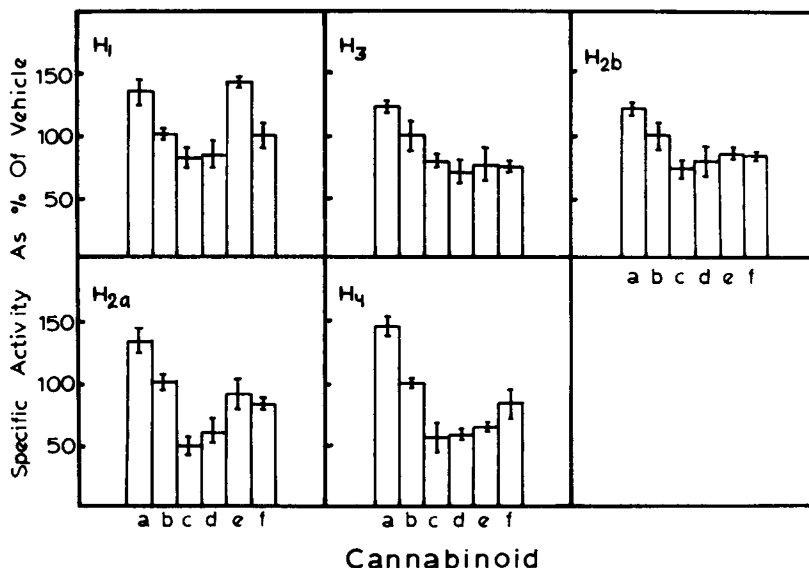


FIG. 32. Specific activities (as percent of vehicle-treated control) of histone proteins (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 15 μCi/ml of ^{3}H-leucine for 15 minutes. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean $\pm$ AD of two independent determinations. Each sample contained approximately 120 μg of protein.

^{3}H-sodium acetate for 30 minutes. Histones were extracted from drug-treated, untreated, and vehicle-treated cells, and were fractionated by electrophoresis in acetic acid-urea-15% polyacrylamide gels. Figure 33 shows the radioactivity and absorbance profiles of the stained gels. These profiles were utilized to calculate the specific activity for each class of acetylated histones—H3, H2B, H2A and H4. Figure 34 shows the specific activities of the acetylated histones expressed as percent of the specific activity of acetylated histones extracted from vehicle-treated cells.

There was a generalized cannabinoid-induced decrease in the specific activities of the acetylated histones when expressed as a percentage of those from vehicle-treated cells (48%–79% for Δ^8-THC, 51%–72% for Δ^9-THC, 70%–96% for 11-OH-Δ^9-THC, 57%–75% for CBN). These changes varied significantly among all four classes of acetylated histones extracted from cells treated with the same cannabinoid and therefore cannot be caused by a decrease in the ^{3}H-acetate made available to the cell. If the latter case were true, one would expect to observe the same amount of change in the acetylation of all four histone classes extracted from cells treated with the same cannabinoid. Therefore our results indicate an interference (a decrease) with the histone acetylation process brought about by cannabinoid treatment of the cells. One possibility is that cannabinoid-induced changes in the structure of the histones modify the availability of acetylation sites and therefore alter the gain or loss of acetate groups. Alternatively, the cannabinoids may be affecting the histone acetylases or deacetylases.

The same experiment was repeated with cells pretreated with cannabinoids for 10 hours

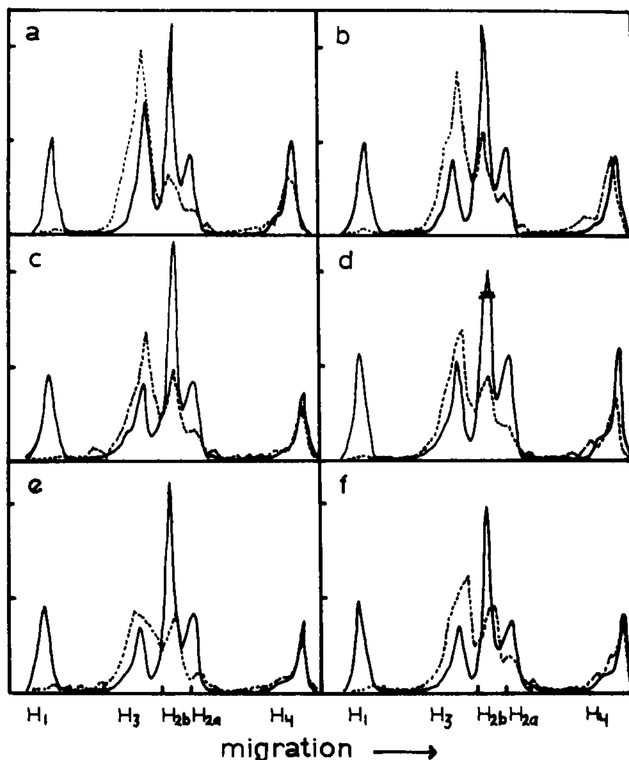


FIG. 33. Absorbance (A_{600} ——) and radioactivity (- - -) profiles of acidic acid-urea-15% polyacrylamide gel electrophoresis of histone proteins (H1, H3, H2B, H2A, H4) extracted from HeLa S_3 cells pulse-labeled with 30 μCi/ml of sodium acetate-^{3}H for 30 minutes after treatment with cannabinoids for 10 hours. The following cannabinoid concentrations were used: 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). Control (a) and vehicle-treated control (b) samples were also studied for comparison. Each mark on the ordinate represents 5% of total gel radioactivity and corresponds to 226 cpm in (a), 211 cpm in (b), 174 cpm in (c), 181 cpm in (d), 195 cpm in (e), 175 cpm in (f). Approximately 120 μg of protein were loaded for each sample.

but with the pretreatment concentrations of cannabinoids present in the pulse-labeling medium. The specific activities of histones extracted from cannabinoid-treated cells displayed a generalized decrease when expressed as percent of the specific activity of acetylated histones extracted from vehicle-treated cells (47%–95% for Δ^8-THC, 58%–94% for Δ^9-THC, 75%–80% for 11-OH-Δ^9-THC, 79%–98% for CBN). It should be emphasized that in acetylation studies, changes in histone acetylation may influence the extractability of histones[17, 18] and thus alter their apparent specific activities.

2. *Phosphorylation*

We initially addressed the possible influence of cannabinoids on phosphorylation of chromosomal proteins as follows. Exponentially growing HeLa S_3 cells were grown in Joklik-modified Eagle's minimal essential medium containing one tenth the normal amount of phosphate. The cells were exposed for 10 hours to cannabinoid concentrations

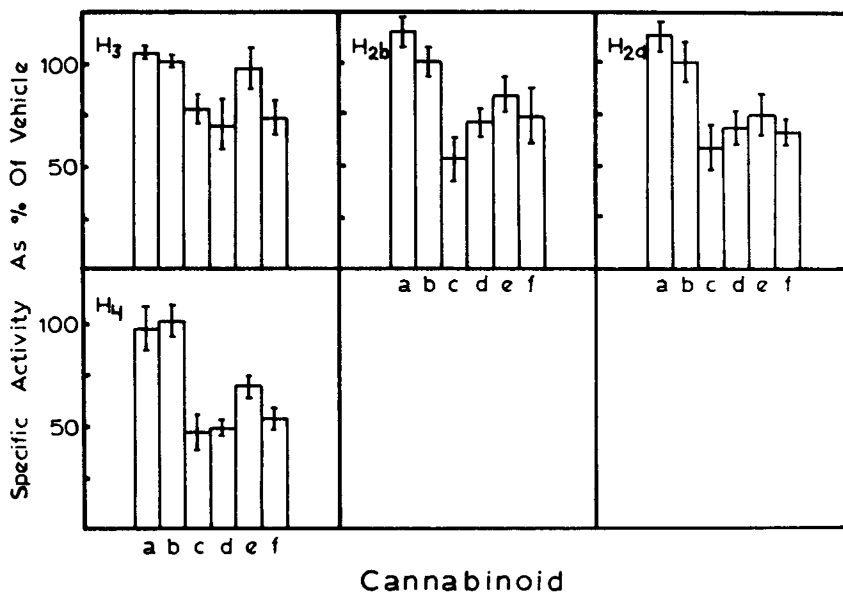


FIG. 34. Specific activities (as percent of vehicle-treated control) of acetylated histone proteins (H3, H2B, H2A, H4) extracted from HeLa S_3 cells pulse-labeled for 30 minutes with 30 μCi/ml of sodium acetate-^{3}H after 10 hours of exposure to cannabinoids. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in acetic acid-urea-15% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean $\pm$ AD of two independent determinations. Each sample contained approximately 120 μg of protein.

of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC, or 15 μM CBN. The cells were resuspended at a 15-fold higher cell density in the same cannabinoid-containing medium and then pulse-labeled with 200 μCi/ml of carrier-free ^{32}P-phosphate for 30 minutes. Total chromosomal proteins were extracted from drug-treated, untreated and vehicle-treated cells and were fractionated in SDS-polyacrylamide slab gels. Figure 35 shows the radioactivity and absorbance profiles of Coomassie Blue-stained gels. These profiles were utilized to calculate the specific activity for each molecular weight group of proteins. Figure 36 shows that the specific activity of these proteins exhibited varying fluctuations for the different molecular weight groups when expressed as percent of the specific activity of proteins extracted from vehicle-treated cells (95%–125% for Δ^8-THC, 34%–57% for Δ^9-THC, 75%–110% for 11-OH-Δ^9-THC, 51%–83% for CBN). There were no generalities in these fluctuations, except that Δ^9-THC exerted the largest depression in the specific activities of most molecular weight groups. Although the highest molecular weight group 1 exhibited the largest cannabinoid-induced increases in specific activities (up to 826% of vehicle for Δ^9-THC and 286% of vehicle for Δ^8-THC), these extremely large increases could be due to the presence of ^{32}P-labeled nucleic acids which might have co-electrophoresed with chromosomal proteins[19]. The radioactivity and absorbance profiles of Fig. 35 support this possibility by showing high levels of radioactivity (perhaps due to the presence of ^{32}P-labeled nucleic acid) and low amounts of protein in the high molecular weight group 1. An alternative explanation would be the magnification of experimental errors in calculating the cpm per unit area ratio for such large cpm values

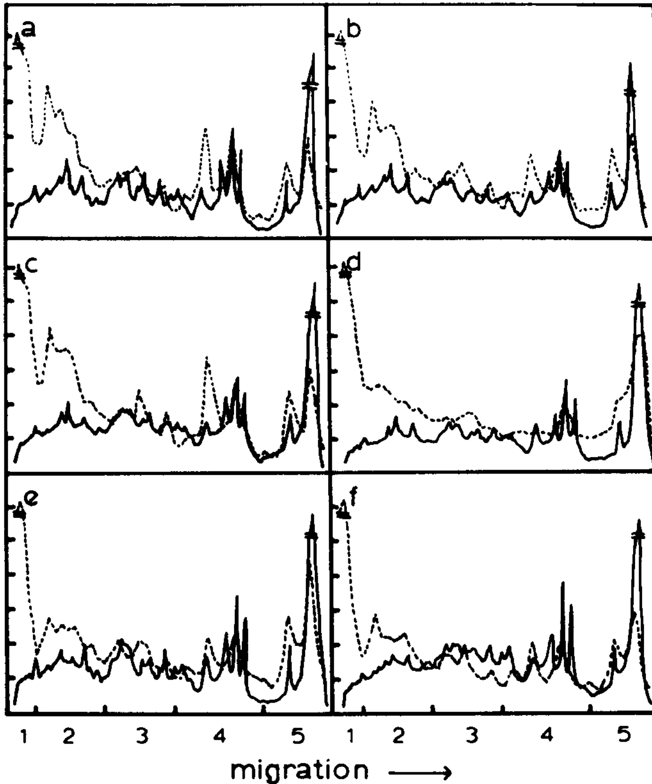


FIG. 35. Absorbance (A_{600} ——) and radioactivity (- - -) profiles of polyacrylamide gel electrophoresis of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 μCi/ml of ^{32}P for 30 minutes. The following cannabinoid concentrations are used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). The abscissa is divided into five molecular weight groups: (1) 200,000–150,000, (2) 150,000–90,000, (3) 90,000–55,000, (4) 55,000–30,000, (5) 30,000–17,000. Each mark on the ordinate represents 1% of total gel radioactivity and corresponds to 150 cpm in (a), 135 cpm in (b), 127 cpm in (c), 56 cpm in (d), 117 cpm in (e), and 126 cpm in (f). Approximately 80 μg of protein were loaded for each sample.

associated with a very small area (small amount of protein) of the absorbance profile. The overall fluctuations in specific activities of drug-treated cells suggest a cannabinoid-induced alteration in the phosphorylation and perhaps dephosphorylation processes of total chromosomal proteins. These changes in phosphorylation varied significantly among all five molecular weight groups of chromosomal proteins extracted from cells treated with the same cannabinoid and therefore cannot be attributed to a generalized decrease in the amount of ^{32}P made available to the cell.

To assess further the possibility that ^{32}P-labeled nucleic acid might have co-electrophoresed with the chromosomal proteins, the same amounts of chromosomal proteins from the experiments just described were fractionated in SDS-polyacrylamide gels, but the gels were treated with 5% TCA in order to hydrolyze ^{32}P-labeled nucleic acids. There was a significant decrease in the amount of radioactivity present in the high molecular weight group of chromosomal polypeptides (group 1) indicating that there may have been ^{32}P-labeled nucleic acid present in this group in the previous experiment.

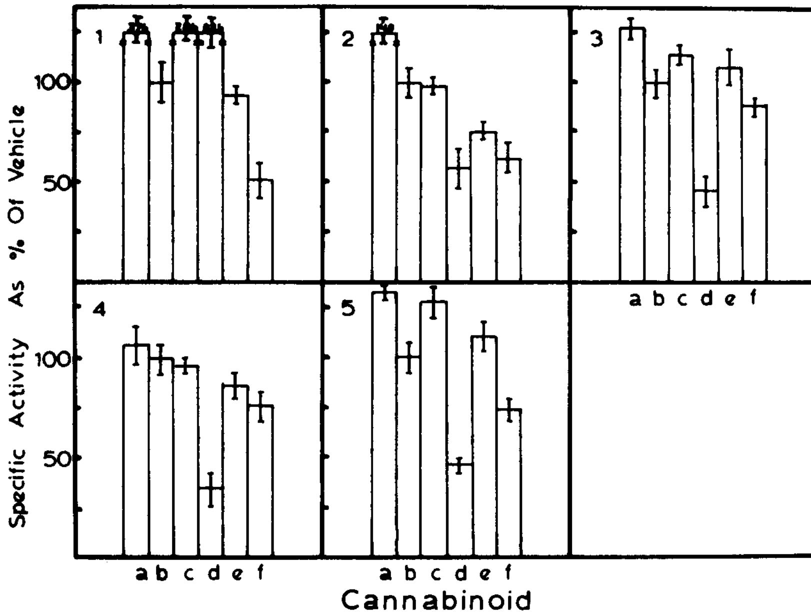


FIG. 36. Specific activities (as percent of vehicle-treated control) of different molecular weight groups of total chromosomal proteins extracted from HeLa S_3 cells exposed for 10 hours to cannabinoids and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 μCi/ml of ^{32}P. The following concentrations of cannabinoids were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), 15 μM CBN (f). Samples were electrophoresed in 8.75% polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each of five molecular weight groups: (1) 200,000–150,000, (2) 150,000–90,000, (3) 90,000–55,000, (4) 55,000–30,000, (5) 30,000–17,000. Results are plotted as the mean $\pm$ AD of two independent determinations. Each sample contained approximately 80 μg of protein.

However, acid treatment did not alter the amount of radioactivity present in the gel regions corresponding to groups 2–5 chromosomal polypeptides. It therefore appears that, indeed, cannabinoids are influencing phosphorylation of several molecular weight classes of chromosomal polypeptides (groups 2–5) and that although there also appears to be some cannabinoid-induced alterations in the phosphorylation of the higher molecular weight chromosomal proteins (class 1), the presence of nucleic acid in this higher molecular weight region of our acrylamide gels precludes a quantitative assessment of the phenomenon.

To investigate the effect of cannabinoids on histone phosphorylation, exponentially growing HeLa S_3 cells were grown in Joklik-modified Eagle's minimal essential medium containing one tenth normal amount of phosphate. The cells were exposed for 10 hours to cannabinoid concentrations of 10 μM Δ^8-THC, 30 μM Δ^9-THC, 15 μM 11-OH-Δ^9-THC or 15 μM CBN. They were then resuspended at a 15-fold higher cell density in the same cannabinoid-containing medium and then pulse-labeled with 200 μCi/ml of carrier-free ^{32}P-phosphate for 30 minutes. Histones were extracted with dilute mineral acid from drug-treated, untreated and vehicle-treated cells as described previously[17] and were fractionated in acetic acid-urea-15% polyacrylamide slab gels. As expected, only histones H1 and H2B were phosphorylated to a significant extent. Figure 37 shows the specific activities of the phosphorylated histones, expressed as percent of the specific activity of phos-

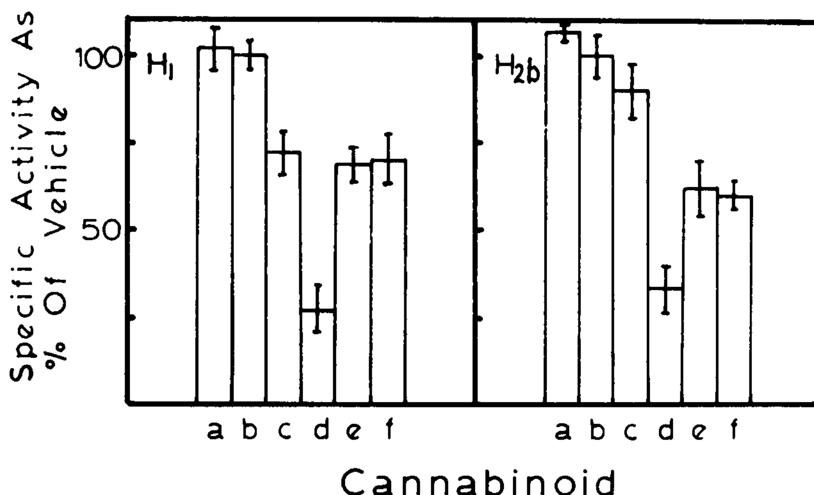


FIG. 37. Specific activities (as percent of vehicle-treated control) of phosphorylated histone proteins (H1, H2B) extracted from HeLa S_3 cells exposed to cannabinoids for 10 hours and then pulse-labeled in the presence of the same cannabinoid concentrations with 200 μCi/ml of ^{32}P for 30 minutes. The following cannabinoid concentrations were used: control (a), vehicle-treated control (b), 10 μM Δ^8-THC (c), 30 μM Δ^9-THC (d), 15 μM 11-OH-Δ^9-THC (e), and 15 μM CBN (f). Samples were electrophoresed in 15% acetic acid-urea polyacrylamide slab gels and the absorbance and radioactivity profiles were used to calculate the specific activity of each histone protein. Results are plotted as the mean $\pm$ AD of two independent determinations. Each sample contained approximately 135 μg of protein.

phorylated histones extracted from vehicle-treated cells. We observed a generalized cannabinoid-induced decrease in the specific activities of the phosphorylated histones when expressed as percent of those from vehicle-treated cells (73%–90% for Δ^8-THC, 27%–35% for Δ^9-THC, 61%–71% for 11-OH-Δ^9-THC, 58%–71% for CBN). As was the case with total chromosomal protein phosphorylation, Δ^9-THC exerted the largest decrease in the specific activities of the phosphorylated histones. The changes in specific activities varied significantly between both classes of phosphorylated histones treated by the same cannabinoid and cannot be caused by a generalized decrease in the ^{32}P made available to the cell. Therefore, our results indicate an interference (decrease) with histone phosphorylation brought about by cannabinoid treatment of the cells. It is possible that the cannabinoids, and especially Δ^9-THC, cause decreases in phosphorylation by interfering with the nuclear protein kinases responsible for phosphorylation[15, 16, 44] or by increasing the activity of the dephosphorylating enzymes. Alternatively, the cannabinoids may divert utilization of ^{32}P-phosphate into other levels of cellular metabolism, such as into the synthesis of cyclic AMP.

Since cannabinoids influence (decrease) phosphorylation of histones and nonhistone chromosomal proteins, it is reasonable to consider that cannabinoid-induced post-translational modifications of chromosomal proteins may, at least in part, be involved with alterations in genome structure and function. However with the data presently available it would be presumptious to postulate the primary level at which the drugs are acting. Yet it is interesting to note that, as with other parameters we have assayed, there is no correlation between the psychoactive effect of cannabinoids and their influence on cellular and molecular processes.

IV. CONCLUSION

From the data presented in this chapter and from results of other laboratories, it seems evident that psychoactive and nonpsychoactive cannabinoids bring about a reduction in the proliferative capacity of eukaryotic cells. Additionally there is an impairment in key macromolecular biosynthetic processes required for cell proliferation. Such biochemical processes are directly or indirectly mediated by modifications in gene expression and it appears that cannabinoids may be modifying the structural and functional properties of the genome.

While these results illustrate cannabinoid-induced alterations in several biological and biochemical processes which are essential for the structural and functional integrity of eukaryotic cells, the essential questions remain unanswered. Specific genes and gene products altered by cannabinoids should be identified. We must further define the cause-and-effect relationships among the many cannabinoid-induced cellular effects. Furthermore, it is imperative to define the primary sites of drug actions.

ACKNOWLEDGEMENTS

These studies were supported by research grant DA-01188 from the National Institute on Drug Abuse. Aspects of the data presented in this chapter will appear in an article in *Biochemical Pharmacology* ("Influence of Δ^9-Tetrahydrocannabinol (Δ^9-THC) on Cell Proliferation and Macromolecular Biosynthesis in Human Cells".)

REFERENCES

1. Berg, D., Barret, K. and Chamberlin, J., Purification of two forms of *Escherichia coli* RNA polymerase and of sigma component. *Methods In Enzymology* (E. Grossman and K. Moldave, eds.). Academic Press, New York, pp. 506–519, Vol. 21, 1971.
2. Blevins, R. D. and Regan, J. D., Δ^9-tetrahydrocannabinol: Effect on macromolecular synthesis in human and other mammalian cells. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 213–222, 1976.
3. Busch, H., *The Cell Nucleus.* (Volumes 4–7). Academic Press, New York, 1978.
4. Cannon, W. D. and Breitman, T. R., Control of deoxynucleotide biosynthesis in *Escherichia coli.* I. Decrease of pyrimidine deoxynucleotide biosynthesis *in vivo* in the presence of deoxythymidylate. *Biochemistry,* **6**,810, 1967.
5. Cannon, W. D. and Breitman, T. R., Control of deoxynucleotide biosynthesis in *Escherichia coli.* II. Effect of deoxythymidylate on the biosynthesis of both deoxynucleotides and ribonucleotide reductase. *Arch. Biochem. Biophys.* **127**,534 (1968).
6. Carchman, R. A., Warner, W., White, A. C. and Harris, L. S., Cannabinoids and neoplastic growth. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 329–345, 1976.
7. Chiu, J. F., Tsai, Y. H., Sakuma, K. and Hnilica, L., Regulation of *in vitro* mRNA transcription by a fraction of chromosomal proteins. *J. Biol. Chem.,* **250**,9431 (1975).
8. DeRobertis, E., Rodriguez de Lores Arnaiz, G., Salganicoff, O., Pellegrino de Iraldi, A. and Zieher, L. M., Isolation of synaptic vesicles and the structural organization of the acetylcholine system within brain nerve endings. *J. Neurochem.,* **10**,225.
9. Detke, S., Stein, J. L. and Stein, G. S., Synthesis of histone messenger RNAs by RNA polymerase II in nuclei from S phase HeLa S_3 cells. *Nucl. Acids Res.,* **5**,1515 (1978).
10. Dewey, W. L., Martin, B. R., Beckner, J. S. and Harris, L. S., A comparison of the subcellular distribution of cannabinoids in the brains of tolerant and nontolerant dogs, rats, and mice after injecting radiolabeled Δ^9-tetrahydrocannabinol. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 349–366, 1976.

11. ELGIN, S. C. R. and WEINTRAUB, H., Chromosomal proteins and chromatin structure. *Ann. Rev. Biochem.*, **44**,725 (1975).
12. HEPPEL, L. A., HARKNESS, D. R. and HILMOE, R. J., A study of the substrate specificity and other properties of the alkaline phosphatase of *Escherichia coli. J. Biol. Chem.*, **237**,841 (1962).
13. HNILICA, L. S., *The Structure and Biological Function of Histones*. CRC Press, Cleveland, 1972.
14. JANSING, R. L., STEIN, J. L. and STEIN, G. S., Activation of histone gene transcription by nonhistone proteins in WI-38 human diploid fibroblasts. *Proc. Natl. Acad. Sci.*, **74**,173 (1977).
15. KISH, V. M. and KLEINSMITH, L. J., Heterogeneity of nuclear protein kinases associated with non-histone chromatin proteins. *J. Cell Biol.* **55**,138a (1972).
16. KISH, V. M. and KLEINSMITH, L. J., Nuclear protein kinases. *J. Biol. Chem.*, **249**,750 (1974).
17. KRAUSE, M. O. and STEIN, G. S., Properties of the genome in normal and SV40 transformed W138 human diploid fibroblasts. II. Metabolism and binding of histones. *Exp. Cell Res.*, **92**,175 (1975).
18. KRAUSE, M. O. and STEIN, G. S., Arginine-rich histone synthesis and acetylation in W138 cells stimulated to proliferate. *Exp. Cell Res.* **100**,63 (1976).
19. KRAUSE, M. O., KLEINSMITH, L. J. and STEIN, G. S., Properties of the genome in normal and SV-40 transformed WI-38 human diploid fibroblasts. III. Turnover of nonhistone chromosomal proteins and their phosphate groups. *Life Sci.*, **16**,1057 (1975).
20. LAEMMLI, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**,680 (1970).
21. MON, M. J., JANSING, R. L., DOGGETT, S., STEIN, J. L. and STEIN, G. S., Influence of Δ^9-tetrahydrocannabinol (Δ^9-THC) on cell proliferation and macromolecular biosynthesis in human cells. *Biochem. Pharmacol.* (in press).
22. MUNSON, A. E., HARRIS, L. S., FRIEDMAN, M. A., DEWEY, W. L. and CARCHMAN, R. A., Antineoplastic activity of cannabinoids. *J. Natl. Cancer Inst.*, **55**,597 (1975).
23. NAHAS, G. and DESOIZE, B., Effet inhibiteur du 5 n-amyl-resorcinol sur la transformation lymphoblastique. *C. R. Acad. Sc. Paris (Series D)* **279**,1607 (1974).
24. NAHAS, G., DESOIZE, B., ARMAND, J.-P., HSU, J. and MORISHIMA, A., Inhibition *in vitro* de la transformation lymphocytaire par divers cannabinoides naturels. *C. R. Acad. Sc. Paris (Series D)*, **279**,785 (1974).
25. NAHAS, G. G., DESOIZE, B., HSU, J. and MORISHIMA, A., Inhibitory effects of Δ^9-tetrahydrocannabinol on nucleic acid synthesis and proteins in cultured lymphocytes. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 299–312, 1976.
26. NAHAS, G. G., MORISHIMA, A. and DESOIZE, B., Effects of cannabinoids on macromolecular synthesis and replication of cultured lymphocytes. *Fed. Proc.* **36**,1748 (1977).
27. NAHAS, G. G., SUCIU-FOCA, N., ARMAND, J.-P. and MORISHIMA, A., Inhibition of cellular mediated immunity in marihuana smokers. *Science*, **183**,419 (1974).
28. PANYIM, S. and CHALKLEY, R., The heterogeneity of histones. I. A quantitative analysis of calf histones in very long polyacrylamide gels. *Biochemistry*, **8**,3972 (1969).
29. PARK, W. D., STEIN, J. L. and STEIN, G. S., Activation of *in vitro* histone gene transcription from HeLa S_3 chromatin by S-phase nonhistone chromosomal proteins. *Biochemistry* **15**,3296 (1976).
30. PAUL, J., GILMOUR, R. S., AFFARA, N., BIRNIE, G., HARRISON, P., HELL, A., HUMPHRIES, S., WINDASS, J. and YOUNG, B., The globin gene: structure and expression. *Cold Spring Harbor Symp. Quant. Biol.* **38**,885 (1973).
31. RANDERATH, K. and RANDERATH, E., Thin-layer separation methods for nucleic acid derivatives. Methods in Enzymology (L. Grossman and K. Moldave, eds.). Academic Press, New York, pp. 323–347, Vol. 12A, 1967.
32. RAZ, A., SCHURR, A. and LIVNE, A., The interaction of hashish components with human erythrocytes. *Biochim. Biophys. Acta*, **274**,269 (1972).
33. RUIZ-CARRILLO, A., WANGH, L. J. and ALLFREY, V. G., Processing of newly synthesized histone molecules. *Science*, **190**,117 (1975).
34. SARMA, M. H., FEMAN, E. R. and BAGLIONI, C., RNA synthesis in isolated HeLa cell nuclei. *Biochim. Biophys. Acta*, **418**,29 (1976).
35. STEIN, G. S. and BORUN, T. W., The synthesis of acidic chromosomal proteins during the cell cycle of HeLa S-3 cells. 1. The accelerated accumulation of acidic residual nuclear protein before the initiation of DNA replication. *J. Cell Biol.*, **52**,292 (1972).
36. STEIN, G. S. and FARBER, J., Role of nonhistone chromosomal proteins in the restriction of mitotic chromatin template activity. *Proc. Natl. Acad. Sci.*, **69**,2918 (1972).
37. STEIN, G. S. and KLEINSMITH, L. J., (eds.) "Chromosomal Proteins and Their Role in the Regulation of Gene Expression." Academic Press, New York, 1975.
38. STEIN, G. and STEIN, J., Chromosomal proteins: their role in the regulation of gene expression. *Bioscience*, **26**,488 (1976).
39. STEIN, G. S., MANS, R. J., GABBAY, E. J., STEIN, J. L., DAVIS, J. and ADAWADKAR, P D., Evidence for fidelity of chromatin reconstitution. *Biochemistry*, **14**,1859 (1975).
40. STEIN, G. S., SPELSBERG, T. C. and KLEINSMITH, L. J., Nonhistone chromosomal proteins and gene regulation. *Science*, **183**,817 (1974).

41. STEIN, G. S., STEIN, J. L. and KLEINSMITH, L. J., Chromosomal proteins and gene regulation. *Sci. Amer.*, **232**(#2):46 (1975).
42. STEIN, G. S., STEIN, J. L. and THOMSON, J. A., Chromosomal proteins in transformed and neoplastic cells: a review. *Cancer Res.*, **38**,1181 (1978).
43. STEIN, G., PARK, W., THRALL, C., MANS, R. and STEIN, J., Regulation of cell cycle stage-specific transcription of histone genes from chromatin by non-histone chromosomal proteins. *Nature*, **257**:764 (1975).
44. THOMSON, J. A., CHIU, J.-F. and HNILICA, L. S., Nuclear phosphoprotein kinase activities in normal and neoplastic tissues. *Biochim. Biophys. Acta* **407**,114 (1975).
45. TSAI, S. Y., TSAI, M. J., HARRIS, S. E. and O'MALLEY, B. W., Effects of estrogen on gene expression in the chick oviduct. *J. Biol. Chem.*, **251**,6475 (1976).
46. WHITE, A. C., MUNSON, J. A., MUNSON, A. E. and CARCHMAN, R. A., Effects of Δ^9-tetrahydrocannabinol in Lewis lung adenocarcinoma cells in tissue culture. *J. Natl. Cancer Inst.*, **56**,655 (1976).
47. ZIMMERMAN, A. M. and MCCLEAN, D. K., Action of narcotic and hallucinogenic agents on the cell cycle. "Drugs and the Cell Cycle" (A. M. Zimmerman, G. M. Padilla and M. L. Cameron, eds.). Academic Press, New York, pp. 67–94, 1973.
48. ZIMMERMAN, A. M. and ZIMMERMAN, S. B., The influence of marihuana on eukaryote cell growth and development. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 195–206, 1976.

DIFFERENCE IN RESPONSE TO VITAMIN C BETWEEN MARIHUANA AND TOBACCO SMOKE EXPOSED HUMAN CELL CULTURES

CECILE LEUCHTENBERGER, RUDOLF LEUCHTENBERGER
and LAURENT CHAPUIS

Department of Cytochemistry, Institut Suisse de Recherches sur le Cancer, Bugnon 21, Lausanne, Switzerland.

Summary. Marihuana smoke evoked in normal human lung and human breast cancer cultures (SK-Br-3) a significantly higher frequency of mitotic abnormalities than did tobacco smoke, regardless of whether the cultures were grown in the absence or the presence of vitamin C.

The response to vitamin C of marihuana smoke exposed breast cancer cultures was completely different from that of tobacco smoke exposed ones. While in the tobacco smoke exposed cultures vitamin C caused a reduction of mitotic abnormalities and led to differentiation, these features were completely absent in marihuana smoke exposed cultures. In marihuana smoke exposed cultures vitamin C accelerated not only the abnormal growth, but also the dedifferentiation of the breast cancer cells.

INTRODUCTION

SMOKING of marihuana cigarettes has become a widespread habit, especially among young people, including children. Surprisingly enough, the unsubstantiated claim that smoking of marihuana is harmless to the lung, was and still is frequently expressed today. For this reason, and also because of our longstanding interest in the experimental exploration of the interrelation between tobacco smoke and pulmonary carcinogenesis[1, 2, 3], we have been engaged since 1970 in experimental investigations comparing effects of tobacco and marihuana smoke, on growth and cell metabolism of animal and human lung tissues[4, 5, 6, 7, 8]. There are particularly three main questions which we have examined:

1. Does smoke from either tobacco or marihuana cigarettes evoke in lung tissues a sequence of morphological and cytochemical alterations resulting in malignant transformation?
2. In case of a carcinogenic effect, what constituents in the smoke are responsible, and what is their mechanism of action?
3. Is it possible to protect the cells against the carcinogenic effect of the smoke?

The present report will be concerned with our recent studies related to aspects of the third

question, namely to our attempts to protect against carcinogenic effects. However, for a better understanding of our approaches to this special problem, and discussion of results, a brief summary of salient features of our previous findings regarding all three questions is presented below.

1. Smoke of either tobacco or marihuana cigarettes accelerates malignant cell transformation in cultured animal lung and enhances atypical growth of cultured human lung tissues[1, 2, 3, 9]. Abnormalities in mitosis, DNA content and number of chromosomes of cultured human lung are more marked after exposure to marihuana smoke than after exposure to tobacco smoke[6, 7, 8].
2. The gas vapor phase from either tobacco or marihuana smoke evokes essentially the same DNA abnormalities, acceleration of premalignant and malignant lesions in human and animal lung cultures as does whole smoke from either tobacco or marihuana cigarettes. There is a correlation between SH reactive components, such as NO, in the smoke and its carcinogenic activity[2, 3].
3. Naturally occurring metabolites, such as L-cysteine or vitamin C protect hamster lung cultures against acceleration of carcinogenesis evoked by either tobacco or marihuana smoke[9].

Furthermore we also observed that after exposure of mouse testis cultures to whole smoke from marihuana cigarettes, the frequency of spermatids with reduced DNA complement was increased. Such an increase in DNA abnormalities was not observed after whole smoke from tobacco cigarettes[7].

These results indicate that marihuana smoke evoked in somatic and in germ cell cultures more marked abnormalities of cell division and DNA metabolism than tobacco smoke. For this reason it seemed of interest to explore the question whether the differences in abnormalities between marihuana and tobacco smoke exposed cultures were only of a quantitative nature, or whether they were accompanied by qualitative differences in cell metabolism. In view of our previous findings that addition of a metabolite, such as vitamin C, will not only prolong significantly the life span of cultured adult human lung, but also favor the tendency to differentiation[10], vitamin C was utilized as a metabolite to study the question. Since in addition vitamin C appeared to have a differentiating effect on a cultured human breast cancer (SK-Br-3)[10], and since this effect was observed when such cultures were injected into nude mice[11], comparative studies with vitamin C were also carried out on marihuana and tobacco smoke exposed breast cancer cultures.

Evidence will be presented that there were significant differences between marihuana smoke and tobacco smoke exposed cultures. Regardless of whether the cultures were prepared from normal adult human lung or from a human breast cancer line (SK-Br-3), and whether they were grown in the absence or presence of vitamin C, the frequency of mitotic abnormalities was always significantly higher after marihuana than after tobacco smoke. Furthermore, vitamin C evoked a significant reduction of mitotic abnormalities and appeared to have a differentiating effect in non exposed and tobacco smoke exposed breast cancer cultures. In marihuana smoke exposed breast cancer cultures, such an effect of vitamin C was not only absent, but vitamin C appeared to have even an opposite effect. Such cultures showed a considerable increase in number and size of cells, and large abnormal spindle formations, suggesting a tendency towards further dedifferentiation of the cancer cells.

MATERIAL AND METHODS

For these studies cultures were prepared from adult normal human lung and grown in normal media (Eagle Dulbecco) (N.M.), or in N.M. + 8 mg vitamin C/liter as previously described[10]. Cultures were also prepared from a human breast cancer line (SK-Br-3)[12], and grown in N.M. or in N.M. + 8 mg vitamin C/liter. 12 sets of cultures were utilized for each of the 7 consecutive experiments with either lung or breast cancer, 2 non-exposed cultures in N.M. and 2 in N.M. + 8 mg vitamin C/liter, 2 marihuana smoke exposed cultures in N.M. and 2 in N.M. + 8 mg vitamin C/liter, and 2 tobacco smoke exposed cultures in N.M. and 2 in N.M. + 8 mg vitamin C/liter. The cultures were exposed under standardized conditions to puffs of fresh cigarette smoke from tobacco or marihuana in a Filtrona CSM12 smoking machine for varying periods, as previously described[7, 8]. Subcultures were prepared and alterations in growth and cell division of the cultures were assessed *in vivo* under phase microscope and in fixed and stained preparations as previously described[4–9].

RESULTS

The salient features of the cytological and growth aspects of human lung and human breast cancer cultures not exposed and exposed to either fresh smoke from tobacco or marihuana cigarettes, grown in N.M. and in N.M. with vitamin C, are presented in Table 1 and Table 2.

There were significant differences in mitotic abnormalities between cultures exposed to marihuana smoke and those exposed to tobacco smoke. Regardless of whether the cultures were grown in the presence or in the absence of vitamin C, smoke from marihuana cigarettes evoked in lung as well as in breast cancer cultures a statistically significant higher frequency of abnormal mitoses than did smoke from tobacco cigarettes (Table 1 and Table 2). Furthermore marihuana smoke exposed breast cancer cultures had a statistically significant greater growth rate than did tobacco smoke exposed cultures, regardless of whether grown in the absence or presence of vitamin C (Table 2).

The greatest difference between tobacco and marihuana smoke was noted in breast cancer cultures grown in the presence of vitamin C. While vitamin C led in non exposed and tobacco smoke exposed cultures to a statistically significant decrease of mitotic abnormalities (Table 2), such a decrease was not observed after vitamin C in marihuana smoke exposed cultures. Furthermore, within a period of 5–7 months, vitamin C led also in non exposed and tobacco smoke exposed cultures to a gradual disappearance of the large cancer cells and to the occurrence of pseudoglandular structures (Figs. 1–3). These pseudoglandular structures gradually disappeared, and more abnormalities of mitoses, such as threepolar spindles were observed when the tobacco smoke exposed cultures were placed back into normal media only, that is without the presence of vitamin C (Fig. 4).

The tendency to differentiation of the cancer cultures was not observed after marihuana smoke, even when grown for 18 months in the presence of vitamin C (Figs. 5a, 5b). Actually after marihuana smoke exposure, vitamin C appeared to have even an opposite effect. Cultures showed more and more an accelerated growth of large atypical cells

TABLE 1. EFFECTS OF VITAMIN C ON CELL DIVISION AND GROWTH OF CULTURED NORMAL ADULT HUMAN LUNG, NON EXPOSED, AND EXPOSED TO FRESH SMOKE FROM MARIHUANA AND TOBACCO CIGARETTES.

Type of media	Type of smoke	Abnormal growth	Acceleration of growth	Mitoses			Alveolar structures (tendency to differentiation)
				Number counted	Abnormalities in %	P values	
Normal	Control	(+)	–	502	5.8		–
	Marihuana	+++	++	622	39.8	PCo < 0.0001 PTo < 0.0005	–
	Tobacco	++	++	308	17.2	PCo < 0.0005	–
Normal + Vitamin C	Control	(+)	++	861	3.4		++
	Marihuana	++	+++	305	21	PToVitC < 0.0005 PCoVitC < 0.0001	++
	Tobacco	+	++	673	11.6	PCoVitC < 0.0005	++

(+): doubtful +: slight ++: moderate +++: marked –: negative.

TABLE 2. EFFECTS OF VITAMIN C ON CELL DIVISION AND GROWTH OF CULTURED HUMAN BREAST CANCER (SK-Br-3) NON EXPOSED, AND EXPOSED TO FRESH SMOKE FROM TOBACCO AND MARIHUANA CIGARETTES.

				Subcultures		Mitoses			
Type of media	Type of smoke	Abnormal growth	Acceleration of growth	mean weekly increase in number of cells $n_1 = 660$	P values	Number counted	Abnormalities in %	P values	Pseudoglandular structures (tendency to differentiation)
Normal	Control	++	+	1 ± 0.3		184	8.2		−
	Marihuana	++	+++	65 ± 7.8	PCo < 0.005 PTo < 0.01	436	21.5	PCo < 0.0005 PTo < 0.005	−
	Tobacco	++	++	16 ± 1.9	PCo < 0.005	237	12.7	PCo < 0.005	−
Normal + Vitamin C	Control	+	++	14 ± 2.3		230	2.2		+++
	Marihuana	++	+++	70 ± 8.5	PToVitC < 0.01 PCoVitC < 0.01	644	18.2	PToVitC < 0.005 PCoVitC < 0.0005	−
	Tobacco	+	++	18 ± 1.7		213	6.1	PTo < 0.025	+++

n_1: number of cultures examined −: negative +: slight ++: moderate +++: marked.

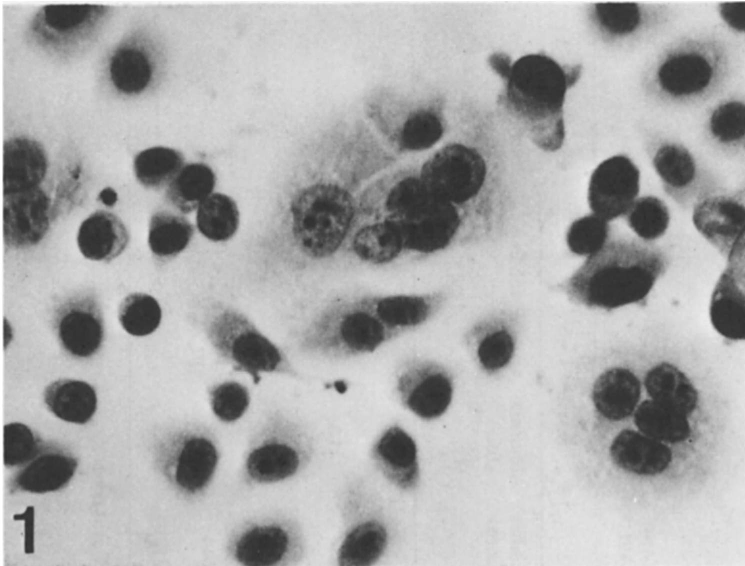


FIG. 1. Light micrograph of human breast cancer culture (SK-Br-3) grown in normal media (N.M.) for 504 days not exposed to smoke. Note small and large atypical epithelial cells. H. & E., × 375.

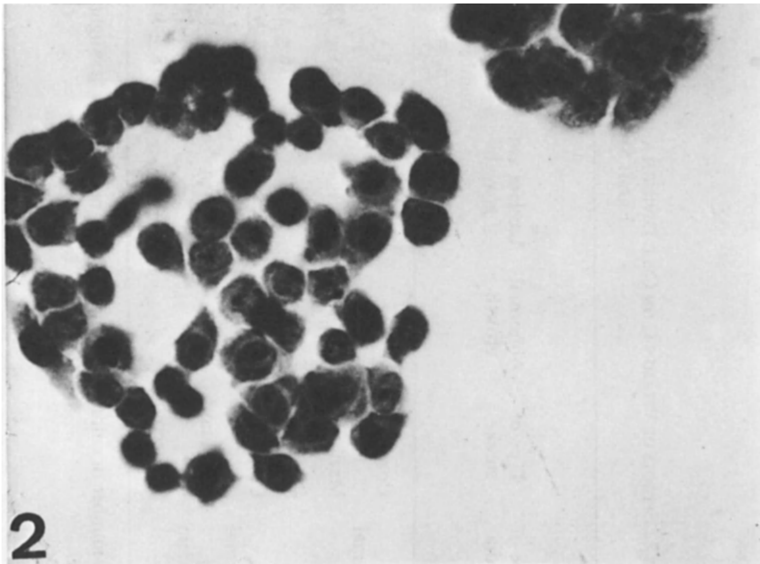


FIG. 2. Light micrograph of human breast cancer culture (SK-Br-3) grown in normal media with vitamin C for 504 days, not exposed to smoke. Note small epithelial cells arranged as pseudoglandular structure. H. & E., × 375.

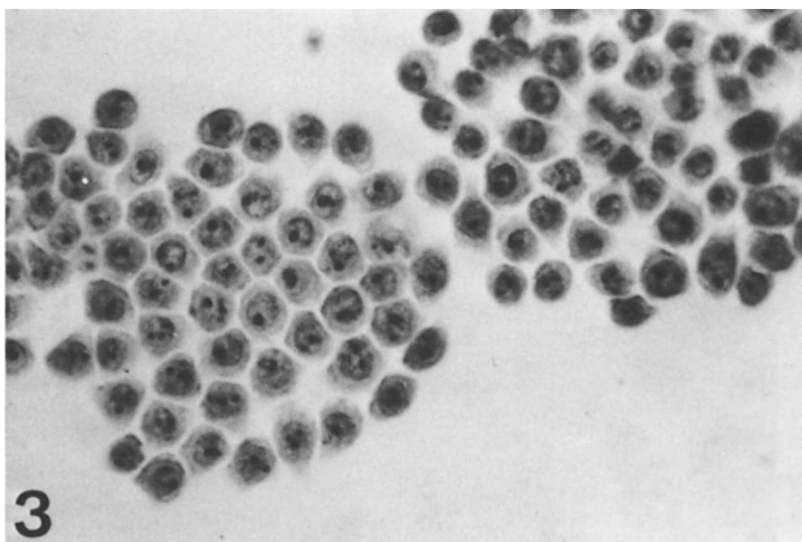


FIG. 3. Light micrograph of human breast cancer culture (SK-Br-3) grown in normal media with vitamin C for 504 days, and after exposure to fresh smoke from tobacco cigarettes. Note small epithelial cells arranged as pseudoglandular structures, similar to that in Fig. 2. H. & E., × 375.

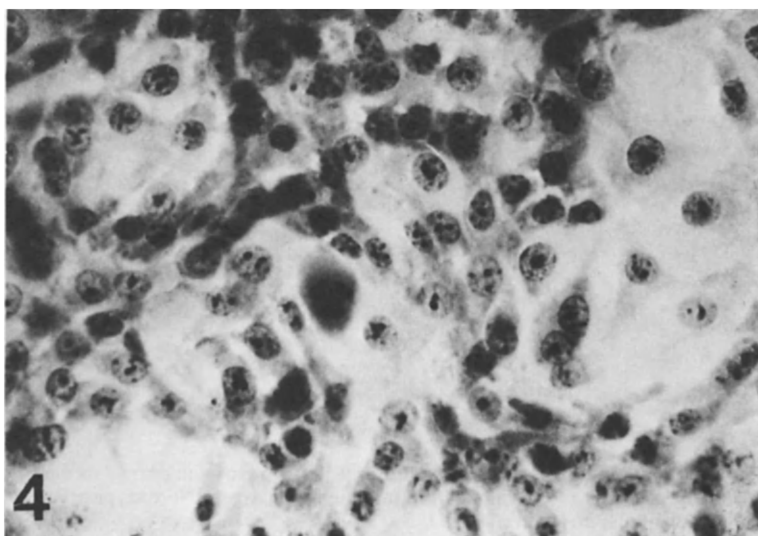
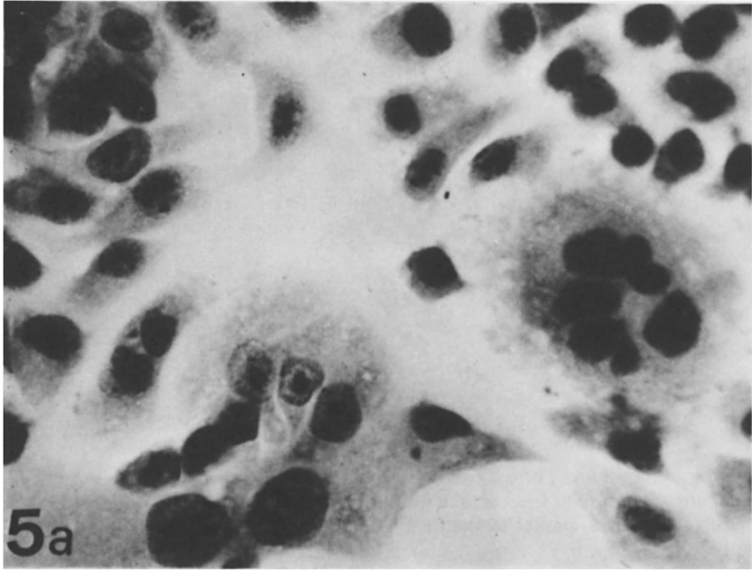


FIG. 4. Light micrograph of human breast cancer culture (SK-Br-3) grown in normal media with vitamin C for 104 days, and placed back for 343 days in normal media (N.M.), and after exposure to fresh smoke from tobacco cigarettes. Note disappearance of pseudoglandular structures and presence of cells of varying size with abnormal mitosis (threepolar spindle). H. & E., × 375.



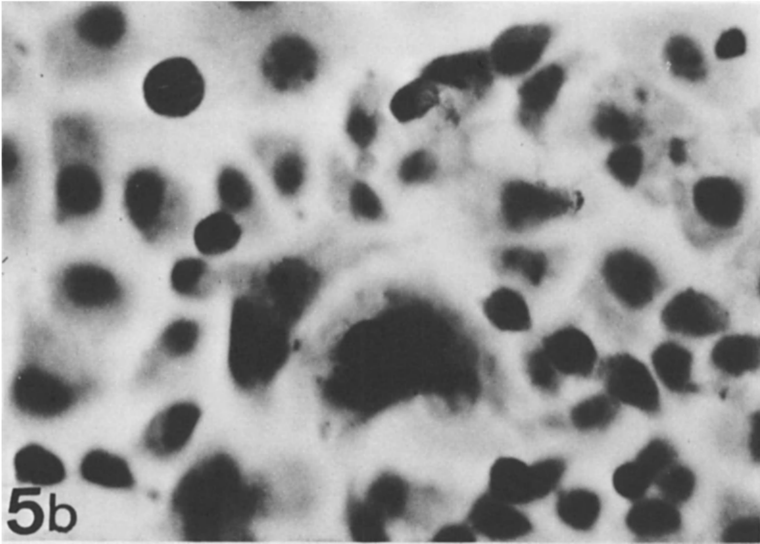


FIG. 5. Light micrographs of human breast cancer cultures (SK-Br-3) grown in normal media with vitamin C for 504 days, and after exposure to fresh smoke from marihuana cigarettes. Note atypical cells of large size and absence of pseudoglandular structures. H. & E., × 375.

(Figs. 5a, 5b), accompanied by occurrence of abnormally large mitosis, especially of threepolar spindles. The frequency of threepolar spindles in breast cancer cultures exposed to marihuana smoke was 7 times larger when grown with vitamin C than in those grown in its absence (22% against 3.2%, $P < 0.0005$).

The results were reproducible in all of the experiments done on the lung cultures and in all of the tobacco smoke and marihuana smoke exposed breast cancer cultures, grown in N.M. and in N.M. + vitamin C. However, in some of the non exposed breast cancer cultures grown with vitamin C, the occurrence of pseudoglandular structures was not seen, although the decrease in large cancer cells and in mitotic abnormalities was always present.

DISCUSSION

It thus would appear that exposure of human lung and breast cancer cultures to fresh smoke from marihuana cigarettes does not only evoke statistically significant higher mitotic abnormalities than tobacco smoke, but also that marihuana smoke will alter the response of breast cancer cultures to vitamin C. While vitamin C led to a reduction of abnormal mitoses and seemed to have a differentiating effect on non exposed and tobacco smoke exposed breast cancer cultures, marihuana smoke appeared to interfere with this regulating effect of vitamin C. In contrast, the acceleration of atypical growth of the marihuana smoke exposed breast cancer cultures, not only in the absence, but also in the presence of vitamin C, would suggest that marihuana smoke evoked a further dedifferentiation of the breast cancer cells. In other words it would seem that marihuana smoke does not only enhance significantly the frequency of mitotic abnormalities in human cells when compared with tobacco smoke, but alters the metabolism of the cells in such a manner that a naturally occurring metabolite, such as vitamin C, is utilized as a promoter of abnormal growth. Although the pathway by which vitamin C evokes a regulation of abnormal growth in non-exposed or tobacco smoke exposed breast cancer cultures, and an acceleration of abnormal growth in marihuana smoke exposed ones is not known, the antagonistic activity induced by marihuana smoke indicates a fundamental metabolic change.

The results obtained on human normal and cancer cultures are in good accordance with those reported previously[5–8]. They support further the concept that smoke from marihuana cigarettes interferes significantly more with DNA metabolism than does tobacco smoke[5–8]. The finding that in the present study this difference was not only observed in normal, but also in cancer cells, and that after marihuana smoke cancer cells showed an opposite response to vitamin C when compared with that of non exposed or tobacco smoke exposed cancer cells deserves special consideration. It would suggest that marihuana smoke does not only enhance DNA abnormalities and carcinogenesis of *normal* cells, but also that marihuana smoke interferes with the differentiating activity of a metabolite, such as vitamin C, on cancer cells.

It is realized that an extrapolation of results obtained on cultured human cancer cells to the human situation is not justified. Nevertheless it is hoped that the results of the present experimental study, implicating especially marihuana smoke in supporting undifferentiated growth of cultured human breast cancer cells, may help to initiate comparative studies on cancer patients smoking tobacco or marihuana cigarettes.

ACKNOWLEDGEMENT

We thank Dr. Olaf J. Braenden, Director of the United Nations Narcotics Laboratory, Geneva, Switzerland, for placing at our disposal the samples of marihuana. We thank also Dr. S. Carrel, Ludwig Institute for Cancer Research, Lausanne, Switzerland, for placing at our disposal the breast cancer line SK-Br-3.

This work was supported in part by grants from A.S.F.C., Switzerland, and W. H. O., Geneva.

REFERENCES

1. Leuchtenberger, C. and Leuchtenberger, R., The experimental exploration of health damaging factors in cigarette smoke, *Sozial u. Präventivmed.* **19**,41–45 (1974).
2. Leuchtenberger, C., Leuchtenberger, R., Zbinden, I. and Schleh, E., SH reactivity of cigarette smoke and its correlation with carcinogenic effects on hamster lung cultures, *Sozial u. Präventivmed.* **21**,47–50 (1976).
3. Leuchtenberger, C., Leuchtenberger, R., Zbinden, I. and Schleh, E., Significance of oxide of nitrogen (NO) and SH reactive components in pulmonary carcinogenesis, *Inserm Symposia Series* **52**,73–80 (1976).
4. Leuchtenberger, C. and Leuchtenberger, R., Morphological and cytochemical effects of marijuana cigarette smoke on epithelioid cells of lung explants from mice, *Nature*, **234**,227–229 (1971).
5. Leuchtenberger, C., Leuchtenberger, R. and Schneider, A., Effects of marijuana and tobacco smoke on human lung physiology, *Nature*, **241**,137–139 (1973).
6. Leuchtenberger, C., Leuchtenberger, R., Ritter, U. and Inui, N., Effects of marihuana and tobacco smoke on DNA and chromosomal complement in human lung explants, *Nature*, **242**,403–404 (1973).
7. Leuchtenberger, C., Leuchtenberger, R., Zbinden, J. and Schleh, E., Cytological and cytochemical effects of whole smoke and from the gas vapour phase from marihuana cigarettes on growth and DNA metabolism of cultured mammalian cells. In: "Marihuana; Chemistry, Biochemistry, and Cellular Effects" ed. G. G. Nahas, Springer-Verlag, New York pp. 243–256, 1976.
8. Leuchtenberger, C. and Leuchtenberger, R., Cytological and cytochemical studies of the effects of fresh marihuana cigarette smoke on growth and DNA metabolism of human lung cultures. In: "The Pharmacology of Marihuana" eds. M. C. Braude and S. Szara, Raven Press, New York, pp. 595–612, 1976.
9. Leuchtenberger, C. and Leuchtenberger, R., Protection of hamster lung cultures by L-cysteine or Vitamin C against carcinogenic effects of fresh smoke from tobacco or marihuana cigarettes, *Br. J. Exper. Pathol.* **58**,625–634 (1977).
10. Leuchtenberger, C. and Leuchtenberger, R., L-cysteine or vitamin C influence cellular growth and prolong survival of normal adult human lung tissue in cultures, *Cell Biol. Intern. Rep.*, **1**,317–324 (1977).
11. Ozzello, L., Leuchtenberger, C. and Hurlimann, J., Manuscript in preparation.
12. Fogh, J. and Trempe, G., New human tumor cell lines. In: "Human Tumor Cells in Vitro" ed. Fogh J., Plenum Press, New York and London, pp. 115–159, 1976.

MARIHUANA AND CELL FUNCTION

R. A. CARCHMAN, D. W. END*, and M. R. PARKER

Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

and

W. WARNER

Department of Physiology, New York University Dental Center, New York, New York 10010

Summary. Δ^9- and Δ^8-tetrahydrocannabinols produce a complex series of cellular responses. These include an enhancement of the inhibition of macromolecular synthesis in the presence of dexamethasone (dx). THC altered the uptake of dx in HTC cells and enhanced the translocation of the dx-receptor complex to the nucleus even at 4°C. Both nuclear and cell membrane effects of THC are demonstrated in several different cell lines in tissue culture. A model is presented which takes into account what is presently known about such cannabinoid cell interactions.

INTRODUCTION

TO DATE at least 37 cannabinoids have been isolated from the marihuana plant[1]. Numerous studies have explored the biological activity of these compounds and certain of these cannabinoids have been demonstrated to have a variety of effects in various systems (for review see Harris *et al.*[2]).

Our experience during the past several years in studying these compounds under *in vitro* conditions has demonstrated the wide range of responses which certain of the cannabinoids can elicit in different cells[3–9]. These responses are not gross toxic manifestations of the drug's activity, but involve specific cellular sites in target cells. Various investigators have postulated that the cellular responses observed with Δ^9-THC, the major phychoactive ingredient in marihuana, are due to its lipophilic membrane interactions[10–16]. Though this is a very attractive notion which can explain some of Δ^9-THC's activity, it falls short of being capable of handling all of the experimental information presently available. The literature abounds with reports demonstrating the breadth of cellular perturbations produced by Δ^9-THC *in vitro* and mirrors the complex interactions observed *in vivo* (for review see Harris *et al.*[2]). It is reasonable to assume that some of the actions of Δ^9-THC are due to membrane alterations and consequent changes in membrane enzymes and cell

* Predoctoral Fellow, National Science Foundation SM 17622901.

product modulators (e.g. prostaglandins, cyclic AMP). Indeed some of our own results lend support to this idea[3, 8, 9]. However, other aspects of our observations cannot be sheltered under this particular model[6, 7–9]. We will present some data which will strengthen our caveat against the use of Occam's razor in trying to reduce cannabinoid cell interactions to a common denominator.

MATERIALS AND METHODS

GROWTH OF CELLS IN TISSUE CULTURE

All cells were maintained in incubators at 37°C under an atmosphere of 5% CO_2, 95% air, 100% humidity. Cells used in this study were grown as previously described: HTC[30], Y-1[7], NB2A[9], C-6[9].

MACROMOLECULAR SYNTHESIS

The uptake and incorporation of radioactive precursors into DNA, RNA and protein were performed on 18 hr fed cultures as previously described[3].

STEROID DISTRIBUTION IN HTC CELLS

Each 75 cm^2 T flask containing HTC cells was incubated for 2 hrs or 48 hrs in a 37°C, 100% humidified 5% CO_2 incubator in the presence of [^{3}H]-dexamethasone (10λ of 1 mCi/ml, final concentration 10 μCi, New England Nuclear) with or without other additions as indicated. After incubation, the medium was decanted and the cells were washed four times with 25 ml Swim's medium (4°C). The cells were removed from the T flask with a rubber policeperson and centrifuged at 100 × g (10 min, 4°C). The cellular pellet was resuspended in 4 ml of Swim's medium and a 1 ml aliquot was dissolved in NCS (Amersham/Searle). This was counted by liquid scintillation spectroscopy and designated as the "whole cell" fraction. The remaining 3 ml of cell suspension was homogenized (4°C) in a hand-held ground glass homogenizer (18 strokes). The resultant homogenate was fractionated by centrifugation[30] and the various fractions were counted by liquid scintillation spectroscopy. The 100,000 × g supernatant was cleared of unbound steroid by adding an excess of dextran-coated charcoal. Data are corrected for aliquot volumes as well as cell number.

CANNABINOID DISTRIBUTION IN NB2A AND C-6 CELLS

Confluent cell cultures (4–5 × 10^6 cells) were incubated for 15 min with 50 μM[^{3}H]-Δ^9-THC (specific activity, 100 μCi/mg). The medium was removed and cell monolayers were washed with two 10 ml portions of ice cold phosphate buffered NaCl. Cells were scraped into 14 ml of ice cold 0.32 M sucrose containing 10 μM $CaCl_2$ and

homogenized in a ground glass Dounce apparatus. The homogenate was centrifuged at 100 × g for 10 min to obtain a low speed pellet, which was subsequently resuspended in 15 ml of 0.32 M sucrose and layered over 3 ml of 2.0 M sucrose for centrifugation (104,000 × g for 60 min) to separate nuclear debris from the crude nuclear pellet.

A mitochondrial fraction was obtained from the low speed supernatant by centrifugation (11,400 × g for 20 min), and the pellet was washed once. The mitochondrial supernatant plus the 5 ml of wash were centrifuged at 104,000 × g for 60 min to pellet the microsomal fraction. Protein and radioactivity for each fraction were determined as previously described.

ELECTRON MICROSCOPY

Samples for electron microscopy were prepared first by dehydrating in ascending concentrations of alcohol. The pellet was then resuspended in propylene oxide and embedded in epon A_{12}. The pellet was then firmed in an oven and cut serially with a diamond knife. These sections were placed on an uncoated grid (200 mesh) and examined in a Hitachi HU-12 electron microscope (magnification 15,000 ×).

RESULTS

Steroid-cannabinoid interactions in biological systems have been observed previously[4, 7, 17–19]. Table 1 presents data which indicate that in HTC cells, a steroid sensitive line[20, 21], the synthetic steroid, dexamethasone, and Δ^8-THC inhibit macromolecular synthesis more effectively in combination than would be predicted by merely summing their individual effects. Δ^8-THC had no significant effect on DNA synthesis and inhibited protein synthesis by 32%. The evaluation of RNA synthesis is complicated by the fact that uptake of cytidine was equally depressed. Dexamethasone slightly depressed DNA and protein synthesis while not effecting RNA synthesis (Table 1). The concomitant addition of Δ^8-THC and dexamethasone inhibited DNA synthesis by 66%, RNA synthesis by 79% and protein synthesis by 66%.

In trying to understand the mechanisms by which cannabinoid/steroid interactions might contribute to the responses presented in Table 1, the effect of Δ^9- and Δ^8-THC on [^{3}H]-dexamethasone uptake and distribution in HTC cells was determined (Table 2). Unlabelled dexamethasone (10^{-5} M) effectively blocked the uptake of [^{3}H]-dexamethasone thus lowering the amount of subcellular associated radioactivity. If the reaction was performed at 4°C the uptake of [^{3}H]-dexamethasone was unaltered while the amount of [^{3}H]-dexamethasone present in the crude nuclear fraction was depressed. These latter two responses are consistent with previous reports[20, 21] as is the observation that the nuclear translocation of the [^{3}H]-dexamethasone-receptor complex can occur after longer periods of incubation (48 hours) (Table 2). Both Δ^9- and Δ^8-THC produced qualitatively similar effects on [^{3}H]-dexamethasone uptake and distribution (Table 2). Under these conditions both cannabinoids increased the uptake of [^{3}H]-dexamethasone (time dependent) and altered the distribution profile of [^{3}H]-dexamethasone. Even at 4°C the cannabinoids facilitated the translocation of the steroid/receptor complex to the nucleus. Finally neither cannabinoid appeared to alter significantly the binding of the

TABLE 1. INTERACTION BETWEEN Δ^8-THC AND DEXAMETHASONE ON HTC CELL MACROMOLECULAR SYNTHESIS IN TISSUE CULTURE

Addition	DNA Synthesis [^{3}H]-thymidine incorporated/ 3.3 x 10^3 cells	RNA Synthesis [^{3}H]-cytidine incorporated/ 3.3 x 10^3 cells	Protein Synthesis [^{3}H]-leucine incorporated/ 3.3 x 10^3 cells
Control	20 ± 6 cpm (100 ± 30)	47 ± 6 (100 ± 13)	18 ± 1.5 (100 ± 8)
		% of control	
Δ^8-THC	73 ± 16	39 ± 2	68 ± 2
Dexamethasone	58 ± 3	93 ± 2	68 ± 1
Δ^8-THC + Dx	34 ± 7	21 ± 2	34 ± 3

HTC cells were grown as previously described[30]. Values presented are mean ± S.E. of 4 plates/grp. Macromolecular synthesis was measured as described by Carchman *et al.*[3]. At 1.5 hrs prior to harvesting cultures, Δ^8-THC (final concentration of 1.5 x 10^{-4} M) was added to cultures of HTC cells either alone or in combination with dexamethasone (final concentration of 3.3 x 10^{-7} M). Simultaneously 1 μCi of [^{3}H]-leucine, [^{3}H]-cytidine or [^{3}H]-thymidine was added to the culture.

steroid to its cytoplasmic receptor. Increases in receptor-associated dexamethasone (48 hours) were probably due to quantitatively similar increases of dexamethasone in all fractions studied.

The depression of macromolecular synthesis by cannabinoids was examined in other cell systems[22–26] to see if it was a general phenomenon. HTC, Y-1[7] and C-6 cells appeared to be rather refractory to cannabinoid effects on macromolecular events (Tables 1 and 3) while NB2A cells showed a specific depression of DNA synthesis. Uridine (or cytidine) uptake and incorporation into RNA were equally depressed while protein synthesis was unchanged.

TABLE 2. THE UPTAKE AND DISTRIBUTION OF [^{3}H]-DEXAMETHASONE BY ADHERENT HTC CELLS ± CANNABINOIDS

	% of control/10^7 cells			
	Whole cells	Supernatant 800 x g	Crude nuclei	Supernatant 100,000 x g
		2-Hour Incubation		
Control	(100) 7,131	(100) 2,135	(100) 3,365	(100) 383
Dexamethasone (10^{-5} M)	11	33	9	13
Δ^9-THC (10^{-4} M)	111	108	195	85
Δ^8-THC (10^{-4} M)	107	122	146	69
		48-Hour Incubation		
Control	(100) 7,508	(100) 2,338	(100) 4,765	(100) 454
Dexamethasone (10^{-5} M)	45	58	33	15
Δ^9-THC (10^{-4} M)	228	204	276	180
Δ^8-THC (10^{-4} M)	206	236	195	230

Experimental protocol is described in the Materials and Methods. The data presented are mean values of 4 experiments; there was less than 10% variability between respective points in this study.

TABLE 3. CANNABINOID DISTRIBUTION IN NB2A AND C-6 CELLS

Cell Fraction	NB2A Distribution (%)	C-6 Distribution (%)
Nuclei	45.1 ± 1.8	20.6 ± 2.1
Nuclear debris	8.8 ± 0.6	10.3 ± 1.2
Mitochondria	13.1 ± 0.8	10.5 ± 0.4
Microsomes	3.5 ± 0.3	7.6 ± 0.6
104,000 × g supernatant	8.8 ± 0.4	28.7 ± 0.9

Cells were incubated with 50 M [^{3}H]-Δ^9-THC for 15 min and fractionated as described under Materials and Methods. Results are the means and standard errors of six determinations.

A partial explanation of these differential cannabinoid effects is seen in the following study in which the uptake and subcellular distribution of [^{3}H]-THC in cannabinoid "sensitive" NB2A and cannabinoid "insensitive" C-6 cells is presented (Table 4). NB2A are approximately 100 times more sensitive to the growth inhibitory effects of THC than are C-6 cells[9] and take up approximately 10 times more cannabinoid. Most of the cannabinoid taken up by the cells of all three types is associated with the crude nuclear fractions.

Further proof of a nuclear locus was obtained in the cannabinoid "sensitive" NB2A cells. Electron microscopic evaluation of nuclei from NB2A cells treated with either vehicle or cannabinoid revealed that THC produced a dramatic condensation of nuclear materials (Plate I) compared to nuclei from vehicle control cells (Plate II).

DISCUSSION

There appears to be a strong structural and biological relationship between steroids and cannabinoids in terms of the latter's ability to mimic[17–19] and/or antagonize[17, 18, 27] some of the former's responses. In addition we have shown that the cannabinoids can inhibit adrenocorticotropic hormone, cholera toxin and cyclic AMP stimulated steroidogenesis in normal cat adrenal and Y-1 mouse adrenal tumor cells[4, 7]. This inhibition was demonstrated to be very specific in that the cannabinoids had no significant effects on cell growth, oxygen consumption, protein synthesis or pregnenolone-induced stimulation of steroidogenesis. It appears that this specific inhibition of steroid production involves an

TABLE 4. UPTAKE OF [^{3}H]-Δ^9-THC BY CELLS IN CULTURE

Cells	Grams × 10^{-6} of Δ^9-THC/10^6 cells
NB2A	2 ± 0.2
Glioma	0.1 ± 0.01

50 μM [^{3}H]-Δ^9-THC was incubated with the cells for 60 minutes. Plates were washed and the radioactivity counted as described in Materials & Methods. Values presented are the means ± S.E. of 5 experiments.

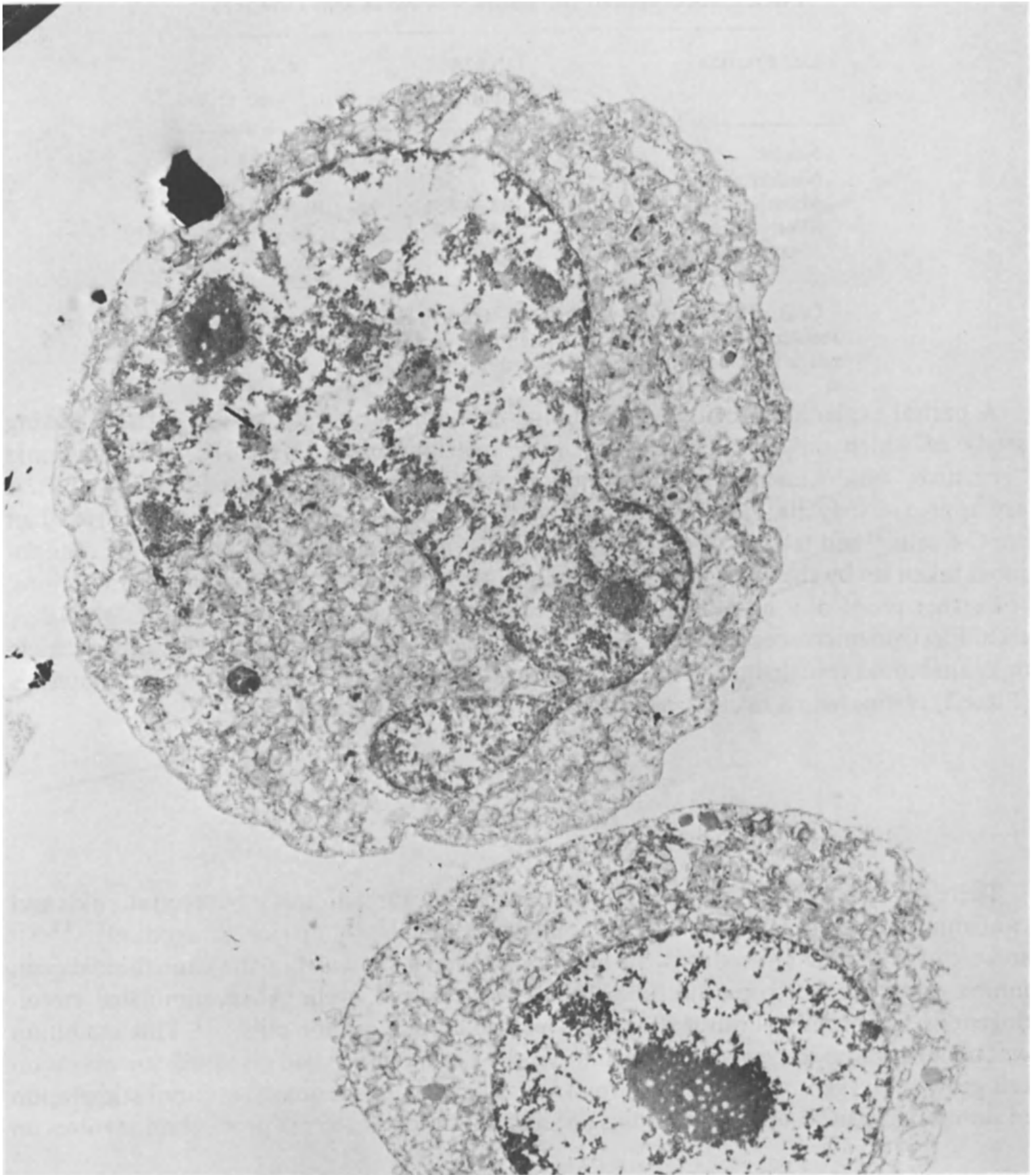


Plate I

Neuroblastoma cells were grown as described in Materials & Methods. Confluent cells were exposed to 10 μl 95% EtOH and fixed for electron microscopy as described in the text.

early step in the steoidogenic pathway, possibly by altering substrate availability (e.g. cholesterol transport or cholesterol esterase) and/or affecting the activity of cell product modulators (e.g. cyclic nucleotides or prostaglandins). Utilizing another "steroid" sensitive cell (i.e. HTC) we observed a strong interaction between the synthetic steroid dexamethasone and both Δ^9- and Δ^8-THC. This potentiation of the inhibition of macromolecular

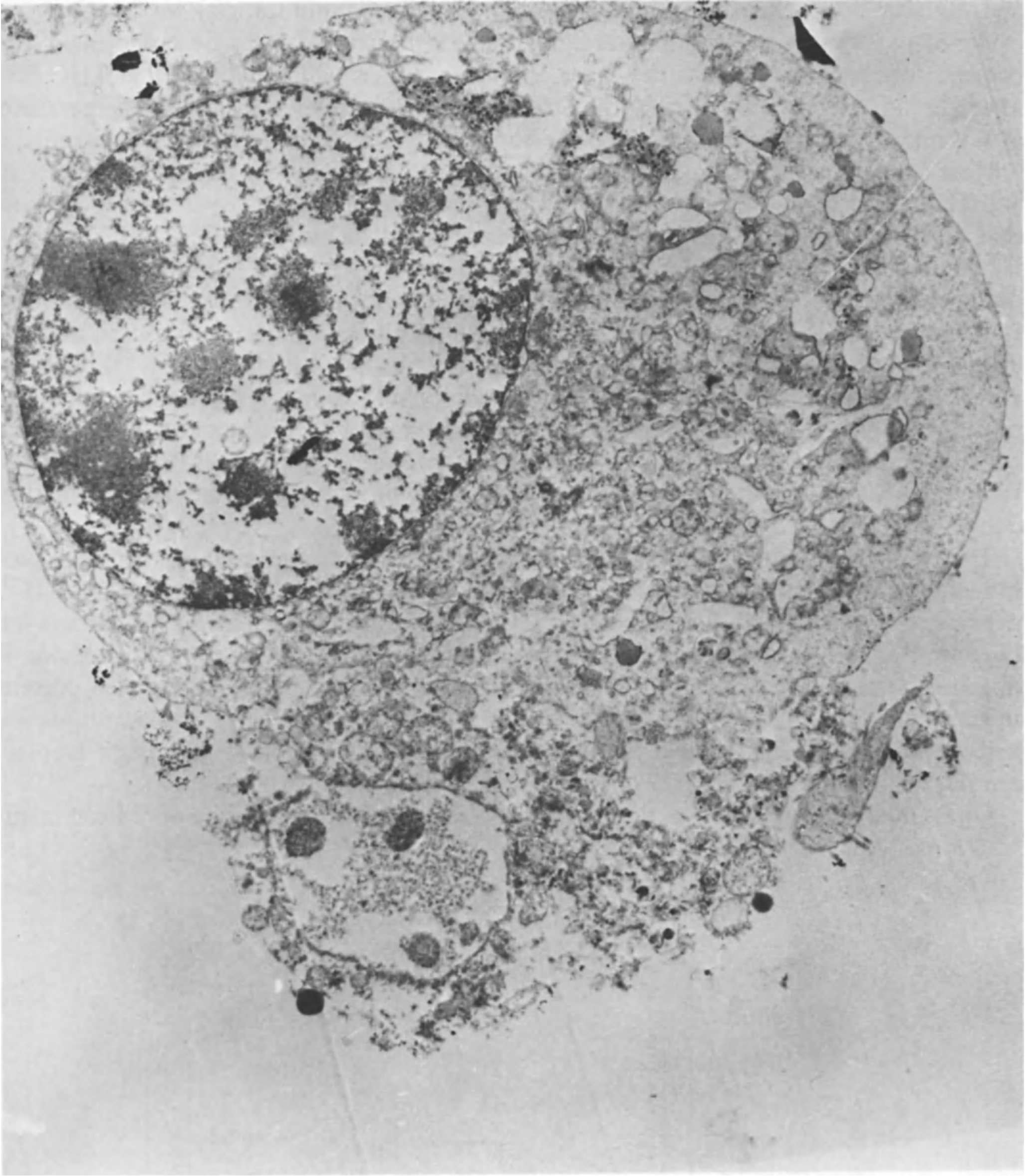


Plate II

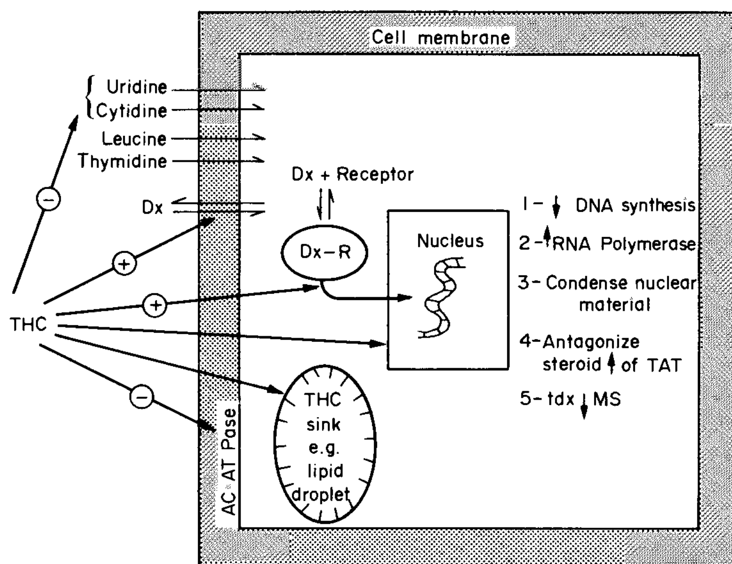
Cells were treated as in Plate I except that 10 μl of an EtOH solution containing Δ^9-THC (final concentration 10^{-5} M) was present.

synthesis in the presence of THC and dexamethasone might explain the partial agonist activity of these cannabinoids in the *in vivo* tyrosine aminotransferase studies of Friedman[17, 18]. While the uptake and distribution studies with [^{3}H]-dexamethasone in the presence of either Δ^9- or Δ^8-THC may help clarify the recent controversy over whether or not THC can directly alter steroid/receptor interactions[28, 29]. In agreement with Okey

and Bondy[28] and Friedman and Wrenn (personal communication) we can find no evidence to support a direct interaction between the steroid/receptor complex in the presence of cannabinoids. On the other hand our studies which indicate that THC can alter steroid uptake and distribution of [^{3}H]-dexamethasone, even at low temperature (4°C), may explain our macromolecular responses. It is possible that THC permits a nuclear concentration of steroid to develop and/or a direct inhibitory interaction of steroid and THC to occur in the nucleus which can depress macromolecular synthesis. It appears that dexamethasone has no effect on the uptake and distribution of [^{3}H]-THC (unpublished observation). Subcellular distribution studies with [^{3}H]-THC indicate that the radiolabel is concentrated in the crude nuclear fractions of NB2A, C-6[9] and HTC cells. Furthermore, Δ^9-THC produces a condensation of nuclear material as ascertained by electron microscopy. Δ^9-THC also causes calf thymus DNA to precipitate, though no effect on the melting temperature of DNA was observed (J. Collins—personal communication). These results are consistent with our observations that THC can induce morphological differentiation in NB2A cells[9], a response also produced by cyclic AMP, nerve growth factor, and by agents which specifically inhibit DNA synthesis (e.g. bromodeoxyuridine) as does THC.

The results from our *in vitro* studies imply that THC can also interact with cell membranes and alter the cellular uptake of certain precursors[3, 4, 8, 9], steroidogenesis[4, 7], dexamethasone uptake and distribution. These cell membranes can also pose barriers for the entrance of THC[9]. A possible explanation of the highly specific inhibition of steroidogenesis in steroid secreting cells may reside in the large, numerous lipid droplets present in such cells. These droplets may represent sinks for trapping these lipophilic compounds and provide insight into their (i.e. cannabinoid) mechanism of action since these droplets are rich in cholesterol esters, the substrates for steroid production.

Our model of cannabinoid action reflects our feelings that both cannabinoid cell mem-



brane and other cellular loci interactions can be directly or indirectly linked to THC's biological activity. Utilizing data obtained primarily from our studies with HTC cells we propose the following model for THC/cell interactions.

The overall cellular response to THC is a consequence of a mosaic interaction between THC and the respective cell. The biological activity of these compounds like the structurally related steroids cannot be based solely on their lipid solubility but must take into consideration other drug/cell complexes with which these agents can bind to and therefore contribute to the biological responses (e.g. receptors).

ACKNOWLEDGEMENT

This work was supported in part by grants DA 01637 and DA 09400. The authors wish to thank Drs. L. S. Harris and W. L. Dewey for their continuing support and assistance in discussing the results during the course of these studies.

REFERENCES

1. MECHOULAM, R., MCCALLUM, N., LEVY, S. and LANDER, N., Cannabinoid chemistry: an overview. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 3-13, 1976.
2. HARRIS, L. S., DEWEY, W. L. and RAZDAN, R. K., Cannabis-its chemistry, pharmacology, and toxicology. "Handbook of Experimental Pharmacology" (G. V. R. Born *et al.*, eds.). Springer-Verlag, New York, pp. 371-429, 1976.
3. CARCHMAN, R. A., HARRIS, L. S. and MUNSON, A. E., The inhibition of DNA synthesis by cannabinoids. *Cancer Res.* **36**,95-100 (1976).
4. CARCHMAN, R. A., WARNER, W., WHITE, A. C. and HARRIS, L. S., Cannabinoids and neoplastic growth. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 329-345, 1976.
5. HARRIS, L. S., MUNSON, A. E. and CARCHMAN, R. A., Antitumor properties of cannabinoids. "Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, Vol. 2, pp. 749-762, 1976.
6. MUNSON, A. E., HARRIS, L. S., FRIEDMAN, M. A., DEWEY, W. L. and CARCHMAN, R. A., Antineoplastic activity of cannabinoids. *J. Natl. Cancer Inst.* **55**,597-602 (1975).
7. WARNER, W., HARRIS, L. S. and CARCHMAN, R. A., Inhibition of corticosteroidogenesis by delta-9-tetrahydrocannabinol. *Endocrinology* **101**,1815-1820 (1977).
8. WHITE, A. C., MUNSON, J. A., MUNSON, A. E. and CARCHMAN, R. A., Effects of Δ^9-tetrahydrocannabinol in Lewis lung adenocarcinoma cells in tissue culture. *J. Natl. Cancer Inst.* **56**,655-658 (1976).
9. END, D. W., THOURSEN, K., DEWEY, W. L. and CARCHMAN, R. A., A comparative study of the disposition of $(-)$-Δ^9-tetrahydrocannabinol in neuroblastoma and glioma cells in tissue culture: relation to cellular impairment. *Molec. Pharmacol.* **13**,864-871 (1977).
10. MELLORS, A., Cannabinoids: effects on lysosomes and lymphocytes. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 283-298, 1976.
11. HUOT, J., Cellular and biochemical alterations induced *in vitro* by Δ^1-tetrahydrocannabinol: effects on cell proliferation, nucleic acids, plasma cell membrane ATPase, and adenylate cyclase. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 313-327, 1976.
12. PATON, W. D. M., PERTWEE, R. G. and TEMPLE, D. M., The general pharmacology of cannabinoids. "Cannabis and Its Derivatives: Pharmacology and Experimental Psychology" (W. D. M. Paton and J. Crown, eds.). Oxford University Press, London, pp. 50-78, 1972.
13. CHARI-BITRON, A., Effects of Δ^1-tetrahydrocannabinol on red blood cell membranes and on alveolar macrophages. "Marihuana: Chemistry, Biochemistry and Cellular Effects." (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 273-281, 1976.
14. GILL, E. W. and JONES, G., Brain levels of Δ^1-tetrahydrocannabinol and its metabolites in mice—correlation with behavior and the effect of the metabolic inhibitors SKF 525A and piperonyl butoxide. *Biochem. Pharmacol.* **21**,2237-2242 (1972).

15. Ho, B. T. and Johnson, K. M., Sites of neurochemical action of Δ^1-tetrahydrocannabinol interaction with reserpine. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 367–382, 1976.
16. Seeman, P., Chau-Wong, M. and Moyyen, S., The membrane binding of morphine, diphenylhydantoin, and tetrahydrocannabinol. *Canad. J. of Physiol. and Pharmacol.* **50**,1193–1200 (1972).
17. Friedman, M. A. and Wrenn, J., Suppression by Δ^9-tetrahydrocannabinol of induction of hepatic tyrosine aminotransferase and tryptophan oxygenase. *Toxicol. Appl. Pharmacol.* **41**,345–352 (1977).
18. Wrenn, J. M. and Friedman, M. A., Evidence for highly specific interactions between Δ^8- and Δ^9-tetrahydrocannabinol and hydrocortisone in the rodent tyrosine aminotransferase system. *Toxicol. Appl. Pharmacol.* **43**,569–576 (1978).
19. Solomon, J., Cocchia, M. A., Gray, R., Shattuck, D. and Vossmer, A., Uterotrophic effect of delta-9-tetrahydrocannabinol in ovariectomized rats. *Science* **192**,559–561 (1976).
20. Thompson, E. B., Tomkins, G. M. and Currain, J. E., Induction of tyrosine α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proc. Nat. Acad. Sci.* **56**,296–303 (1966).
21. Tomkins, G. M., Thompson, E. B., Hayashi, S., Gelehreter, T., Granner, D. and Peterkofsky, B., Tyrosine transaminase induction in mammalian cells in tissue culture. *Cold Spring Harbor Symp. Quart. Biol.* **31**,349–360 (1966).
22. Blevins, R. D. and Regan, J. D., Delta-9-tetrahydrocannabinol: effect on macromolecular synthesis in human and other mammalian cells. *Arch. Toxicol.* **34**,127–135 (1976).
23. Morishima, A., Milstein, M., Henrich, R. T. and Nahas, G., Effects of marihuana smoking, cannabinoids, and olivetol on replication of human lymphocytes: formation of micronuclei. "Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, Vol. 2, pp. 711–722, 1976.
24. Nahas, G., Suciu-Foca, N., Armand, J. P. and Morishima, A., Inhibition of cellular immunity in marihuana smokers. *Science* **183**,419–420, 1974.
25. Nahas, G., DeSoize, B., Armand, J. P., Hsu, J. and Morishima, A., "Pharmacology of Marihuana" (M.C. Braude and S. Szara, eds.). Raven Press, New York, Vol. 1, pp. 177–186, 1976.
26. Zimmerman, A. M. and Zimmerman, S. B., The influence of marihuana on eukaryote cell growth and development. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 195–205, 1976.
27. Kolodny, R. C., Lessin, P., Toro, G., Masters, W. H. and Cohen, S., Depression of plasma testosterone with acute marihuana administration. "Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, Vol. 1, pp. 217–225 (1976).
28. Okey, A. B. and Bondy, G. P., Δ^9-Tetrahydrocannabinol and 17β-estradiol bind to different macromolecules in estrogen target tissues. *Science* **200**,312 (1978).
29. Rawitch, A. B., Schultz, G. S., Ebner, K. E. and Vardaris, M., Competition of Δ^9-tetrahydrocannabinol with estrogen in rat uterine estrogen receptor binding. *Science* **197**,1189 (1977).
30. Rousseau, Guy G., Higgins, Stephen J., Baxter, John D., Gelfand, David and Tomkins, Gordon M., Binding of glucocorticoid receptors to DNA. *J. Biol. Chem.* **250**,6015–6021 (1975).

EFFECT OF (−)Δ^9THC ON ATPase SYSTEMS FROM VARIOUS SOURCES

E. TORO-GOYCO, M. B. RODRÍGUEZ, A. PRESTON and H. JERING

Department of Biochemistry and Nutrition
University of Puerto Rico School of Medicine
San Juan, Puerto Rico 00936

Summary. Chromatographically pure Δ^9- tetrahydrocannabinol (Δ^9THC) and other cannabinoids were tested for inhibitory activity on sodium and potassium stimulated ATPase (ATP phosphohydrolase, EC 3.6.1.3) from various sources. We found out that in addition to inhibiting Na^+ and K^+ dependent ATPases, Mg^{++} ATPases were inhibited as well. Rat brain ATPases were much more sensitive to Δ^9THC than to equal concentrations of ouabain. Inhibition by Δ^9THC was shown to occur both *in situ* and *in vitro*. *In vitro*, an I_{50} of 3×10^{-6} M was calculated for enzyme preparations of specific activity of 1,200–1,800 nmoles P_i/min/mg protein. The drug was found to bind to the enzyme and to cause a marked increase in K_m for Na^+, but no change in the K_m for K^+. These results suggest that Δ^9THC inhibits (Na + K) ATPases by interfering with the phosphorylation in the sequence of reactions leading to ATP hydrolysis. Phosphatidyl ethanolamine was found to reverse the inhibition caused by Δ^9THC in these enzyme preparations.

In Ehrlich ascites tumor cells (10^7 cells/flask) 60 μM Δ^9THC inhibited nucleoside ([^{3}H] thymidine) incorporation without exerting any significant effect on the viability of cells as measured by O_2 consumption. Particulate ATPases isolated from cells exposed to Δ^9THC had significantly lower activity than cells in control media, 123 ± 36 *vs.* 172 ± 16 nmoles P_i/min/mg protein ($p < 0.005$, $n = 16$). Thus a correlation seems to exist between ATPase inhibition and nucleoside uptake. A thorough study of the effect of cannabinoids on membrane bound ATPases deserves to be undertaken.

INTRODUCTION

THE pharmacological effects of Δ^9THC are so diverse that the choice of a single mechanism of action will undoubtedly be open to challenge[1–8]. In biological systems, alterations in a single biochemical pathway may lead to a variety of effects on cellular processes. The large number of metabolic changes observed by alteration of the reactions leading to cyclic AMP formation or catabolism are the best illustration of this point[9]. As an additional example, we may think of changes in any of the energy yielding reactions. These changes have far reaching effects in many cellular processes. Because of the close relationship of ATPase activity and the energy yielding processes we have been interested in finding which effects, if any, do the cannabinoids exert on the enzymes of the cell membrane dealing with transport processes and maintenance of ionic gradients, specifically the membrane bound ATPase systems of the cell.

We have concentrated our efforts in determining alterations in the activity of

$(Na^+ + K^+)$ dependent ATPases. The rationale for concentrating our efforts on this last system is obvious. If the unifying concept that the active pumping of a single substance out of the cell (e.g. Na^+) may furnish the driving force for the active transport of a variety of other substances into the cell is valid[10] then the inhibition of enzymes involved in Na^+ (or K^+) transport may have far reaching effects on the transport of nutrients and a variety of other biosynthetic processes in the cells. An additional reason for choosing ATPases as a model enzyme system for the study of possible effects of cannabinoids is that a number of these compounds are known to seriously hamper the normal function of the central nervous system (cns). The brain is among the richest sources of both ouabain-sensitive and ouabain-insensitive ATPases. Evidence of strong inhibition of ATPase function thus could be useful in the eventual understanding of the mechanism of action of cannabinoids in the cns.

This paper is a summary of our work on ATPases isolated from different sources. Some kinetic aspects of experiments done in cell free preparations are presented. An attempt is made to extrapolate these results to " in situ " experiments. We will explain some metabolic effects on the basis of interference by Δ^9THC with the energy yielding and transport processes in the intact cell.

MATERIALS

The cannabinoids used in this work have been a generous gift from Dr. L. S. Harris.* The purity of these preparations has been ascertained by one dimensional thin layer chromatography performed in two different solvent systems hexane-diethyl ether-acetic acid (90 : 15 : 1) and chloroform-methanol-water (56 : 25 : 4) followed by development in iodine vapors, or sulfuric acid charring. The working cannabinoid solutions were prepared as follows; five mg were dissolved in 1 ml acetone, diluted 1 : 500 in distilled water (final concentration 10 μg/ml). [^{3}H] labelled deoxythymidine was obtained from Schwartz-Mann. Ouabain, phosphatidyl ethanolamine, ATP and *p*-nitrophenyl phosphate were from Sigma: all other chemicals used were reagent grade.

Minimum essential medium without glutamine, with Hank's salts was purchased from Grand Island Biological Company. Protein was determined by the method of Lowry[11]. Statistical analysis of the kinetic data was done by the non-linear regression method of Wilkinson[12] using a program adapted to a 9810A Hewlet-Packard computer. Student's t test[13] was used for the statistical evaluation of the results in the experiments in situ in which changes in the specific activity of $(Na^+ + K^+)$ ATPases of Ehrlich ascites cells exposed to Δ^9THC were measured.

PREPARATION OF ATPases

ATPases have been obtained from various sources; one source has been the electric organ (electroplax) of the electric eel—*Electrophorus electricus*. The rationale for using this source is the high specific activity of this preparation and limited amount of ouabain insensitive ATPases present in them (4–5%). The enzyme was prepared essentially as

* Department of Pharmacology, Medical College of Virginia, Richmond.

suggested by Albers[14] with the modification that the tissue was disrupted by homogenization. The specific activity of these preparations was around 1500 nmoles P_i/min/mg prot.

ATPases were also obtained from adult rat brain (Sprague-Dawley rats weighing from 250–300 gm). The method of preparation was essentially that of Ahmed and Judah[15]. The specific activity of these preparations was 1,000–1,200 nmoles P_i/min/mg prot., with 20–25% of the activity being ouabain insensitive. Rat brain ATPases were isolated prior to and at various intervals after intraperitoneal injections of Δ^9THC (50 mg/kg). For intraperitoneal injections into rats, Δ^9THC was emulsified in 10% Tween-80 in normal saline.

Ehrlich ascites tumor cells grown in Swiss albino mice were used as a third source of ATPases. The procedure has been previously described[16]. Intact cells were exposed to 100 μg Δ^9THC for 50 min prior to disruption and isolation of the ATPases. The specific activity of these enzyme preparations was much lower than that of the ones isolated from electroplax or rat brain (172 $\pm$ 16 nmoles P_i/min/mg protein). The predominant ATPase in these preparations has been found to be ouabain insensitive Mg^{++} ATPase[17]. The purpose of this assay was to search for any relationship between ATPase inhibition and nucleoside uptake in intact cells.

ENZYME ASSAYS (ATPases)

Enzyme activity was measured as inorganic phosphorus liberated, using Sigma equine ATP as substrate. No attempt was made to remove the vanadate found by other investigators[18, 19] to be present in these substrate preparations and to be strongly inhibitory to ATPases. Incubations were performed at 37°C. The total amount of the incubation mixture was 1 ml. The procedure for the incubations has been outlined elsewhere[20]. In those assays where ouabain, Δ^9THC or other cannabinoids were used as inhibitor the blanks contained the corresponding amounts of inhibitor in their respective solvents. As a rule, the assay mixture contained 10–20 μg protein, 1 μg Δ^9THC (or cannabinoid) and from 0–10 μmoles ATP. ($Na^+ + K^+$) ATPase activity was determined as the difference of activities between total ATPase and Mg^{++} ATPase. For measurement of Mg ATPase activity, K^+ was omitted and 0.1 M ouabain was added.

K^+ dependent phosphatases were assayed as described by Nagai[21] using *p*-nitrophenylphosphate as the substrate. Its activity was calculated as difference between the activity measured in the presence of Mg^{++} plus K^+ (5 and 10 mM) and the activity detected with Mg^{++} alone. These incubations were performed for 15 min at 37°C and stopped by the addition of 0.05 M NaOH. The *p*-nitrophenol (pNP) liberated was read at 410 nm.

DETERMINATION OF THE BINDING OF Δ^9THC TO PARTICULATE MICROSOMAL ATPase FROM RAT BRAIN

Determination of the binding of Δ^9THC to particulate ATPase is not technically easy due to the strong binding of Δ^9THC to nitrocellulose filters. The separation of the unbound and bound THC must be accomplished by centrifugation. To accomplish this, 5 μC

of $[^3H]\Delta^9THC$ (specific activity 42 mC/mg) were dissolved in 1 ml acetone and 50 μl added to each of 5 test tubes containing 5 ml of an incubation mixture containing the ordinary constituents (40 mM ATP, 150 mM Na^+, 10 mM K^+, 5 mM Mg^{++}, buffer pH 7.4). The tubes were incubated at 37°C for 20 min. The contents of the tubes were transferred to 5.5 ml lusteroid tubes and centrifuged for 1 h at 20,000 × g in a Spinco Model L 350 Preparative ultracentrifuge, using a SW 39 swinging bucket rotor. The tubes were removed and each cut in 5 fractions with the aid of a tube cutter. The volumes were collected and aliquots of 0.1 ml suspended in Aquasol-2 (New England Nuclear) and counted in a Picker liquid scintillation counter.

RESULTS AND DISCUSSION

EFFECT OF VARIOUS CONCENTRATIONS OF Δ^9THC ON ATPase ACTIVITY

Figure 1 shows that both ouabain sensitive and insensitive ATPases are inhibited by Δ^9THC. In our assays concentrations of 1 μg Δ^9THC/ml, (3×10^{-6} M) cause slightly over 50% inhibition of the NaK ATPase. We found this to be a concentration of Δ^9THC suitable for studying the effects of Na^+ and K^+ on enzyme activity when the concentration of these cations is varied in the assay medium. Notice that as Δ^9THC concentration increases, inhibition tails-off, suggesting that if inhibitory activity of Δ^9THC is due to binding to the particulate enzyme, it follows saturation kinetics.

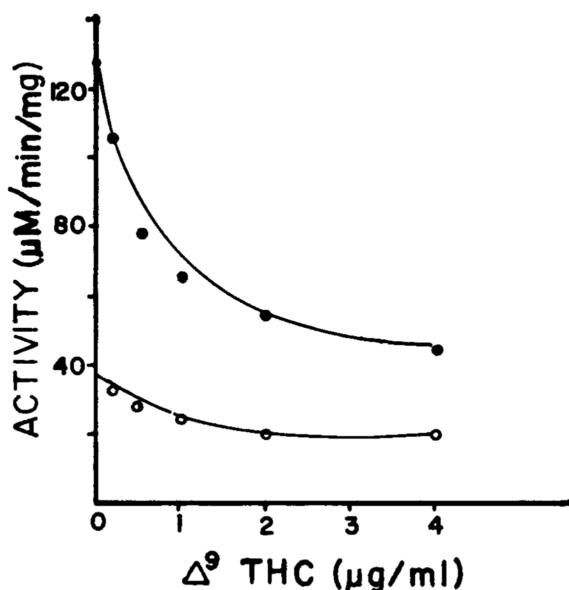


FIG. 1. Effect of varying concentrations of Δ^9THC on the enzymatic activity of brain ATPases: ●——●——● total ATPase activity; ○——○——○ ouabain insensitive ATPase activity. Ouabain insensitive activity was determined as the residual activity after the addition of 0.1 mM ouabain and omission of K^+ from the incubation mixture.

EFFECT OF Δ^9THC ON ATPases AT VARYING CONCENTRATIONS OF Na^+ AND K^+

Optimum ionic concentrations for ATPase activity were: 150 mM Na^+; 10 mM K^+; 5 mM Mg^{++}. Figures 2 and 3 illustrate the effects of Δ^9THC on ATPase activity when the concentrations of the essential cations, K^+ and Na^+, respectively were varied, all other experimental conditions remaining constant. Figure 2 shows that in the absence of Δ^9THC, enzymatic activity decreased when K^+ concentrations were changed from its optimum value, leaving all other experimental conditions constant. The inhibitory effect of the drug was more pronounced at optimum concentrations of K^+, but it did not seem to potentiate the inhibitory activity of K^+ when the concentrations of the ion deviated significantly from the optimum. Figure 3 illustrates the results obtained in the absence and presence of the drug when the Na^+ concentrations were varied, while other experimental conditions remained optimal for activity.

In Fig. 4 a Lineweaver-Burk plot constructed using varying concentrations of ATP at optimal concentration of Na^+, K^+ and Mg^{++} shows that Δ^9THC is a non-competitive inhibitor of ATP. The affinity of the enzyme for the substrate is not altered. The capacity of the enzyme to hydrolyze the substrate, however, is diminished, leading to a markedly

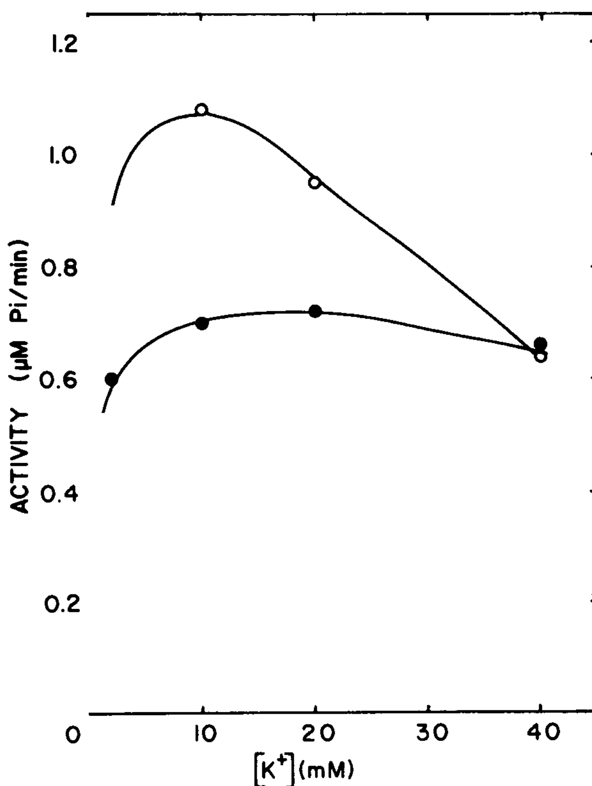


FIG. 2. Electric eel ($Na^+ + K^+$) ATPase activity in the absence (○——○——○) and in the presence (●——●——●) of 1 μg Δ^9THC at varying concentrations of K^+. Incubation performed with optimum concentrations of Na^+ and Mg^{++}, (as chloride salts). Maximum inhibition due to Δ^9THC occurs at 10 mM K^+. Total protein in incubation mixture: 10 μg.

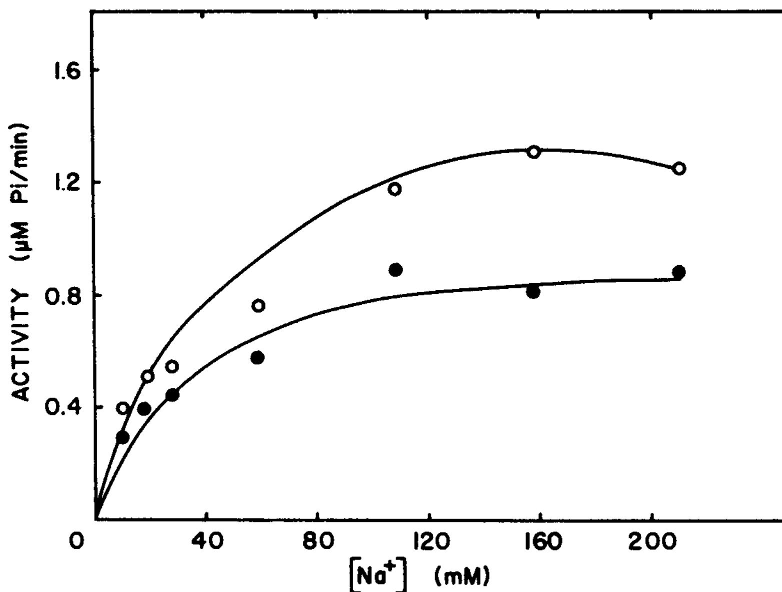


FIG. 3. Electric eel ($Na^+ + K^+$) ATPase activity in the absence (○——○——○) and in the presence (●——●——●) of 1 μg Δ^9THC at varying concentrations of Na^+, and optimum concentrations of K^+ and Mg^{++}. The sharpest reduction in enzymatic activity due to Δ^9THC occurs at 150 mM Na^+, which is the optimal for activity in the absence of the drug. Total protein in incubation mixture: 10 μg.

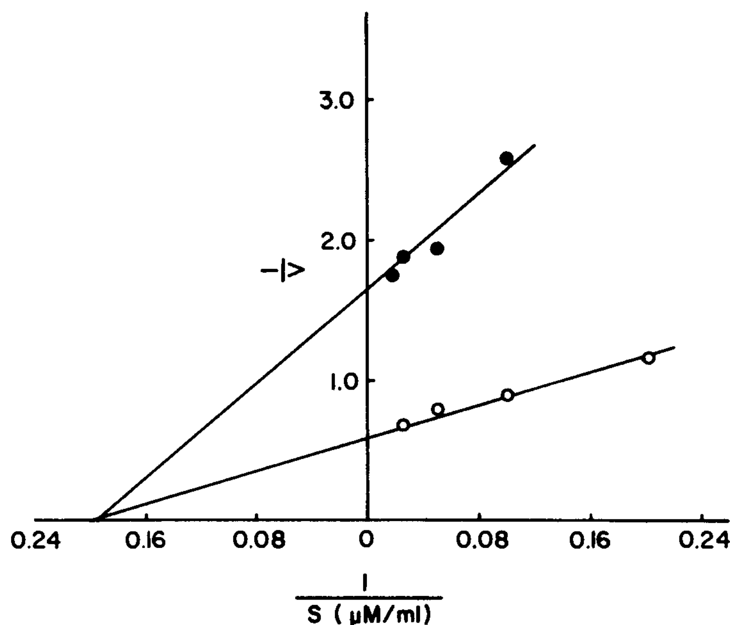


FIG. 4. Double reciprocal plot indicating non-competitive effects of Δ^9THC on ATP. Electric eel ATPase, 95% sensitive to ouabain, used for this experiment. Incubation performed using optimal concentrations of cations. For further details refer to text.

lowered rate of reaction. The value for V_m in the absence of inhibitor was 1.31 ± 0.1 μmoles P_i/min while in the presence of 30 μM Δ^9THC it was 0.96 ± 0.10 μmoles P_i/min, a highly significant difference ($P < .001$).

IN VITRO EFFECT OF SEVERAL INHIBITORS ON RAT BRAIN ATPase ACTIVITY

Table 1 summarizes the effect of various inhibitors on rat brain ATPases. Notice that amounts of Δ^9THC thirty times lower than those of ouabain show a comparable inhibitory activity and a combination of the two inhibitors increases the inhibition of the crude enzyme preparation. This is due to the fact that in addition to inhibiting ouabain sensitive ATPases Δ^9THC inhibits ouabain insensitive ATPases as well. Kinetic evidence of the inhibition is presented in Fig. 5 where it is shown that the maximum enzymatic activity is diminished for both ouabain sensitive and insensitive ATPases in the presence of Δ^9THC.

TABLE 1. INHIBITORY ACTIVITY OF VARIOUS SUBSTANCES ON THE ($Na^+ + K^+$) ATPASE ACTIVITY OF RAT BRAIN*

Contents of mixture	Specific activity (nmoles P_i/min/mg prot.)	Inhibition (%)
A. Enzyme preparation X (10 μg protein)	880	—
B. A + 0.1 μmoles ouabain	340	61.4
C. A + 0.003 μmoles Δ^9THC	442	50.0
D. A + B + C	193	78.4
E. Enzyme preparation Y (20 μg protein)	1010	—
F. E + 1 μmole ouabain	260	74.5
G. E + 10 μmoles serotonin	950	5.9
H. E + 0.03 μmoles Δ^9THC	335	66.8
I. E + 1 μmole ouabain + 0.003 μmoles Δ^9THC	60	94.3

* Assays for activity performed as described in the text. Two different enzyme preparations (identified as X and Y) prepared on different dates.

KINETIC STUDIES ON THE BINDING OF Na^+ AND K^+; EFFECT OF Δ^9THC

Since ATPases have binding sites for Na^+ and K^+, kinetic studies can be performed under saturating concentrations of ATP and optimal concentrations of K^+ to determine the effects of varying concentrations of Na^+ on enzyme activity and on reversal of the inhibition caused by Δ^9THC. Figure 6 illustrates this type of study. In the presence of Δ^9THC, both the maximum rate of reaction (V_m) and the affinity of the enzyme for Na^+ (K_m app) were significantly diminished.

A series of experiments performed at saturating concentrations of ATP, optimal concentrations of Na^+ and varying concentrations of K^+ yielded the results shown in Fig. 7. The affinity of the enzyme for K^+ (K_m app) ($2.63 + 0.30$ mM in the absence; 2.60 ± 0.32 mM)

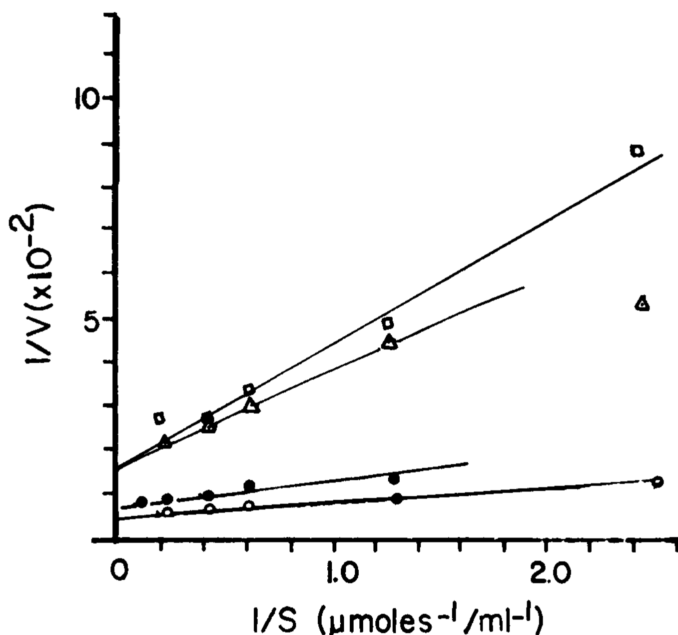


FIG. 5. Double reciprocal plot illustrating the inhibitory effects of Δ^9THC in concentrations of 3×10^{-6} M on brain ATPase preparations. ○——○——○ ($Na^+ + K^+$) ATPase activity; ●——●——● ($Na^+ + K^+$) ATPase activity in the presence of Δ^9THC; △——△——△ ouabain insensitive ATPase activity; □——□——□ ouabain insensitive ATPase activity in the presence of Δ^9THC. Total specific ATPase activity of preparation 1200 nmoles min/mg protein. For further details refer to text.

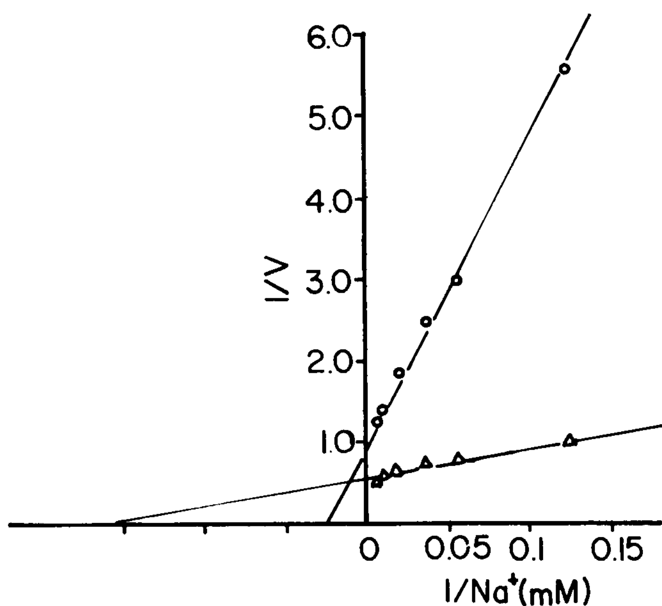


FIG. 6. Double reciprocal plot illustrating the effects of varying concentrations of K^+ on the specific activity of ($Na^+ + K^+$) ATPases in the absence (△——△——△) and in the presence (○——○——○) of Δ^9THC. The experiments were performed in the presence of saturating concentrations of ATP as substrate. A non-competitive inhibition for K^+, because of not significant changes in K_m in the absence and in the presence of Δ^9THC is apparent. For further details refer to the text.

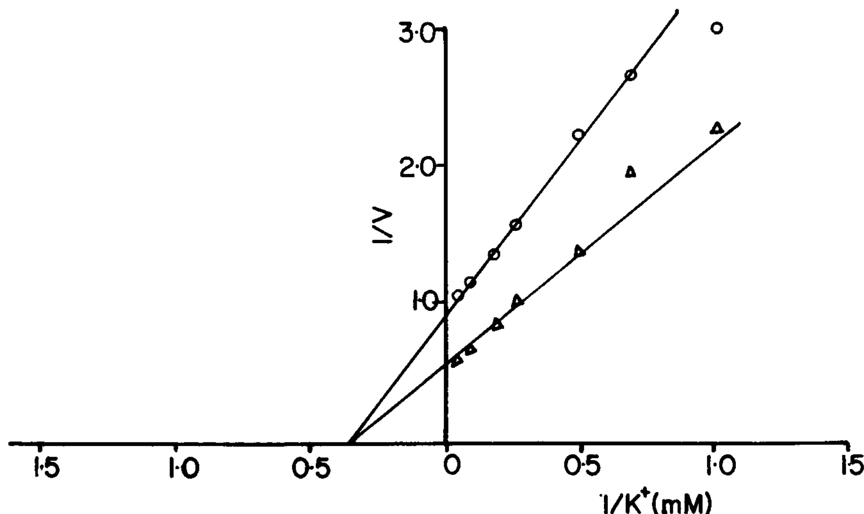
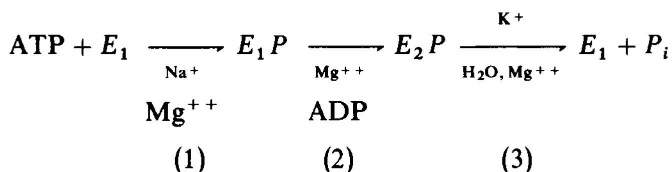


FIG. 7. Inhibition of the incorporation of ^{3}H thymidine to acid insoluble radioactivity in Ehrlich ascites tumor cells. THC concentrations causing 65% inhibition in ^{3}H thymidine incorporation also caused a significant decrease on (Na^+ + K^+) ATPases activity in the same cells. For further details, refer to Table 6 and to the text. △——△——△; no Δ^9THC; ○——○——○ 1 μg Δ^9THC.

in the presence of the drug did not seem to be altered, but the maximum rate of reaction (V_m) was significantly diminished (1.66 ± 0.13 μmoles P_i/min *vs.* 1.10 ± 0.06 μmoles P_i/min).

EFFECT OF Δ^9THC ON THE K^+ DEPENDENT PHOSPHATASE

A considerable body of knowledge exists to substantiate the claim that the hydrolysis of ATP occurs via the reactions indicated below[22]



$$\mathrm{ATP} + E_1 \xrightarrow[\mathrm{Na^+}]{} E_1P \xrightarrow[\mathrm{Mg^{++}}]{} E_2P \xrightarrow[\mathrm{H_2O,\,Mg^{++}}]{\mathrm{K^+}} E_1 + P_i$$

$$\underset{(1)}{\mathrm{Mg^{++}}} \qquad \underset{(2)}{\mathrm{ADP}} \qquad (3)$$

Thus it is theoretically possible to identify the step where Δ^9THC exerts its inhibitory action. An appropriate substrate can replace the theoretical E_2P intermediate and be hydrolyzed by the enzyme system. The substrate that can replace the theoretical E_2P intermediate is *p*-nitrophenylphosphate, yielding on hydrolysis *p*-nitrophenol. The enzyme catalyzing step (3) above is named the K^+-dependent phosphatase. This is precisely the step inhibited by ouabain in the ouabain-sensitive system[23].

Table 2 shows the results obtained after incubation of K^+-dependent phosphatase

TABLE 2. ASSAY OF K^+ DEPENDENT PHOSPHATASE WITH *p*-NITROPHENYL PHOSPHATE AS SUBSTRATE

Δ^9THC in mixture (μg)	Activity (μM P_i/min/mg)
0	0.16
2	0.23
5	0.25
10	0.24

activity in the presence of Δ^9THC. The drug slightly increases the activity of the phosphatase, thus suggesting that Δ^9THC has no appreciable effect on step (3) of the scheme shown above and that probably it exerts its inhibitory effect by blocking step (1) in the hydrolysis of ATP, that is, the Na^+ dependent phosphorylation of the enzyme system. The results shown in Fig. 6 agree with this contention since in the presence of the drug the enzyme shows a definite decrease in the affinity for Na^+.

REVERSAL OF ATPase INHIBITION BY PHOSPHATIDYL ETHANOLAMINE

NaK ATPases are inbedded in the cell membranes. It is known that a significant portion of the particulate active enzyme is phospholipid and that the activity of the enzyme decreases as the phospholipid matrix is removed from the particle[24]. It has been reported that sphingolipids and their derivatives have an activating effect on rat brain deoxycholate solubilized ATPases[25]. We conducted a series of experiments on the effect of a specific phospholipid, phosphatidyl ethanolamine on the activity of rat brain ATPases prior to and after exposure to Δ^9THC. Table 3 summarizes the results. The results show that although phospholipids per se do not increase enzymatic activity, they restore the activity to enzyme preparations inhibited by Δ^9THC. The mechanism by which the reactivation of the enzyme occurs is not known. It can be speculated that because of the highly lipophilic nature of THC phospholipids can compete for the drug with the particulate enzyme.

TABLE 3. EFFECTS OF PHOSPHATIDYL ETHANOLAMINE ON THE INHIBITION CAUSED BY Δ^9THC ON RAT BRAIN ATPASES

Content of mixture	Specific activity (μM P_i/min/mg prot.)	% inhibition
Enzyme (10 μg protein)	1.58	—
Enzyme (10 μg protein) + 30 nmoles P.E.	1.52	3.7
Enzyme + 3 nmoles Δ^9THC	0.55	65.1
Enzyme + Δ^9THC + 30 nmoles P.E.	1.28	19.0

Incubation performed at 37°C. Activity determined as P_i liberated.
P.E. solutions prepared in a 98 : 2 water acetone mixture (v/v).
P.E. added to enzyme and incubated for 10 min prior to addition of Δ^9THC.

ATPase INHIBITION BY OTHER CANNABINOIDS

Several cannabinoids have been shown to exert inhibitory effects on DNA synthesis in various cell lines analogous to those of Δ^9THC[26]. We tested a number of these cannabinoids for ATPase inhibitory activity on rat brain ATPases. The results are shown in Table 4. The inhibitory effects were of the same order of magnitude, although these were slightly higher when CBN was used as inhibitor.

TABLE 4. *In Vitro* EFFECTS OF VARIOUS CANNABINOIDS ON RAT BRAIN ATPASE ACTIVITY

Cannabinoid added*	Total ATPase†	$(Na^+ + K^+)$ ATPase	% $(Na^+ + K^+)$ ATPase inhibited
None	1345	1114	—
Δ^9THC	867	645	42.1
Δ^8THC	810	582	47.8
CBD	869	652	41.5
CBN	688	466	58.2

* Assay performed as described under METHODS. The amount of each cannabinoid used was 0.5 μg.

† Enzyme activity expressed as nmoles P_i/min/mg protein.

IN SITU EFFECTS OF Δ^9THC ON RAT BRAIN ATPases

Table 5 shows a comparison of brain ATPase activity in rats exposed to a dose of 50 mg/kg of Δ^9THC 2 h before being sacrificed and control rats sacrificed at the same time. The differences in specific activity found 1604 ± 151 nmoles P_i/min/mg protein for

TABLE 5. EFFECTS OF INTRAPERITONEAL INJECTIONS OF Δ^9THC ON RAT BRAIN ATPASES

Animal No.	Specific activity of enzyme preparation (nmoles P_i/min/mg protein) Control	Experimental
1	1627	1232
2	1748	1027
3	1683	1177
4	1658	1085
5	1597	1438
6	1313	1458
	1604 ± 151	1236 ± 179
	$t = 3.84$, $P < .005$	

The drug was prepared as a suspension in saline containing 10% Tween-80. Doses of 50 mg/kg were injected i.p. to adult Sprague-Dawley rats weighing from 200–250 g. Controls were injected with the same volume of solvent. The rats were killed by decapitation two hours thereafter. Brains were removed and the ATPases isolated following the method of Ahmed and Judah (*Biochem. Biophys. Acta* **93**,603, 1964). The preparations were 75–80% sensitive to 1 mM ouabain.

the controls as compared to 1236 ± 179 nmoles P_i/min/mg protein for the experimental animals were highly significant ($t = 3.84$, $p < .005$). The time of two hours after injection was used because after that period, the animals were already showing signs of a heavy narcotic state. No attempt was made to estimate the Δ^9THC levels in the circulation of these animals.

BINDING OF Δ^9THC TO THE PARTICULATE ENZYME

The results shown in Table 6 indicate that Δ^9THC definitely binds to the tube fraction containing the protein. No attempt, however, was made to characterize this binding as to specific or non-specific. These results can be interpreted as indicating that complexing of Δ^9THC with the substrate prior to binding with the enzyme can be ruled out as a possible mechanism of inhibition.

TABLE 6. BINDING OF Δ^9THC TO PARTICULATE RAT BRAIN ATPASE

Tube No.	Contents*	CPM/ml per fraction†			
		b	c	d	e
I	0.1 μC ^{3}H Δ^9THC + 50 μg enzyme	0	0	55	176
II	0.1 μC ^{3}H Δ^9THC + 50 μg enzyme	0	0	49	285
III	0.1 μC ^{3}H Δ^9THC + 50 μg P.E.	0	0	0	70
IV	0.1 μC ^{3}H Δ^9THC	0	0	0	0

* The contents in the incubation mixture in addition to the ones listed were: 750 μmoles Na^+, 100 μmoles K^+, 25 μmoles Mg^{++} (as chloride salts).

† Each lusteroid tube was cut in five fractions. The upper fraction was used as reference. The remaining fractions were labelled b, c, d, e from top to bottom. CPM were found by substrating cpm in the upper fraction from cpm in the corresponding fractions.

P.E. = phosphatidyl ethanolamine.

CORRELATION OF RESULTS *IN VITRO* WITH RESULTS *IN SITU*

Using the results shown in Fig. 8 and in Table 7 we will attempt to correlate the *in vitro* effects of Δ^9THC with the *in situ* effects. Figure 8 shows the inhibition of the incorporation of [^{3}H] thymidine into TCA insoluble material by Ehrlich ascites tumor cells. A total of 100 μg of the drug in 5 ml of the incubation mixture decreases [^{3}H]thymidine incorporation by 65% when compared to control cells. Table 7 shows the total ATPase activity assayed in aliquots of THC treated and untreated cells. The amount of THC per 10^7 cells used was the same as in the incorporation experiments. It can be seen that there is a statistically significant decrease in the activity of the ATPases in cells exposed to the drug. It is important to emphasize that the bulk of the ATPase activity in Ehrlich ascites tumor cells is Mg^{++} dependent[17].

These data show conclusively that the *in vitro* effects of Δ^9THC (and hence other active cannabinoids) on ATPases are intimately related to the *in situ* effects. Inhibition of ATPases by Δ^9THC has been reported by various investigators[27–30]. The diversity of

TABLE 7. EFFECT OF 100 μg Δ^9THC ON THE ACTIVITY OF A CRUDE ($Na^+ + K^+$) ATPASE PREPARATION FROM EHRLICH ASCITES TUMOR CELLS*

Sample number	Specific activities (nmoles P_i/min/mg protein) Control	Δ^9THC treated
1	150	78
2	181	80
3	161	121
4	195	171
5	198	179
6	165	142
7	160	118
8	164	126
9	175	95
	172 ± 16	123 ± 36

degrees of freedom = 16
$t = 3.71$, $P < .005$

* The amount of cells used for each experiment was 10^7 cells. The cells were incubated with Δ^9THC in 3 ml minimum essential medium of 37°C prior to their disruption. After breakage of the cells, the crude enzyme preparations were assayed for activity as described in the text.

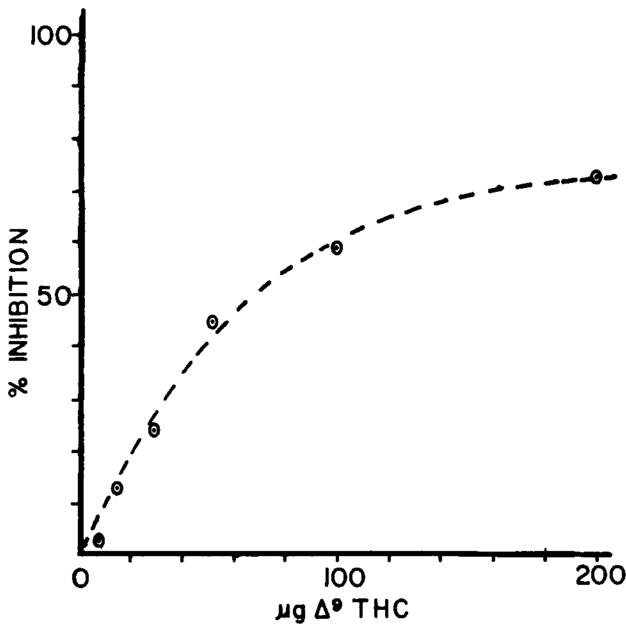


FIG. 8. Inhibition by Δ^9-THC of the incorporation of [^{3}H]thymidine into TCA insoluble material by Ehrlich ascites tumor cells.

metabolic effects thus could be attributed in part to inhibition of ATPases. Current work in our laboratory, to be discussed later[31] suggests that diminished amino acid and nucleoside uptake by one particular cell line (Reuber hepatoma cells) can be attributed to inhibition of enzymes associated with transport processes.

In conclusion the mechanism by which cannabinoids inhibit ATPase activity deserve to be thoroughly studied, in view of the possibly important biological significance of such inhibition.

ACKNOWLEDGEMENTS

This work was supported in part by U.S.P.H. Grants #00-203-51-01 and DA 00490 and Grant 5P30 CA 16598-02 from the National Cancer Institute. We thank Dr. J. del Castillo for the electric eels, and Mr. J. Caloca and Mrs. E. Torres for the illustrations and typing.

REFERENCES

1. OSGOOD, P. E., and PARS, H. G., *Fed. Proc.* **35**,643 (1976).
2. CARCHMAN, R. A., WARNER, W., WHITE, A. C., and HARRIS, L. S., In: "Marihuana: Chemistry, Biochemistry and Cellular Effects." G. G. Nahas, (ed.) 329–345, Springer-Verlag, N.Y., 1976.
3. NAHAS, G. G., DE SOIZE, B., HSU, J., MORISHIMA, A., and SRINIVASAN, P. R., *Proc. of the 6th International Congress of Pharmacol. (Helsinski)*, p. 495 (1975).
4. DE SOIZE, B. and NAHAS, G. G., *C.R. Acad. Sci. (Paris)*, 281–284 (1975).
5. CARCHMAN, R. A., HARRIS, L. S., and MUNSON, A. E., *Cancer Research* **36**,95–100 (1976).
6. TEN HAM, M., and JONG, Y., *Proc. of the 6th International Congress of Pharmacol. (Helsinski)*, p. 504 (1975).
7. SOFIA, R. D., DIXIT, B. N., and BARRY, H., *Life Sci.*, **10**,425–436 (1971).
8. GREENBERG, J. H., SAUNDERS, M. E. and MELLORS, A., *Science*, **197**,475–477 (1977).
9. ROBINSON, G. A., BUTCHER, R. W., and SUTHERLAND, E. W., "Cyclic AMP," Academic Press, New York, 1971.
10. LEHNINGER, A. L., "Biochemistry," 1st Ed. Ch. 27, Worth Publishers Inc., New York, 1970.
11. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.* **193**,265–275 (1951).
12. WILKINSON, G. N., *Biochem. J.* **80**,324–332 (1961).
13. SNEDECOR, G. W., "Statistical Methods Applied to Experiments in Agriculture and Biology," Chapter 2, 5th ed., The Iowa State College Press, Ames, 1956.
14. ALBERS, R. W., FAHN, S., and KOVAL, G. J., *Proc. Nat. Acad. Sci. U.S.A.* **50**,474–481 (1963).
15. AHMED, K., and JUDAH, D., *Biochem. Biophys. Acta* **93**,603–613 (1964).
16. TORO-GOYCO, E., RODRÍGUEZ, M. B., and PRESTON, A. M., *Molecular Pharmacol.* **14**,130–137 (1978).
17. RONQUIST, G. and AGREN, G. K., *Cancer Res.* **35**,1402–1406 (1975).
18. BEAUGE, L. A. and GLYNN, I. M., *Nature* **268**,355–356 (1977).
19. CANTLEY, L. C., JOSEPHSON, L., WARNER, R., YANAGISAWA, M., LECHENE, C. and GUIDO HI, G., *J. Biol. Chem.* **252**,7421–7423 (1977).
20. GARCÍA-CASTIÑEIRAS, S., WHITE, J. I., RODRÍGUEZ, L. D., and TORO-GOYCO, E., *Biochem. Pharmacol.* **26**,589–594 (1977).
21. NAGAI, K., IZUMI, F., and YOSHIDA, M., *J. Biochem. (Tokyo)* **59**,295–303 (1966).
22. DAHL, J. L. and HOKIN, L. E., *Ann. Rev. Biochem.* **43**,327–356 (1974).
23. SIEGEL, G. J. and JOSEPHSON, L., *J. Biochem.* **25**,323–335 (1972).
24. BRIGHT, H. J., *Biochemistry* **6**,1191–1197 (1967).
25. CAPUTTO, R., MACCIONI, A. H. R., and CAPUTTO, B. L., *Biochem. Biophys. Res. Comm.* **74**,1046–1052 (1977).
26. CARCHMAN, R. A., HARRIS, L. S. and MUNSON, A. E., *Cancer Res.* **36**,95–100 (1977).
27. LAURENT, B., ROY, P. E., and GAILIS, L., *Can. J. Physiol. Pharmacol.* **52**,1110–1113 (1974).
28. HUOT, J. In Nahas, G. G., *et al.*, ed. "Marihuana, chemistry, biochemistry and cellular effects", Springer-Verlag, New York, 1976.
29. HUOT, J. In Radouco-Thomas, S., ed. "Pharmacologie, toxicologie et a bus des psychotomiméfigues (hallucinogens) Les Presses del' Universití Laval, Quebec, 1973.
30. TORO-GOYCO, E., and RODRÍGUEZ, M. B., *Molecular Pharmacol.* (in press).
31. JERING, H., and TORO-GOYCO, E., in these proceedings. pp. 161–169.

(–)TRANS-Δ^9-TETRAHYDROCANNABINOL: SYNAPTOSOMAL PHOSPHOLIPIDS, SYNAPTOSOMAL PLASMA MEMBRANE (Na^+-K^+)-ATPase, AND THIOPENTONE ANAESTHESIA IN MICE

R. MALOR, D. M. JACKSON and G. B. CHESHER

Department of Pharmacology, University of Sydney, N.S.W. 2006, Australia

Summary. Delta-9-THC did not change the kinetic behavior of (Na^+-K^+) ATPase of cortical synaptosomal membranes of mice treated an hour before with 10 mg/kg I.P. Tolerance did not develop to the prolongation by THC of thiopentone anesthesia. Mice pretreated with THC (10 mg/kg I.P.) one hour before sacrifice did not present a significant change in distribution of the major phospholipids. One should be cautious about interpreting the effect of THC on (Na^+-K^+) ATPase changes as increase in membrane fluidity.

THE high membrane-buffer partition coefficient[48] of (–) trans-Δ^9-tetrahydrocannabinol (THC) implies that the drug should be preferentially adsorbed to the more hydrophobic regions of the cell—i.e. the plasma membrane and the membranes of the cytoplasmic organelles. Since the non-psychoactive polar metabolites translocate rapidly to the aqueous cytosol[15, 31, 38] it would seem probable that the effects of THC will be primarily on membranes and might involve changes in membrane structure and/or changes in the function and integration of the membrane-bound enzyme systems and receptors. Many of these enzymes and receptors not only have specific lipid requirements for activation, but also their kinetics may reflect changes in their immediate lipid microenvironment[1, 20, 29, 32, 33, 42]. THC has been reported *in vitro* to inhibit ATPase from rat liver mitochondria[11], (Na^+-K^+)-ATPase and Mg^{2+}-ATPase from rat brain synaptosomes and microsomes[46], (Na^+-K^+)-ATPase from rat ileum[34], to inhibit (Na^+-K^+)-ATPase and Mg^{2+}-ATPase from rat brain synaptosomes[28] and (Na^+-K^+)-ATPase from rat brain, Electrophorus, and Ehrlich ascites cells[51]. THC has also been reported to greatly reduce the phospholipid content of rat brain synaptosomes and mitochondria[47]. THC also produced mitochondrial uncoupling and above about 70 nmoles THC/mg mitochondrial protein, large amplitude swelling which was not energy dependent, and led to a leakage of the matrix enzymes[39]. There was evidence that this could have been due to THC sensitizing the more cardiolipin-rich areas of the mitochondria to Mg^{2+}-induced destabilization. THC increased the fluidity of phospholipid-

cholesterol liposomes[24, 35], reduced the enthalpy of phospholipid phase change[6] (see also Ref. 7), and eliminated the discontinuity in the Arrhenius plot for microsomal *O*-demethylase[8]. Should a similar pattern of membrane changes be found in the intact animal they may well be relevant to such properties of THC as its potentiation of general anaesthetics[44] and its metabolic drug interactions[41]. The present report concerns our own studies on THC and its effects, *in vivo*, on (Na^+-K^+)-ATPase derived from a mouse cortical synaptosomal plasma membrane fraction, on mouse synaptosomal phospholipids and on thiopentone-induced anaesthesia in mice.

THC AND (Na^+-K^+)-ATPase (EC 3.6.1.3.)

Adult male albino mice were used. The cerebral cortices were removed, placed immediately in 0.32 M sucrose at 4°, and trimmed of white matter by blunt disection. The synaptosomal subfractions were isolated[30] and the synaptosomal plasma membrane fraction collected. The identity, homogeneity, and freedom from contamination by other cell membranes was confirmed by electron microscopy and by marker enzymes. (Na^+-K^+)-ATPase was assayed essentially by the method of Bloj *et al.*[9] but using a 5 or 10 min incubation time to achieve a better estimate of initial rates. Activity was measured by determining the liberated inorganic phosphate[5] and the (Na^+-K^+)-ATPase activity calculated as the difference between the total and the Mg^{2+} activated enzymes. Ouabain was not included as there is a THC-ouabain interaction on brain (Na^+-K^+)-ATPase[51]. The enzyme was always assayed immediately upon isolation, since freezing or lyophilizing may alter the membrane structure.

TABLE 1. THE EFFECT OF THC (10 mg/kg I.P. 1 H PRETREATMENT) ON THE KINETIC BEHAVIOUR OF (Na^+-K^+)-ATPASE FROM MOUSE CORTICAL SYNAPTOSOMAL PLASMA MEMBRANES.

Treatment group	V_{max} (μm P_i/10 min/mg protein)	K_m (ATP, M × 10^{-5})	Hill coefficient	percent activation
Vehicle	3.192 ± 0.298 (9)*	4.928 ± 0.744 (13)	0.93 ± 0.03 (8)	36 ± 5 (5)
THC	3.025 ± 0.222 (10)	4.064 ± 0.365 (16)	0.97 ± 0.04 (6)	37 ± 3 (4)

* The number of experiments is in parentheses.

In the absence of F^-, the enzymes from both THC- (10 mg/kg i.p. 1 h) and vehicle-treated animals behaved identically towards the substrate (Table 1). These findings are at variance with both the *in vitro* reports from other workers (see references above) and the *in vivo* results of Poddar and Ghosh[46]. The latter authors found THC to inhibit, in a dose-related manner, (Na^+-K^+)-ATPase from synaptosomes. *In vitro* experiments involve not only the separate effects of the vehicle and the cannabinoid but also the possibility of an interaction between them (see Refs. 13 and 23). The problem of vehicle interaction, however, has also been observed *in vivo* with many of the vehicles commonly used for the administration of cannabinoids. Thus, (a) Tween 80-saline, and ethanol-saline[14] can change monoamine oxidase from various tissues, (b) sesame oil (orally) can deplete brain noradrenaline without changing brain 5-hydroxytryptamine[3], and (c) propylene glycol–Tween-80–saline and ethanol-saline also shows an interaction with "stress" as determined

by corticosterone replacement[37]. It may well be that the difference between the results in Table 1 and those of Poddar and Ghosh[46] reflects the different vehicles used as well as different pretreatment times. In a parallel experiment, preparations from undosed mice were compared to those from mice dosed with THC plus vehicle or vehicle alone. No differences in the enzymes' behaviour were apparent. The synaptosomal fraction used by Poddar and Ghosh[46] for their kinetic study would be expected to consist of a mixture of organelles including synaptosomal plasma membrane, synaptic vesicles, intraterminal mitochondria and microsomal material included within the cytoplasm retained by the synaptosome. All these organelles have some form of relevant ATPase activity. Differential enzyme sensitivity to THC might explain the discrepancy between the two sets of results. The possibility of heterogeneity is strengthened by the finding of Laurent and Roy[34] that *in vitro*, THC decreased membrane fluidity with respect to microsomal (Na^+-K^+)-ATPase.

TABLE 2. EFFECT OF THC CHALLENGE (10 mg/kg I.P. 1 H) BOTH WITHOUT AND WITH PRIOR DAILY THC PRETREATMENT (10 mg/kg/d, P.O.) ON THE KINETIC PARAMETERS OF THE INHIBITION OF MOUSE CORTEX SPM (Na^+-K^+)-ATPase BY F^-.

Treatment	Challenge drug	Number of exps.	Hill coefficient	$K_{0.5}$ mM F^-
Acute	Vehicle	7	−1.70 ± 0.20*	2.62 ± 0.47
	THC	8	−1.02 ± 0.12*	2.37 ± 0.64
Chronic 1 day	Vehicle	1	−1.76 ± 0.20	2.17 ± 0.27
	THC	1	−1.71 ± 0.25	1.88 ± 0.32
Chronic 21 day	Vehicle	1	−1.37 ± 0.23	2.31
	THC	1	−1.30 ± 0.18	2.93

* $p < 0.02$

The inhibition of (Na^+-K^+)-ATPase by F^- revealed a difference in the kinetic behaviour of the enzymes from the THC- and vehicle-treated animals (Table 2). There was a decrease in the Hill coefficient to a value which was not significantly ($p < 0.02$) different from 1.0. This indicated that there was no co-operativity between the effector (F^-) and the substrate binding sites after a single acute dose of THC. This may be interpreted in at least two ways:

(a) THC may have interacted with the enzyme in such a way as to prevent access of the ligand to the regulatory binding site or to prevent interaction with the substrate binding site. Either of these would result in the loss of co-operativity. This change could be analogous to the effect of cholesterol in restricting conformational changes in reconstituted (Na^+-K^+)-ATPase[32].

(b) An analogy might be drawn with the kinetic behaviour of erythrocyte (Na^+-K^+)-ATPase[9, 20], where it was found that the absolute value of the Hill coefficient was highly negatively correlated with the ratio of unsaturated to saturated fatty acids (and the double bond index) of the membrane phospholipids. In other words THC has produced a large increase in membrane fluidity, at least in the microenvironment of the enzyme. This interpretation is in general agreement with the theories based on fluidity studies with spin labelled liposomes[35].

In two experiments (Table 2) mice were pretreated once daily with THC (10 mg/kg p.o.) for either 1 or 21 d and challenged on day 2 or 22 with THC (10 mg/kg i.p.) or vehicle. Possible vehicle effects were not controlled for in these experiments. The enzyme from THC-treated animals did not differ from that of vehicle-treated animals (Table 2). This might be regarded as "membrane tolerance". A similar form of tolerance has been reported previously[43] for oxygen uptake by brain homogenates with both ADP-succinate and endogenous substrates. Tolerance has been shown to develop equally rapidly to a range of behavioural parameters[2].

Because: (1) it has been postulated that THC may function as a partial anaesthetic[44, 48]; (2) anaesthetics in general produce an increase in membrane fluidity[49]; and (3) the data in Table 2 might be interpreted as resulting from a marked change in membrane fluidity due to a single acute dose of THC, it seemed of interest to establish whether tolerance to THC prolongation of anaesthesia[12, 22, 40] was developed as quickly. To investigate this, mice were dosed with THC (10 mg/kg i.p.) or vehicle. One hour later thiopentone was administered (40 mg/kg i.p.) and the duration of anaesthesia measured (Table 3). To control for hypothermia the environment was maintained at 30°.

TABLE 3. THE DURATION OF ANAESTHESIA OF MICE PREMEDICATED WITH THC (10 mg/kg P.O.) OR VEHICLE 24 H BEFORE A CHALLENGE DOSE OF THC (10 mg/kg I.P.) OR VEHICLE. 1 H AFTER THE CHALLENGE DOSE, MICE WERE ADMINISTERED THIOPENTONE (40 mg/kv I.V.) AND THE DURATION OF ANAESTHESIA DETERMINED.

Premedicant	Challenge	Mean sleeping time ± s.e.m. (min)	n*
THC	THC	117.9 ± 12.8	16
THC	Vehicle	58.4 ± 11.8	16
Vehicle	THC	89.9 ± 12.7	19
Vehicle	Vehicle	22.6 ± 7.3	20

* n is the number of animals used.

Tolerance did not develop to the prolongation by THC of thiopentone anaesthesia. Indeed after a single dose of THC there was still significant prolongation of the anaesthetic's action.

SYNAPTOSOMAL PHOSPHOLIPIDS

Phospholipids form an integral part of the structure of biological membranes. As well as being responsible for the electrochemical properties of the membrane many of the membrane bound enzymes have quite specific lipid requirements. (Na^+-K^+)-ATPase must be associated with particular phospholipids, specified in both base and fatty acyl chains for maximum activation[25, 32]. We therefore examined the effect of THC on the composition of the major phospholipids from the total synaptosomal fraction of mouse cortex. The phospholipids were extracted immediately the fraction was isolated and non-lipid material removed in the standard manner[21]. The phospholipids were separated by thin layer chromatography on silica gel H[54]. Pretreatment with THC (10 mg/kg i.p.) 1 h prior to

TABLE 4. THE DISTRIBUTION OF SYNAPTOSOMAL PHOSPHOLIPIDS FROM MOUSE CEREBRAL CORTEX. THE ANIMALS WERE PRETREATED WITH EITHER THC (10 mg/kg I.P.) OR VEHICLE. THE DATA CAME FROM 4 SEPARATE EXPERIMENTS FOR EACH GROUP.

Phospholipid	Percentage total lipid phosphorus (± s.e.m.)	
	Vehicle	THC
Sphingomyelin	4.33 ± 0.56	3.38 ± 0.41
Phosphatidyl choline	38.95 ± 1.88	40.61 ± 0.41
Phosphatidyl inositol	3.39 ± 0.31	3.45 ± 0.49
Phosphatidyl serine	12.76 ± 1.19	12.11 ± 0.47
Phosphatidyl ethanolamine*	37.76 ± 0.82	38.36 ± 1.03
Phosphatidic acid + Cardiolipin	1.67 ± 0.62	1.33 ± 0.49

* This actually represents the sum of phosphatidyl ethanolamine and ethanolamine plasmalogen. Recovery from the plate was between 89–102% of the applied phospholipid phosphorus.

sacrifice produced no change in the distribution of the major phospholipids (Table 4). Furthermore, these data are in close agreement with the published phospholipid distribution for guinea pig and rabbit synaptosomes[19, 27].

Our findings are in conflict with those of Sarkar and Ghosh[47] who reported that THC (10 mg/kg i.p. 6 h) produced a marked reduction in rat brain synaptosomal phosphatidyl choline (reduced by 30%), phosphatidyl ethanolamine (24%), and phosphatidyl serine (48%). There was an increase in sphingomyelin but this did not reach statistical significance. There may be several reasons for the difference between the results in Table 4 and those of Sarkar and Ghosh[47]. These might include species, THC vehicle, pretreatment time, their use of silica gel G which gives only fair chromatographic resolution of phospholipids, and an unexplained 58% recovery of phospholipids from the THC-treated animals as against 79% for the vehicle-treated group. The recovery in our experiments was 95% ± 2% ($n = 13$). (This was calculated as the sum of the phosphates of the separate spots taken from the chromatogram, corrected for the consistently very low plate blank, expressed as a percentage of the amount of phospholipid phosphorus applied to the plate).

DISCUSSION

Both the allosteric behaviour of (Na^+-K^+)-ATPase (Table 2) and the anaesthetic action of thiopentone (Table 3) have been related to the nature of the structure and order of the membrane lipids[9, 20, 32, 36]. Lee[36] has suggested that certain of the anaesthetic properties of the barbiturates are due to the action of the drug in changing the lipid microenvironment (or lipid annulus) immediately surrounding the sodium channel to a rigid configuration. The channel proteins are restrained to an inactive configuration resulting in the reduction of the sodium current. Despite this, the development of a rapid tolerance to the effect of THC on (Na^+-K^+)-ATPase and the failure to develop tolerance to the action of THC in prolonging thiopentone anaesthesia could well both be due to actions on the membrane lipids. The behaviour of the enzyme relates largely to the fluidity and crystalline

structure of its phospholipid annulus[20, 45, 53]. Furthermore, from the way in which thiopentone and THC differ structurally and in charge distribution (the ring system of THC is not coplanar[4] and THC has no ionizable groups and is totally hydrophobic), it would not be unreasonable to suggest that the location of THC in the phospholipid annulus is different to that of the barbiturates. This would result in the two drugs causing different structural perturbations in the annulus, thereby acting on different ligand binding sites of the enzyme. Moreover we have investigated only one specific enzyme in one particular brain region.

In measuring the duration of the thiopentone anaesthesia, what was in fact being obtained was a behavioural correlate which was the sum total of the anaesthetic action on the CNS. Although the synaptosome contains the enzymes of lipid synthesis[16], transacylation[50], and ethanolamine and serine phosphatide base exchange[26], it still would have been surprising if rapid tolerance had developed in all of the membrane systems and brain regions where there might be a functional interaction between the two drugs.

The cyclic AMP stimulated protein kinase of the synaptosome is located within the synaptosomal plasma membrane and localized at the synaptic cleft[55]. Since (Na^+-K^+)-ATPase and the intrinsic protein kinases have been shown to influence, or co-regulate each other[52], the results presented in Table 2 may provide a rationale for the complexity of the effect of THC on brain cyclic AMP formation[17, 18].

One should be cautious about interpreting the effect of THC on (Na^+-K^+)-ATPase changes in co-operativity as reflecting an increase in membrane fluidity. This is especially so when one considers the data from the erythrocyte and the liposome studies of Bloj *et al.*[9], Farias *et al.*[20] and Lawrence and Gill[35]. The cholesterol-phospholipid ratio for erythrocyte membranes is about 0.9, Lawrence and Gill[35] made their liposomes with a ratio of 1.0, but the ratio for synaptosomal plasma membrane (as used in the present study) lies between 0.43 and 0.48[10]. And it is important to note here that membrane fluidity, and thus the behaviour of many membrane enzymes like (Na^+-K^+)-ATPase, depends not only upon the degree of unsaturation of the phospholipid fatty acids but also on the relative proportion of cholesterol present in the lipid closely associated with the enzyme[45]. If THC is exerting an action on the membrane of the nerve terminals it would be of great interest to see the liposome work repeated with a lower cholesterol-phospholipid ratio. It is quite possible that were the ratio reduced to about 0.5 the liposomes might not have been stereoselective.

ACKNOWLEDGEMENTS

We wish to thank Dr. Monique Braude of NIDA for gifts of Δ^9-THC, Ms. B. Chan for technical assistance, and the National Health and Medical Research Council for support of one of us (R.M.).

REFERENCES

1. Abood, L. G., Salem, N., Macneil, M., Bloom, L. and Abood, M. E., Enhancement of opiate binding by various molecular forms of phosphatidyl serine and inhibition by other unsaturated lipids. *Biochim. Biophys. Acta* **468**,51 (1977).

2. ANDERSON, P. F., JACKSON, D. M., CHESHER, G. B. and MALOR, R., Tolerance to the effects of Δ^9-tetrahydrocannabinol in mice on intestinal motility, temperature and locomotor activity. *Psychopharmacologia (Berl.)* **43**,31 (1975).
3. ANTON, A. H., SERRANO, A., BEYER, R. D. and LAVAPPA, K. S., Tetrahydrocannabinol, sesame oil and biogenic amines: a preliminary report. *Life Sci.* **14**,1741 (1974).
4. ARCHER, R. A., BOYD, D. B., DEMARCO, P. V., TYMINSKI, I. J. and ALLINGER, N. L., Structural studies of cannabinoids. A theoretical and proton magnetic resonance analysis. *J. Amer. Chem. Soc.* **92**,5200 (1970).
5. ATKINSON, A., GATENBY, A. D. and LOWE, A. G., The determination of inorganic orthophosphate in biological systems. *Biochim. Biophys. Acta* **320**,195 (1973).
6. BACH, D., RAZ, A. and GOLDMAN, R., The effect of hashish compounds on phospholipid phase transition. *Biochim. Biophys. Acta* **436**,889 (1976).
7. BACH, D., RAZ, A. and GOLDMAN, R., The interaction of hashish compounds with planar lipid bilayer membranes (BLM). *Biochem. Pharmacol.* **25**,1241 (1976).
8. BACH, D., BURSUKER, I. and GOLDMAN, R., Differential scanning calorimetry and enzymic activity of rat liver microsomes in the presence and absence of Δ^1-tetrahydrocannabinol. *Biochim. Biophys. Acta* **469**,171 (1977).
9. BLOJ, B., MORERO, R. D., FARÍAS, R. N. and TRUCCO, R. E., Membrane bound fatty acids and regulation of membrane bound enzymes. *Biochim. Biophys. Acta* **311**,67 (1973).
10. BRECKENRIDGE, W. C., GOMBOS, G. and MORGAN, I. G., The lipid composition of adult rat brain synaptosomal plasma membranes. *Biochim. Biophys. Acta* **266**,695 (1972).
11. CHARI-BITRON, A. and BINO, T., Effect of Δ^1-tetrahydrocannabinol on ATPase activity of rat liver mitochondria. *Biochem. Pharmacol.* **20**,473 (1971).
12. CHESHER, G. B., JACKSON, D. M. and STARMER, G. A., Interaction of cannabis and general anaesthetic agents in mice. *Brit. J. Pharmacol.* **50**,593 (1974).
13. CHIU, P., KARLER, R. and CRAVEN, C., The influence of Δ^9-tetrahydrocannabinol, cannabinol and cannabidiol on tissue oxygen consumption. *Res. Comm. Chem. Path. Pharmacol.* **12**,267 (1975).
14. CLARKE, D. E. and JANDHYALA, B., Acute and chronic effects of tetrahydrocannabinols on monoamine oxidase activity: possible vehicle/tetrahydrocannabinol interaction. *Res. Comm. Chem. Path. Pharmacol.* **17**,471 (1977).
15. COLBURN, R. W., NG, L. K. Y., LEMBERGER, L. and KOPIN, I. J., Subcellular distribution of Δ^9-tetrahydrocannabinol in rat brain. *Biochem. Pharmacol.* **23**,873 (1974).
16. COTMAN, C. W., MCCAMAN, R. E. and DEWHURST, S. A., Subsynaptosomal distribution of enzymes involved in the metabolism of lipids. *Biochim. Biophys. Acta* **249**,395 (1971).
17. DOLBY, T. W. and KLEINSMITH, L. J., Effects of Δ^9-tetrahydrocannabinol on the levels of cyclic adenosine 3′,5′-monophosphate in mouse brain. *Biochem. Pharmacol.* **23**,1817 (1974).
18. DOLBY, T. W. and KLEINSMITH, L. J., Cannabinoid effects on adenylate cyclase and phosphodiesterase activities of mouse brain. *Canad. J. Physiol. Pharmacol.* **55**,934 (1977).
19. EICHBERG, J., WHITTAKER, V. P. and DAWSON, R. M. C., Distribution of lipids in subcellular particles of guinea pig brain. *Biochem. J.* **92**,91 (1964).
20. FARÍAS, R. N., BLOJ, B., MORERO, R. D., SINERIZ, F. and TRUCCO, R. E., Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. *Biochim. Biophys. Acta* **415**,231 (1975).
21. FOLCH, J., LEES, M. and SLOANE STANLEY, G. H., A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**,497 (1957).
22. FRIZZA, J., CHESHER, G. B., JACKSON, D. M., MALOR, R. and STARMER, G. A., The effect of Δ^9-tetrahydrocannabinol, cannabidiol, and cannabinol on the anaesthesia induced by various anaesthetic agents in mice. *Psychopharmacol.* **55**,103 (1977).
23. GILBERT, J. C., PERTWEE, R. G. and WYLLIE, M. G., Effects of Δ^9-tetrahydrocannabinol and cannabidiol on Mg^{2+}-ATPase of synaptic vesicles prepared from rat cerebral cortex. *Brit. J. Pharmacol.* **59**,599 (1977).
24. GILL, E. W. and LAWRENCE, D. K., The physicochemical mode of action of tetrahydrocannabinol on cell membranes. "Pharmacology of Marihuana" (M. Braude and S. Szara, eds.). Raven Press, New York, 1976.
25. GOLDMAN, S. S. and ALBERS, R. W., Sodium-potassium-activated adenosine triphosphatase. IX. The role of phospholipids. *J. Biol. Chem.* **248**,867 (1973).
26. GORACCI, G., BLOMSTRAND, C., ARIENTI, G., HAMBERGER, A. and PORCELLATI, G., Base-exchange enzymic system for the synthesis of phospholipids in neuronal and glial cells and their subfractions: a possible marker for neuronal membranes. *J. Neurochem.* **20**,1167 (1973).
27. HAMBERGER, A. and SVENNERHOLM, L., Composition of gangliosides and phospholipids of neuronal and glial cell enriched fractions. *J. Neurochem.* **18**,1821 (1971).
28. HERSHKOWITZ, M., GOLDMAN, R. and RAZ, A., Effect of cannabinoids on neurotransmitter uptake, ATPase activity and morphology of mouse brain synaptosomes. *Biochem. Pharmacol.* **26**,1327 (1977).
29. INGELMAN-SUNDBERG, M., Protein-lipid interactions in the liver microsomal hydroxylase system. "Microsomes and Drug Oxidations" (V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney, eds.). Pergamon Press, London, 1977.
30. ISRAËL, M. and FRACHON-MASTOUR, P., Fractionnement du cortex cérébral du rat, distribution subcellulaire de la 5′ nucléotidase et des cholinesterases. *Archives d'Anatomie Microscopique* **59**,383 (1970).

31. JAKUBOVIC, A. and McGEER, P. L., Intracellular distribution of ^{3}H-Δ^8-tetrahydrocannabinol in rat organs after i.v. administration. *Res. Comm. Chem. Path. Pharmacol.* **9**,197 (1974).
32. KIMELBERG, H. K. and PAPAHADJOPOULOS, D., Effects of phospholipid acyl chain fluidity, phase transitions, and cholesterol on ($Na^+ + K^+$) stimulated adenosine triphosphatase. *J. Biol. Chem.* **249**,1071 (1974).
33. KLEEMAN, W., Protein-lipid interactions: freeze-fracture and spin label studies. "Structural and Kinetic Approach to Plasma Membrane Functions." (C. Nicolau and A. Paraf, eds.). Springer-Verlag, Berlin, p. 77, 1977.
34. LAURENT, B. and ROY, P. E., Alteration of membrane integrity by Δ^1-tetrahydrocannabinol. *Int. J. Clin. Pharmacol.* **12**,261 (1975).
35. LAWRENCE, D. M. and GILL, E. W., The effects of Δ^1-tetrahydrocannabinol and other cannabinoids on spin-labelled liposomes and their relationship to mechanisms of general anaesthesia. *Molec. Pharmacol.* **11**,595 (1975).
36. LEE, A. G., Interactions between phospholipids and barbiturates. *Biochim. Biophys. Acta* **455**,102 (1976).
37. LIST, A. F., BARTRAM, S. F., NAZAR, B. L. and HARCLERODE, J., Interactions of Δ^9-tetrahydrocannabinol, adrenal steroids, and ethanol. *J. Pharm. Pharmacol.* **27**,606 (1975).
38. MAGOUR, S., COPER, H., FÄHNDRICH, C. and HILL, R., Relationship between the subcellular distribution of delta-9-tetrahydrocannabinol and its metabolites in rat brain and the duration of the effect on motor activity. *Life Sci.* **18**,575 (1976).
39. MAHONEY, J. M. and HARRIS, R. A., Effect of Δ^9-tetrahydrocannabinol on mitochondrial processes. *Biochem. Pharmacol.* **21**,1217 (1972).
40. MALOR, R., JACKSON, D. M. and CHESHER, G. B., The effect of Δ^9-tetrahydrocannabinol, cannabidiol and cannabinol on ether anaesthesia in mice. *J. Pharm. Pharmacol.* **27**,610 (1975).
41. MITRA, G., PODDAR, M. K. and GHOSH, J. J., *In vivo* and *in vitro* effects of Δ^9-tetrahydrocannabinol on rat liver microsomal drug metabolizing enzymes. *Toxicol. Appl. Pharmacol.* **35**,523 (1976).
42. NARASIMHULU, S., Relationship between the membrane lipids and substrate-cytochrome P-450 binding reaction in bovine adrenocortical microsomes. "Microsomes and Drug Oxidations" (V. Ullrich, I. Roots, A. Hidebrandt, R. W. Estabrook and A. H. Conney, eds.). Pergamon Press, London, p. 119, 1977.
43. NAZAR, B. L., HARCLERODE, J., ROTH, R. I. and BUTLER, R. C., Acquisition of tolerance to Δ-9-THC as measured by the response of a cellular function. *Life Sci.* **14**,2513 (1974).
44. PATON, W. D. M., PERTWEE, R. G. and TEMPLE, D., The general pharmacology of cannabinoids. "Cannabis and Its Derivatives" (W. D. M. Paton and J. Crown, eds.). Oxford University Press, London, p. 50, 1972.
45. PAPAHADJOPOULOS, D., COWDEN, M. and KIMELBERG, H., Role of cholesterol in membranes: effects on phospholipid-protein interactions, membrane permeability and enzymic activity. *Biochim. Biophys. Acta* **330**,8 (1973).
46. PODDAR, M. K. and GHOSH, J. J., Neuronal membrane as site of action of Δ^9-tetrahydrocannabinol. "Pharmacology of Marihuana" (M. Braude and S. Szara, eds.). Raven Press, New York, p. 159, 1976.
47. SARKAR, C. and GHOSH, J. J., Effect of delta-9-tetrahydrocannabinol administration on the lipid constituents of rat brain subcellular fractions. *Neurochem.* **24**,381 (1975).
48. SEEMAN, P., CHAU-WONG, M. and MOYYEN, S., The membrane binding of morphine, diphenylhydantoin, and tetrahydrocannabinol. *Canad. J. Physiol. Pharmacol.* **50**,1193 (1972).
49. SEEMAN, P., The membrane action of anaesthetics and tranquillisers. *Pharmacol. Rev.* **24**,583 (1972).
50. SUN, G. Y., The turnover of phosphoglycerides in the subcellular fractions of mouse brain: a study using [1-^{14}C] oleic acid as precursor. *J. Neurochem.* **21**,1083 (1973).
51. TORO-GOYCO, E., RODRÍGUEZ, M. B. and PRESTON, A. M., On the action of Δ^9-tetrahydrocannabinol as an inhibitor of sodium and potassium-dependent adenosine triphosphatase. *Molec. Pharmacol.* **14**,130 (1978).
52. TRIA, E., LULY, P., TOMASI, V., TREVISANI, A. and BARNABEI, O., Modulation by cyclic AMP *in vitro* of liver plasma membrane ($Na^+ - K^+$)-ATPase and protein kinases. *Biochim. Biophys. Acta* **343**,297 (1974).
53. WARREN, G. B. and METCALFE, J. C., The molecular architecture of a reconstituted calcium pump. "Structural and Kinetic Approach to Plasma Membrane Functions" (C. Nicolau and A. Para, eds.). Springer-Verlag, Berlin, p. 188, 1977.
54. WEINHOLD, P. A. and VILLEE, C. A., Phospholipid metabolism in the liver and lung of rats during development. *Biochim. Biophys. Acta* **106**,540 (1965).
55. WELLER, M. and MORGAN, I. G., Localization in the synaptic function of the cyclic AMP stimulated intrinsic protein kinase activity of synaptosomal plasma membranes. *Biochim. Biophys. Acta* **433**,223 (1976).

BIOCHEMICAL ALTERATIONS INDUCED BY CANNABINOIDS IN THE LEYDIG CELLS OF THE RAT TESTIS *IN VITRO*: EFFECTS ON TESTOSTERONE AND PROTEIN SYNTHESIS

A. JAKUBOVIC, E. G. McGEER and P. L. McGEER

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada.

Summary. The interference of various cannabinoids with protein synthesis and steroidogenic response of isolated Leydig cells of the rat testes during incubation with human chorionic gonadotropin (hCG) or with dibutyryl-cAMP *in vitro*, was studied. The hCG-stimulated testosterone production was significantly reduced, in a dose-related fashion, by SP-111A (the water soluble derivative of Δ^9-THC) and the reduction was significant even at 1.0 μM concentration (0.31 μg THC/ml). Comparable dose-related inhibitions were also caused by water insoluble cannabinoids where the order of potency in inhibiting testosterone synthesis was found to be: 8β-OH-Δ^9-THC $\geqq$ 11-OH-Δ^9-THC > CBN = CBD = CBG $\geqq$ Δ^9-THC = Δ^8-THC. With 15 μM cannabinoids the inhibition was about 80% and the total amount of androgen produced was even below the basal nonstimulated production by the Leydig cells. The stimulation of testosterone production by dibutyryl-cAMP was also inhibited in a comparable concentration-dependent fashion by the cannabinoids. However, in the absence of added hCG or dibutyryl-cAMP, there was no effect of any of the tested cannabinoids on steroidogenesis by interstitial cells.

Even though protein synthesis in the hCG stimulated Leydig cells, as measured by L-[U-^{14}C]leucine incorporation, was practically the same as found in nonstimulated cells, 15 μM concentrations of cannabinoids reduced the label in the protein by 40–80%. The reduction by cannabinoids was even more marked (70–90%) in cells stimulated by dibutyryl-cAMP and there was also a significant decrease in the radioactivity in the soluble fraction and $^{14}CO_2$ evolved. Cannabinoids had a similar effect on L-[U-^{14}C]leucine metabolism and incorporation in nonstimulated Leydig cells.

When spermatogonial cells, isolated by collagenase treatment from rat testicular tubules, were incubated in the presence of 5 μM cannabinoids there was a significant decrease of ^{14}C-leucine and ^{14}C-uridine incorporation into the protein and RNA fraction, respectively. The inhibiting effects of cannabinoids on metabolic processes in these experiments are greater than those found with testicular cells suspensions and testicular slices.

The high inhibitory effect on testosterone production and on vital biosynthetic processes by cannabinoids with low psychotogenic activity indicates that there is no correlation between euphoric and cytotoxic effects. The present results, obtained with relatively low doses of cannabinoids, strengthens our previous conclusions that the effect of cannabinoids on testosterone production, spermatogenesis and related processes may be primarily through direct action on testicular cells, especially Leydig cells, and are probably mediated by the inhibitory effects on cellular RNA and protein synthesis.

WE HAVE previously reported[10] that psychotogenically active as well as inactive cannabinoids significantly reduce the biosynthesis of protein, nucleic acids and lipids in rat testicular tissue slices *in vitro* and that these effects are magnified when testicular cell suspensions

are used[11]. We report now that with spermatogonial and Leydig cell preparations these inhibitory effects of cannabinoids are even more enhanced[11], and are apparently unrelated to the euphoric effects or other indirect actions involving the CNS.

High doses of cannabis reduce copulatory behavior in male rats[2, 21]. Furthermore, high doses of cannabis extracts produce a complete arrest of spermatogenesis and regression of Leydig cell tissue and of accessory sex organs in mice[4, 5]. Cannabis also produces a dose-related decrease in the development of testis, prostate and seminal vesicles[23, 24] and of testosterone in rats[17] and mice[3]. Reduced sperm counts, impaired potency, and a dose-related decrease of plasma testosterone have also been reported in human heavy cannabis users[15], although in one study no change in testosterone levels were found after chronic marijuana smoking[20]. Δ^9-Tetrahydrocannabinol (THC) was shown to be retained for more than 7 days in humans following a single dose[16]. In rats, THC was found to be retained in the testis for a week after only 15 min exposure and inhalation of the smoke of a single THC-containing cigarette[7]. The mechanism of the apparent interference of cannabis with gonadal function is unknown. The data available so far do not suggest that alterations in testicular function and steroidogenesis are secondary to a central action via the hypothalamic-pituitary-gonadal axis[6]. However, direct interference of cannabinoids with vital macromolecular synthesis in testicular cells may be the cause of decreased spermatogenesis and steroidogenesis as well as impaired gonadal function[10, 11].

We began this study to obtain more information on the mechanism(s) involved in the interference of cannabinoids with testicular steroidogenesis. We therefore, investigated in rat Leydig cells, *in vitro*, the interference of cannabinoids with protein synthesis and with the basal, gonadotropin and dibutyryl-cAMP stimulated production of testosterone.

METHODS

INTERSTITIAL AND SPERMATOGONIAL CELL PREPARATIONS

The cell preparations were made by a modification of the method of Janszen *et al.*[13]. Two testes excised from adult male Wistar rats (280–320 g) were decapsulated. The testes were incubated in a 50 ml plastic bottle containing 7 ml of Krebs-Ringer bicarbonate-glucose buffer with 7 mg collagenase. The bottle was flushed with 95% O_2 : 5% CO_2, capped and agitated for 25–30 min at 37°C in a water bath. The interstitial cells were separated from the tubular mass by adding 15 ml 0.9% NaCl to the tube. After 10 min the supernatant, containing the Leydig cells, was taken up into a plastic 50 ml syringe. The cell suspension was further purified by centrifugation through a 13% Ficol solution and then through a 6% dextran solution in 0.9% NaCl[13]. The sediment was resuspended in Krebs-Ringer phosphate buffer with 10 mM glucose and immediately used for incubations. Each testis produced 10 ml of suspension.

The sediment remaining after withdrawal of the interstitial cells was shaken with 20 ml of 0.9% NaCl. After a 10 min period of settling, the supernatant was discarded, and the sedimented testicular tubules were chopped with a McIlwain Tissue Chopper. The spermatogonial cells were then extracted with Krebs-Ringer phosphate buffer containing glucose, as described in our previous publication[11].

DETERMINATION OF TESTOSTERONE PRODUCTION AND PROTEIN SYNTHESIS IN RAT LEYDIG CELL PREPARATIONS

Incubations were carried out in liquid scintillation counting vials. 2 ml of the interstitial cell suspension was added to the vials plus 0.1 ml of stimulant. The stimulant consisted either of 600 mU (227 ng) of chorionic gonadotropin (hCG) from human pregnancy urine or 2.1 μmoles of dibutyryl-cAMP (final concentration 1 mM). SP-111A was introduced in approximately 50 μl of aqueous solution and the other cannabinoids in 0.5 or 1.0 μl of 95% alcohol (final concentration of alcohol about 4 or 8 mM). The vials were flushed for 3 min with O_2, capped and incubated for 3 h in a water bath at 37°C.

After incubation, the cells were centrifuged and the supernatant was stored at −15°C. Testosterone, produced by the Leydig cells and released into the medium, was determined by direct radioimmunoassay in an aliquot of the supernatant diluted with 0.9% NaCl.

For determination of protein synthesis, 2 ml of interstitial cells were incubated under the same conditions but in Warburg flasks at 37°C, under oxygen, for 2 h. One μC of L-[$U^{14}C$]leucine (20 μCi/μmol) was then added from the side arm of the flask and the incubation was continued for 1 h. Incubation was then terminated, and the fractionation and determination of radioactivity in various fractions carried out as previously described[11].

DETERMINATION OF PROTEIN AND NUCLEIC ACID SYNTHESIS IN SPERMATOGONIAL CELL SUSPENSIONS

Three ml aliquots of spermatogonial cell suspensions were incubated with labeled leucine or uridine in Warburg flasks under oxygen for 60 min with or without added cannabinoids. Incubation, fractionation and determination of radioactivity in various fractions was carried out as previously described[11].

RESULTS

EFFECT ON PROTEIN AND NUCLEIC ACID SYNTHESIS IN SPERMATOGONIAL CELLS

The concentration-dependent effects of various cannabinoids on both ^{14}C-leucine metabolism and incorporation into spermatogonial cells are given in Table 1. Five μM concentrations of the drugs did not induce changes in the radioactivity found either in the soluble fraction or the evolved $^{14}CO_2$, but there was a marked reduction in protein synthesis as measured by label incorporated into proteins. The inhibitory effects were more pronounced with higher concentrations of cannabinoids, with a significant decrease of label in both the protein and soluble fractions. The inhibitory effect was greatest with 11-OH-Δ^9-THC.

The dose-dependent effects of the water-soluble derivative of Δ^9-THC, SP-111A (1-[4-(morpholino)butyryloxy]-3-*n*-pentyl-6,6,9-trimethyl-10a,6a,7,8-tetrahydrodibenzo[*b,d*] pyran hydrobromide)[25] on ^{14}C-uridine incorporation into RNA are indicated in Fig. 1.

The 1 μM concentration did not induce any significant alterations in the radioactivity found in either the soluble fraction or RNA. However, 5 μM SP-111A caused a significant reduction of the label in both fractions, and the decrease reached about 40% at 10 μM concentration.

TABLE 1. EFFECT OF CANNABINOIDS ON L-[1-^{14}C]LEUCINE METABOLISM AND INCORPORATION IN RAT SPERMATOGONIAL CELLS.

Cannabinoids	μM	Percent of control dpm		
		$^{14}CO_2$	Soluble fraction	Protein
Δ^9-THC	5	80 ± 2	93 ± 3	59 ± 5*
	50	86 ± 2	46 ± 3*	40 ± 3*
11-OH-Δ^9-THC	5	79 ± 4	82 ± 3	50 ± 2*
	50	66 ± 2*	39 ± 1*	18 ± 1*
8β-OH-Δ^9-THC	5	81 ± 2	92 ± 4	62 ± 2*
CBN	5	87 ± 1	78 ± 5	68 ± 3*
	100	70 ± 8	33 ± 1*	16 ± 3*
SP-111A	100	78 ± 1	46 ± 1*	23 ± 1*

Values are expressed as percentage (mean ± S.E.) of dpm of parallel control incubations. $n = 3$. Control values in dpm/mg protein were: $^{14}CO_2$ 860 ± 46; soluble fraction 1527 ± 14; protein 1046 ± 55. *$p < 0.01$.

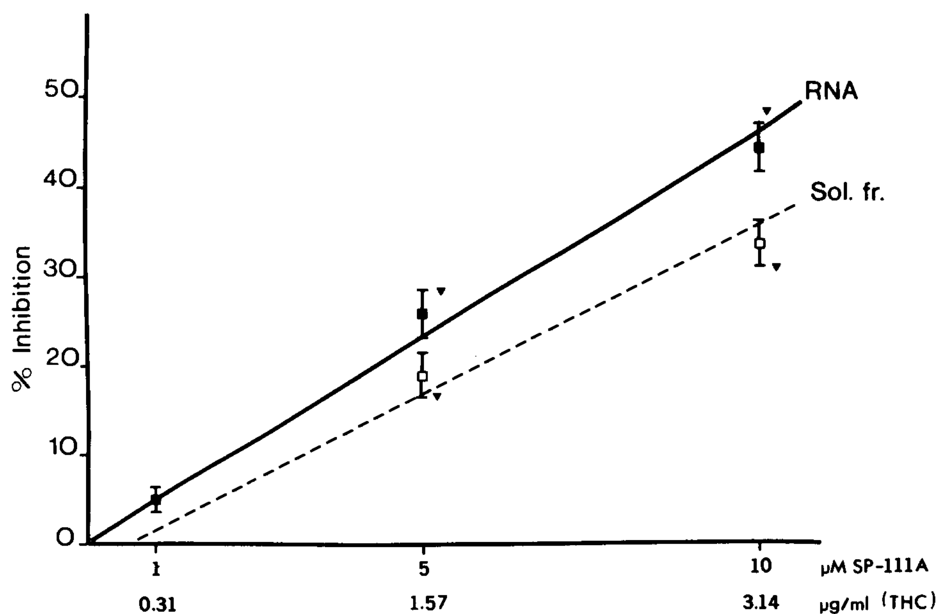


FIG. 1. Effect of SP-111A on [2-^{14}C]uridine metabolism and incorporation in rat spermatogonial cells. 0.33 μCi [2-^{14}C]uridine (20 μCi/μmole) was the substrate. Values are expressed as percentage (mean ± S.E.) of dpm of parallel control incubations. $n = 3$. Control values in dpm/mg protein: Soluble fraction 1203 ± 60; RNA 769 ± 30; ▾$p < 0.01$.

TESTOSTERONE PRODUCTION BY LEYDIG CELLS IN RESPONSE TO hCG STIMULATION

The gonadotropin-stimulated interstitial cell suspension was used for study of the interference of cannabinoids with steroidogenesis. As shown in Fig. 2, the addition of SP-111A produced a concentration-dependent inhibition of androgen production in response to hCG. Even at 1.0 μM concentration (0.31 μg/ml of THC in SP-111A) the decrease was significant. The inhibition reached its maximum (over 80% inhibition) at about 50 μM SP-111A. The testosterone production by the stimulated Leydig cells exposed to 50–200 μM concentration of SP-111A was below the basal androgen synthesis, that is the synthesis by non-stimulated control in the absence of drugs.

The dose responsiveness of stimulated Leydig cells to various water insoluble cannabinoids is shown in Fig. 3. The addition of 0.15 μM (about 0.05 μg/ml) of 11-OH-Δ^9-THC and 8β-OH-Δ^9-THC produced a significant (30% and 50% respectively) reduction of testosterone production. Except for Δ^9- and Δ^8-THC, all cannabinoids significantly decreased testosterone synthesis at 1.5 μM concentration. The inhibitory effect of 11-OH-Δ^9-THC and 8β-OH-Δ^9-THC always exceeded that of Δ^9- and Δ^8-THC. The addition of 8 mM alcohol (1 μl) to control incubations had no effect on testosterone synthesis (Fig. 3). Here again, the testosterone produced at 15 μM concentration of cannabinoids, other than Δ^9-THC, was less than that of basal cellular synthesis.

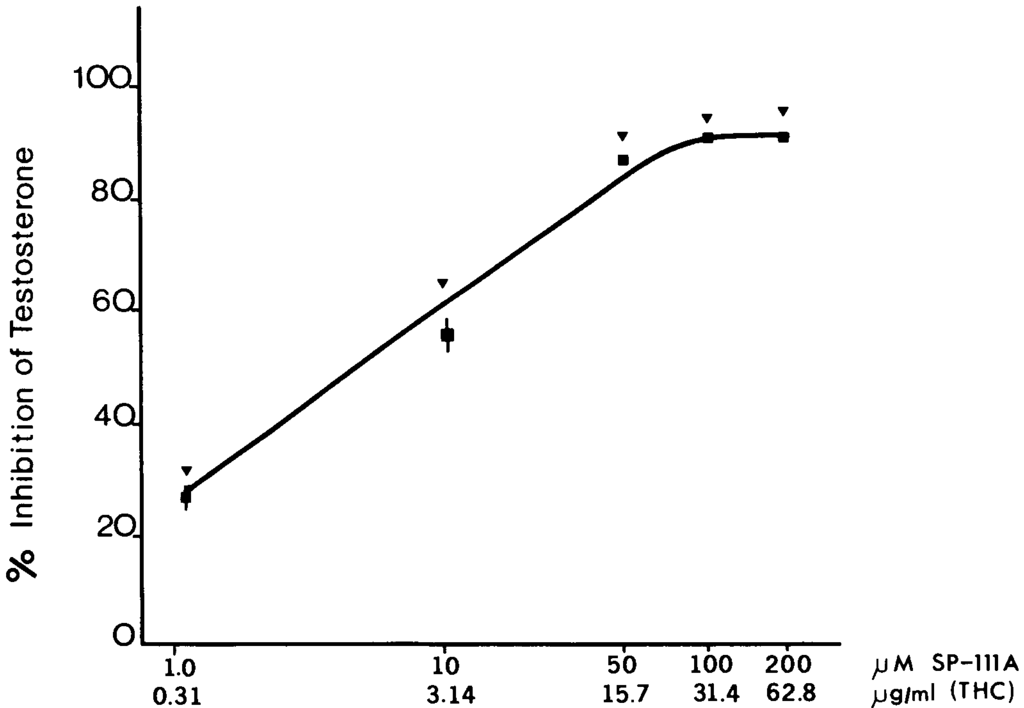


FIG. 2. Effect of SP-111A upon testosterone production by rat Leydig cells in the presence of hCG. Results expressed as percentage (mean ±S.E., no S.E. shown if less than area occupied by the symbol) of total testosterone in the incubation medium of parallel controls without SP-111A. $n = 3$. Control testosterone production in the 3 hr incubation was 22 ± 0.5 ng. Testosterone production in the absence of hCG was 6.5 ± 0.3 ng. ▼$p < 0.01$.

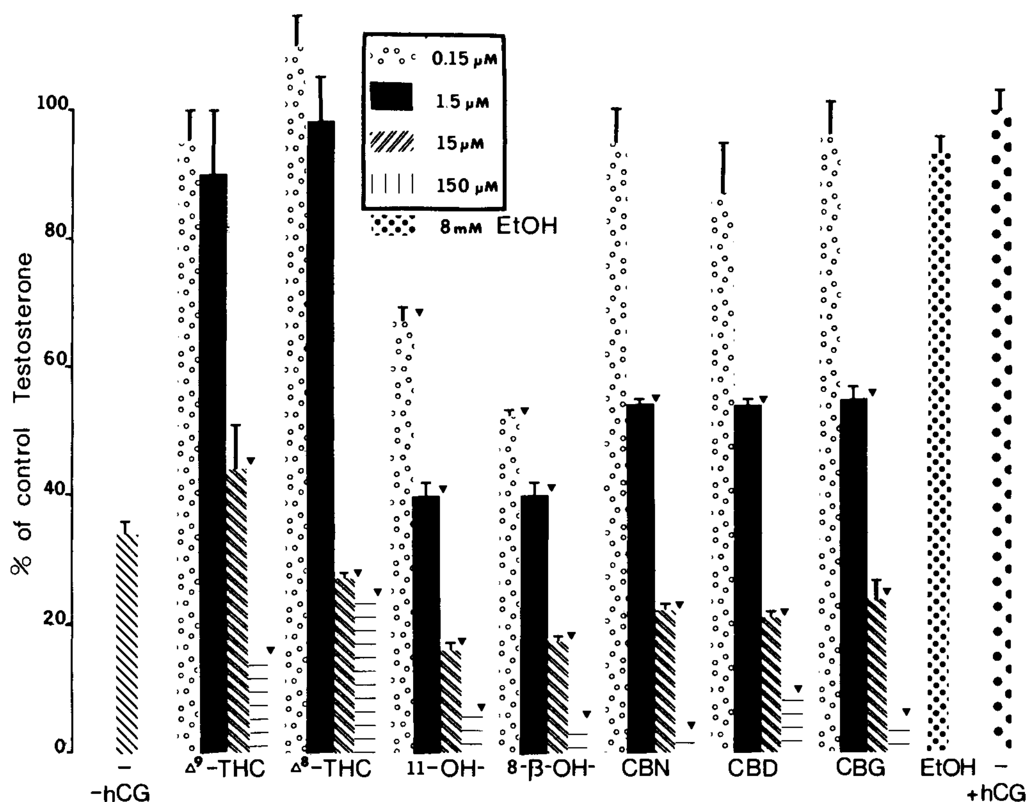


FIG. 3. Effect of cannabinoids and ethanol upon testosterone production by rat Leydig cells in the presence of hCG. Results expressed as percentages (mean ±S.E.) of total testosterone in the incubation medium of parallel controls without cannabinoids. Each set of incubations had its control group. $n = 3$. Average control testosterone production in the 3 hr incubations was 26 ± 0.6 ng, $n = 12$. Testosterone production without hCG was 7.6 ± 0.3 ng, $n = 7$. ▼$p < 0.01$.

TESTOSTERONE PRODUCTION BY LEYDIG CELLS IN RESPONSE TO DIBUTYRYL-cAMP STIMULATION

In further studies, the stimulation of testosterone production by dibutyryl-cAMP was also found to be inhibited in a dose-related fashion by SP-111A (Table 2). The inhibition was significant at 1 µM, and reached about 75% at 10 µM SP-111A.

Figure 4 demonstrates that at 1.5 µM, all water insoluble cannabinoids tested markedly reduced testosterone synthesis in dibutyryl-cAMP stimulated interstitial cell incubations. The effect of 8β-OH-Δ⁹-THC was more marked than that of the others, with a 50% inhibition being observed at 0.15 µM concentration (Fig. 4).

BASAL TESTOSTERONE PRODUCTION BY LEYDIG CELLS

In this experiment, the effect of various cannabinoids on nonstimulated (without exogenous hCG or dibutyryl-cAMP) testosterone production by interstitial cells was

examined. The results in Table 3 indicate that none of the tested cannabinoids even at a 15 μM concentration had a significant effect on the basal androgen production.

TABLE 2. EFFECT OF SP-111A UPON TESTOSTERONE PRODUCTION BY RAT LEYDIG CELLS IN THE PRESENCE OF 1 mM DIBUTYRYL-cAMP.

SP-111A μM	(as μg THC/ml)	Percent of control
0.1	(0.03)	98 ± 2
1	(0.31)	79 ± 2*
10	(3.14)	22 ± 1*

Results are expressed as percentage (mean ±S.E.) of total testosterone in the incubation medium of parallel control incubations. $n = 3$. Control testosterone production was 67 ± 1.3 ng. Basal testosterone production, in the absence of cAMP was 6.9 ± 0.3 ng. *$p < 0.01$.

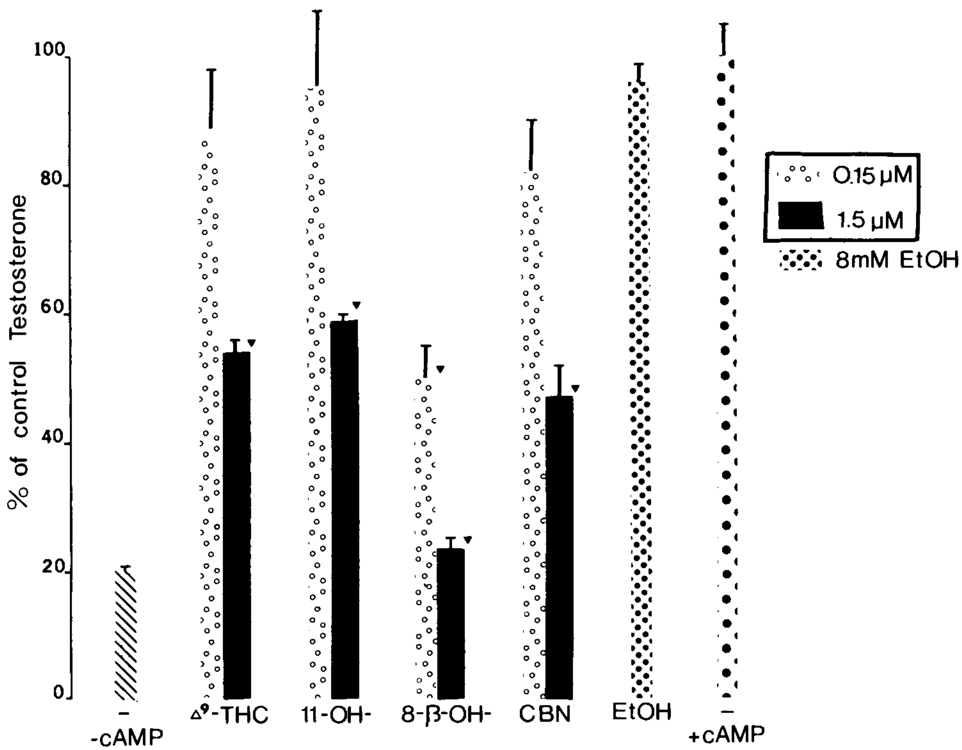


FIG. 4. Effect of cannabinoids upon testosterone production by rat Leydig cells in the presence of dibutyryl-cAMP. Results expressed as percentage (mean ±S.E.) of total testosterone in the incubation medium of parallel controls at the end of 3 h incubation, $n = 3$. Control testosterone production was 28 ± 1.8 ng. ▼$p < 0.01$.

TABLE 3. EFFECT OF CANNABINOIDS UPON BASAL TESTOSTERONE PRODUCTION BY RAT LEYDIG CELLS.

Cannabinoids 15 μM	Percent of control
SP-111A	115 ± 6
Δ^9-THC	106 ± 6
Δ^8-THC	116 ± 8
11-OH-Δ^9-THC	120 ± 5
8β-OH-Δ^9-THC	86 ± 6
CBN	80 ± 4
CBD	96 ± 6
CBG	114 ± 3

The results represent percentage (mean $\pm$S.E.) of total testosterone in the incubation medium of parallel control incubations. $n = 3$. Control testosterone production was 7.5 ± 0.4 ng.

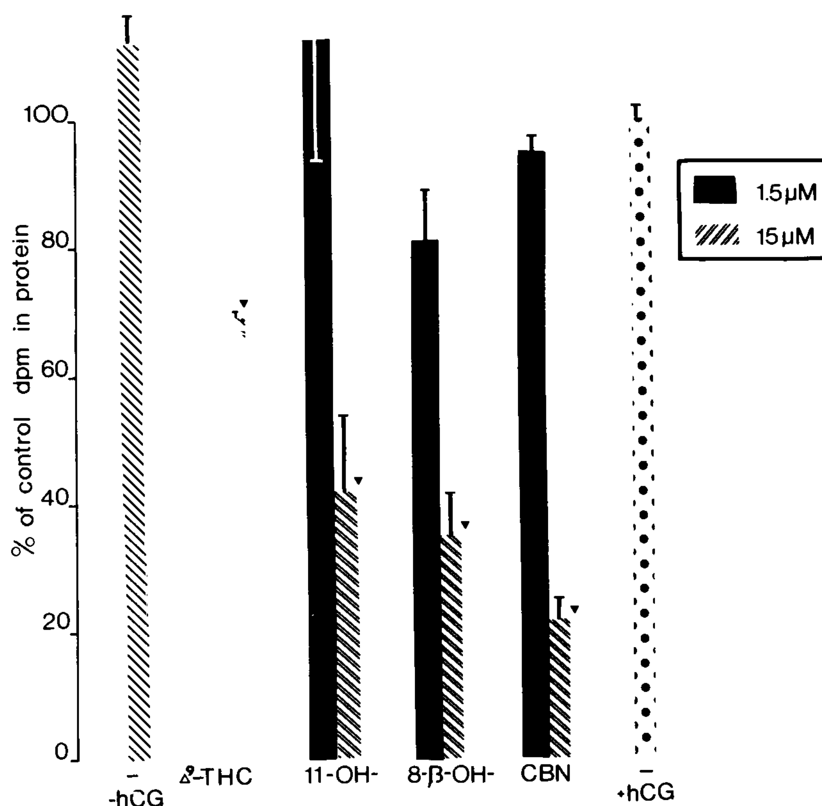


FIG. 5. Effect of cannabinoids on L-[U-^{14}C]leucine incorporation into protein by rat Leydig cells in the presence of hCG. Incubation as described in Table 4 except that hCG was the stimulant. Values are expressed as percentage (mean $\pm$S.E.) of dpm in parallel control incubations, $n = 3$. $^{\blacktriangledown}p < 0.01$.

L-[U-^{14}C]LEUCINE METABOLISM AND INCORPORATION INTO PROTEIN BY LEYDIG CELLS IN THE PRESENCE OR ABSENCE OF hCG OR DIBUTYRYL cAMP

It can be seen from Fig. 5 that hCG had very little effect on the amount of label incorporated into protein when compared with control Leydig cells incubated without added hCG. Addition of 1.5 μM of various cannabinoids did not affect protein synthesis. With 15 μM cannabinoids, however, the incorporation of label into protein was markedly reduced. The highest inhibition (75%) was produced by CBN, and the lowest by Δ^9-THC (30%) (Fig. 5). The radioactivity in the soluble fraction was only slightly decreased (0–20%).

Addition of 15 μM of various cannabinoids to the Leydig cells in the presence of dibutyryl cAMP (Table 4), brought about a 70–90% inhibition of the radioactivity incorporated into protein. Similarly, there was in most instances marked reduction of the label found in both the soluble fraction and the evolved $^{14}CO_2$.

TABLE 4. EFFECT OF CANNABINOIDS ON L-[U-^{14}C]LEUCINE METABOLISM IN RAT LEYDIG CELLS IN THE PRESENCE OF DIBUTYRYL-cAMP.

15 μM	Percent of control dpm		
	$^{14}CO_2$	Soluble fraction	Protein
SP-111A	33 ± 8*	35 ± 10*	11 ± 1*
Δ^9-THC	82 ± 1*	45 ± 5*	32 ± 3*
11-OH-Δ^9-THC	63 ± 2*	39 ± 5*	27 ± 2*
8β-OH-Δ^9-THC	18 ± 4*	30 ± 2*	8 ± 1*
CBN	49 ± 8*	34 ± 1*	15 ± 1*

Cells were incubated with 1 mM dibutyryl-cAMP in the absence or presence of cannabinoids for 2 h (see Methods). 1 μCi of L-[U-^{14}C]leucine as labeled substrate (20 μCi/μmol) was then added and incubation continued for 1 h. Results are expressed as percentage (mean ±S.E.) of dpm parallel control incubations $n = 3$. Control values in dpm/cells in incubation vessel were: $^{14}CO_2$ 5161 ± 112; soluble fraction 13, 143 ± 415; protein 4157 ± 146. *$p < 0.01$.

The effect of cannabinoids on protein synthesis by nonstimulated interstitial cells was also studied. The dose-related inhibition produced by SP-111A is shown in Fig. 6. Concentrations lower than 10 μM did not induce any alterations in the radioactivity in either the soluble or protein fraction. At 15 μM, however, there were reductions of 50% and 25% respectively, of the label in protein and soluble fractions.

The water insoluble cannabinoids at 15 μM concentration caused an inhibition of 80–95% in protein synthesis, 60–70% decrease of radioactivity in the soluble fraction, and a marked reduction of $^{14}CO_2$ evolved (Table 5). The effect of Δ^9-THC on incorporation of ^{14}C-leucine label into protein was less than that of other cannabinoids tested (Fig. 5, Tables 4 and 5).

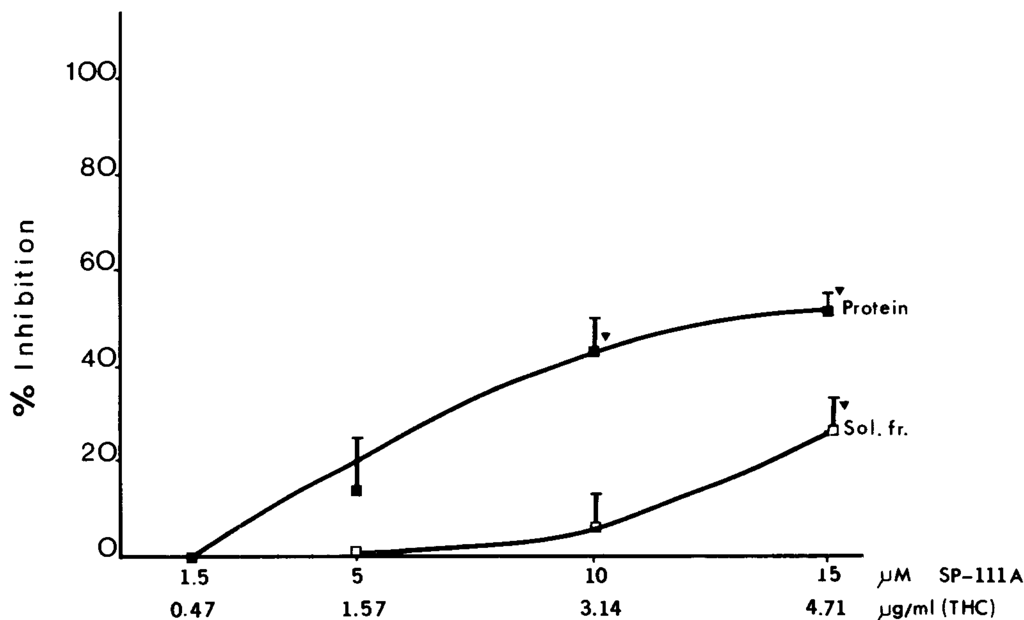


FIG. 6. Effect of SP-111A on L-[U-^{14}C]leucine metabolism in rat Leydig cells. Results are expressed as percentage (mean ± S.E., no S.E. shown if less than area occupied by the symbol) of parallel incubations. Control values in dpm/cells in incubation vessels were: soluble fraction 7620 ± 519; protein 3735 ± 403. $n = 3$. $^{▼}p < 0.01$.

TABLE 5. EFFECT OF CANNABINOIDS ON L-[U-^{14}C]LEUCINE METABOLISM IN RAT LEYDIG CELLS.

Cannabinoids 15 μM	Percent of control dpm		
	$^{14}CO_2$	Soluble fraction	Protein
Δ^9-THC	79 ± 12	43 ± 5*	19 ± 4*
11-OH-Δ^9-THC	31 ± 4*	37 ± 4*	9 ± 1*
8β-OH-Δ^9-THC	17 ± 3*	31 ± 4*	6 ± 1*
CBN	23 ± 2*	30 ± 8*	7 ± 1*
CBD	34 ± 7*	33 ± 2*	7 ± 1*

Incubations as described in Table 4, but without dibutyryl-cAMP. Results are expressed as percentage (mean ± S.E.) of dpm of parallel control incubations $n = 3$. Control values in dpm/cells in incubations vessel were: $^{14}CO_2$ 3735 ± 91, soluble fraction 11, 659 ± 1119; Protein 4309 ± 654. $^*p < 0.01$.

DISCUSSION

The results of the present investigation show that various cannabinoids inhibit protein and RNA synthesis in spermatogonial cells of rat testicular seminiferous tubules isolated by collagenase treatment. The incorporation of both ^{14}C-leucine (Table 1) and ^{14}C-uridine

(Fig. 1) into macromolecules was significantly reduced in the presence of 5 μM concentrations of various cannabinoids. In similar experiments with whole testicular cell suspensions[11], or with testicular slices[10, 11], the same magnitude of inhibition was not produced even with 100 μM concentrations of cannabinoids. Thus the degree of inhibition for a given dose of cannabinoids was: seminiferous tubule cells ⋙ testicular cell suspensions ≫ testicular slices. This indication that the degree of inhibition increases with purification of the testicular cell population is similar to the observation[22] that the inhibitory effects of THC on synthetic processes in cell cultures decrease with increasing concentrations of serum in the medium.

This direct interference of cannabinoids with biosynthetic processes in sperm-forming cells may be one important factor in the decreased spermatogenesis reported in THC-treated animals, including humans. Another contributory factor is probably the direct effect shown here on testosterone production by Leydig cells.

Cannabinoids at 15 μM concentrations had no significant effect on the basal production of testosterone by Leydig cells (in the absence of added hCG or dibutyryl-cAMP). The stimulated production is, however, undoubtedly much more analogous to the *in vivo* situation so that the very marked effects (Figs. 2, 3, 4) of low concentrations of cannabinoids on such stimulated testosterone synthesis are of great importance. It is of particular interest that many of the cannabinoids at 15 μM concentrations reduced testosterone production in the presence of dibutyryl-cAMP or hCG to even less than the basal production seen in the absence of these stimulants. Although the mechanism of stimulation of testosterone production by dibutyryl-cAMP may differ from the mechanism of gonadotropin (hCG) stimulation, the inhibition by cannabinoids was similar in the two systems and much greater than that reported recently[3] for mouse testes *in vitro*.

The great sensitivity of Leydig cell preparations in their steroidogenic response to several stimulants (e.g. LH, hCG, dibutyryl-cAMP) is well documented[1, 12, 14, 18, 19] but the precise mechanism(s) of the stimulation is unknown. It has been shown, however, that the increased testosterone production induced in interstitial cells can be reduced by inhibitors of protein and nucleic acid synthesis, such as puromycin and cycloheximide[1]. It has been suggested, therefore, that the testicular steroidogenesis is related to and dependent on continuous production of newly synthesized RNA and protein in Leydig cells[1, 12, 14, 19]. It seems probable therefore that the inhibitory effects of cannabinoids on stimulated testosterone production are related to their effects in inhibiting protein synthesis. These are marked at 15 μM concentrations (Table 4, Fig. 5) and in some instances involve also significant decreases in the radioactivity in the soluble fraction and in $^{14}CO_2$ evolved. These results indicate that cannabinoids could interfere with Leydig cell functions by altering transport mechanisms and/or metabolic processes.

Cannabinoids also inhibit protein synthesis by Leydig cells under non-stimulated conditions (Table 5, Fig. 6) so that it is somewhat perplexing that they do not interfere with the basal testosterone production under such conditions. However, this basal production is also not inhibited by cycloheximide and puromycin[1]. It has been suggested that although the stimulants such as LH[1] and hCG (Fig. 5) have no detectable effect on the total label of ^{14}C-leucine incorporated into protein by Leydig cells, they may stimulate synthesis of a specific protein which is required for the increased testosterone production and whose synthesis can be inhibited by actinomycin D[14] or cycloheximide[12]. Similar reasoning could be used to explain the effects of cannabinoids.

The present results clearly demonstrate that the steroidogenic response of Leydig cell

preparations to gonadotropin and dibutyryl-cAMP represent a sensitive system for the study of cannabinoid interactions with fundamental biochemical processes. The inhibition of testosterone synthesis in stimulated Leydig cells by some cannabinoids is greater than that produced under similar conditions by cycloheximide, puromycin or actinomycin D[1, 19]. The high inhibitory effect on testosterone production by cannabinoids with low psychogenic activity (e.g. 8β-OH-Δ^9-THC, CBN, CBG, CBD) in comparison with psychogenically active cannabinoids (e.g. Δ^9- and Δ^8-THC) indicates that there is no correlation between the euphoric effects and the ability to interfere with vital cellular biosynthetic processes. It seems probable, however, that various cell types might be affected in a similar manner by cannabinoids and these actions might well play a major role in some of the longer term, relatively subtle effects of marihuana usage. This probability is strengthened when one realizes that the concentrations of cannabinoids found effective in these experiments are of the same order of magnitude as those found in rat testes after a single 15 min exposure of the rat to the smoke of one ^{3}H-Δ^9-THC cigarette[7].

ACKNOWLEDGEMENTS

We thank Deborah Money for excellent technical assistance. This work was supported by a grant from the British Columbia Ministry of Health, Alcohol and Drug Commission and Non-Medical Use of Drugs Directorate, Health and Welfare Canada.

Résumé. Nous avons étudié l'interférence des differents cannabinoïdes avec la synthèse des proteines et la réponse steroidogénique des cellules de Leydig, isolées du testicule de rat, incubées avec l'hormone chorio gonadotrophique humaine (hCG) ou avec du dibutyryl-cAMP *in vitro.*

La production de testostérone stimulée par le hCG a été réduite de façon significative, en rapport avec les doses de SP-111A (le dérivé hydrosolube du Δ^9-THC) et la diminution a été importante même à une concentration de 1 μM de (0,31 μG THC/ml). Des inhibitions comparables selon les doses, ont été provoquées par des cannabinoïdes non hydrosolubes ; dans ce cas, le degré d'inhibition de la synthèse de testosterone a été de: 8β-OH-Δ^9-THC $\geq$ 11-OH-Δ^9-THC > CBN = CBD = CBG $\geq \Delta^9$-THC = Δ^8-THC. Avec 15 μM de cannabinoïde, l'inhibition a été d'environ 80% et la quantité totale d'androgène obtenue a été même inférieure à la production de base des cellules de Leydig non stimulées.

Selon les concentrations et de façon comparable, la stimulation de production de testostérone par le dibutyryl cAMP a été aussi inhibées par les cannabinoïdes.

Toutefois, sans l'addition de hCG ou de dibutyryl cAMP, les cannabinoïdes utilisés n'ont eu aucun effet sur la steroidogénèse des cellules interstitielles.

Même si la synthèse des protéins dans les cellules de Leydig stimulées par le hCG mesurée par l'incorporation de L-[U-^{14}C]leucine, a été pratiquement la même que celle notée dans des cellules non stimulées, une concentration de 15 μM de cannabinoïdes a réduit le marquage des proteines de 40% à 80%.

La diminution par les cannabinoïdes a été même plus prononcée (70% à 90%) dans les cellules stimulées par le dibutyryl cAMP et il y a eu aussi une diminution significative de la radioactivité dans la fraction soluble et le $^{14}CO_2$ produit.

Quand les cellules spermatogoniales, isolées des tubules testiculaires du rat par le traitement à la collagénase, ont été incubées en presence de 15 μM de cannabinoïdes, il y a eu une diminution significative de l'incorporation de la ^{14}C-leucine et de la ^{14}C-uridine dans la fration proteinique et acide ribonucleique (RNA) respectivement. Les effets inhibiteurs de cannabinoïdes sur les processus métaboliques dans ces expériences sont plus grandes que ceux obtenus avec des suspensions de cellules et coupes testicularies.

L'important effet d'inhibition par les cannabinoïdes sur la production de testostérone et sur les processus vitaux de biosynthèse, avec une activité psychogénique basse, indique qu'il n'y a aucune corrélation entre les effets euphoriques et cytotoxiques.

Les présents résultats, obtenus avec des doses relativement basses de cannabinoïdes, renforcent nos conclusions précédentes à savoir que les effets des cannabinoïdes sur la production de testostérone, la spermatogénèse et leurs processus associés, peuvent être d'abord une action directe sur les cellules testiculaires particulièrement les cellules de Leydig; cette action est probablement produite par l'intermédiaire des effets inhibiteurs sur la synthèse de l'acide ribonucleique (RNA) et proteinique.

REFERENCES

1. Cooke, B. A., Janszen, F. H. A., Clotscher, W. F. and Van de Molen, H. J., Effect of protein-synthesis inhibitors on testosterone production in rat testis interstitial tissue and Leydig-cell preparations. *Biochem. J.* **150**,413 (1975).
2. Corcoran, M. E., Amit, Z., Malsbury, C. W. and Daykin, S., Reduction in copulatory behaviour of male rats following hashish injections. *Res. Commun. Chem. Pathol. Pharmacol.* **7**,792 (1974).
3. Dalterio, S., Bartke, A. and Burstein, S., Cannabinoids inhibit testosterone secretion by mouse testes in vitro. *Science* **196**,1472 (1977).
4. Dixit, V. P., Sharma, V. N., and Lohiya, N. K., The effect of chronically administered cannabis extract on the testicular function in mice. *Eur. J. Pharmacol.* **26**,111 (1974).
5. Dixit, V. P. and Lohiya, N. K., Effects of cannabis extracts on the response of accessory sex organs of adult male mice to testosterone. *Ind. J. Physiol. Pharmacol.* **19**,98 (1975).
6. Hembree, W. C. III, Zeidenberg, P. and Nahas, G. G., Marihuana's effect on human gonadal function. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.) Springer-Verlag, New York, p. 521, 1976.
7. Ho, B. T., Fritchie, G. E., Kralik, P. M., Englert, L. F., McIsaac, W. M. and Idänpään-Heikkilä, J. Distribution of tritiated-1-Δ^9-tetrahydrocannabinol in rat tissues after inhalation. *J. Pharm. Pharmacol.* **22**,538 (1970).
8. Huot, J., Cellular and biochemical alterations induced *in vitro* by Δ^1-tetrahydrocannabinol: Effects on cell proliferation, nucleic acids, plasma cell membrane ATPase, and adenylate cyclase. "Marihuana: Chemistry, Biochemistry and Cellular Effects (G. G. Nahas, *et al.*, eds.). Springer-Verlag, New York, p. 313, 1976.
9. Jakubovic, A. and McGeer, P. L., Inhibition of rat brain protein and nucleic acid synthesis by cannabinoids *in vitro. Can. J. Biochem.* **50**,654 (1972).
10. Jakubovic, A. and McGeer, P. L., *In vitro* inhibition of protein and nucleic acid synthesis in rat testicular tissue by cannabis. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, *et al.*, eds.). Springer-Verlag, New York, p. 223, 1976.
11. Jakubovic, A. and McGeer, P. L., Biochemical changes in rat testicular cells *in vitro* produced by cannabinoids and alcohol: metabolism and incorporation of labeled glucose, amino acids, and nucleic acid precursors. *Toxicol. Appl. Pharmacol.* **41**,473 (1977).
12. Janszen, F. H. A., Cooke, B. A. and Van der Molen, H. J., Specific protein synthesis in isolated rat testis Leydig cells. *Biochem. J.* **162**,341 (1977).
13. Janszen, F. H. A., Cooke, B. A., Van Driel, M. J. A. and Van der Molen, H. J., Purification and characterization of Leydig cells from rat testes. *J. Endocr.* **70**,345 (1976).
14. Janszen, F. H. A., Cooke, B. A., Van Driel, M. J. A. and Van der Molen, H. J., Regulation of the synthesis of lutropin-induced protein in rat testis Leydig cells. *Biochem. J.* **170**,9 (1977).
15. Kolodny, R. C., Masters, W. H., Kolodner, R. M. and Toro, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *N. Engl. J. Med.* **290**,872 (1974).
16. Lemberger, L., Tamarkin, N. R., Axelrod, J. and Kopin, I. J., Delta-9-tetrahydrocannabinol: metabolism and disposition in long-term marihuana smokers. *Science* **173**,72 (1971).
17. Maskarinec, M. P., Shirley, G., Novotny, M., Brown, D. J. and Forney, R. B., Different effects of synthetic Δ^9-tetrahydrocannabinol and cannabis extract on steroid metabolism in male rats. *Experientia* **34**,88 (1978).
18. Mendelson, C., Dufau, M. and Catt, H. K., Gonadotropin binding and stimulation by cyclic adenosine 3′: 5′-monophosphate and testosterone production in isolated Leydig cells. *J. Biol. Chem.* **250**,8818 (1975).
19. Mendelson, C., Dufau, M. and Catt, K., Dependence of gonadotropin-induced steroidogenesis upon RNA and protein synthesis in the interstitial cells of the rat testis. *Biochem. Biophys. Acta* **411**,222 (1975).
20. Mendelson, J. H., Kuehnle, J., Ellingboe, J. and Babor, T. F., Plasma testosterone levels before, during and after chronic marihuana smoking. *N. Engl. J. Med.* **291**,1051 (1974).
21. Merari, A., Barak, A. and Plaves, M., Effects of $\Delta^{1(2)}$-tetrahydrocannabinol on copulation in the male rat. *Psychopharmacologia* **28**,243 (1973).

22. NAHAS, G. G., DESOIZE, B., HSU, J. and MORISHIMA, A. A., Inhibitory effects of Δ^9-tetrahydrocannabinol on nucleic acid synthesis and protein in cultured lymphocytes. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.). Springer-Verlag, New York, p. 229, 1976.
23. OKEY, A. B. and TRUANT, G. S., Cannabis demasculinizes rats but is not estrogenic. *Life Sci.* **17**,1113 (1975).
24. THOMPSON, G. R., MASON, M. M., ROSENKRANTZ, H. and BRAUDE, M. C., Chronic oral toxicity of cannabinoids in rats. *Toxicol. Appl. Pharmacol.* **25**,373 (1973).
25. ZITKO, B. A., HOWES, J. F., RAZDAN, R. K., DALZEL, B. C., DALZEL, H. C., SHEEHAN, J. C. and PARIS, H. G., Water-soluble derivatives of Δ^1-tetrahydrocannabinol. *Science* **177**,442 (1972).

INFLUENCE OF DELTA-9-TETRAHYDROCANNABINOL ON CONTRACTION RATE AND ENZYMATIC ACTIVITY OF EMBRYONIC HEART CELLS

H. Choisy, G. Choisy, H. Millart and H. Legris,
with technical assistance of
J. Pisani and M. Aranthabe

Summary. The authors have studied the action of delta-9-tetrahydrocannabinol (3.2 to 13 × 10^{-6} M) on the enzymatic activities and on the rhythm of chicken embryonic myocardial cells. Some specific enzymatic activities, as well as glucose consumption, lactate production and the contraction rates have been measured after 6 to 72 hours exposure to THC. Following 6 and 18 h exposure THC induces an increase of GOT, LDH and HBD activities, a decrease of glucose consumption, lactate production and of the contraction rate of the myocardial cells. After 72 hours exposure, there is a general decrement of all of the factors studied.

THC might inhibit synthesis of short half-life proteases.

INTRODUCTION

Delta-9-THC (THC) has marked chronotropic effects on the mammalian heart: decreasing heart rate in certain species (dog, rodents) (Harris, 1971), increasing it in man (Hollister, 1971). The purpose of this investigation was to study the *in vitro* effect of THC on the contraction rate of embryonic chicken heart cells and on the activities of some of their basic enzymes.

MATERIALS AND METHODS

1. CULTURE OF EMBRYONIC CHICKEN HEART CELLS

The White Leghorn hen race which is genetically stable and often used for cell cultures was selected. The hearts are removed from the eggs 11 days after start of incubation, at a time which is optimal for the recording of heart rate. Trypsination is performed in order to obtain many unaltered cells, and according to a technique previously described (De Haan, 1967).

In order to obtain a confluent layer of cells beating synchronously the density was about 2.6×10^5 cells per ml. The following medium was used:

Medium M 199 (BioMérieux)	20%
Earle's balanced salt solution without potassium	75%
Calf serum	5%

This medium differs from most synthetic proposed media by its low potassium concentration. When the medium is renewed, it contains calf serum (1%) and Medium NCTC 135 GIBCO (4%).

Observation of cells

Cells are observed at 37°C, using an inverted microscope. Myocardial cells are much more numerous than fibroblasts, most of the latter being separated by their greater adherence to the glass.

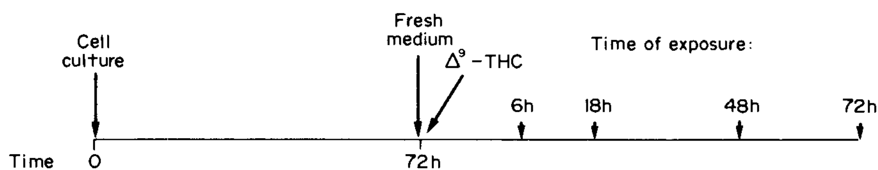
Preparation of THC

An alcoholic solution of THC provided by NIDA diluted in culture medium was used, in concentrations of 3.2 to 13×10^{-6} M.

Measurement of rhythmic activity

For each cell culture, five different areas are observed, always in the same place during the experiment. In each field, the concentrations are observed during 30 seconds, and the results expressed in contractions per minute.

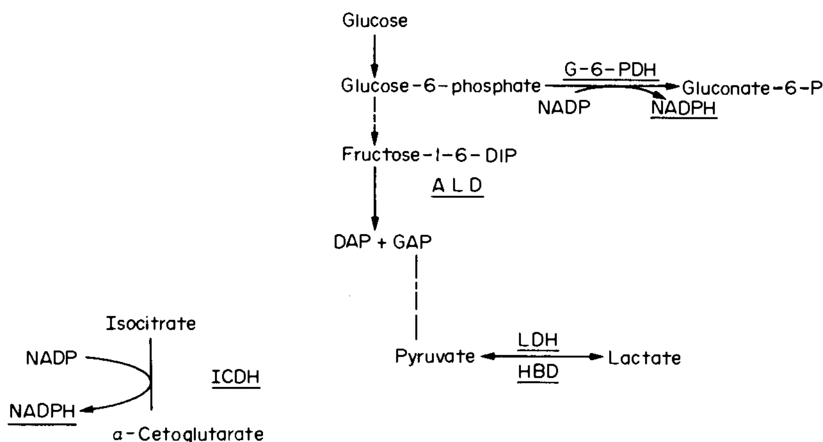
The medium is changed after three days, then, the cells are exposed to the drug. In order to measure the enzymatic activities, cells are removed from the flask after either 6h, 18h, 48h, or 72h. During this time, the medium is unchanged. The beating rates are measured before, during and after addition of THC according to the following schedule:

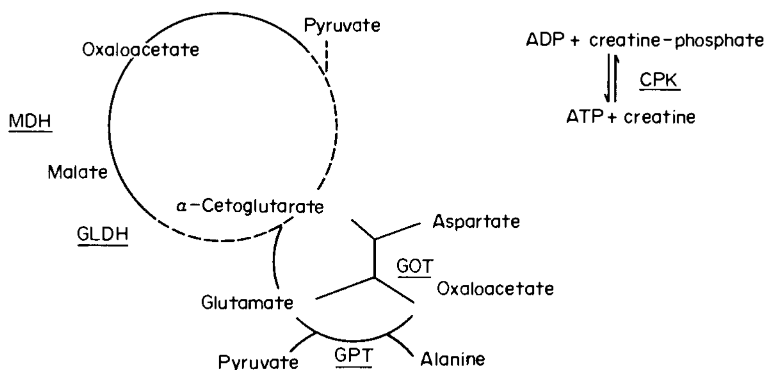


The measurements represent the differences between the rate before (T_0) and after addition of the drug. In order to express the data in a uniform way, changes in rate were expressed in percentage of the initial rate, before THC addition.

2. ENZYMATIC ACTIVITIES

The following diagram shows the metabolic role of the enzymes studied in this investigation.





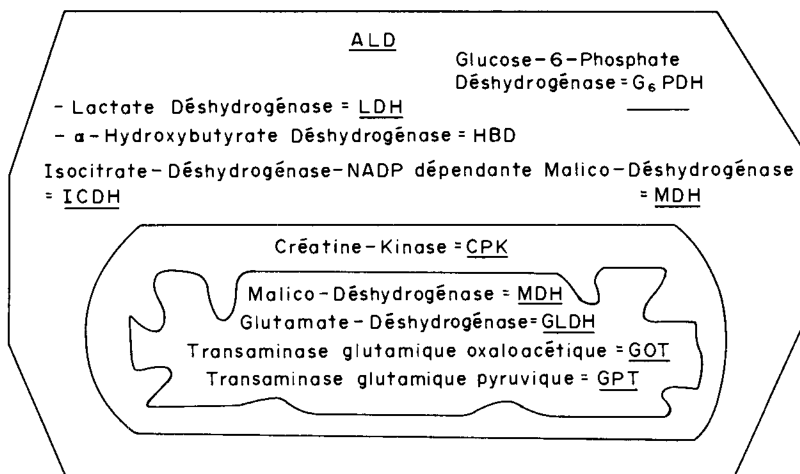
Most of these enzymes are not exclusively found in the heart, except HBD, isoenzyme-1 of LDH, which prevails in cardiac tissue. All the enzymes we have measured in this study are underlined in the diagram below which shows their cell localization.

They are NAD (Nicotinamide Adenine Dinucleotide) or NADP (Nicotinamide Adenine Dinucleotide Phosphate) enzymes. That is why we have measured the variations of optical density of reduced or oxidized form. Thus, there is a uniformity of the method of measurement and also a great rapidity in its performance.

Aldolase (ALD), Glucose-6-Phosphate dehydrogenase (G6PDH), Lactate dehydrogenase (LDH), Hydroxy-butyrate dehydrogenase (HBD), NADP dependent Isocitrate dehydrogenase (ICDH) and a part of Malate dehydrogenase (MDH) are soluble in hyaloplasm.

Glutamate dehydrogenase, Aspartate aminotransferase and Alanine aminotransferase are localized in the mitochondria.

Creatine phosphokinase (CPK) is found between the two layers of the membranes of the mitochondria.



Extraction of enzymes

Cell homogenates are obtained following treatment with a Potter piston homogenizer, heat and hypotonic solutions. With this procedure, the enzymes are in a single compartment, but the rapidity of the measurement performed at low temperature, limits eventual interactions.

Measurement of enzymatic activities

All the determinations are performed with Boehringer Mannheim test at 37°C, after micromethod adaptation, using an Eppendorf 1101 M photometer with automatic sampler. Each enzymatic activity is expressed in international milliunits per ml (mU/ml) and per protein concentration of homogenate which is obtained after centrifugation. The age of the culture and the change of culture medium influence enzymatic activity. The method we have used takes into account the different factors which influence myocardial cells and modify their enzymatic activity (Collombel and Mathieu, 1977). The different factors which interfere in enzymatic determination were carefully controlled: temperature, duration, specificity. Reproducibility of methods was tested.

Glucose and lactate measurements

We have also measured glucose consumption, lactate production and lactate/glucose ratio. Results are expressed in nanomoles per μg protein.

RESULTS

A. RHYTHMIC ACTIVITY (Figs. 1 and 2)

After 3 days of culture the average rate is 120 per minute (from 100 to 160 per minute). An uninterrupted cell layer is needed for the establishment of a synchronous rhythm. THC induces a dose related decrease in rate after 19 or 45 hours exposure (Fig. 1). At 3.2×10^{-5} M, the beats entirely and quickly stop.

Figure 2 shows the evolution of rhythmic activity with time. In control cultures, after 19

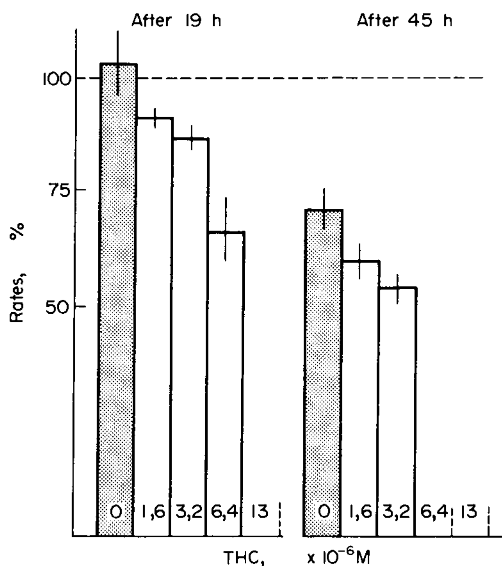


FIG. 1. Influence of concentrations of THC on contraction rate (as a percentage of control at time T_0).

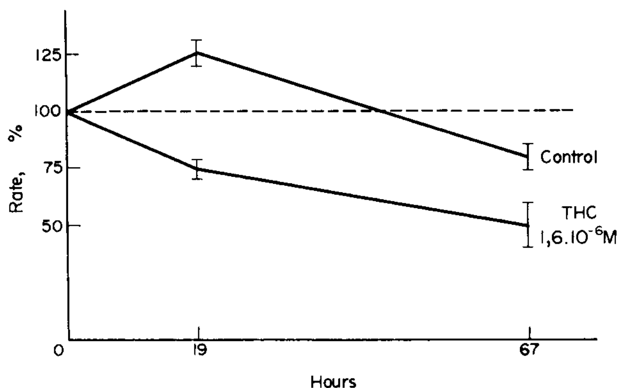


FIG. 2. Influence of THC on contraction rates, after 19 and 67 hours (as a percentage of that at time T_0).

hours, rate is increased by 24% (due to the change of medium at time T_0). Then, there is a decrease after 67 hours. In cultures exposed to THC a steady decrease rate is observed with time. In other experiments we have observed that decrease in heart rate is only apparent after 2 hours of exposure with THC.

It would appear that following THC exposure, there is a progressive decrease in active pacemaker cells but these may not be quantified with present technique.

B. ENZYMATIC ACTIVITY

We have measured glucose consumption, lactate production and the ratio lactate/glucose, because it is difficult to obtain cell cultures in exactly the same conditions. The results are expressed in nanomoles per μg protein and take into account the duration of each experiment. With short exposures (6 h), reactions of enzymatic adaptation are observed, while longer exposures (18, 48 and 72 h) induce paradoxical variations of specific enzymatic activities. In order to interpret such variations, in several experiments a protein synthesis inhibitor, Daunorubicin (DNRB) was used. A concentration of 9.7×10^{-7} M, the E.D.50, which induces a 50% decrease of protein concentration after 72 h, was selected.

1. 6-hour exposure: Fig. 3a and 3b

The ratio lactate/glucose (Fig. 3a) shows a predominant anaerobic metabolism of cell culture. Daunorubicin diminishes the ratio lactate/glucose, increasing glucose consumption, without modifying lactate production while THC slightly increases this ratio. Protein level is unchanged.

THC (3.2×10^{-6} and 13×10^{-6} M) increases specific GOT, LDH and HBD activities (Fig. 3b, Table 1), Daunorubicin also. These drugs in combination induce a variation of enzymatic activities in the same way but without clear potentiation.

In order to interpret these results, one must consider the spontaneous evolution of these enzymatic activities in the same way but without clear potentiation.
any rate, the enzymatic activities with delta-9-THC and/or Daunorubicin never exceed the activity levels at time T_0.

2. 18-hour exposure: Figs. 4a and 4b

THC induces a decrease of glucose consumption. Table 2 indicates:

an unchanged protein concentration,
a decrease of MDH and ALD activities with the lower dose, then, a normalization with the higher doses,
an increase of LDH, HBD and GOT activities, with increasing THC concentrations. (Fig. 4b).

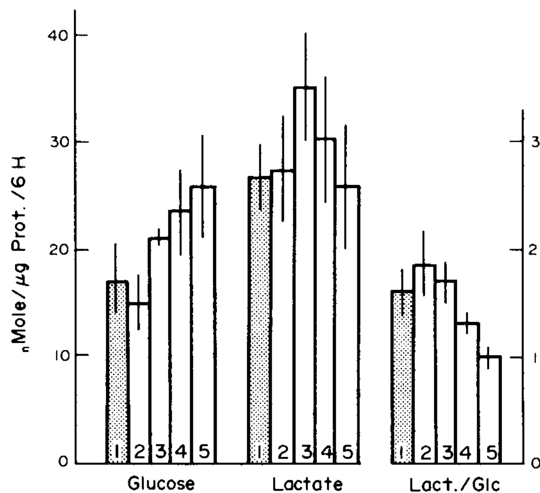


FIG. 3A. Influence of THC, after 6 h, on glucose consumption and lactate production.
(1) Control, (2) THC 3.2×10^{-6} M, (3) THC 13×10^{-6} M, (4) THC 13×10^{-6} M + DNRB 9.5×10^{-7} M, and (5) DNRB 9.5×10^{-7} M.

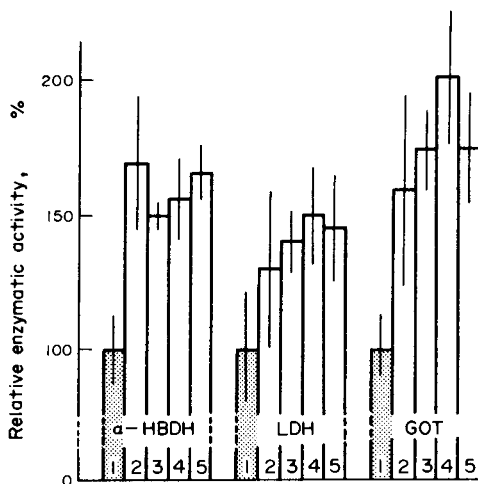


FIG. 3B. Influence of THC, after 6 H, on α-HBDH, LDH and GOT (as a percentage of control).
(1) Control, (2) THC 3.2×10^{-6} M, (3) THC 13×10^{-6} M, (4) THC 13×10^{-6} M + DNRB 9.5×10^{-7} M, and (5) DNRB 9.5×10^{-7} M.

TABLE 1. COMPARISON OF ENZYMATIC AND RHYTHMIC ACTIVITIES OF CARDIAC CELLS, AFTER EXPOSURE TO THC, WITH AND WITHOUT DAUNORUBICIN (DNRB) DURING 6 HOURS.

	Control		THC	THC	13 × 10^{-6} M	9.5 × 10^{-7} M
	T_0	T_{6h}	3.2 × 10^{-6} M	13 × 10^{-6} M	9.5 × 10^{-7} M	DNRB
μg protein/ml	140_{12}	175_{5}	190_{15}	190_{10}	185_{15}	175_{15}
CPK	129_{10} 3900	100_{18} 3000	125_{12} 3750	130_{10} 3900	136_{20} 4080	141_{5} 4230
MDH	115_{20} 2300	100_{10} 2000	112_{18} 2250	118_{12} 2350	126_{8} 2520	104_{4} 2080
LDH	165_{25} 5550	100_{20} 3350	130_{30} 4380	142_{12} 4780	149_{18} 5000	146_{20} 4900
α-HBDH	166_{20} 3450	100_{14} 2070	169_{25} 3500	150_{5} 3100	157_{15} 3250	166_{10} 3450
Aldolase	120_{6} 110	100_{4} 92	106_{8} 98	109_{10} 100	109_{7} 100	101_{12} 93
GOT	218_{8} 360	100_{10} 165	160_{35} 265	174_{15} 285	202_{25} 335	175_{20} 290
Rates at T_{6h}		116_{3}	120_{2}	100_{2}	115_{15}	105_{8}

1. The enzymatic activities are expressed:
   a. in mUI/mg proteins (lower number on the left)
   b. in percentage of the enzymatic activity of control cultures T_{6h} (upper number with standard deviation).
2. The rhythmic activity is expressed in number of beats per minute.

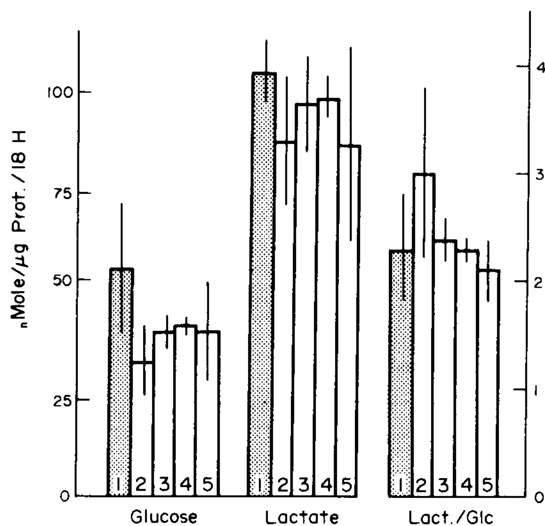


FIG. 4A. Influence of THC, after 18 h, on glucose consumption and lactate production.
(1) Control, (2) THC 1.6 × 10^{-6} M, (3) THC 3.2 × 10^{-6} M, (4) THC 6.4 × 10^{-6} M, (5) THC 13 × 10^{-6} M.

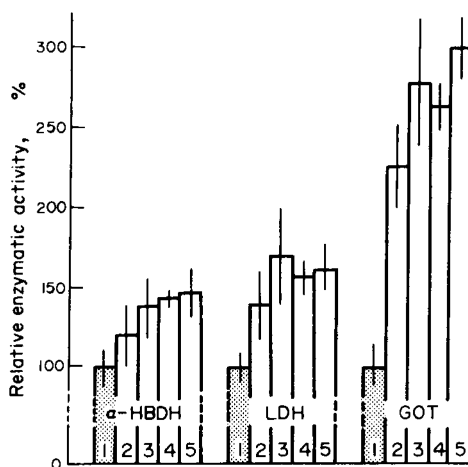


FIG. 4B. Influence of THC, after 18 H, on α-HBDH, LDH and GOT (as a percentage of control).
(1) Control, (2) THC 1.6 × 10^{-6} M, (3) THC 3.2 × 10^{-6} M, (4) THC 6.4 × 10^{-6} M, (5) THC 13 × 10^{-6} M.

TABLE 2. COMPARISON OF ENZYMATIC AND RHYTHMIC ACTIVITIES OF CARDIAC CELLS, AFTER EXPOSURE TO THC DURING 18 HOURS.

	Control T_0	Control T_{18h}	THC			
			1.6×10^{-6} M	3.2×10^{-6} M	6.4×10^{-6} M	13×10^{-6} M
μg protein/ml	85_5	105_{10}	130_5	120_{15}	120_4	125_{15}
CPK	94_{15} 5760	100_{10} 6170	92_6 5680	100_{15} 6170	97_7 5950	111_{13} 6850
MDH	83_{20} 4200	100_{20} 5000	69_5 3470	101_{20} 5070	110_{10} 5550	116_{20} 5800
LDH	90_{20} 5100	100_{10} 5700	134_{20} 7600	169_{30} 9650	155_{10} 8850	162_{15} 9200
α-HBDH	92_{20} 3450	100_{10} 3750	120_{20} 4500	134_{20} 5000	141_5 5250	147_{15} 5500
Aldolase	71_{10} 145	100_{10} 200	65_{15} 135	80_{10} 165	80_5 165	88_{10} 180
GOT	145_{20} 300	100_{15} 200	225_{25} 450	280_{40} 560	263_{15} 535	300_{30} 600
Rate	T_0	100	100	100	100	100
	T_{18h}	89_1	79_5	66_1	65_2	68_3

1. The enzymatic activities are expressed:
   a. in mUI/mg proteins (lower number on the left)
   b. in percentage of the enzymatic activity of control cultures T_{18h} (upper number with standard deviation).
2. The rhythmic activity is expressed in percentage of activity at time T_0.

The spontaneous evolution of the enzymatic activities during the time course of this experiment indicates: decrease of GOT activity, increase of ALD and MDH activities with smaller increases of LDH, HBD and CPK.

CPK activity is unchanged by THC but the contraction rate decreases significantly with the lower dose and the number of active pacemakers cells also decreases.

In another experiment of similar duration, the effects of THC (13×10^{-6} M) and Daunorubicin (DNRB: 9.5×10^{-7} M) alone or in combination, were compared. Protein level is unchanged with THC, but decreased with Daunorubicin (−25%). THC in combination with DNRB partially inhibits the decrease in protein concentration induced by DNRB (−14%).

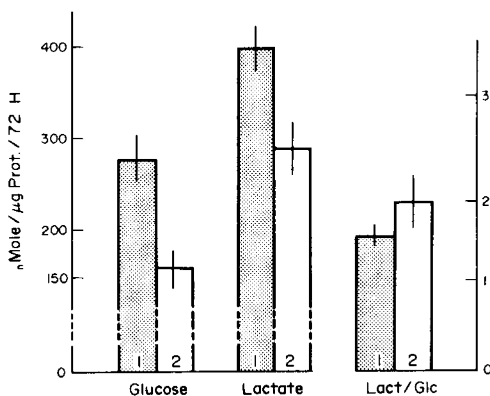


FIG. 5A. Influence of THC, after 72 h, on glucose consumption and lactate production. (1) Control, (2) THC 1.6×10^{-6} M.

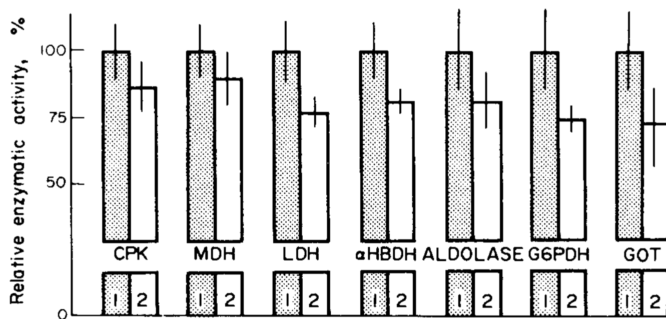


FIG. 5B. Influence of THC, after 72 H, on enzymatic activities (as a percentage of control). (1) Control, (2) THC 1.6×10^{-6} M.

With DNRB, there is a decrease of LDH (−48%) and HBD (−25%) activities in spite of a diminution of protein level, while THC does not decrease LDH and HBD activities. On the contrary, THC slightly increases LDH activity (+17%).

3. 72-hour exposure: Fig. 5a and 5b

With THC (1.6×10^{-6} M), there is a decrease of glucose consumption (−41%) and lactate production (−28%), and the ratio lactate/glucose is increased. Protein level is unchanged (Table 3). GOT, G6PDH, ALD, HBD, LDH, MDH, and CPK activities are all decreased.

TABLE 3. COMPARISON OF ENZYMATIC AND RHYTHMIC ACTIVITIES OF CARDIAC CELLS, AFTER EXPOSURE TO THC DURING 72 HOURS.

		Temoin	THC 1.6×10^{-6} M
μg protein/ml		245_{30}	260_{25}
CPK		100_{12} 3430	86_{10} 2960
MDH		100_{11} 6130	89_{10} 5450
LDH		100_{12} 6000	77_{5} 4600
α-HBDH		100_{10} 2550	82_{5} 2100
Aldolase		100_{15} 160	82_{10} 130
G6PDH		100_{10} 8	75_{5} 6
GOT		100_{15} 70	73_{13} 50
Rate	T_0	100	100
	T_{19h}	124_7	73_4
	T_{69h}	79_7	50_{12}

1. The enzymatic activities are expressed:
   a. in mUI/mg proteins (lower number on the left)
   b. in percentage of the enzymatic activity of control cultures (upper number with standard deviation).
2. The rhythmic activity is expressed in percentage of activity at time T_0.

DISCUSSION

In the 6-hour experiments, LDH, HBD and GOT activities are increased. This may be due to an increased synthesis or to a decreased catabolism of these enzymes.

The inhibiting action of Daunorubicin on the enzymatic catabolism has been observed and in this experiment, THC has the same effects as Daunorubicin. However, these drugs have an opposite effect on the ratio lactate/glucose.

When the cells are exposed to THC during 18 hours, there is a strict anaerobic metabolism. Lactate production does not come only from anaerobic glycolysis, but also from aminoacid desamination; it implicates transamination reactions, as shown by an increase of GOT activity. The increases of LDH and HBD activities could be due to a biosynthesis induced by the increase of pyruvate which mainly comes from the degradation of glucose, the consumption of which is decreased.

LDH, HBD and GOT catabolism inhibition might be associated with a biosynthesis of these three enzymes. In contrast with results observed after 6 hours, LDH activity is inhibited by Daunorubicin and increased by THC after 18 hours.

Daunorubicin, a protein synthesis inhibitor, will inhibit the synthesis of very short half-life proteases which induce enzymatic catabolism. This effect is particularly clear when the enzymatic activities remain unchanged in time, when they decrease.

In the experiment with a long exposure to THC, this drug induces a non specific inhibition of enzymatic activities, a decrease of the aerobic metabolism, and a decrease of glucose consumption. These different results lead us to suggest the following hypothesis: THC might inhibit the membrane transport of glucose and other substrates, as well as synthesis of proteases which induce GOT, LDH and HBD catabolism. As a result, for a short period of time, the activity of these enzymes, which are mainly implicated in anaerobic metabolism, is increased.

When the cells are exposed to THC for a longer time, the enzymatic biosynthesis would be decreased by lack of substrate availability, and the proteases synthesis inhibition by THC could not maintain enzymatic activities. Furthermore, it must be noted that the half-life of these enzymes is shorter than the total protein pool because their activity is measured as a function of protein concentration which hardly changes.

THC is not a direct protein synthesis inhibitor, like Daunorubicin but would indirectly decrease protein synthesis as discussed in this monograph by Desoize, Stein, Toro-Goyco, Carchman and by Issidorides. The mechanism of the depressant effect on the beating rates of the myocardial cells remains to be clarified.

REFERENCES

1. Harris, L. S., General and behavioral pharmacology of Delta-9-THC. *Pharmacol. Rev.*, **23**,285–294 (1971).
2. Hollister, L. E., Actions of various marihuana derivatives. *Pharmacol. Rev.* **23**,349–358 (1971).
3. De Haan, R. L., Regulation of spontaneous activity and growth of embryonic chick heart cells in tissue culture. *Devel. biol.*, **116**,216–249 (1967).
4. Collombel, Ch. et Mathieu, M., Problèmes posés par les mesures d'activités enzymatiques dans les tissus et les cellules cultivées. *Problèmes Actuels de Biochemie*, Ed. MASSON, (Paris), Sème série, p. 179, (1977).
5. Cartier, P., Leroux, J. P., et Marchand, J. Cl., Techniques de dosage des enzymes glycolytiques tissulaires. *Ann. Biol. clin.*, **25**,1–2, 109–136 (1967).

B. In vivo Preparations

EFFECTS OF CANNABIS ON LUNGS

HARRIS ROSENKRANTZ and ROBERT W. FLEISCHMAN

Department of Biochemical Pharmacology, EG & G Mason Research Institute, Worcester, Massachusetts 01608, U.S.A.

Summary. Because of reported changes in lung function and morphology in cannabis users and animals exposed to marihuana smoke, a rat model was developed for standardized automatic chronic exposure to marihuana smoke. The smoking apparatus was regulated to present a 50-ml puff volume simultaneously from each of 3 NIDA cigarettes in a 2-sec puff for a 30-sec exposure interval followed by 30-sec of fresh air each min. Estimated Δ^9-THC doses in marihuana smoke were 0.4–5 mg/kg and were relevant to those used by man. The doses were attained by varying the number of puffs from 4 to 16 which correlated with carboxyhemoglobin levels between 15 and 48% and plasma Δ^9-THC levels of 34–313 ng/ml. The latter were similar to those found in human cannabis consumers (20–300 ng/ml). Behavioral signs of CNS-inhibition and CNS-stimulation were in accord with dosages and development of tolerance was observed. No pulmonary pathology could be attributed to marihuana smoke when exposure was for 14–57 days. In contrast, exposures for 87–360 days induced a dose-related focal alveolitis or pneumonitis which progressed from extensive mobilization of alveolar macrophages and foreign body cell inflammation to pronounced focal proliferative aberrations associated with focal granulomatous and cholesterol clefts. In all chronic studies, the effects of marihuana smoke were distinguished from those of placebo and tobacco smoke. The lung pathology was due to a combination of non-specific smoke particulates and cannabinoids.

THE architecture of lungs has evolved to achieve an efficient exchange of gases coincidentally with defence mechanisms adequate to remove reasonable quantities of particulates. The design of the microanatomy of the pulmonary tree was probably not intended to cope with the continuous onslaught of smoke ingredients. Therefore, in the light of the devastating finding on human tobacco smokers[5, 10, 46] and pulmonary lesions observed in animals[40, 44], it does not require too active an imagination to anticipate adverse effects of cannabis smoke. Indeed on the basis of the deeper inspirations that marihuana consumers exploit in order to achieve a " high ", it can be anticipated that a greater quantity of particulates enter the respiratory tract from cannabis than from tobacco smoke[2, 4, 56].

Other observations enhance the anticipation that cannabis may have potential deleterious effects on the lungs. Potential carcinogenic activity of marihuana smoke has been reported[33, 54] although conflicting results have been obtained as to mutagenicity[65, 100]. In rats, marihuana smoke increased the number and size of alveolar macrophages[19, 57] and induced aryl hydrocarbon hydroxylase activity[57]. In dogs bronchiolitis and squamous metaplasia were evident after chronic marihuana inhalation[79]. Both human and mouse lung explants exhibited a derangement in DNA metabolism after exposure to

marihuana smoke[50, 51]. Membrane transport of alveolar macrophages was disturbed by Δ^9-tetrahydrocannabinol (Δ^9-THC)[11]. Isolated guinea pig lungs responded to Δ^9-THC with the release of prostaglandin-like material[38] and norepinephrine[97]. In this same species, Δ^9-THC did not alter the bronchoconstriction induced by histamine[1].

In man, chronic marihuana and hashish use has been associated with laryngitis, pharyngitis, cough, hoarseness, bronchitis and squamous metaplasia[12, 31, 90]. Subchronic use of marihuana has caused pneumonitis[71] and pulmonary impairment as measured by a variety of lung function tests[8, 36, 60, 86, 87, 96].

It is also worthwhile mentioning the fact that passive inhalation of cannabinoids can occur in the non-user in the presence of marihuana smokers[105] and that there is a relatively strong affinity of cannabinoids for lung tissue irrespective of the route of administration[6, 20, 32, 41]. Rat lung has been shown to metabolize Δ^9-THC[63].

In order to more precisely assess the potential pulmonary changes evoked by marihuana smoke in animals, it was mandatory to perform a chronic study with doses relevant to man. Standard procedures of exposure, dose monitoring, duration of treatment and species selection had to be carefully considered. These criteria were addressed in several preliminary studies before a one-year marihuana inhalation study was conducted. One of the major problems in investigating the pulmonary effects of cannabis in animal paradigms is that of simulating the human smoking technique. Superimposed on this requirement is the need to have a sufficient number of animals of a convenient size. There is an obvious advantage in being able to expose multiple animals simultaneously to an identical smoke atmosphere. Such restrictions almost make it inevitable to utilize a rodent species.

Rodent species are obligatory nose breathers and are protected by a relatively efficient nasal filtration apparatus which can play a role in the dose of cannabinoids reaching the blood. This inadvertent regulation of dose can be avoided by administration of cannabis smoke via a tracheal tube. However, this inhalation technique departs from human smoking simulation. In addition to the potential of recurrent infection at the site of incision, more and larger smoke particulates can penetrate deeper into the lungs than could occur in human marihuana smoking. Furthermore, the physical (i.e., smoke cooling and particulate condensation) and chemical (oxidation/reduction interactions) alterations of smoke components that usually occur more rostrally would not take place when tracheotomy is used[27]. Therefore, it seems more justifiable to accept passive nasal inhalation in place of tracheotomy.

Additional factors that may influence simulation of human marihuana smoking in the use of a smoking machine relate to variation of marihuana potency, cannabinoid transformation during pyrolysis and the effect of non-cannabinoid smoke constituents. The potency of marihuana based on the Δ^9-THC content has been reported to vary from approximately 0.5 to 5.0 percent and was directly related to the geographical source of marihuana[16, 49]. In general, little or no transformation of non-psychogenic cannabinoids to Δ^9-THC occurs during the smoking process in smoking machines or during human consumption[42, 61, 70, 81, 84]. Pyrolytic and butt entrapment losses of Δ^9-THC have been documented to be approximately 50%[2, 18, 56, 75, 95]. Characteristic profiles of marihuana components have been obtained and compared with tobacco ingredients and a similar complexity of constituents exists[37, 65, 66]. Of considerable importance is the lack of an adequate placebo cigarette; the extraction process used for the removal of cannabinoids no doubt eliminates other lipid constituents as well.

Within the guidelines of the restrictions discussed above, essentially four toxicological

studies were performed using the inhalation route and regulating the doses of Δ^9-THC in marihuana smoke to be of relevance to man. The approximate duration of treatments were either 24, 90, 180 or 360 days. In some instances a recovery period was part of the experimental design and in one instance, three daily exposures were compared with the usual single daily exposure. In all studies the Fischer 344 rat from the same breeder was utilized and the same automatic smoking apparatus was used throughout. In most of the chronic studies smoke dosage was monitored by carboxyhemoglobin (COHb) levels and cannabinoids by the determination of plasma Δ^9-THC concentrations. Although a complete toxicological assessment was performed in most inhalation studies, particular attention was devoted to changes in lung morphology.

METHODS AND MATERIALS

The earlier subacute (14–28-day) and subchronic (87-day) studies were conducted at an older facility with fewer air exchanges and wider excursions in room temperature. Newer facilities with greater control of the environment were used for the most recent subchronic (90-day) and chronic (180–360-day) investigations. In all studies, animals were housed adjacent but separately from the room in which exposure to smoke was performed. Furthermore, the same inhalator was utilized for all studies and the same professional and, in large part, the same technical staff were involved in the inhalation studies.

ANIMAL HOUSING AND CARE

All Fischer 344 rats weighing approximately 90–110 gm were procured from the same source (Charles River Breeding Laboratories, Wilmington, MA). One shipment of animals was obtained for each study and animals of doubtful health were eliminated from the experiment. The Fischer rat was selected for the inhalation studies because of modest growth rate, low incidence of spontaneous pulmonary disease, immunogenetic homogeneity and the availability of extensive marihuana toxicity data on this rat strain.

Two rats of the same sex were housed in wire-mesh suspension cages of dimensions 24 × 18 × 17 cm. Commercial rodent chow pellets and water were freely available. Each animal room had a 12-hr light/dark circadian cycle to minimize the effects of hormonal surges. Ambient room temperature was generally 23 ± 2°C and in the new facilities, there were 6–8 fresh air changes each hour. Animals were transferred from rooms adjacent to the room in which smoking was performed and returned as soon as possible after exposure to smoke or sham treatment. Control and treated groups were housed in separate cage racks with sufficient intervening space to minimize transfer of expired air between the groups.

CANNABINOID MATERIALS AND DOSES

All marihuana and placebo cigarettes were supplied by the National Institute on Drug Abuse. Because the content of Δ^9-THC varied considerably between certain lot numbers, the concentration of Δ^9-THC of each cigarette shipment is given here: for marihuana cigarettes, QCD-72974 contained 2.5 percent Δ^9-THC; QCD-72978 and 72979, 2.1 percent

Δ^9-THC; QCD-83814, 1.2 percent Δ^9-THC; QCD-83813, 0.9 percent Δ^9-THC and RTI 2055-53, 2.8 percent Δ^9-THC. The content of each of the other major cannabinoids for all shipments was less than 0.2 percent. Placebo cigarettes were essentially devoid of all cannabinoids: SSC-69417, less than 0.05 percent Δ^9-THC; QCD-83812, 0 percent Δ^9-THC and RTI 2055-34, 0 percent Δ^9-THC. In general, for each inhalation study, marihuana cigarettes with the same Δ^9-THC content were used throughout the study. Cigarettes were stored at 5°C but before use, they were placed at 60 percent humidity and 23°C for 24–48 hr before use.

The dosages of marihuana applied in each study were based on the content of Δ^9-THC. Doses were estimated by a mathematical formula derived from known measurements of tidal volumes, respiration rates, duration and number of exposure periods (puffs) and concentrations of Δ^9-THC in the smoke during its presence in the smoke chamber of the automatic inhalator[73, 74]. Corrections for Δ^9-THC losses due to pyrolysis, entrapment in the cigarette butt, filtration by nasal turbinates and not absorbed in the lung were made as previously described[9, 73, 74]. It has been determined that rodent Δ^9-THC doses correlate more closely to those of man when related to body surface area.

It has been estimated from data in the literature that the inhalation of cannabis smoke in humans would provide a Δ^9-THC dose approximately between 0.1–3.5 mg/kg. This dosage range is based on the assumption that the Δ^9-THC content of the marihuana is between 1 and 5 percent and that 1–6 marihuana cigarettes are consumed per day. On the basis of body surface area, these doses would be approximated by Δ^9-THC inhalation doses between 1 and 20 mg/kg in rats[73]. As a point of reference, the acute inhalation LD 50 in Fischer rats was approximately 40 mg/kg[77]. In all the rat inhalation studies to be discussed, the range of Δ^9-THC doses used was between 0.4 and 5.0 mg/kg.

Although dosimetry was not monitored in the earlier subacute inhalation studies, in the more recent investigations, COHb levels were monitored by suborbital bleeding and the use of a spectrophotometric procedure[17]. The error was about 12 percent. In this manner equivalency of smoke exposure could be documented for control and treated animals and COHb concentrations could be related to dosage in terms of the numbers of puffs used. It was by varying the number of puffs that each estimated Δ^9-THC dose could be achieved. At the termination of the more recent investigations, blood specimens were obtained and submitted for the determination of plasma Δ^9-THC levels by radioimmunoassay (RIA, courtesy of S. J. Gross and J. R. Soares at the University of California, Los Angeles and V. Marks at the University of Surrey) or gas chromatography (GC, courtesy of Battelle Columbus Laboratories).

TREATMENT PROTOCOLS

The same automatic inhalator was used for every inhalation experiment. The smoking apparatus was regulated to present a 50-ml puff volume simultaneously from each of 3 cigarettes (a total puff volume of 150 ml) in a 2-sec puff period and the smoke was retained in the smoke chamber for 30 sec immediately followed by a 30-sec purge interval with fresh air from an air compressor. These events were repeated each minute, providing 1 puff/min/cigarette. The smoking conditions were a compromise of the smoking techniques of naive and experienced marihuana smokers. Cannabis consumers usually inspire a puff

volume of 50–200 ml during a 2–10-sec period and retain the smoke in their lungs for 15–60 sec before expiration[74].

Depending upon the Δ^9-THC content of the marihuana, an approximate number of puffs were selected to attain the necessary Δ^9-THC doses. For example, using marihuana with a content of 2 percent Δ^9-THC, 4 puffs provided a Δ^9-THC dose of approximately 0.7 mg/kg, 8 puffs were equivalent to 2 mg/kg and 16 puffs to 4 mg/kg. In order to achieve the 16-puff dosage, new cigarettes were substituted for the ones burned for 8 puffs without interrupting the automatic cycle of events. In all but one study, there was a single daily exposure and smoking was performed either 5, 6 or 7 days per week. It had been determined that the COHb generated during a single exposure to smoke is cleared from the blood of rats, mice, hamsters, guinea pigs and rabbits by 2 hr (Rosenkrantz, unpublished data).

Multiple rats were simultaneously exposed to smoke through attachment of a circular array of 4–5 cone-shaped lucite animal holders to each side of the smoke chamber. The sequence of exposure for the various groups of animals was randomized each day to minimize the effect of the time of the day at which treatment took place. The sexes were separately exposed and all animals were conditioned to the stresses of confinement and noise of the smoking apparatus in the absence of smoke for 1–2 days before commencement of the experiment.

The experimental designs of the inhalation studies encompassed a 28-day subacute trial, an 87-day exposure regimen followed by a 20-day recovery period, triple versus single daily exposure for 30 days, a 1-year study plus a 30-day recovery period and a 90-day study plus a 30-day recovery period in which the effects of marihuana smoke were compared with tobacco smoke. Tobacco cigarettes were the IRI reference cigarette fabricated by the University of Kentucky Tobacco and Health Research Institute. The tobacco cigarettes were stored and placed in a humidor in an identical manner like that for marihuana cigarettes.

In the 28-day experiment, 24 rats/sex/dose received a single daily exposure to marihuana (2.1 percent Δ^9-THC) smoke at approximate Δ^9-THC doses of 0.7, 2.1 or 4.2 mg/kg for 5 days/week. Placebo groups received only 8 puffs (compared with 16 puffs for the high dose) because a larger number of puffs caused carbon monoxide lethality. It has been established that placebo cigarettes generate approximately 30 percent more carbon monoxide (CO) than marihuana or tobacco cigarettes during pyrolysis (Rosenkrantz, unpublished data). One-half the rats of each group were necropsied after 14 treatments and the other half after 28 treatments.

In the 87-day study, 40 rats/sex/dose were exposed to Δ^9-THC doses of 0.7, 2 or 4 mg/kg for 6 days/week. Placebo groups were given 8 puffs each day. One-fourth of the animals were necropsied on days 27, 57 and 87 of treatment and the remainder, who had been exposed to smoke or sham for 87 days, were sacrificed after a 20-day recovery period.

The exposure protocol for the study in which a single daily exposure was compared with 3 exposures/day included Δ^9-THC doses of 0.7 and 2 mg/kg and 8 puffs of placebo given 5 days/week for 30 days. There were 10 rats/sex/dose/exposure regimen. Rats that received triple daily exposure were permitted to recover for 1.5–2.0 hr after each exposure to assure clearance of COHb before the next treatment.

The 1-year inhalation study was predominantly performed with marihuana containing the lowest content of Δ^9-THC (0.9 percent) and therefore, the numbers of puffs were altered to achieve the necessary doses. As a precaution, because of projected prolonged

treatment, the high-dosed groups contained more animals than other groups. In addition, sham-treated groups were included in the study to provide information on spontaneous lung changes. Marihuana smoke was reproducibly presented as 5 puffs (0.4 mg/kg) or 8 puffs (0.8 mg/kg) to 30 rats/sex/dose for 7 days/week or 15 puffs (1.5 mg/kg) to 50 rats of each sex. The placebo group consisted of 30 rats of each sex, who were exposed to 12 puffs of placebo smoke. For both the high-dosed marihuana groups and placebo groups, exposure was begun with a lesser number of puffs until the desired number of puffs was reached in the first week of treatment. Such gradual acclimatization to smoke diminished the chances of early mortality potentially related to CO intoxication. A second control group of 30 rats/sex were sham treated for 12 min each day in the absence of smoke. Necropsies were performed on approximately 25 percent of the animals of each group after 180 days and on 50 percent of rats after 360 days of treatment. The remaining 25 percent of animals treated for 360 days were permitted to recover for 30 days.

In order to attain some idea of the specificity of lung changes observed after exposure to marihuana smoke, a study was carried out with tobacco smoke side by side with marihuana and placebo smoke. In this experiment 40 rats/sex/dose were exposed to Δ^9-THC doses of approximately 2.5 mg/kg (8 puffs) or 5 mg/kg (16 puffs) for 7 days/week. The placebo dose was 10 puffs and the largest number of non-lethal doses of tobacco smoke that could be used was 12 puffs[64]. Approximately 25 percent of the animals in each group were sacrificed after approximately 30, 60 or 90 days of treatment or after 90 days of exposure followed by a 30-day recovery interval.

PATHOLOGY PROCEDURE

In most studies a representative number of rats in each group were sacrificed by decapitation while others were anesthetized with sodium pentobarbital. The latter procedure was used to facilitate collection of blood from the abdominal aorta and for lung instillation with fixative to maintain pulmonary architecture. In the latter instance, Bouin's fixative was introduced via a tracheal polyethylene cannula or a 15-gauge gavage needle of 4.5-cm length[7]. Approximately 5-ml fixative restored the lungs to a normal expanded volume. The trachea was ligated and the lungs placed in 10 percent neutral buffered formaldehyde. Paraffin blocks were prepared as 6 μm sections and stained with H & E for histopathological evaluation. Sections of all lobes of the lungs were made and the numbers of alveolar macrophage foci or clusters in the lobes were counted. A focus or cluster was considered to be a circumscribed group of 6 or more macrophages. Based on these counts, a semiquantitative grading related to severity of pneumonitis was achieved: 1–10, very slight; 11–100, slight; 101–200, moderate; 201–300, marked; 301–400, severe; > 401, very severe.

RESULTS

In performing the present cannabis inhalation studies in rats, 3 aspects were given particular attention: (1) standardization of exposure so that simulation of human marihuana smoking could be reproduced from experiment to experiment; (2) monitoring of dosimetry through cigarette consistency, levels of CO and COHb, animal survival rates, limitations of daily and weekly exposure, plasma Δ^9-THC levels and behavioral responses;

and (3) determination of a potential temporal pattern of morphological changes in lungs. Of importance was the fact that the same automatic smoking machine was utilized in all the inhalation studies.

STANDARDIZATION OF SMOKE EXPOSURE

The physical characteristics of active cannabis inhalation in the human were compared with passive marihuana inhalation in rats (Table 1). Since the weight and length of cannabis cigarettes prepared by cannabis users themselves are known to vary considerably, no definitive data on these parameters could be evaluated on illicit cannabis. In controlled studies on human volunteers, the standard NIDA cigarettes were used which had similar dimensions to those employed in animal studies. It is pointed out that the cigarette posture in the animal experiments was horizontal to conform with the smoking position of cigarettes in man. Other smoking machines orient cigarettes in a vertical position[4, 27]. Although "fresh" smoke was delivered over a 2-sec period, some ageing of the smoke could occur since there was approximately a 17-cm distance from the butt end of cigarettes to the center of the smoke chamber. In general, a compromise of puff volume as generated by naive and experienced cannabis users was simulated and a puff volume of 150 ml was retained in all animal experiments. Similarly, a mean exposure interval of 30 sec was a compromise between the shorter inspiration period of naive users compared with exper-

TABLE 1. PHYSICAL CHARACTERISTICS OF HUMAN MARIHUANA SMOKING AND SIMULATION IN AN AUTOMATIC SMOKING MACHINE[a]

Parameter[b]	Active human inhalation			Passive rodent inhalation	
	Marihuana		Tobacco	Marihuana	Placebo
	Naive	Experienced			
Cigarette weight (mg)	998 ± 19	998 ± 19	1114 ± 13	998 ± 19	861 ± 17
Cigarette posture	horizon.	horizon.	horizon.	horizon.	horizon.
Butt distance to mouth (cm)	0	0	0	17	17
Puffs per 85 mm cigarette length	8–10	6–9	11–12	8–10	8–9
Puff volume (ml)	35–50	50–200	35	150	150
Puff duration (sec)	2	2–10	2	2	2
Exposure interval (sec)	15–20	30–45	15	30	30
Puff frequency (puffs/min)	1	1	1	1	1
Interval between puffs (sec)	58	50–58	58	58	58
Fresh air purge (sec)	40–45	15–30	45	30	30

a. The same modified Walton inhalator was used for all marihuana inhalation studies (Homberger *et al.*, 1967; Rosenkrantz and Braude 1974).

b. Marihuana and placebo cigarettes were those prepared by the National Institute on Drug Abuse and the tobacco cigarettes were those prepared as a reference standard by the University of Kentucky.

ienced cannabis users. Essentially, 1 puff/min and a sufficiently long purge period to clear smoke from the smoke chamber was instituted to mimic frequency of human inspiration and expiration of smoke.

REPRODUCIBILITY OF SMOKE GENERATION AND DOSIMETRY

Since different crop harvests of cannabis were used for preparing the standard NIDA cigarettes, the consistency of cigarettes was assessed by mass, total particulate matter, burning time as estimated by residual butt length after 8 puffs and the concentration of CO generated (Table 2). The coefficient of variation of cigarette weight was between 4–9 percent for marihuana, 4–8 percent for placebo and 3 percent for tobacco. Except for the largest variation in total particulate matter for marihuana with 2.0 percent Δ^9-THC (mean of 2 different batches), the coefficient of variation was 7–13 percent for all types of cigarettes. Greatest variability occurred for residual butt length probably due to the effect of non-homogeneity of cannabis on burning time (the presence of larger stem fragments sometimes punctured the cigarette wrapping). The coefficient of variation for marihuana was 10–26 percent, for placebo 10–18 percent and tobacco 12 percent. Usually, placebo cigarettes burned faster due to smaller mass and less homogeneous packing. CO generation was largest for placebo and similar for marihuana and tobacco.

TABLE 2. MARIHUANA CIGARETTE PUFF TRAITS AND EXPOSURE CONSISTENCY TO SMOKE IN AN AUTOMATIC INHALATOR[a]

Cigarette type and Δ^9-THC content[b]	Cigarette weight (mg)	Total particulates (mg/3 cigs/8 puffs)	Butt length (mm)	Carbon monoxide (%)
Marihuana, 2.6%	923 ± 84	125 ± 10	20 ± 5	1.2 ± 0.2
Marihuana, 2.0%	994 ± 38	112 ± 25	27 ± 7	1.2 ± 0.2
Marihuana, 0.9%	1002 ± 37	106 ± 7	30 ± 3	1.2 ± 0.2
Placebo A, 0%	814 ± 64	69 ± 7	17 ± 3	1.6 ± 0.2
Placebo B, 0%	746 ± 32	78 ± 9	22 ± 3	1.6 ± 0.2
Tobacco IRI, 0%	1106 ± 33	79 ± 10	25 ± 3	1.3 ± 0.2

a. All cigarette smoking was performed on the same modified Walton automatic smoking machine (Rosenkrantz and Braude, 1974); 50-ml puff volume per cigarette.

b. National Institute on Drug Abuse cigarette codes were: marihuana with 2.8% Δ^9-THC, RTI 2055-53; marihuana with 2.0% Δ^9-THC, QCD-72984 and 5; marihuana with 0.9% Δ^9-THC, QCD-83813 and 4; placebo A was RTI 2055-34; and placebo B was QCD-83812.

The number of puffs used to achieve the desired Δ^9-THC doses had to be selected in relationship to CO intoxication and were monitored by the determination of COHb levels (Table 3). In the absence of acclimatization to smoke, lethality of both sexes given 1–2 doses of 16 or more puffs from 3 marihuana cigarettes was associated with mean COHb concentrations between 35–47 percent. In the instance of placebo smoke, approximately one-half the animals died under similar exposure conditions with mean COHb levels being between 47–58 percent. In general, COHb levels were dose related. Prolonged exposures for 8–20 days at 14–16 puffs generated mean COHb levels between 28–40 percent for both

TABLE 3. INTERRELATIONSHIP OF EXPOSURE DURATION, LETHALITY AND CARBOXYHEMOGLOBIN LEVELS IN RATS EXPOSED TO MARIHUANA (1.2% Δ⁹-THC) OR PLACEBO SMOKE IN AN AUTOMATIC INHALATOR[a]

Type of smoke	Exposure duration		Lethality (deaths/group)		Carboxyhemoglobin (%)	
	Puffs/day	Days	Males	Females	Males	Females
Marihuana (QCD-83814)	28	1	6/10	6/10	46 ± 5	47 ± 5
	16	1	2/10	2/10	39 ± 12	35 ± 12
	16	2	3/10	2/10	40 ± 15	42 ± 16
	16	10	0/40	0/40	35 ± 12	33 ± 11
	16	20	4/35	2/35	30 ± 6	28 ± 9
	12	3	0/10	0/10	32 ± 12	37 ± 10
	12	20	0/10	0/10	20 ± 6	22 ± 6
	8	3	0/10	0/10	15 ± 4	20 ± 2
	8	20	0/10	0/10	26 ± 4	26 ± 4
	4	20	0/10	0/10	12 ± 2	14 ± 2
Placebo (QCD-83812)	16	1	6/15	6/15	55 ± 15	58 ± 20
	16	2	5/10	4/10	50 ± 16	47 ± 17
	14	8	2/40	3/40	40 ± 8	40 ± 4
	14	20	0/35	0/35	25 ± 4	28 ± 9
	12	2	0/14	0/14	31 ± 10	28 ± 4
	12	20	0/8	0/8	39 ± 9	30 ± 7
	8	2	0/6	0/6	25 ± 5	25 ± 3
	8	20	0/6	0/6	21 ± 2	15 ± 2
	4	20	0/6	0/6	12 ± 2	17 ± 2

a. Smoking was performed 7 days per week without acclimatization to smoke.

types of cigarettes and resulted in some deaths. However, signs of cyanosis were discerned for placebo-treated rats and not animals exposed to marihuana smoke.

The relationship of delayed lethality was further explored by varying the weekly exposure schedule with and without acclimatization (Table 4). At estimated doses of 2 mg/kg (8 puffs) and 4 mg/kg (16 puffs), a sex-related mortality correlated with the time of death and number of weekly treatments. Among the males given the high dose, 17, 33 and 67 percent succumbed within 5 weeks after 5, 6 or 7 days/week exposure, respectively, when not acclimatized to smoke. Despite acclimatization, some delayed deaths, albeit significantly less, occurred. The cause of these delayed deaths may not have been due to CO toxicity. Delayed lethality has been reported for monkeys[91] given Δ⁹-THC intravenously and in rats treated orally[78]. In any case, it seemed appropriate for the performance of chronic inhalation studies to increase the number of puffs progressively until the desired dose is reached in the first week. Such acclimatization to smoke minimizes extensive losses of rodents during the course of the investigation.

Although COHb concentrations could be monitored during the experiment, sequential bleedings for the determination of plasma Δ⁹-THC levels were not feasible because of the volume of plasma needed for analysis. Therefore, such analyses were conducted at the termination of the study. Blood specimens were obtained between 5–25 min after the animals were removed from the inhalator. It was known that peak Δ⁹-THC levels occurred within 10–15 min[3, 21, 72, 82, 99] but this timeframe was not always possible to achieve and contributed to the variability in results (Table 5). However in each experiment in which plasma Δ⁹-THC was determined, a dose relationship was found. Those animals exposed to marihuana smoke clearly absorbed Δ⁹-THC. This cannabinoid was present in

TABLE 4. DELAYED LETHALITY TOXICITY IN RATS EXPOSED TO MARIHUANA SMOKE (2.1% Δ^9-THC) WITH AND WITHOUT ACCLIMATIZATION TO SMOKE[a]

No. rats, sex	Δ^9-THC (mg/kg × days/wk)	Time and number of deaths without acclimatization: Wk. 1	2	3	4	5	Mortality: Not acclimat. (%)	Acclimat. (%)
15M	2 × 5	0	0	0	0	0	0	0
15F	2 × 5	0	0	0	0	0	0	0
12M	4 × 5	1	1	0	0	0	17	4
12F	4 × 5	0	0	0	0	0	0	0
50M	2 × 6	0	1	0	0	0	2	0
50F	2 × 6	0	1	0	0	0	2	0
30M	4 × 6	2	1	4	2	1	33	10
30F	4 × 6	0	0	1	0	1	7	2
50M	2 × 7	0	1	3	0	1	10	0
50F	2 × 7	0	1	1	0	3	10	0
12M	4 × 7	2	1	3	1	1	67	10
12F	4 × 7	2	0	1	0	0	25	7

a. To achieve the estimated Δ^9-THC doses, 8 or 16 puffs during a single daily exposure were used.

TABLE 5. DOSIMETRY: Δ^9-TETRAHYDROCANNABINOL ESTIMATED DOSE, LEVELS IN MARIHUANA SMOKE AND PLASMA AND CARBOXYHEMOGLOBIN CONCENTRATIONS IN RAT INHALATION STUDIES[a]

Cigarette type and Δ^9-THC content	Treatment schedule: 1/day (days)	Number puffs	Δ^9-tetrahydrocannabinol levels: Estimated dose (mg/kg)	In smoke (μg/ml)	In plasma (ng/ml)	COHb levels (%)
Marihuana, 2.8%	90	8	2.5	2.4	34 ± 12[b]	25 ± 4
	90	16	5.0	2.4	70 ± 24[b]	48 ± 7
	recov.	0	0	0	0	9 ± 9
Placebo, 0%	90	10	0	0	0	27 ± 12
	recov.	0	0	0	0	10 ± 7
Marihuana, 2.0%	20	4	0.4	0.7	76 ± 52[c]	15 ± 4
	20	8	1.1	2.2	179 ± 82[c]	23 ± 7
	20	16	2.2	2.2	313 ± 135[c]	39 ± 12
Placebo, 0%	20	12	0	0	14 ± 19[c]	37 ± 15
Marihuana 0.9%	360	5	0.4	0.4	36 ± 9[b]	17 ± 5
	360	8	0.8	0.8	95 ± 61[b]	31 ± 8
	360	15	1.5	0.8	175 ± 130[b]	47 ± 17
	recov.	0	0	0	0	6 ± 6
Placebo, 0%	360	12	0	0	25 ± 15[b]	51 ± 19
	recov.	0	0	0	0	6 ± 6

a. All inhalation studies performed in the same automatic smoking machine.
b. Analyses by gas chromatography by courtesy of Battelle Columbus Laboratories.
c. Analyses by radioimmunoassay performed by Gross and Soares (Gross *et al.*, 1974).

blood at mean ranges of 15–50 ng/ml after 8 puffs and approximately 50–130 ng/ml after 16 puffs when analyses were performed on days 30, 60 and 90 of treatment (marihuana with a Δ^9-THC content of 2.8 percent). No Δ^9-THC was detected in blood after a 30-day recovery period or in blood specimens from placebo-exposed rats. Not shown in Table 5 are negative values of plasma Δ^9-THC on rats exposed to tobacco smoke. In the 20-day subchronic study, mean plasma Δ^9-THC levels of 76, 179 and 303 ng/ml correlated with 4, 8 and 16 puffs, respectively. The slight positive reaction for placebo reflected either residual Δ^9-THC in placebo-extracted marihuana or limits of the RIA procedure, or passive transfer of cannabinoid[105]. After 360 days of exposure to marihuana smoke, mean plasma Δ^9-THC levels were 36, 95 and 175 ng/ml for 5, 8 and 15 puffs, respectively (marihuana Δ^9-THC content was 0.9 percent). A 30-day recovery interval was sufficient to deplete plasma Δ^9-THC. It can also be seen in the data of Table 5 that the concentration of Δ^9-THC in marihuana smoke (as determined in the smoke chamber) correlated with the Δ^9-THC content of the marihuana cigarettes.

The range of circulating Δ^9-THC concentrations achieved in the present animal studies were similar to those reported for human consumers of cannabis and for other animals beside rats (Table 6). Utilizing a variety of analytical procedures, a general range for the human was 40–200 ng/ml and relevant doses in animal studies yielded similar blood values[15, 22, 24, 29, 47, 48, 59, 76, 89, 99, 104].

TABLE 6. CIRCULATING TETRAHYDROCANNABINOL LEVELS IN VARIOUS SPECIES EXPOSED TO MARIHUANA SMOKE OR INTRAVENOUS Δ^9-THC[a]

Species	Cannabinoid form	Dose mg/kg	Number of treatments	Plasma THC[b] (ng/ml)	Literature reference	
					method	reference
Human	Marihuana	0.08–0.17	1–3	20–40	Tracer	2, 21, 100
	Marihuana	0.08–0.16	1	30–200	Mass frag.	71, 80
	Marihuana	0.05–0.08	1-chronic	200–500	RIA[e]	29, 30, 59
Rabbit	Marihuana[c]	40–50	1	800–900	GC	d
	Δ^9-THC	0.08 (i.v.)	1	200–250	RIA[e]	90
Mouse	Δ^9-THC	1 (i.v.)	1	25–50	Tracer	24
	Marihuana	2–4	10	100–200	GC	75
Rat	Marihuana	2–4	30–90	50–200	GC	d
	Marihuana	2–4	10–20	100–300	RIA[e]	74
	Δ^9-THC	5 (i.v.)	1	70–270	Tracer	47

a. In some instances doses were estimated because of lack of information.
b. Mean ranges given because published data were in units that had to be converted to ng/ml.
c. Marihuana was impregnated with pure Δ^9-THC to provide a content of approximately 10 percent Δ^9-THC in reformed marihuana cigarettes.
d. Rosenkrantz, unpublished data.
e. Including cross-reacting compounds.

Another aspect of dosimetry was the manifestation of behavioral changes as proof of cannabinoid entrance into tissues. In a previously reported inhalation study in rats using an estimated Δ^9-THC dose of 4 mg/kg, involuntary vertical jumping ("popcorn response") occurred[19]. The "popcorn" response commenced after the first week of exposure and was exhibited by approximately 70 percent of male rats by the third week. After a transient tolerance developed to this marihuana manifestation, a second peak of the "popcorn" response took place in the majority of male rats after 8 weeks of treatment.

Thereafter, tolerance developed to this behavioral symptom and was nearly complete by the tenth week. It was also observed that the "popcorn" response correlated with delayed lethality.

Fighting aggression also occurred at the Δ^9-THC dose of 4 mg/kg[53]. Among male rats, fighting commenced in the third week of exposure to marihuana smoke and was exhibited by approximately 90 percent of the males between weeks 6 and 7 at which time the onset of tolerance to this symptom occurred. There were 2 phases of fighting aggression among female rats. The first peak was observed in week 6 and involved approximately 30 percent of the females and the second peak was near week 11 and approximately 55 percent of females participated in fighting. Some tolerance developed to aggression between the intervals of peak activity and was complete by week 14 for both sexes.

In the present inhalation studies, the estimated doses of Δ^9-THC used did not evoke the "popcorn" response or fighting aggression (Table 7). However, behavioral aberrations were clearly observed. CNS-inhibition was dose related and prolonged at the higher doses (4–5 mg/kg) given over 90 days. At the higher doses, tolerance developed to CNS-inhibition (mainly manifested as depression of voluntary activity) within 1–4 weeks. CNS-stimulation was elicited by the lower doses and tolerance developed to hypersensitivity and hyperactivity also within a few weeks. That some of the suppression in voluntary

TABLE 7. BEHAVIORAL MANIFESTATIONS OF RATS EXPOSED TO MARIHUANA, PLACEBO OR TOBACCO SMOKE UNDER VARIOUS TREATMENT SCHEDULES[a]

Cigarette type and Δ^9-THC content	Treatment schedule					Major behavioral change[b]	
	Number of puffs	Number of daily expos.	Number of days/wk	Duration of treatment (days)	Estimated Δ^9-THC dose (mg/kg)	CNS-inhibition (%)	CNS-stimulation (%)
Marihuana, 2.8%	8	1	7	90	2.5	50	5
	16	1	7	90	5.0	70	5
Placebo, 0%	10	1	7	90	0	16	1
Tobacco, IRI, 0%	12	1	7	90	0	25	0
Sham treated	0	1	7	90	0	0	0
Marihuana, 2.1%	4	1	6	87	0.7	10	30
	8	1	6	87	2.0	30	36
	16	1	6	87	4.0	75	18
Placebo, 0%	8	1	6	87	0	10	5
Marihuana, 2.1%	4	3	5	30	0.7	35	45
	8	3	5	30	2.0	80	15
Placebo, 0%	8	3	5	30	0	50	10
Sham treated	0	3	5	30	0	0	0
Marihuana, 0.9%	5	1	7	360	0.4	0	5
	8	1	7	360	0.8	45	20
	15	1	7	360	1.5	60	10
Placebo, 0%	12	1	7	360	0	20	0
Sham treated	0	1	7	360	0	0	0

a. All inhalation studies were performed in the same automatic smoking apparatus.
b. Tolerance developed to CNS-inhibition except for continued mild depression at the high dose in 10–14 days after which CNS-stimulation appeared for a few weeks.

activity was in part also due to smoke ingredients other than cannabinoids was certain since both placebo and tobacco smoke caused some CNS-inhibition. The CNS-inhibition induced by placebo and tobacco smoke diminished rapidly compared with marihuana smoke. Of course, it should be recalled that a lesser number of puffs had to be used for placebo and tobacco because of their individual irritating qualities.

MORPHOLOGICAL LUNG CHANGES

The histopathology findings on lungs of rats exposed to marihuana 5 days/week for 14 or 28 days were not related to inhalation of marihuana smoke. The incidence of focal hemorrhages, edema and focal aggregates and severity of these lesions were similar in marihuana and placebo groups. The sacrifice procedure of decapitation contributed to the induction of focal hemorrhages and edema. A similar finding was made for marihuana and placebo groups given 3 daily exposures. However, there was an increase in the number of alveolar macrophages compared with groups exposed once per day. The macrophages were randomly distributed in alveoli. Only rarely was an aggregate of macrophages seen surrounding bronchioles, alveolar ducts or within alveolar spaces beneath the pleura. A rare polymorphonuclear leukocyte was admixed with the macrophages. There was a lesser severity of these morphological changes in sham control lungs but the difference between smoke-exposed groups and sham controls was negligible.

Inhalation of marihuana smoke 6 days/week for 87 days produced a dose-related focal pneumonitis not observed at 27 or 57 days. Foci of various degrees of compacted aggregates of alveolar macrophages were discerned and were semiquantitated by actual counts of such macrophage aggregates. Representative photomicrographs have been published elsewhere(19). The aggregates of alveolar macrophages were admixed with a few polymorphonuclear leukocytes and lymphocytes. Foci of these cells, normally mobilized during inflammatory processes, occurred around respiratory bronchioles, alveolar ducts and within alveolar spaces. Approximately 2, 4 and 10 percent of lungs were involved at Δ^9-THC doses of 0.7, 2 and 4 mg/kg, respectively. Similar changes were seen in approximately 1 percent of lungs of placebo controls. No other pulmonary pathology was discerned. The alveolar macrophages were polygonal with small round to oval nuclei containing single or multiple nucleoli and the chromatin was marginated. The cytoplasm was finely, granular eosinophilic to yellow-brown and occasionally contained fragments of cellular debris. Proliferation of alveolar lining cells was minimal and metaplastic changes were absent in the bronchiolar and alveolar epithelium. Despite a 20-day recovery period, the morphological changes were persistent. No pulmonary lesions were observed in non-sham untreated control rats.

When the exposure schedule was 7 days/week, similar morphological lesions were found in rat lungs exposed to marihuana, placebo or tobacco smoke. The changes were more evident at 90 days of treatment and persisted after a 30-day recovery period. The lesions were slightly more severe in animals exposed to tobacco smoke than marihuana or placebo smoke. In general, the alveolar macrophages were a darker brown from lungs of tobacco-smoked than marihuana-smoked rats. For all groups, the lesions were characterized by a random multifocal distribution with exudation of yellow-brown and foamy pink alveolar macrophages in less than 1 percent of lung tissue. The macrophages, polymorphonuclear leukocytes and other mononuclear cells were found in the alveolar spaces. Varying degrees

of hyperplasia of granular pneumocytes were noted. Interstitial thickening and infiltration of mobilized cells into alveolar walls were striking features of lungs exposed to any type of smoke.

Prolongation of treatment to approximately 360 days of consecutive exposure to marihuana smoke evoked more intense morphological changes in the respiratory system. The changes were dose related, more severe than those seen at 90 or 180 days of exposure, and were clearly differentiated from those elicited by placebo smoke. In addition to the greater accumulation of macrophages in lungs from rats exposed to marihuana smoke, a greater cross-sectional area of the lungs were affected. At Δ^9-THC doses of 0.4, 0.8 and 1.5 mg/kg, 1, 5 and 10 percent of lung sections exhibited inflammatory aberrations. In contrast to placebo-exposed or sham-treated lungs, lungs exposed to marihuana smoke had greater numbers of foamy pink and yellow-brown alveolar macrophages. More neutrophils, mononuclear inflammatory cells and debris were present. Focal hyperplasia of alveolar lining cells and thickening of alveolar walls and pleura occurred. There was an accumulation of amorphous masses of light-blue mucinous material admixed with the macrophages. There were foci of marked architectural disarray with granulomatous inflammation and cholesterol clefts. The presence of cholesterol was demonstrated histochemically. The inflammatory process was also revealed by the accumulation of multinucleated and Langhan's giant cells and focal aggregates of alveolar lipophages and mast cells. Many alveoli were filled with a homogenous pink material. Yellow-brown pigmented macro-

TABLE 8. SEMIQUANTITATIVE MICROSCOPIC COUNTS OF FOCI OF ALVEOLAR MACROPHAGES IN FISCHER RATS EXPOSED TO MARIHUANA OR PLACEBO SMOKE OR SHAM TREATED

Treatment schedule 1/day × days	Estimated Δ^9-THC dose (mg/kg)	Number of foci of alveolar macrophages[a]					
		nos. with focal pneumonitis[b]		range of nos. foci/lung		mean nos. of foci/lungs[c]	
		male	female	male	female	male	female
Non-sham controls	0	0/12		0		0	
Placebo	0	4/8	5/8	1–7	1–5	4	1
Marihuana × 87	0.7	3/8	4/8	2–8	2–5	4	4
Marihuana × 87	2.0	8/8	8/8	10–24	7–59	16	24
Marihuana × 87	4.0	7/7	7/7	6–53	12–90	23	30
Sham treated × 180	0	3/6	0/6	3–10	0–5	6	3
Placebo × 180	0	6/6	11/11	3–13	7–28	6	18
Marihuana × 180	0.4	11/11	12/12	2–13	1–15	5	6
Marihuana × 180	0.8	12/12	12/12	11–80	15–84	38	45
Marihuana × 180	1.5	12/12	11/11	85–201	61–168	119	111
Sham treated × 360	0	2/12	4/12	0–1	1–2	1	1
Placebo × 360	0	8/9	8/8	0–9	1–19	4	11
Marihuana × 360	0.4	12/12	11/11	2–18	5–24	7	14
Marihuana × 360	0.8	11/11	11/11	21–118	17–104	47	54
Marihuana × 360	1.5	26/26	11/11	56–168	57–286	109	119

a. The number of foci were based on a count of single sections from right and left lobes of each lung of each rat.
b. The values represent the number of affected animals over the number evaluated.
c. The values were calculated by dividing the total numbers of foci by the number of affected animals.

phages were also observed in large peribronchial lymphoid aggregates. Representative photomicrographs of these chronic morphological changes induced by marihuana smoke will be presented elsewhere (Fleischman *et al.*, *Toxicol. Appl. Pharmacol.*, in Press).

In an effort to semiquantitate the inflammatory effects, the numbers of foci of yellow-brown alveolar macrophage aggregates were counted in single sections of right and left lobes of both lungs (Table 8). Both the range (severity) of numbers of foci and the mean numbers of foci were dose related. Although focal pneumonitis occurred in the majority of rats exposed to marihuana or placebo smoke, the effect of the former was definitively distinguished from the latter on the basis of macrophages aggregate counts and the presence of cholesterol clefts. Not shown in the data of Table 8, are counts obtained on lungs after recovery periods. In fact, the numbers of macrophage aggregates not only persisted during recovery but increased. It should be mentioned that the numbers of cholesterol clefts also were dose related and both parameters, macrophage aggregates and cholesterol clefts, tended to have a greater frequency in female rat lungs compared with male lungs.

DISCUSSION

To accomplish sufficient credibility for the assessment of the effects of cannabis smoke on lung morphology, great care was exercised in the standardization of smoke exposure. The same automatic smoking machine, adjusted to provide reproducible puff conditions, was utilized in all the inhalation studies. The same rat strain was used throughout and groups of animals exposed to marihuana smoke were compared with several types of control groups: placebo-exposed, tobacco-exposed, sham treated and a few shelf controls. The range of doses of marihuana smoke, based on the concentration of Δ^9-THC in the smoke reaching the animals, were relevant to those consumed by man. This fact was substantiated by documentation of circulating Δ^9-THC levels at different intervals of time during the course of smoke inhalation.

The potential contribution of CO intoxication was monitored by COHb levels and measurement of the latter also substantiated reproducibility of exposure conditions. CO toxicity was minimized by generally using a single daily exposure and by introducing fresh air for one-half of each exposure cycle[26, 102]. Cyanotic signs were essentially absent from marihuana- and tobacco-exposed groups. In contrast, placebo-exposed rats exhibited cyanosis at the high COHb levels which often led to their demise. It has been demonstrated that placebo cigarettes produced greater quantities of CO during pyrolysis. This fact plus the loss of other ingredients during the extraction of cannabinoids make these placebo cigarettes less than desirable for comparative purposes with marihuana. The number of puffs of placebo cigarettes always had to be less than those for marihuana. CO production is known to vary with puff volume, moisture content, cigarette paper porocity and airflow resistance but is independent of puff duration[4, 27, 69].

Acclimatization to any type of smoke considerably improved the survival rate of animals during chronic exposure. However, delayed lethality did occur in marihuana-exposed animals which could not unequivocally be attributed to CO intoxication but would appear to be related to cannabinoid toxicity. In one study, such delayed lethality correlated with adverse behavioral aberrations. There were no deaths in sham-treated groups or shelf controls. In general, tolerance developed to CNS-inhibition and subsequent CNS-

stimulation although behavioral depression was prolonged in the chronic studies. Survivors in all treated groups behaved normally during recovery periods. No signs characteristic of withdrawal symptoms were observed. Growth rates were slightly suppressed at the higher doses but did not contribute to debilitation.

The primary histological changes were in the alveoli and did not appear until at least 87 days of exposure were performed. The morphological alterations increased in severity when exposure to smoke was continued for approximately 180 and 360 days. Of considerable importance was that neither a 20- or 30-day recovery interval was sufficient for reversal of the pulmonary pathology. A time and dose-related focal alveolitis or pneumonitis occurred. Pulmonary irritation progressed from extensive mobilization of alveolar macrophages and foreign body cell inflammation to more pronounced focal proliferative aberrations. After chronic exposure, the most striking morphological lesions were focal granulomatous inflammation and cholesterol clefts.

The presence of cholesterol was histochemically demonstrated[68, 92]. It is speculated that cholesterol and other lipids may play a role in the removal of Δ^9-THC from lungs. The extensive network of alveolar capillaries does provide a source of cholesterol and lipids which may enter the alveolar sacs to be phagocytosed by macrophages with subsequent crystallization[25]. In man accumulation of cholesterol within foamy alveolar macrophages has been observed in a condition of unknown etiology called chronic pneumonitis of cholesterol type[93] and in areas distal to bronchial obstruction[83]. Cholesterol granulomas associated with pulmonary hypertension have also been reported[25]. Tobacco smoke has increased serum cholesterol in man and dog[43, 85]. In rabbits with experimentally produced hypercholesterolemia, a bacterial pneumonia with lung alveoli filled with lipid and cholesterol laden macrophages has been reported[101]. Cholesterol pneumonia has also been experimentally produced in rats instilled intratracheally with antimony trioxide[28].

Chronic respiratory disease was not ascertained and symptoms of emphysema and proliferative inflammatory lesions within larger airways were not observed after exposure to marihuana or placebo smoke. Single cases of alveolar/bronchiolar carcinoma and adenoma were found in 2/26 high-dosed, chronically-treated male rats but such tumors have occasionally been detected in the untreated Fischer rats. Carcinogenicity of both marihuana and tobacco are suspect[62, 65, 88].

The pulmonary pathology evoked by marihuana smoke in rats were similar to those seen in man and animals exposed to tobacco smoke[19, 44, 45, 46, 80, 103]. Accumulation of alveolar macrophages is a common response to pulmonary irritation and may reflect increased mobilization, enhanced cell adherence and decreased macrophage removal by the mucociliary blanket[19]. In dogs macrophage infiltration into the walls of terminal air-passages was common to both marihuana and tobacco smoke but the former initiated more intense bronchiolitis and squamous metaplasia[79]. Both marihuana and tobacco smoke induced aryl hydrocarbon hydroxylase activity in rodent lungs[34, 39, 57, 58, 67, 94]. Both functional and structural changes have been elicited in alveolar macrophages in man and rodents after cannabis use[13, 14, 23, 35, 55]. Tobacco smoke has been shown to effect substrate transport in alveolar macrophages[52]. It would appear that the beneficial effects of marihuana in the treatment of asthma may be offset by the adverse effects reported on lungs[87, 98].

It has been concluded that because of the affinity of cannabinoids for lung tissue, their storage and biotransformation in this organ leads to adverse morphological changes. The

dose-related inflammatory and proliferative lesions became discernible after 90 days of exposure and were irreversible following a 30-day recovery period. Placebo and tobacco smoke had similar, but less intense, changes indicating that smoke particulates and gaseous components contributed to pulmonary irritation. However, in all the inhalation studies on rats, the marihuana-exposed animals could be distinguished on the basis of pulmonary pathology from those animals exposed to placebo or tobacco smoke. This finding indicated that cannabinoids themselves may have a direct undesirable effect on pulmonary function. It is recommended that a two-year inhalation study be performed under the same conditions to ascertain whether the morphological aberrations would intensify. Some animals should be permitted to recover for periods longer than 30 days to establish whether the lesions are reversible. Such a prolonged chronic investigation may also shed further light on the potential carcinogenicity of cannabis.

ACKNOWLEDGEMENT

The cooperation of Dr. M. C. Braude in these investigations is greatly appreciated. Deep gratitude is expressed to Dr. L. Hagopian for Δ^9-THC analyses in smoke, to John R. Baker for performing morphology evaluation and to the staffs of toxicology and histology for competent assistance. Supported by Contract HSM 42-71-79.

REFERENCES

1. ACKERMAN, N. R., The lack of an effect of Δ^9-THC on pulmonary smooth muscle function in the guinea pig. *Toxicol. Appl. Pharmacol.* **41**,321–328 (1977).
2. AGURELL, S. and LEANDER, K., Stability, transfer and absorption of cannabinoid constituents of cannabis (hashish) during smoking. *Acta Pharm. Suecia* **8**,391–402 (1971).
3. AGURELL, S., GUSTAFSSON, B. and HOLSTEDT, B., Quantitation of Δ^1-tetrahydrocannabinol in plasma from cannabis smokers. *J. Pharm. Pharmacol.* **25**,554–558 (1973).
4. Atomic Energy Commission, Inhalation Carcinogenesis. A.E.C. *Symp. Ser.* **18**,1–90 (1970).
5. AZEN, S. P., LINN, W. S., HACKNEY, J. D., JONES, M. P. and SCHOENTGEN, S., A comparison of eight lung function indices in smoking and non-smoking office workers. *Lung* **154**,213–221 (1977).
6. BAKER, J. R. and ROSENKRANTZ, H., A simple method for demonstrating tetrahydrocannabinols in fresh or fixed frozen sections. *J. Histochem. Cytochem.* **20**,827–832 (1972).
7. BAKER, J. R. and ROSENKRANTZ, H., Volumetric instillation of fixatives and inert substances into mouse lungs. *Stain Technol.* **51**,107–113 (1976).
8. BELLVILLE, J. W., GASSER, J. C., MIYAKE, T. and AQLEH, K., Tolerance to the respiratory effects of marihuana in man. *Pharmacol. Exp. Therap.* **197**,326–331 (1976).
9. BINNS, R., BEVEN, J. L., WILTON, L. V. and LUGTON, W. G. D., Inhalation toxicity studies on cigarette smoke. II. Tobacco smoke inhalation dosimetry studies on small laboratory animals. *Toxicology* **6**,197–206 (1976).
10. BURROWS, B., KNUDSON, R. J., CLINE, M. C. and LEBOWITZ, M. D., Quantitative relationships between cigarette smoking and ventilatory function. *Am. J. Resp. D.* **115**,196–205 (1977).
11. CHARI-BITRON, A., Effect of Δ^1-tetrahydrocannabinol on red blood cell membranes and on alveolar macrophages. In "Marihuana: Chemistry, Biochemistry and Cellular Effects." G. G. Nahas, ed., Springer-Verlag, New York, pp. 273–281, 1976.
12. CHOPRA, G. S., Studies on psycho-clinical aspects of long-term marihuana use in 124 cases. *Int. J. Addict.* **8**,1015–1026 (1973).
13. CUTTING, M., WATSON, A., GOODENOUGH, S., SIMMONS, G., LAGUARDA, R. and HUBER, G., The effect of exposure to marihuana smoke on the bacteriocidal activity of pulmonary alveolar macrophages. *Clin. Res.* **22**,501A (1974).
14. CUTTING, M., GOODENOUGH, S., SIMMONS, G., WATSON, A., LAGUARDA, R. and HUBER, G., Marijuana and pulmonary antibacterial defenses: depression of alveolar macrophage function following experimental exposure to marijuana smoke. *Chest* **66**,321–322 (1974).

15. DETRICK, R. and FOLTZ, F., Quantification of Δ^9-tetrahydrocannabinol in body fluids by gas chromatography/chemical ionization mass spectrometry. *Res. Monogr. Ser. Natl. Inst. Drug. Abuse*, DHEW Pub. No. (ADM) 76-339, **7**,88–95 (1976).
16. DOORENBOS, N. J., FETTERMAN, P. S., QUIMBY, M. W. and TURNER, C. E., Cultivation, extraction and analysis of cannabis sativa *L. Ann. N. Y. Acad. Sci.* **191**,3–12 (1971).
17. DUBOWSKI, K. M., Measurement of hemoglobin derivatives. In "Hemaglobin: Its Precursors and Metabolites," Sunderman, F. W. and Sunderman, F. W., Jr., eds., J. B. Lippincott, Philadelphia, pp. 49–60, 1964.
18. FEHR, K. O. and KALANT, H., Analysis of cannabis smoke obtained under different combustion conditions. *Can. J. Physiol. Pharmacol.* **50**,761–767 (1972).
19. FLEISCHMAN, R. W., HAYDEN, D. W., BRAUDE, M. C. and ROSENKRANTZ, H., Chronic marihuana inhalation toxicity in rats. *Toxicol. Appl. Pharmacol.* **34**,467–477 (1975).
20. FREUDENTHAL, R. I., MARTIN, J. and WALL, M. E., Distribution of Δ^9-tetrahydrocannabinol in the mouse. *Brit. J. Pharmacol.* **44**,244–249 (1972).
21. GALANTER, R., WYATT, R. J., LEMBERGER, L., WEINGARTNER, H., VAUGHAN, T. B. and ROTH, W. T., Effects on humans of delta-9-tetrahydrocannabinol administered by smoking. *Science* **176**,934–936 (1972).
22. GARRETT, E. R. and HUNT, C. A., Separation and sensitive assay of THC in biological fluids by HPLC and GLC. *Res. Monogr. Ser.—Natl. Inst. Drug. Abuse*, DHEW Pub. No. (ADM) 76–339, **7**,33–41 (1976).
23. GAUL, C. C. and MELLORS, A., Delta-9-tetrahydrocannabinol and decreased macrophage migration inhibition activity. *Res. Comm. Chem. Pathol. Pharmacol.* **10**,559–564 (1975).
24. GILL, E. W. and LAWRENCE, D. K., Blood and brain levels of Δ^1 tetrahydrocannabinol in mice. The effect of 7-hydroxy-Δ^1-tetrahydrocannabinol. *Biochem. Pharmacol.* **23**,1140–1143 (1974).
25. GLANCY, D. L., FRAZIER, P. D. and ROBERTS, W. C., Pulmonary parenchymal cholesterol-ester granulomas in patients with pulmonary hypertension. *Am. J. Med.* **45**,198–210 (1968).
26. GOLDSMITH, J. R. and LANDAW, S. A., Carbon monoxide and human health. *Science* **162**,1352–1359 (1968).
27. GORI, G. E., Proceedings of the tobacco smoke inhalation workshop on experimental methods in smoking and health research. *DHEW Pub. No. (NIH) 75-906*, pp. 1–83 (1974).
28. GROSS, P., BROWN, J. H. V. and HATCH, T. F., Experimental endogenous lipoid pneumonia. *Am. J. Pathol.* **28**,211–221 (1972).
29. GROSS, S. J. and SOARES, J. R., Separate radioimmune measurements of body fluid Δ^9-THC and 11-nor-9-carboxy-Δ^9-THC. *Res. Monogr. Ser.—Natl. Inst. Drug Abuse, DHEW Pub. No. (ADM) 76-339* **7**,10–14 (1976).
30. GROSS, S. J., SOARES, J. R., WONG, S. L. R. and SCHUSTER, R. E., Marihuana metabolites measured by radioimmune technique. *Nature* **252**,581–582 (1974).
31. HENDERSON, R. L., TENNANT, F. S. and GUERRY, R., Respiratory manifestations of hashish smoking. *Arch. Otolaryng.* **95**,248–251 (1972).
32. HO, B. T., FRITCHIE, G. E., KRALIK, P. M., ENGLERT, L. F., MCISSAC, W. M. and IDANPAAN-HEIKKILA, J. E., Distribution of tritiated-1-Δ^9-tetrahydrocannabinol in rat tissues after inhalation. *J. Pharm. Pharmacol.* **22**, 538–539 (1970).
33. HOFFMANN, D., BRUNNEMANN, K. D., GORI, G. B. and WYNDER, E. L., On the carcinogenicity of marihuana smoke. *Res. Adv. Phytochem.* **9**,63–81 (1975).
34. HOLT, P. G. and KEAST, K., Induction of aryl hydrocarbon hydroxylase in the lungs of mice in response to cigarette smoke. *Experientia* **29**,1004 (1973).
35. HUBER, G. L., SIMMONS, G. A., MCCARTHY, C. R., CUTTING, M. B., LAGUARDA, R. and PEREIRA, W., Depressant effect of marihuana smoke on antibactericidal activity of pulmonary alveolar macrophages. *Chest* **68**,769–773 (1975).
36. JOHNSTONE, R. E., LIEF, P. L., KULP, R. A. and SMITH, T. C., Combination of Δ^9-tetrahydrocannabinol with oxymorphone or pentobarbital. Effects on ventilatory control and cardiovascular dynamics. *Anesthesiology* **42**,674–684 (1975).
37. JONES, L. A. and FOOTE, R. S., Cannabis smoke condensate. Identification of some acids, base and phenols. *J. Argic. Food Chem.* **23**,1129–1131 (1975).
38. KAYMAKCALAN, S., ERCAN, Z. S., and TURKER, R. K., The evidence of the release of prostaglandin-like material from rabbit kidney and guinea pig lung by (−)-trans-delta-9-tetrahydrocannabinol. *J. Pharm. Pharmacol.* **27**,564–568 (1975).
39. KELLEHER, P. C., THANASSI, N. M. and MOEHRING, J. M., Prolyl hydroxylase in pulmonary alveolar macrophages. *Febs Letter* **81**,125–128 (1977).
40. KENDRICK, J., NETTESHEIM, P., GUERIN, M., CATON, J., DALBEY, W., GRIESEMER, R., RUBIN, I. and MADDOX, W., Tobacco smoke inhalation studies in rats. *Toxicol. Appl. Pharmacol.* **37**,557–569 (1976).
41. KENNEDY, J. S. and WADDELL, W. J., Whole-body autoradiography of the pregnant mouse after administration of ^{14}C-Δ^9-THC. *Toxicol. Appl. Pharmacol.* **22**,252–258 (1972).
42. KEPHALAS, T. A., KIBURIS, J., MICHAEL, C. M., MIRAS, C. J. and PAPADAKIS, D. P., Some aspects of cannabis smoke chemistry. In "Marihuana: Chemistry, Biochemistry and Cellular Effects," G. G. Nahas ed., Springer-Verlag, New York, pp. 39–49, 1976.

43. KERSHBAUM, A., BELLET, S. and KHORSANDIAN, R., Elevation of serum cholesterol after administration of nicotine. *Am. Heart J.* **69**,206–210 (1965).
44. KETKAR, M. B., REZNIK, G. and MOHR, U., Pathological alterations in syrian golden hamster lungs after passive exposure to cigarette smoke. *Toxicology* **7**,265–273 (1977).
45. KILBURN, K. H., Effects of tobacco smoke on biological systems. *Scand. J. Resp. Dis. Suppl.* # 9, pp. 63–78 (1974).
46. LARSON, P. S. and SILVETTE, H., Experimental and clinical studies: a comprehensive account of the world literature. In, "Tobacco," Supplement III, Williams and Wilkins Co., Baltimore, Md., pp. 1–798, 1975.
47. LEIGHTY, E. G., Metabolism and distribution of cannabinoids in rats after different methods of administration. *Biochem. Pharmacol.* **22**,1613–1621 (1973).
48. LEMBERGER, L., Tetrahydrocannabinol metabolism in man. *Drug Metab. Dispos.* **1**,461–468 (1973).
49. LERNER, M. and ZEFFERT, J. T., Determination of tetrahydrocannabinol isomers in marijuana and hashish. *Bull. Narcotics* **20**,53–54 (1968).
50. LEUCHTENBERGER, C. and LEUCHTENBERGER, R., Cytological and cytochemical studies of the effects of fresh marihuana cigarette smoke on growth and DNA metabolism of animal and human lung cultures. In "Pharmacology of Marihuana," M. C. Braude and S. Szara, eds., Raven Press, New York, **2**,595–616, 1976.
51. LEUCHTENBERGER, C., LEUCHTENBERGER, R., ZBINDEN, J. and SCHLEH, E., Cytological and cytochemical effects of whole smoke and of the gas vapor phase from marihuana cigarettes on growth and DNA metabolism of cultured mammalian cells. In "Marihuana: Chemistry, Biochemistry and Cellular Effects," G. G. Nahas ed., Springer-Verlag, New York, pp. 243–256, 1976.
52. LOW, R. B. and BULMAN, C. A., Substrate transport by the pulmonary alveolar macrophage: effects of smoke components. *Am. Rev. Resp. Dis.* **116**,423–431 (1977).
53. LUTHRA, Y. K., ROSENKRANTZ, H., HEYMAN, I. A. and BRAUDE, M. C., Differential neurochemistry and temporal pattern in rats treated orally with Δ^9-tetrahydrocannabinol for periods up to six months. *Toxicol. Appl. Pharmacol.* **32**,418–431 (1975).
54. MAGUS, R. D. and HARRIS, L. S., Carcinogenic potential of marihuana smoke condensate. *Fed. Proc.* **30**,279 (1971).
55. MANN, P. E. G., COHEN, A. B., FINLEY, T. N. and LADMAN, A. J., Alveolar macrophages. Structural and functional differences between non-smokers and smokers of marijuana and tobacco. *Lab. Invest.* **25**,111–120 (1971).
56. MANNO, J. E., KIPLINGER, G. F., HAINE, S. E., BENNETT, I. F. and FORNEY, R. B., Comparative effects of smoking marihuana or placebo on human motor and mental performance. *Clin. Pharmacol. Therap.* **11**,808–815 (1970).
57. MARCOTTE, J., SKELTON, F. S., COTE, M. G. and WITSCHI, H., Induction of aryl hydrocarbon hydroxylase in rat lung in marijuana smoke. *Toxicol. Appl. Pharmacol.* **33**,231–245 (1975).
58. MARCOTTE, J. and WITSCHI, H., Induction of pulmonary aryl hydrocarbon hydroxylase by marihuana. *Res. Comm. Chem. Pathol. Pharmacol.* **4**,561–568 (1972).
59. MARKS, V., TEALE, J. D. and KING, L. J., Radioimmunoassay of cannabis products in blood and urine. In "Marihuana: Chemistry, Biochemistry and Cellular Effects." G. G. Nahas ed., Springer-Verlag, New York, pp. 71–85, 1976.
60. MENDELSON, J., BABOR, T., KEUHNLE, J., ROSSI, A., BERNSTEIN, J., MELLO, N. and GREENBERG, I., Behavioral and biologic aspects of marijuana use. *Ann. N.Y. Acad. Sci., Chronic Cannabis Use* **282**,202–204 (1976).
61. MIKES, F. and WASER, P. G., Marihuana components: effects of smoking on Δ^9-tetrahydrocannabinol and cannabidiol. *Science* **172**,1158–1159 (1971).
62. MIZUSAKI, S., OKAMOTO, H., AKIYAMA, A. and FUKUHARA, Y., Relation between chemical constituents of tobacco and mutagenic activity of cigarette smoke condensate. *Mutat. Res.* **48**,319–324 (1977).
63. NAKAZAWA, K. and COSTA, E., Metabolism of Δ^9-tetrahydrocannabinol by lung and liver homogenates of rats treated with methylcholanthrene. *Nature* (London) **234**,48–49 (1971).
64. NETTESHEIM, P., GUERIN, M. R., KENDRIK, J., RUBIN, I., STOKELY, J., CREASIA, D., MADDOX, W. and CATON, J. E., Control and maximization of tobacco smoke dose in chronic animal studies. In "Proceedings of the Tobacco Smoke Inhalation Workshop on Experimental Methods in Smoking and Health Research." *DHEW Pub. No. (NIH) 75-906*. G. B. Gori, ed., pp. 17–26 (1975).
65. NOVOTNY, M., LEE, M. L. and BARTLE, K. D., A possible chemical basis for the higher mutagenicity of marihuana smoke as compared to tobacco smoke. *Experientia* **32**,280–282 (1976).
66. NOVOTNY, M., LEE, M. L., LOW, C. E. and RAYMOND, A., Analysis of marijuana samples from different origins by high-resolution gas liquid chromatography for forensic application. *Anal. Chem.* **48**,24–29 (1976).
67. OKAMATO, T., CHAN, P. C. and SO, B. T., Effect of tobacco, marihuana and benzo(a)-pyrene on aryl hydrocarbon hydroxylase in hamster lung. *Life Sci.* **11**,733–741 (1972).
68. PEARSE, A. G. E., Histochemistry Theoretical and Applied. Little, Brown and Co., Boston, p. 300, 1960.
69. PRIGGE, E., Influence of carbon monoxide on lung growth in young rats. *Toxicol. Letters* **1**,191–194 (1978).
70. QUARLES, W., ELLMAN, G. and JONES, R., Toxicology of marihuana: conditions of conversion of cannabidiol to THC upon smoking. *Clin. Toxicol.* **6**,211–216 (1973).

71. RENAULT, P. F., SCHUSTER, C. R., FREEDMAN, D. X., SIKIC, B., NEBEL DE MELLO, D. and HALARIS, A., Repeat administration of marihuana smoke to humans. *Arch. Gen. Psychiat.* **31**,95–102 (1974).
72. ROSENFELD, J. J., BOWINS, B., ROBERTS, J., PERKINS, J. and MACPHERSON, A. S., Mass fragmentographic assay for Δ^9-tetrahydrocannabinol in plasma. *Anal. Chem.* **46**,2232–2234 (1974).
73. ROSENKRANTZ, H., The immune response and marihuana. In "Marihuana: Chemistry, Biochemistry and Cellular Effects." G. G. Nahas ed., Springer-Verlag, New York, pp. 441–456, 1976.
74. ROSENKRANTZ, H. and BRAUDE, M. C., Acute, subacute and 23-day chronic marihuana inhalation toxicities in the rat. *Toxicol. Appl. Pharmacol.* **28**,428–441 (1974).
75. ROSENKRANTZ, H., FLEISCHMAN, R. W. and BAKER, J. R., Pulmonary pathology in rats exposed to marihuana smoke for one year. *17th Ann. Meet. Sco. Toxicol. Abst. 158*, p. 139 (1978).
76. ROSENKRANTZ, H., FLEISCHMAN, R. W. and BAKER, J. R., Embryotoxicity of marihuana by inhalation. *Fed. Proc.* **37**,737 (1978).
77. ROSENKRANTZ, H., HEYMAN, I. A. and BRAUDE, M. C., Inhalation, parenteral and oral LD 50 values of Δ^9-tetrahydrocannabinol in Fischer rats. *Toxicol. Appl. Pharmacol.* **28**,18–27 (1974).
78. ROSENKRANTZ, H., SPRAGUE, R. A., FLEISCHMAN, R. W. and BRAUDE, M. C., Oral Δ^9-tetrahydrocannabinol toxicity in rats treated for periods of up to six months. *Toxicol. Appl. Pharmacol.* **32**,339–417 (1975).
79. ROY, P. E., MAGNAN-LAPOINTE, F., HUY, N. D. and BOUTET, M., Chronic inhalation of marihuana and tobacco in dogs pulmonary pathology. *Res. Comm. Chem. Pathol. Pharmacol.* **14**,305–317 (1976).
80. RYLANDER, R., Pulmonary cell responses in inhaled cigarette smoke. *Arch. Environ. Health* **29**,329–333 (1974).
81. SHOYAMA, Y., YAMAGUCHI, A., SATO, T., YAMAUCHI, T. and NISHIOKA, I., Cannabis. IV. Smoking test. *Yakugaku Zasshi* **89**,842–845 (1969).
82. SKINNER, R. F., Determination of submicrogram amounts of Δ^9-THC (tetrahydrocannabinol) and its metabolites. *Proc. West. Pharmacol. Soc.* **15**,136–138 (1972).
83. SPENCER, H., Pathology of the Lung (Excluding Pulmonary Tuberculosis). W. B. Saunders Co., Philadelphia, **1**,469–502 (1977).
84. SPRONK, H. J. W. and LOUSBERG, R. J. J. CH., Pyrolysis of cannabidiol. Structure elucidation of a major pyrolytic conversion product. *Experientia* **33**,705–706 (1977).
85. SVACHA, A. J., WESSON, S. C. and WASLIEN, C. I., Effect of EEG intake and tobacco smoking on serum cholesterol. *Fed. Proc.* **33**,690 (1974).
86. TASHKIN, D. P., SHAPIRO, B. J., LEE, Y. E. and HARPER, C. E., Subacute effects of heavy marihuana smoking on pulmonary function in healthy men. *N. Eng. J. Med.* **294**,125–129 (1976).
87. TASHKIN, D. P., SHAPIRO, B. J., RAMAMNNA, L., TAPLIN, G. V., LEE, Y. E. and HARPER, C. E., Chronic effects of heavy marihuana smoking on pulmonary function in healthy young males. In "Pharmacology of Marihuana," M. C. Braude and S. Szara, eds., Raven Press, New York, **1**,291–295, 1976.
88. TAYLER, T. and PIPER, D. W., The carcinogenic effect of cigarette smoke. The effect of cigarette smoke on human gastric mucosal cells in organ culture. *Cancer* **39**,2520–2530 (1977).
89. TEALE, J. D., FORMAN, E. J., KING, L. J. and MARKS, V., Radioimmunoassay of cannabinoids in blood and urine. *Lancet* **2**,533–555 (1974).
90. TENNANT, F. S., PREBLE, M., PRENDERGAST, T. J. and VENTRY, P., Medical manifestations associated with Hashish. *J. Amer. Med. Assoc.* **216**,1965–1969 (1971).
91. THOMPSON, G. R., FLEISCHMAN, R. W., ROSENKRANTZ, H. and BRAUDE, M. C., Oral and intravenous toxicity of Δ^9-tetrahydrocannabinol in rhesus monkeys. *Toxicol. Appl. Pharmacol.* **27**,648–665 (1974).
92. THOMPSON, S. W., "Selected histochemical and histopathological methods," Charles C. Thomas, Springfield, pp. 334–342 and 356–357 (1966).
93. THORNTON, N., OTKEN, L. B. and SELMONOSKY, C. A., Cholesterol pneumonia. *Am. J. Dis. Child* **119**,454–456 (1970).
94. TRAURIG, H. H., *In situ* response of lung macrophages and hydrolase activity to cigarette smoke. *Lab. Invest.* **37**,314–326 (1977).
95. TRUITT, E. B., JR., Biological disposition of tetrahydrocannabinols. *Pharmacol. Rev.* **23**,273–278 (1971).
96. VACHON, L., The smoke in marihuana smoking. *N. Eng. J. Med.* **294**,160–161 (1976).
97. VACHON, L., MATHE, A. A. and WEISSMAN, B., Effect of Δ^9-THC on the catechol amine content of the guinea pig lung. *Res. Comm. Chem. Pathol. Pharmacol.* **13**,345–348 (1976).
98. VACHON, L., MIKUS, P., MORRISSEY, W., FITZGERALD, M. and GAENSLER, E., Bronchial effect of marihuana smoke in asthma. In "Pharmacology of Marihuana," M. C. Braude and S. Szara eds., Raven Press, New York, **2**,777–784, 1976.
99. VALENTINE, J. L., BRYANT, P. J., GUTSHALL, P. L., GAN, O. H. M., THOMPSON, E. D. and NIU, H. C., HPLCS-MS determination of Δ^9-tetrahydrocannabinol in human body samples. *Res. Monogr. Ser.—Natl. Inst. Drug Abuse, DHEW Pub. No. (ADM) 76-339*, **7**,96–106 (1976).
100. VANWENT, G. F., Mutagenicity testing of 3 hallucinogens—LSD psilocybin and delta-9-THC using micronucleus test. *Experientia* **34**,324–325 (1978).

101. WADDELL, W. R., SNIFFEN, R. C. and WHYTEHEAD, L. L., Influence of blood lipid levels on inflammatory response in lung and muscle. *Am. J. Pathol.* **30**,757–769 (1954).
102. WALD, N., IDLE, M. and SMITH, P. G., Carboxyhaemoglobin levels in smokers of filter and plain cigarettes. *The Lancet* **15**,110–112 (1977).
103. WARR, G. A., MARTIN, R. R., HOLLEMAN, C. L. and CRISWELL, B. S., Classification of bronchial lymphocytes from non smokers and smokers. *Am. Rev. Resp. Dis.* **113**,96–100 (1976).
104. WILLINSKY, M. D., KALANT, H., MERESZ, O., ENDRENYI, L. and WOO, N., Distribution and metabolism in vivo of carbon 14-labeled tetrahydrocannabinol in the rat. *Eur. J. Pharmacol.* **27**,106–119 (1974).
105. ZEIDENBERG, P., BOURDON, R. and NAHAS, G. G., Marihuana intoxication by passive inhalation: documentation by detection of urinary metabolites. *Am. J. Psychiat.* **134**,76–77 (1977).

AN EXPERIMENTAL ANIMAL MODEL FOR QUANTIFYING THE BIOLOGIC EFFECTS OF MARIJUANA ON THE DEFENSE SYSTEM OF THE LUNG*

GARY L. HUBER, VAL E. POCHAY, JOHN W. SHEA, WILLIAM C. HINDS, ROBERT R. WEKER, MELVIN W. FIRST, and G. CLINTON SORNBERGER

Department of Medicine, Mount Auburn Hospital, Harvard University, Cambridge, Massachusetts

and

Department of Environmental Health Sciences, Harvard School of Public Health, Boston, Massachusetts

Summary. An animal model was developed as an inhalation bioassay for acute and chronic delivery of tobacco and marijuana smoke in physiologic doses to the lungs of experimental animals. Although generating identical aerosols, the pyrolyzation characteristics of marijuana and tobacco differed considerably. Both products impaired host intrapulmonary antibacterial defenses, with marijuana appearing in comparable dosages to be significantly more toxic to the alveolar bactericidal activity of the lung than tobacco smoke. The effect of both smoking products on mucociliary clearance by the airways, in this model system, appeared to be negligible. Direct biologic comparisons between the effects of the two smoking products must be guarded, however, as dosimetry cannot be precisely equated because of their opposing physiologic effects on the lung.

INTRODUCTION

ALTHOUGH marijuana and its derivatives have been widely used for centuries, their biologic effects on man are not yet fully understood. Following the identification of the psychoactive component of the cannabinoid products by Mechoulam and associates in Israel in 1964 (Gaoni and Mechoulam, 1964), most marijuana-related research focused on better understanding the chemistry, biopsychological influences, and potentially adverse health effects of tetrahydrocannabinol (THC) and related compounds (Braude and Szara, 1976). In contrast, relatively little effort has been expended to define the effects of marijuana on those primary organs by which the user consumes the drug. For centuries, particularly in Eastern cultures, the drug has been used by ingestion. In the past several decades in North America and Western Europe, however, it has been consumed primarily by

* Presented in part to le 2[e] Symposium International sur Marijuana, 7[e] Congress International de Pharmacologie, Reims, France. July 22–23, 1978. Supported in part by Public Health Service grant NIDA-R01-DA00566-05 from the National Institute on Drug Abuse.

smoking, with a resultant exposure of the host to a very large number of pyrolyzed components of marijuana. Thus, the effect of the smoke in marijuana smoking on the consumer must be determined (Vachon, 1976).

When used by smoking, the lung is the primary target organ and the first line of resistance to inhaled marijuana. An understanding of the effects of marijuana smoke on the pulmonary defense system is crucial to identifying any associated potential hazards of smoking. Unfortunately, as long as possession of marijuana and related cannabinoids is illegal, extensive epidemiologic information on their possible relationship to disease in man, including their role in the development of pulmonary disorders, will be difficult to obtain. Under these conditions, it is necessary to develop animal models as bioassays for evaluating some of the biologic effects of marijuana on the lung. Such models must utilize smoke of comparable nature to that generated by human smokers. Although all animal models have certain limitations, their judicious utilization can identify specific hypotheses for further study and eventual clarification in man. The purpose of this communication is to report the development of an inhalation experimental animal bioassay for assessing the biologic effects of marijuana on the defense system of the lung. In that tobacco, like marijuana, is consumed primarily by inhalation of the pyrolyzed product, comparative evaluations between the biologic effects of marijuana and tobacco smoke were made.

Both acute and chronic inhalation studies have been undertaken in our laboratory. In this communication, we will describe the smoke-generating apparatus and inhalation system employed in our studies, the methods of monitoring smoke delivery and retained dosimetry, and one of several bioassays used to quantify the response of the pulmonary defense system following exposure to marijuana and tobacco smoke. More detailed reports of research on the pulmonary alveolar macrophage, the key host defense cell of the lung, and other components of the lung defense network have been or will be published separately.

METHODS

ANIMALS

Pathogen-free, male albino rats of the CD strain (Charles River Breeding Laboratory, Wilmington, Massachusetts), weighing 125 ± 5 gms were used in all experiments. The animals were housed individually, and upon receipt from the supplier were adapted for at least one week prior to study in quarters at constant humidity and temperature. All control and experimental animals were weight-matched at the beginning of the experiments. Food (RMH 3000 Oval Rat Pellets, Charles River Breeding Laboratories, Wilmington, Massachusetts) and water were provided for ad libitum consumption.

ANIMAL EXPOSURES

A general view of our smoke-generating machine is shown in Fig. 1. A more detailed illustration of the cigarette-holding collets, puff chambers, and other components of the smoke delivery system is shown in Fig. 2. The animal exposure rack, (with four of the five

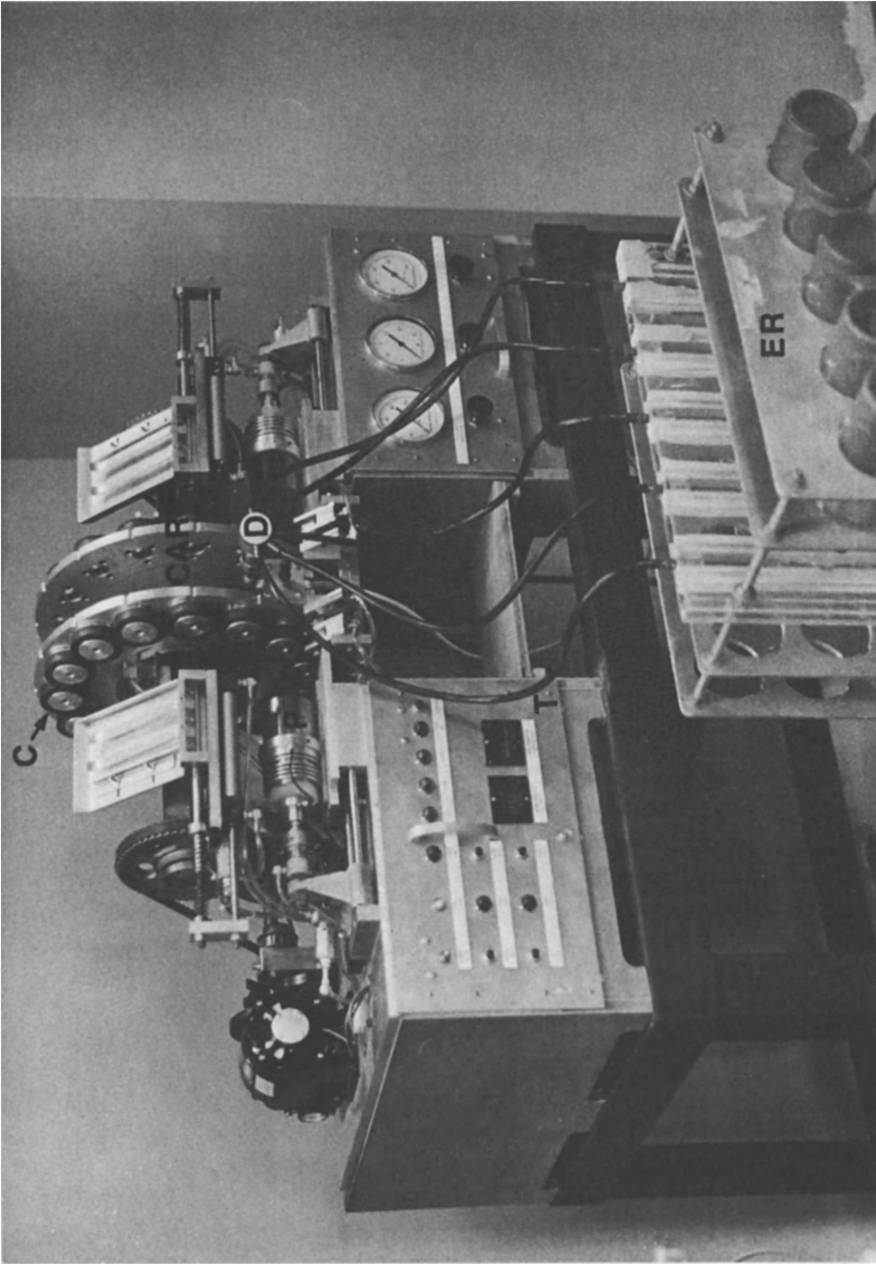


FIG. 1. Multiportal smoke-generating machine and exposure system. The cigarette carousels (CAR) rotate at one revolution per minute, presenting cigarette-holding collets (C) with cigarettes or blank position to the puff chambers (P) with a dwell time of two seconds. Smoke exits via a distribution valve (D), and is connected to the animal rack (ER) by "aged" polyurethane tubing (T).

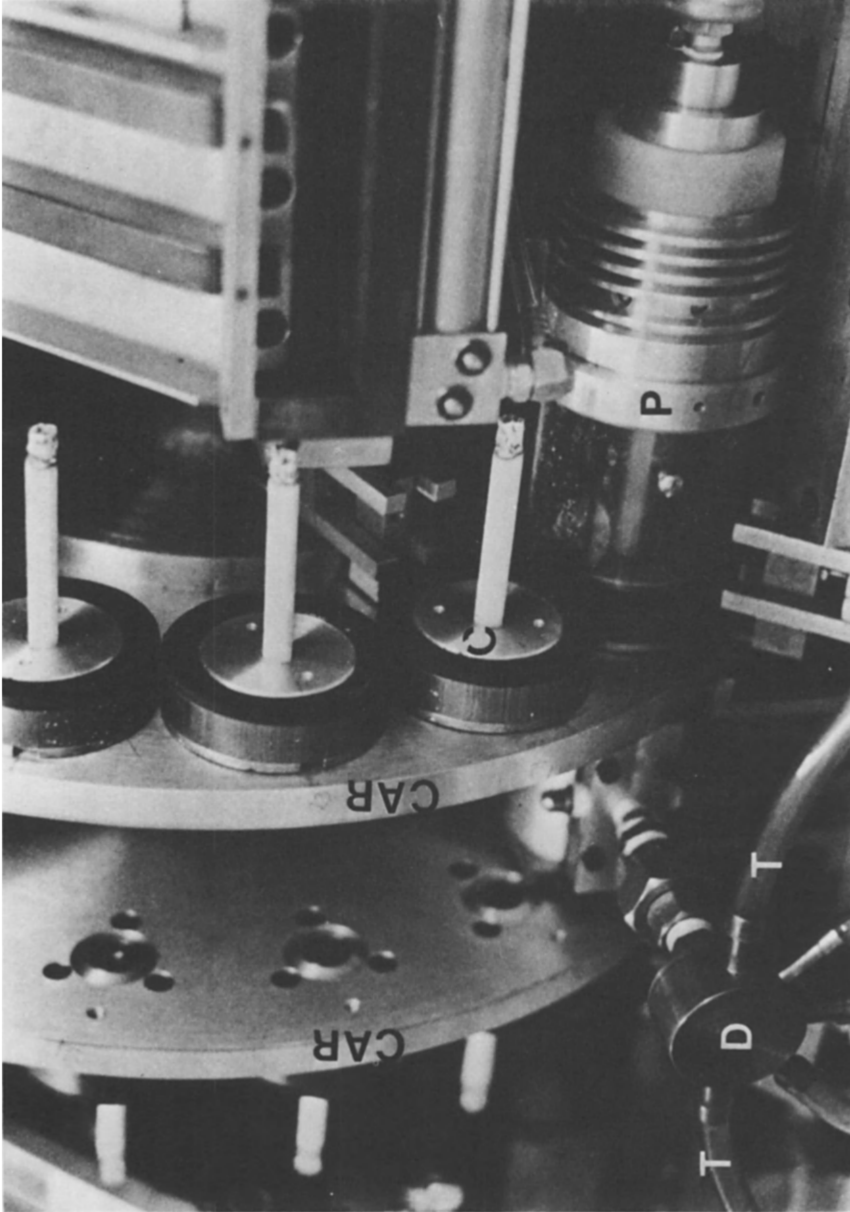


FIG. 2. Detail of smoke-generating machine, The puff chamber (P) delivers a 35 ml standard puff volume. Cigarettes are held by collets (C) arranged on a pair of rotating carousels (arrows). The freshly generated smoke is diluted 1 : 10 with a supply of carbon monoxide-free air (A) and partitioned to the channels of the animal exposure rack by the distribution valve (D). The smoke-generating machine is connected to the exposure rack by polyurethane tubing (T).

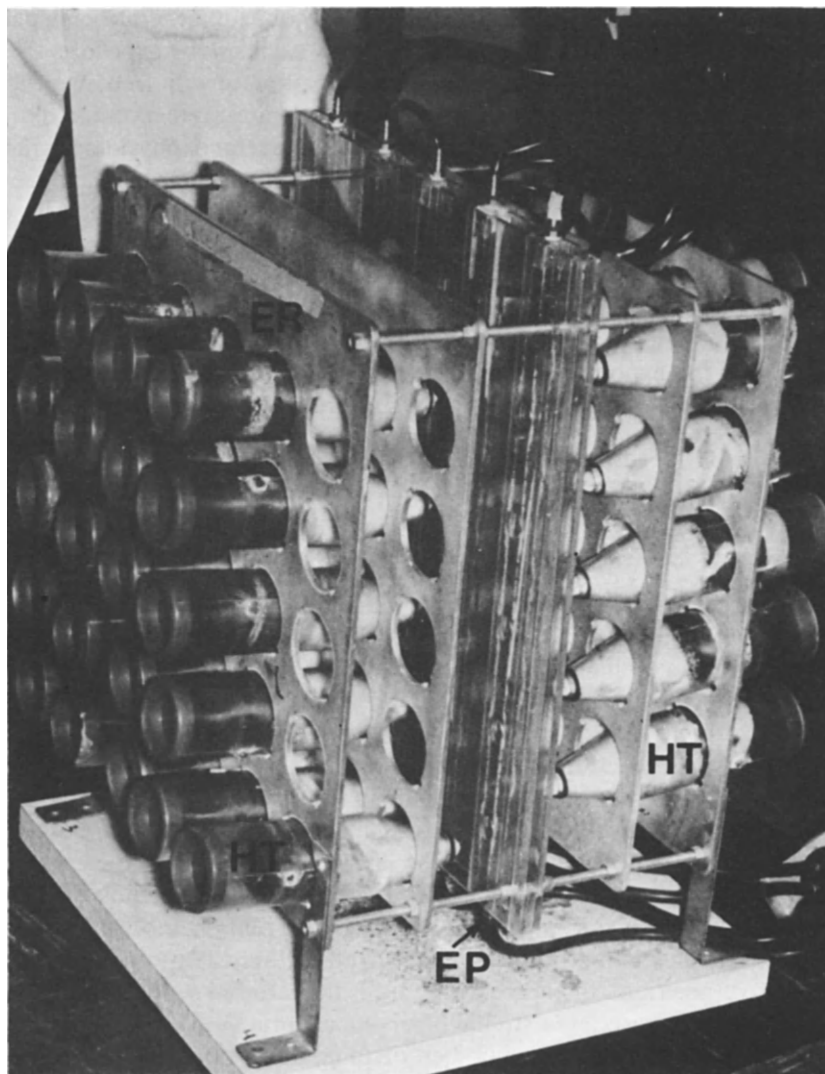


FIG. 3. Animal holding tubes (HT) in the animal exposure rack (ER). Smoke is delivered to the animals in tiers through one of five vertical channels (the channel on the right is empty). Exposure sampling sites are at the distribution valve of the smoke-generating machine and the effluent portal (EP) of each exposure channel.

channels loaded), is shown in Fig. 3. The five-channel exposure rack* is connected to the smoke-generating machine by polyurethane tubing, (Tygon tubing, internal diameter 5/16 inch, R-3603, Norton Plastics and Synthetics, Akron, Ohio) which has been "aged" by repeated passages of smoke to bring its walls into equilibrium with the gas phase vapors during animal exposure. In our studies, freshly generated smoke was stabilized with 10 volumes of air dilution and delivered directly to the exposure channels of the animal

* The animal holding tubes, animal exposure rack, and sampling probe were developed in our laboratories. Plans of these components of our inhalation system can be obtained by writing to the co-authors (First, Hinds) at the Harvard School of Public Health, 677 Huntington Avenue, Boston, Mass., 02115.

exposure rack so that animals inhaled it within 2–4 seconds of generation, depending on their position in the smoke conducting channels. For each smoke exposure, the animals were gently placed in holding tubes* (Fig. 4) and held in place with a sponge rubber plug at the rear of the tube. Animal heart rates and respiratory rates were monitored on selected animals with needle electrodes connected to an amplifier and physiologic monitoring system (MK4 monitor, Narco Bio-Systems, Ridgefield, Connecticut). To reduce the possibility of cross-animal infection, all tubes were thoroughly cleaned between exposure periods. Only the nose of the animal protruded from the holding tube, restricting exposure to smoke within the exposure channel and preventing any significant deposition of smoke on the fur. For all exposures, animals were placed in position in the exposure rack on a random basis. Smoke delivery levels were monitored both before entry into and at the effluent portals of the exposure channels in the animal rack. Additional samples were obtained at selected positions in the animal exposure rack, equivalent in position to the snouts of the animals, using the sampling probe* depicted in Fig. 5.

SMOKE GENERATION

Marijuana or tobacco smoke for all experimental exposures was generated with the 30 port automated smoking machine (Eastern Scientific, Providence, Rhode Island) shown in Fig. 1. The performance characteristics of this smoke-generating apparatus have been reported previously (Schultz and Wagner, 1975). With this system, an intermittent or continuous smoke stream can be generated for animal exposures. In all of our experiments, fresh whole smoke was generated by a reverse puff mechanism employing a square-wave puff-profile. The puff volume was 35 ml, the puff duration was 2 sec, and the puff frequency once per minute for each cigarette in the smoking machine. Draw resistance of the research cigarettes was determined by measuring the pressure drop across the cigarette, using standard techniques (Schultz and Wagner, 1975). Puff-volume of the smoking machine was calibrated routinely with a spirometer, flow through the puff-chambers determined from rotometer readings, and puff-duration checked with a stop watch on a regular basis. By filling consecutive positions in the rotating cigarette carousel with either marijuana or tobacco experimental cigarettes, a stream of smoke of variable duration (two seconds per cigarette) could be produced. The number of puffs obtained from each cigarette was recorded automatically by the machine.

CIGARETTES TESTED

Research marijuana cigarettes, coded as SSC 72991 and containing 0.9 grams of marijuana per 85 mm unit with 2.02% Δ-9-THC content, were obtained through the National Institute on Drug Abuse, Bethesda, Maryland. Research tobacco cigarettes (2R1 reference cigarettes) were obtained from the University of Kentucky Tobacco and Health Research Program, Lexington, Kentucky. All cigarettes were preconditioned for 48 hours at 24°C

* The animal holding tubes, animal exposure rack, and sampling probe were developed in our laboratories. Plans of these components of our inhalation system can be obtained by writing to the co-authors (First, Hinds) at the Harvard School of Public Health, 677 Huntington Avenue, Boston, Mass., 02115.

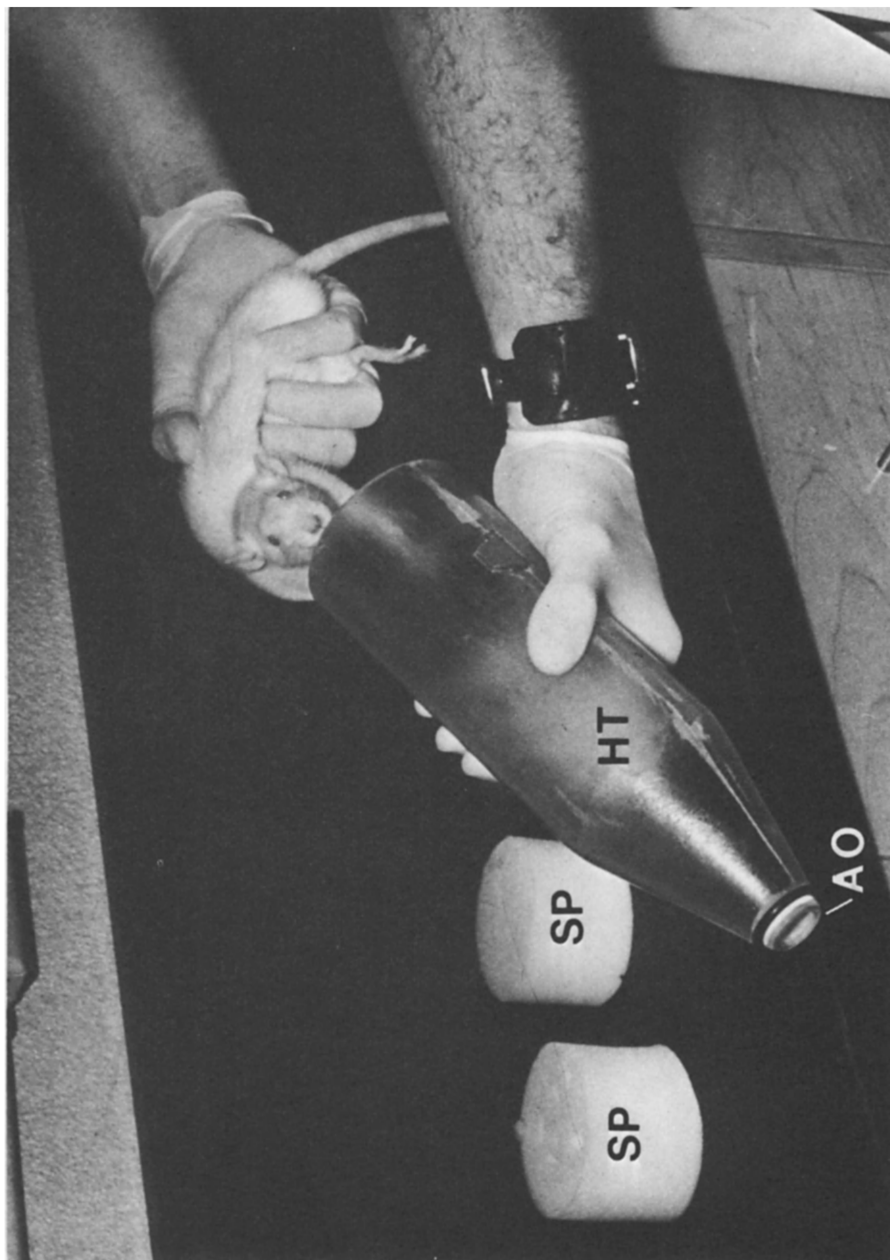


FIG. 4. Animal placement holding tube (HT). Only the animal snout protrudes into the exposure channel through the anterior opening (AO) of the holding tube preventing smoke deposition on the animal fur and subsequent ingestion by preening. Animals are held in place and the rear of the holding tube sealed with sponge rubber plugs (SP).

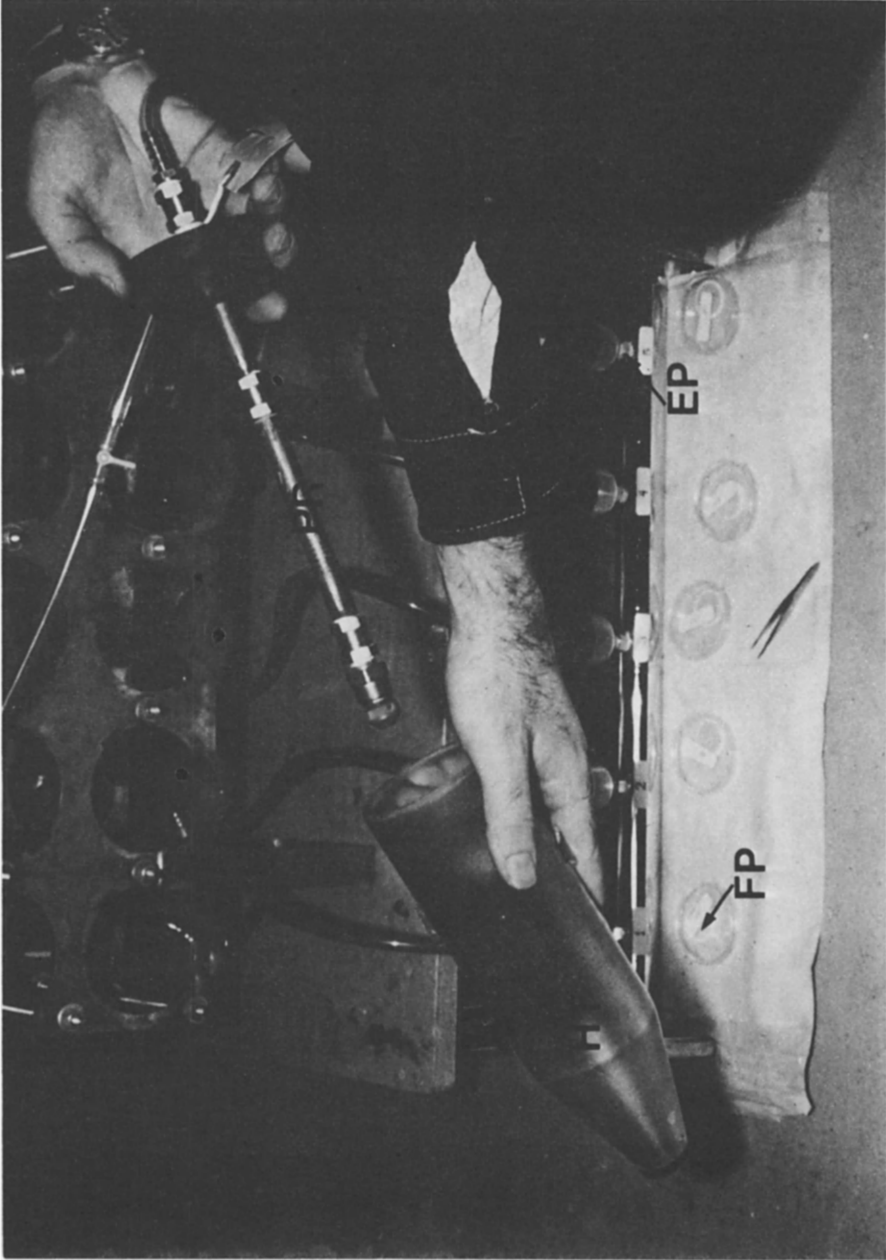


FIG. 5. Probe (PR) for sampling exposure concentrations at variable positions of the animal snouts through the holding tube (HT) in the exposure channel. Additional samples are collected on to filter pads (FP) at the effluent portals (POR) of the animal exposure rack.

and 60% relative humidity prior to use. The cigarettes were weighed and the length measured prior to use. Over the smoke-delivery sequence, individual cigarettes were puffed to a constant length, and the remaining butt length measured.

SMOKE AEROSOL CHARACTERIZATION

Comparative studies on the aerosol particle size for marijuana and tobacco smoke were undertaken. All studies were performed at a dilution of one part smoke to ten parts air, utilizing the 30-port smoking machine under the same conditions of experimental animal exposure. Prior to measurement of aerosol size distributions, an ultraviolet (UV) absorbance spectrum was determined on a weighed sample of marijuana smoke extracted in methanol (Sinclair, 1950). Based on these determinations, a wave length of 260 nm was selected for all measurements of marijuana smoke particulate mass. The same UV wave length was used for measuring the tobacco smoke aerosol, as both materials have a reasonably flat spectrum in this region. Specific particle size distributions were determined, using a concentric aerosol centrifuge that we have previously described (Hinds, 1978). This centrifuge partitions the aerosol distribution into 11 aerodynamic size ranges, with the mass collected in each range measured by UV absorbance. Centrifuge winnowing air contained 10.5% or 21.9% carbon dioxide to suppress Rayleigh-Taylor instability and cloud settling effects at the 1 : 10 smoke dilution (Hinds, 1978). Puffs three through seven of each product, as generated in the 30 port smoking machine, were sampled in a 10 minute exposure sequence, with each sample extracted in 4 ml of methanol within 30 min. Absorbance measurements were made within 4 hrs of sampling. To assure that there were no gross differences in chemical composition for different particle size ranges, UV scans were prepared for $> 1.23\ \mu m$, $0.456–0.515\ \mu m$, and $0.267–0.275\ \mu m$ size distributions.

EXPOSURE AND DOSIMETRY MEASUREMENTS

In this report, exposure and dosimetry measurements were obtained during exposures in which 10 consecutive ports in the rotating carousel were filled with either tobacco or marijuana cigarettes, the standard smoke dosage employed in all of our chronic studies. In selected experiments, marijuana research cigarettes or reference tobacco cigarettes were each laced with 2000 μgm of decachlorobiphenyl (DCBP), an inert water-insoluble chlorinated hydrocarbon tracer of the particulate phase of the smoke (Lewis *et al.*, 1973; Lewis *et al.*, 1972; Guerin and Nettesheim, 1976). The labeled smoke generated from the laced cigarettes was analyzed in both the delivery system (exposure) and in the lungs of the experimental animals (dosimetry). DCBP has no known significant acute absorption from the lung, has no short-term toxicity to mammals, does not react chemically with tobacco or marijuana smoke components in standard pyrolyzation procedures, and on the basis of work in progress by our staff appears to be uniformly distributed in the particulate phase of the smoke.

Total particulate matter (TPM) for each smoking product was analyzed by standard gravimetric techniques (Lewis *et al.*, 1973), and the burning rates and the TPM per puff were calculated. Samples of the air-diluted smoke obtained at various positions in the animal rack were collected at a rate of 200 ml per minute (approximating the minute

volume of the treated animals) onto absolute filters (Phipps and Bird Co., Richmond, Virginia), which were weighed before and after sampling. In addition, continuous samples of TPM entering and leaving the exposure channels were obtained over the course of burning the cigarettes to a constant butt length, and loss of smoke to the channel walls calculated. The resultant value, corrected for puff volume and number of puffs per cigarette, was converted into the average concentration of particulate matter to which the animals were exposed at any given time in the delivery system, expressed as weight per unit volume (μgm/cc) of particulate matter passing through the chamber with each bolus of smoke generated. From these measurements, the exposure concentrations and accumulative particulate deliveries to the experimental animals were calculated for all acute and chronic marijuana and tobacco studies.

DCBP was extracted in hexane from all weighed smoke channel filter paper samples, quantified by gas chromatographic analyses, and expressed as the ratio of DCBP to TPM. DCBP content in the lungs of exposed animals was also analyzed by standard procedures (Lewis *et al.*, 1973). From the ratio of DCBP to total particulate matter measured on the filter samples and from the amount of DCBP tracer present in the whole lung of the animals, at the time of sacrifice, (within 5 min of the final smoke exposure), the amount of retained intrapulmonary total particulate matter, corrected for physical clearance between the time from the initiation of the exposure to the time of sacrifice (Lewis *et al.*, 1973), was calculated for each animal. The data generated were used to estimate an equivalent human consumption. By using three *scaling variables* (body weight, lung weight, or lung volume) to calculate corresponding experimental animal-to-human *scaling factors* (Stahl, 1967), a range of human *marijuana equivalent* or *tobacco equivalent* dosages was then calculated as the product of particulate matter retained by the exposed experimental animal lungs times the corresponding scaling factors.

As an indicator of exposure to the gas phase of the smoking products, carbon monoxide was measured at the distribution valve of the smoke-generating machine. These samples were collected continuously at a rate of approximately 300 ml per minute during the entire exposure sequence, including the lighting puff. Particulate matter was removed by passage through an absolute filter, and the resultant gas phase quantified with an Ecolyzer Model 2100 (Energetic Science, Inc., New York, New York). Gas phase dosimetry, in turn, was determined by analyzing carboxyhemoglobin in arterial blood sampled from the descending aorta of lightly anesthetized smoke-treated animals immediately (within 1–3 min) following one accumulative 10 cigarette exposure, using a CO-Oximeter (Instrumentation Laboratories, Watertown, Massachusetts) fitted with a special grid calibrated for rat hemoglobin. Particulate matter collected from both smoking products was treated with 10 ml of hexane to extract nicotine and the solution analyzed by gas chromatography with a carbowax column and a flame ionization detector (Wagner *et al.*, 1977; Lyerly and Greene, 1976).

BIOASSAYS

Using this animal inhalation model, the biologic effects on the lung following acute and chronic exposures to marijuana smoke are being evaluated by several techniques in our laboratory. As one measure of the integrity of the intact system in the living host, the inactivation of an aerosolized challenge of bacteria within the lung was quantified. The

results of the effects of acute exposures to marijuana smoke on intrapulmonary bacterial inactivation will be included in this report. The effects of chronic exposures to marijuana smoke on this system, as well as our more detailed studies on the metabolism and structure of the alveolar macrophage, will be reported separately. Studies involving chronic exposure to tobacco smoke and alveolar macrophage metabolism (Drath *et al.*, 1978), macrophage structure (Davies *et al.*, 1977; Davies *et al.*, 1978), and antibacterial defenses (Shea and Huber, 1978; Huber *et al.*, 1977a) have been reported previously.

In the studies reported herein, experimental and matched control animals received an intrapulmonary challenge of coagulase positive *Staphylococcus aureus* (FDA-209P, phage type 42D), labelled with a radioisotope of phosphorus (^{32}P, New England Nuclear Corporation, Boston, Massachusetts) by previously described techniques (Green and Goldstein, 1966). The radioisotope is tightly bound to the DNA of the bacteria and serves as a marker of their physical clearance (LaForce *et al.*, 1973). The methods of aerosol inoculation and related analyses have been published (Laurenzi *et al.*, 1964; Goldstein, Green, Seamens, 1970; Huber *et al.*, 1977a). In an initial series of experiments involving groups of 35 control or non-smoke exposed animals, the simultaneous estimation of physical clearance of the inhaled microorganisms and a quantitation of intrapulmonary bacterial *in situ* inactivation or killing were determined at zero, 6, 12, 18, and 24 hours after aerosol inoculation. At each of these time periods, seven randomly selected, aerosol-inoculated animals were sacrificed with intraperitoneal pentobarbital (50 mg/kg). Animals were exsanguinated by aortic transection and their lungs aseptically exposed. The trachea was transected at the sixth cartilage ring above the carina and the thoracic viscera removed en bloc. Nonpulmonary tissue was stripped away and the lungs thoroughly homogenized in nutrient broth. After serial dilution, aliquot homogenates were cultured in triplicate on pour plates. An additional aliquot of the homogenate was solubilized with quaternary ammonium hydroxide and radiophosphorus activity counted in a deep-well scintillation spectrometer. From these experiments, data were obtained to construct physical clearance and intrapulmonary bacterial inactivation curves as a function of time, according to the group mean method previously described (Laurenzi *et al.*, 1964) and the following formulae:

$$\text{Bacteria inactivated } (\%) = \left(1 - \frac{\text{staphylococcal viable } t_x}{\text{staphylococcal viable } t_0}\right) 100\% \qquad \text{(1a)}$$

$$\text{Radioisotope cleared } (\%) = \left(1 - \frac{^{32}\text{P radioactivity } t_x}{^{32}\text{P radioactivity } t_0}\right) 100\% \qquad \text{(1b)}$$

Where t_x is the time at 6, 12, 18, or 24 hrs post-inoculation and t_0, or time zero, is the time at which the aerosol inoculation was completed.

Using the experimental design diagrammatically presented in Fig. 6, animals in the smoke-exposure studies were exposed in groups of 36 to the airborne challenge of bacteria for 30 minutes in an aerosol generator apparatus (Green and Goldstein, 1966; Laurenzi *et al.*, 1964; Goldstein, Green and Seamens, 1970). Immediately following staphylococcal aerosolization, one-third of randomly chosen animals were sacrificed with intraperitoneal pentobarbital (50 mg/kg), and processed as described above. One-half of the remaining animals were set aside as bacterial-inoculated shelf controls. The remainder of the animals were exposed for 10 minutes at the beginning of five consecutive hours to the smoke exposure regimens described below. Control and smoke-exposed animals were sacrificed

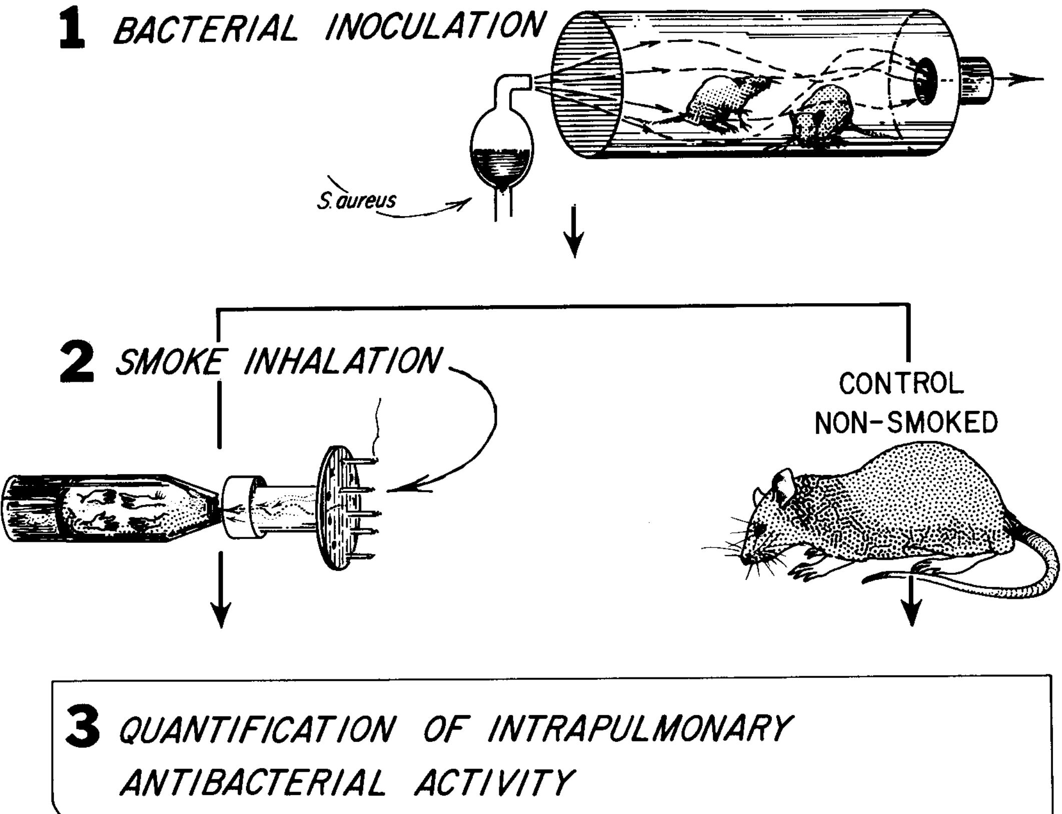


FIG. 6. Diagrammatic representation of the experimental design used in the acute smoke exposure studies. Animals were (1) inoculated with an aerosol of ^{32}P-labeled *S. aureus* and (2) randomly divided into those undergoing experimental smoke inhalation or those serving as non-smoked controls. Intrapulmonary antibacterial activity was quantified (3) by calculation of intrapulmonary bacterial inactivation values and rate constants.

six hours after intrapulmonary bacterial deposition, and radioisotope activity quantified in each homogenate.

The *inactivation* of the aerosolized staphylococcal challenge was assumed, for purposes of the following calculations, to be the result of two processes: (1) *physical clearance* by mucociliary transport from the lung of microorganisms landing on or transferred to the airways and (2) *intrapulmonary bactericidal activity* involving the *in situ* killing of staphylococci, primarily by alveolar macrophages (Kass, Green and Goldstein, 1966; Green, 1968; Huber *et al*., 1977b).

The total bacterial inactivation by the lung and airways was modeled by a negative exponential decay process, given by the following equation:

$$B_t = B_0 e^{-(k_1 + k_2)t} \tag{2}$$

where B_t is the number of viable staphylococci after an elapsed time t, B_0 is the number of staphylococci culturable at time zero, t is the time parameter, k_1 is the rate factor for the process of physical clearance, and k_2 is the rate factor for intrapulmonary bactericidal activity.

The *combined rate factor* for the total bacterial inactivation by the lung and airways was estimated separately in the control and exposed animals by calculating the per cent of the initial deposited amount of bacteria remaining in the lungs at six hours post exposure, using the formula:

$$k_1 + k_2 = -\frac{1}{6}\left(\text{Ln}\left(\frac{\text{viable intrapulmonary staphylococci (6 hr)}}{\text{viable intrapulmonary staphylococci (0 hr)}}\right)\right) \tag{3}$$

The *physical clearance rate factor* of the inhaled bacterial challenge from the lung was estimated separately in the control and exposed animals by calculating the per cent of the initial amount of radioisotope remaining in the lungs at six hours post exposure, using the following formula:

$$k_1 = -\frac{1}{6}\left(\text{Ln}\left(\frac{\text{intrapulmonary isotope activity (6 hr)}}{\text{intrapulmonary isotope activity (0 hr)}}\right)\right). \tag{4}$$

The *rate factor for intrapulmonary bactericidal activity* was estimated by the difference:

$$k_2 = (k_1 + k_2) - k_1 \tag{5}$$

Corresponding to each rate process the "half-life" or $t_{1/2}$ associated with each rate factor was determined, using the formula:

$$t_{1/2}(\text{hours}) = \frac{\text{Ln}(0.5)}{-k} \tag{6}$$

An index of the amount of impairment in the net intrapulmonary bacterial inactivation produced by exposure to either tobacco or marijuana smoke was determined by calculation of the *time impairment index*, where the

$$\text{Time impairment index} = \left(\frac{k_{\text{control}}}{k_{\text{smoke-exposed}}} - 1\right) \times 100\%. \tag{7}$$

The time impairment index is the percentage of additional time required by the smoke-exposed animals to inactivate a given proportion of intrapulmonary bacteria, relative to the time required by the unexposed animals to inactivate the same proportion in six hours.

SMOKE EXPOSURE REGIMENS

In acute exposures, animals were exposed for 10 minutes at the beginning of each of five consecutive hours to the smoke from 3, 6, 10, 15 or 30 marijuana or tobacco cigarettes, providing a delivery period of 6, 12, 20, 30 or 60 seconds of smoke inhalation alternating with 54, 48, 40, 30 or zero seconds of fresh air, respectively, in each exposure sequence. This provided over a five-hour period accumulative exposure to the smoke from 15, 30, 50, 75 or 150 marijuana or tobacco cigarettes. All exposure conditions met the recommended guidelines of the Hunter Committee for experimental smoke delivery to laboratory animals (Hunter, 1975).

In all chronic studies, 10 consecutive ports in the rotating cigarette carousel were loaded with research marijuana or tobacco cigarettes and 20 ports were left empty, providing 8 to 10 cycles of 20 seconds of smoke exposure alternating with 40 seconds of fresh air over an 8 to 10 minute period. Total exposure time was a function of cigarette burning rate as, in as far as possible, all cigarettes were smoked to a consistent butt length. Animals were exposed to smoke in the chronic studies three times each day, seven days per week, in blocks of 30 consecutive days of experimental treatment. The thrice daily exposures used in the chronic smoke inhalation regimen were completed during a three-to-five hour period. During each period of study, in both acute and chronic investigations, the animals were returned to their cages between exposures. Food and water were available for ad libitum consumption at all times during chronic exposure regimens; only water and not food were provided during all of the acute six-hour study periods. Matched shelf-controls, or in some instances sham-smoked animals, were studied in parallel in comparable groups.

STATISTICAL ANALYSES

Each experiment was repeated three times. The arithmetic mean and standard error of the mean were calculated for all data, and comparisons were made using the Student t-test for unpaired data.

RESULTS

The smoke-generating machine used in these studies has proved to be a highly reliable apparatus, operating daily with a minimum of "down time". During more than a two-year period involving a variety of acute and chronic studies in our laboratory, consecutive daily exposures have been maintained without loss of a single exposure sequence. There was very little variation in the results generated from periodic chemical and physical measurements designed to monitor machine performance. The research marijuana smoking cigarettes were of the same length as the reference tobacco cigarettes, but weighed less. They were considerably more variable in packing consistency and friability, and left a gummy residue in the smoke exposure system. The machine had to be cleaned once every day or two following marijuana exposures, as compared to weekly cleanings with tobacco smoking. The data derived from pyrolyzation of both cigarettes are presented in Table 1. The reference 2R1 tobacco research cigarettes consistently burned to a butt length of 22–25 mm with ten 35 ml puffs, and provided a nicotine and particulate delivery compar-

able to that obtained by analytical smoking machines (Benner, 1970). Marijuana cigarettes (total particulate delivery: 12.8 mg TPM), on the other hand, burned more rapidly and delivered on a per cigarette basis approximately half the total particulate matter of tobacco cigarettes (24.0 mg TPM). Nicotine was not detectable in marijuana smoke (detection level < 0.3 mg per cigarette).

TABLE 1. MARIJUANA AND TOBACCO PYROLYZATION

Parameter	Marijuana (NIDA)	Tobacco (2R1)
Cigarette length (mm)	85	85
Cigarette weight (gm)	0.79	1.09
Puffs/cigarette	8.0	10.0
Butt length (mm)	12.0	23.0
Burning rate (mm/min)*	9.1	6.2
Corrected burning rate (mm/min/gm cig)*	11.6	5.7
Total particulate matter (mg)†	12.8	24.0
TPM/puff (mg)	1.6	2.4
DCBP/TPM (in diluted smoke)	0.0036	0.012
Nicotine (mg) per cigarette	ND‡	2.4

* Combined burning rate for mainstream and sidestream smoke generation per minute, corrected for puffs per cigarette.

† Total particulate matter (TPM) in mainstream smoke per cigarette.

‡ ND-nondetectable (limit of detection: 0.3 mg/cigarette.)

The variation in carbon monoxide exposure levels generated from the two products is presented in Table 2. Following an initial puff during which the cigarettes were lighted, there was a small but consistent incremental increase in the amount of carbon monoxide

TABLE 2. VARIATION IN CARBON MONOXIDE EXPOSURE LEVELS

Puff	Marijuana (NIDA)†	Tobacco (2R1)†
Lighting*	830 ± 140	1600 ± 35
1	2000 ± 110	2700 ± 150
2	2400 ± 150	3400 ± 120
3	2200 ± 80	3600 ± 190
4	2400 ± 120	3800 ± 200
5	2900 ± 330	3800 ± 120
6	3000 ± 260	4100 ± 150
7	3000 ± 230	4300 ± 150
8	3000 ± 300	4600 ± 210
9	—	5100 ± 140
10	—	5200 ± 110
Average exposure concentration‡	2600 ± 140 ppm	4100 ± 240 ppm

* Carbon monoxide (ppm) delivered to the animal exposure system during the lighting puff of the cigarette.

† All data expressed as average carbon monoxide concentration (ppm) ± one standard error of the mean for analysis on five individual cigarettes.

‡ Average concentration of carbon monoxide (ppm) generated per puff for each cigarette, excluding the lighting puff.

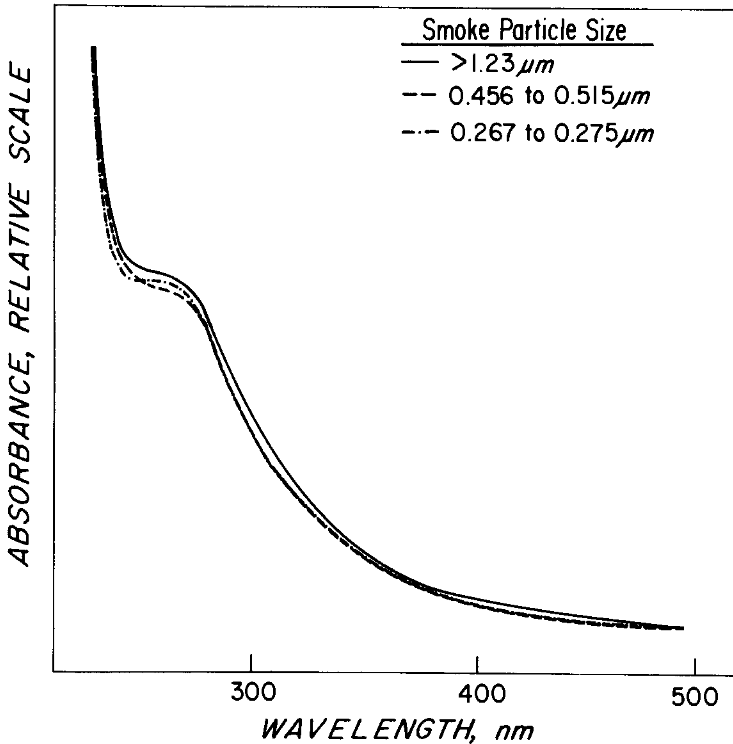


FIG. 7. Absorbance spectra for three particle sizes of marijuana smoke. Absorbance, on a relative scale, is expressed as a function of ultraviolet wavelength, in nm. Absorbances for particle sizes of 0.267–0.275 nm, 0.456–0.515 nm, and greater than 1.23 nm were determined.

released with each successive puff. Although the trend was generally the same with marijuana, the progression was less consistent after the fifth puff. The average exposure concentration of carbon monoxide for tobacco (4100 ppm) was about twice that for marijuana (2600 ppm).

The absorbance spectra for three particle sizes of marijuana smoke, as separated in the aerosol centrifuge, are shown in Fig. 7. The differences in absorbance of TPM at 260 nm, a range where marijuana and tobacco both have a relatively flat spectrum, were negligible, indicating that quantification of the relative absorbance of sizes by photometry introduced no significant error in the results. Log probability plots of the size distribution results for three experiments with marijuana smoke and one experiment with reference tobacco smoke are shown in Fig. 8. The size distribution lines in these figures were fitted to the data visually, with primary emphasis on points in the 10% to 90% range. The mass median aerodynamic equivalent diameters and the geometric standard deviations for marijuana and tobacco smoke are presented in Table 3. These data were derived from the "best-fit" lines of the aerosol measurements (Hinds, 1978). The mass median aerodynamic diameters, corrected for coagulation, are also presented in Table 3. This correction is based on the time delay between smoke generation and measurement, as well as the initial number

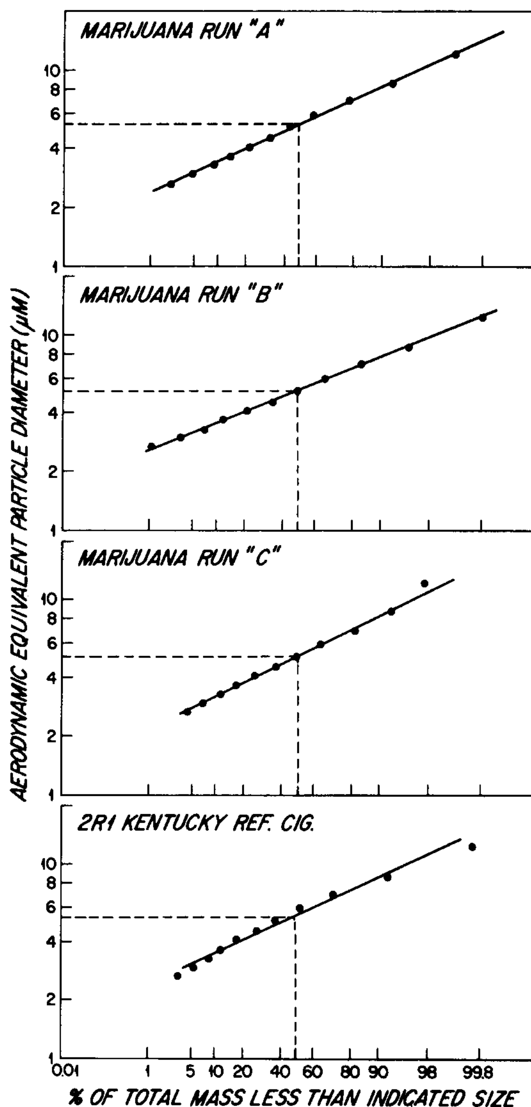


FIG. 8. Composite log probability plots of size distribution results measured with an aerosol centrifuge for marijuana and tobacco smoke generated in the smoking machine employed in all biological studies. Particle size diameters were measured in μm and expressed as the percentage of total mass less than the indicated size. All measurements were determined at a 10 : 1 dilution of the whole smoke in air.

concentrations of particles leaving the cigarettes (Keith and Derrick, 1960). Calculation of the mass median diameters at the moment the smoke was formed was based on simple monodisperse coagulation theory (Hinds, 1978). Virtually identical results were obtained on analyses of marijuana and tobacco smoke.

The data derived from exposure and dosimetry analyses are summarized in Table 4. The average concentration of particulate matter in the exposure channels for each puff was about 50% higher for tobacco (6.2 μgm/cc) than for marijuana (4.1 μgm/cc). The total exposure of animals to marijuana total particulate matter was approximately half that of

TABLE 3. SMOKE AEROSOL MEASUREMENTS

Measurement	Marijuana (NIDA)	Tobacco (2R1)
Mass median aerodynamic diameter*	0.53 μm	0.56 μm
Corrected aerodynamic diameter†	0.48 μm	0.50 μm
Geometric standard deviation	1.41	1.41

* Mass median aerodynamic diameter obtained from probability plots (Fig. 8).
† Mass median aerodynamic diameter corrected for coagulation to time zero.

tobacco. In the absence of animals there was an average loss of approximately 3% (range: 2%–5%) of the particulate matter of each smoking product to the walls of the delivery tubing and the channels in the exposure rack. Although the same amount of tracer was added to each cigarette, the ratio of decachlorobiphenyl to total particulate matter (DCBP/TPM in Table 1) in the pyrolyzed product was quite different. On the average, approximately twice as much tobacco particulate matter was recoverable from the lungs of experimental animals, compared to marijuana (Table 4). Less than 5% or so of the whole smoke delivered to the exposure system was retained by the exposed animals; there was no significant differential as a function of animal placement in the exposure rack. The DCBP tracer was not recoverable from the trachea. Both tracer and staphylococcal deposition per lobe of lung were proportional to the weight of the lobe. Carboxyhemoglobin was not detectable in the blood of shelf controls at a significant level (COHb $< 0.05\%$). There was a mean carboxyhemoglobin concentration of $5.6\% \pm 0.6\%$ in animals exposed to whole marijuana smoke, and $4.9\% \pm 0.5\%$ in animals exposed to whole tobacco smoke (Table 4).

TABLE 4. EXPOSURE AND DOSIMETRY ANALYSES

Smoke Exposure Levels	Marijuana (NIDA)	Tobacco (2R1)
Carbon monoxide (ppm)*	2600 ± 140	4100 ± 240
TPM per unit volume (μgm/cc)†	4.1	6.2
Animal Dosage Retained		
Carboxyhemoglobin (% COHb)	5.6 ± 0.6	4.9 ± 0.5
TPM per lung (μgm/lung)‡	157.0 ± 11.0	343.0 ± 12.9

* Average CO (ppm) concentration in the smoke during the intermittent exposure periods.
† Concentration of total particulate matter (TPM) in the animal exposure rack with the passage of smoke during intermittent exposure periods.
‡ Amount of total particulate matter (TPM) retained per lung for one accumulative 10 cigarette exposure, based on recovery of DCBP tracer and calculation of equivalent weight of TPM from ratio of DCBP to TPM (Table 1).

The extrapolation of lung deposition equivalents is presented in Table 5. Values for human and rat body weights, lung weights, and total lung capacity were obtained from the literature (Stahl, 1967). By volume displacement, our rat lungs had a total lung capacity of 8.5 ml (unpublished data), compared to the reported value of 5.9 ml (Stahl, 1967). Scaling factors calculated from published data were comparable for comparisons of body weight (560) and lung weight (526), and higher when lung volumes were used (819). If the total

TABLE 5. EXTRAPOLATION OF OBSERVED ANIMAL LUNG DEPOSITION VALUES TO HUMAN EQUIVALENTS

Scaling Variable	Scaling Factor*	Marijuana Equivalent†	Tobacco Equivalent†	Cigarette Equivalents‡
Weight calculations				
Body weight (kg)	70/0.125 = 560	264	588	22.0
Lung weight (gm)	758/1.44 = 526	248	552	20.7
Volume calculations				
Lung capacity (ml)‖	4830/5.9 = 819	386	843	31.6
Lung capacity (ml)§	4830/8.5 = 568	277	585	21.9

* Reference values (Stahl, 1967), expressed as the man to animal ratio.
† Total particulate matter deposition equivalent in man (mg TPM per lung per day).
‡ Assuming a total particulate delivery of 28.8 mg per cigarette to the human consumer, with a 70% inhalation retention value, the animal tobacco equivalent was converted to cigarette equivalents (comparable data could not be calculated for marijuana because of lack of data in human consumers).
‖ Literature value (Stahl, 1976).
§ Average measured value of 125 gm rat lung in our laboratory (Huber, unpublished data).

lung capacity measured in our animals (8.5 ml) was used instead of the published value (5.9 ml), the scaling factor for volume comparisons (568) is comparable to scaling factors developed from weight comparisons. Using a reported value of 38.2 mg of total particulate delivery, and as assumed for use by man, for 10 puffs of the tobacco reference cigarette, and assuming a retention of 70% of the inhaled particulate matter within the lungs of man, based on measured smoke size (Hatch and Gross, 1964) we calculated a retention of 26.7 mg of particulate matter, to the human tobacco smoker, for each cigarette consumed (Benner, 1970). The human tobacco equivalents, as presented in Table 6, then can be converted by comparison to equivalent cigarettes consumed by man. In that human marijuana dosimetry and relative retention values are not known, comparable calculations

TABLE 6. INTRAPULMONARY BACTERIAL INACTIVATION

	Controls	Marijuana (NIDA)	Tobacco (2R1)
Physical Clearance			
Radioisotope cleared (%)	17.6 ± 7.0	11.1 ± 8.0	17.0 ± 4.8
$t_{1/2}$ (hrs)	20.3	31.4	22.4
Bactericidal Activity			
Bacteria killed (%)*	64.7	41.0	55.3
$t_{1/2}$ (hrs)	3.5	7.5	4.3
Net Bacterial Inactivation			
Bacteria inactivated (%)	75.2 ± 3.4	49.5 ± 3.0	68.0% ± 3.1
$t_{1/2}$ (hrs)	2.95	6.09	3.6
Time impairment index (%)	—	106	22

* Calculated as the proportion of the combined rate factor due to the corresponding rate process, in reference to the percent of initial bacteria deposited.

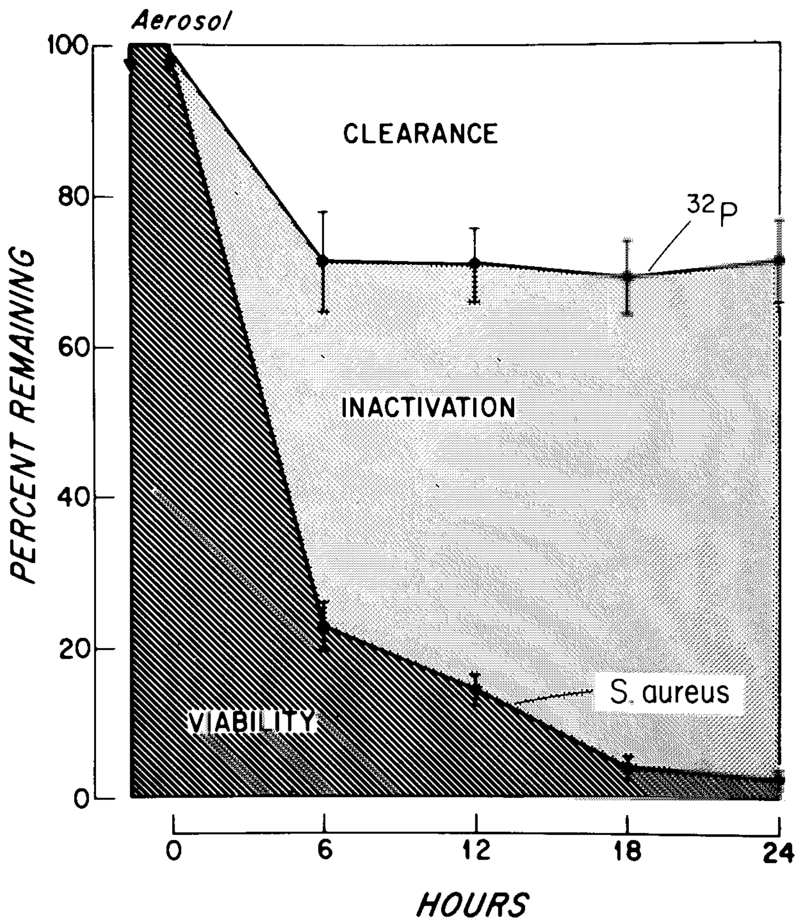


FIG. 9. Inactivation of *S. aureus* by rat lungs, with the percent of viable bacteria and radiophosphorus activity remaining in the lung expressed as a function of time (hours). The rapid decline in intrapulmonary bacterial viability, with only a relatively small change in isotope clearance, indicates that bacterial inactivation is primarily due to *in situ* killing deep within the lung rather than to physical removal.

could not be performed for the research marijuana cigarettes or in reference to the more commonly employed "joints" or "reefers."

As shown in Fig. 9, there was a more rapid exponential decline in the percent staphylococci that could be cultured from the lungs of control animals. In contrast, after an initial small decline in isotope clearance (presumably due to removal of those organisms landing on the airways), there was a less rapid change in radiophosphorus activity. On the basis of these and other related studies in our laboratory, comparative inactivation times of zero and 6 hrs were selected for comparative analyses in the smoke-exposure studies.

The dose-related effect of acute experimental exposure to marijuana smoke is presented in Fig. 10, and the effect of comparable exposures to tobacco smoke are shown in Fig. 11. In these graphs, the calculated individual exposure concentrations or accumulative deliveries were expressed as the concentration of marijuana or tobacco smoke particulate matter (μgm/cc) delivered to the exposure chamber with each bolus of smoke times the seconds of consecutive exposure each minute (2 sec/puff) times the number of puffs per

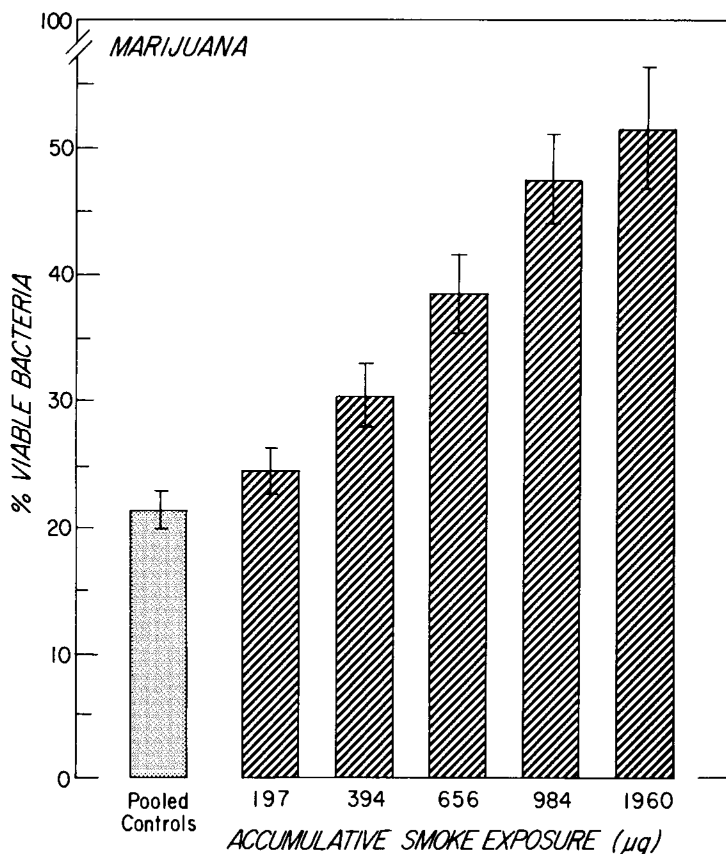


FIG. 10. The effect of whole marijuana smoke on intrapulmonary bacterial inactivation at six hours post-inoculation in the intact animal. Accumulative smoke exposures (µgm) were calculated for smoke delivered from the pyrolyzation of 3, 6, 10, 15, or 30 cigarettes in the cigarette carousel.

cigarette (8 puff/cig for marijuana and 10 puffs/cig for tobacco) for 3, 6, 10, 15, or 30 marijuana or tobacco cigarettes. Acute exposure to increasing concentrations of marijuana smoke induced a dose-dependent depression of intrapulmonary antibacterial defenses, with an apparent maximum or plateau effect associated with exposure to a concentration of 2000 µgm in the delivery system. Higher concentrations could not be generated without increasing acute animal mortality. In general, similar results were obtained with exposure to tobacco smoke, except that in the lower exposure levels a stimulatory effect on the antibacterial defenses of the lung appeared to occur and the threshold of the dose-related response was higher (Fig. 11).

Intrapulmonary bacterial inactivation indices derived from animals exposed to an accumulative concentration of marijuana (984 µgm) and tobacco (1900 µgm) smoke at levels high enough to impair pulmonary antistaphylococcal defenses (Figs. 10 and 11) without significant mortality were calculated, and are summarized in Table 6. Control animals cleared, as determined by quantification of radioisotope activity remaining in the lung, 17.6% ± 7.0% of the initially deposited isotope-labeled staphylococci over the 6 hr period of evaluation, representing a half-life for physical clearance of 20.3 hours. Physical clearance of the aerosolized bacterial challenge was not significantly altered by acute exposure

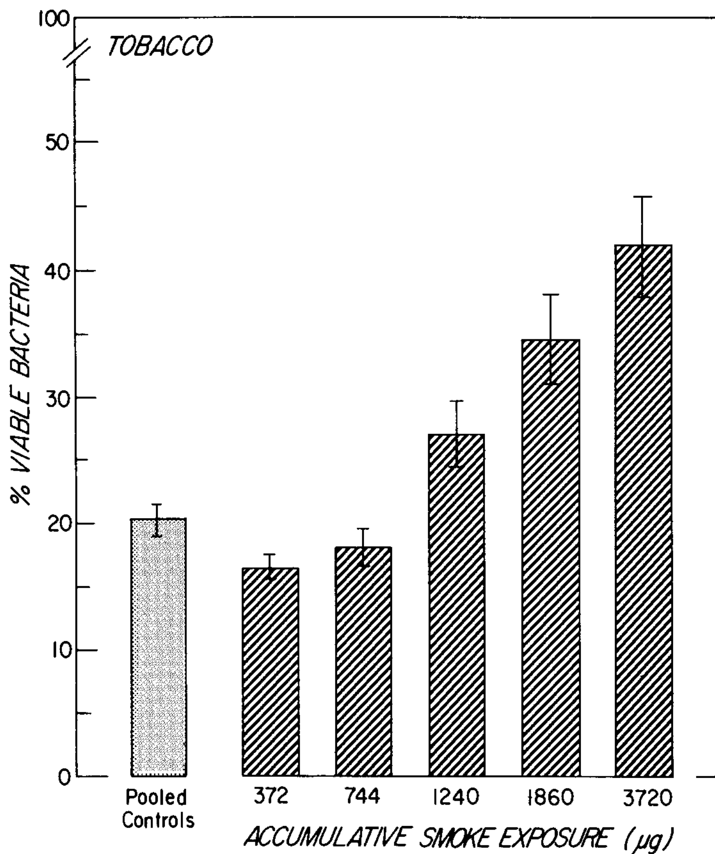


FIG. 11. The effect of whole tobacco smoke on intrapulmonary bacterial inactivation at six hours post-inoculation in the intact animal. Accumulative smoke exposures (μgm) were calculated for smoke delivered from the pyrolyzation of 3, 6, 10, 15, or 30 cigarettes in the cigarette carousel.

to smoke from either product, with 11.1% ± 8.0% ($t_{1/2}$ = 31.4 hrs) of the isotope activity cleared from the lungs of marijuana treated animals and 17.0% ± 4.8% ($t_{1/2}$ = 22.4 hrs) radioactivity removed after exposure to tobacco smoke. Although there was a trend towards reduced clearance following exposure to marijuana smoke, when compared to controls the difference in these values was not statistically significant.

Control animals inactivated 75.2% ± 3.4% of the inhaled viable organisms over the 6 hr period of study. Sham-treated animals did not differ significantly from control values. This inactivation is equivalent to a net half-life for staphylococcal survival of 3.0 hrs, incorporating both the processes of physical clearance and intrapulmonary bactericidal activity. When the rate of physical clearance, as estimated by radioisotope remaining, was subtracted from the total inactivation rate, the half-life for staphylococcal survival in non-exposed animals due to intrapulmonary bactericidal activity alone was 3.5 hrs. In like manner, the half-life for bacterial survival due to intrapulmonary staphylococcal killing in animals exposed to marijuana smoke was 7.5 hrs ($P < 0.01$), while the half-life for staphylococcal survival in the lungs of animals exposed acutely to tobacco was 4.3 hrs ($P < 0.01$). A comparison of the effects of exposure to either marijuana or tobacco smoke using the time impairment index resulted in an increase in the percentage of addition time of 106%

and 22% for total intrapulmonary bacterial inactivation associated with exposure to marijuana and tobacco smoke, respectively. Restricting the impairment index to *in situ* bactericidal activity resulted in similar impairment indices of 117% and 24% for exposure to marijuana and tobacco respectively.

There was no animal mortality in acute studies at exposure levels at or less than 1900 μgm of tobacco (15 cigarettes in the smoke-generating machine) or 980 μgm of marijuana (Figs. 10 and 11). When animals were exposed to continuous smoke from 30 cigarettes (2000 μgm marijuana and 3700 μgm tobacco accumulative exposures), 43% and 58% mortality rates were encountered for marijuana and tobacco exposure treatments, respectively. Average heart rates were 290 beats/min in marijuana exposed animals and 96 beats/min with exposure to tobacco smoke. Respiratory rates were comparably depressed in animals exposed acutely to marijuana and tobacco; with chronic exposure, adapted responses developed.

Animal mortality in our chronic studies ranged from approximately 10% or less for periods of exposure of 30 consecutive days up to about 15% accumulative mortality over six months of daily experimental treatment. Although not always determinable, the cause of death was usually related to a specific traumatic event associated with a smoke-exposure sequence. In the chronic studies, which will be reported in detail independently, controls gained 342% their initial body weight over an initial one-month exposure period. Animals exposed to marijuana or tobacco smoke weighed approximately 80% ($P < 0.001$) as much as shelf-matched controls of the same age, and there was no significant difference between the effect of tobacco and marijuana on weight gain.

DISCUSSION

The data presented support the concept that the smoke generating machine used in these studies is a reliable system for experimentally exposing animals under relatively stress-free conditions to physiologic concentrations of marijuana and tobacco smoke. This apparatus, which was developed by industry (Schultz and Wagner, 1975), is not widely used, presumably because of its very high price. Nevertheless, its high cost is justified because daily reproducibility of performance in smoke generation is crucial to ongoing investigations evaluating smoking products in a comparative manner. Delivery time from smoke generation to deposition within the animal lungs in our system, which is also important, was comparable to that encountered in human smokers.

The quality of the marijuana cigarettes that we have received for our research program has varied significantly. As reflected in the cigarette weights, the marijuana was not as tightly packed as tobacco. Preconditioning the cigarettes at standard conditions of temperature and relative humidity was essential in order to obtain reproducible information on their pyrolyzation characteristics. We gravimetrically measured total particulate matter as a monitor of the aerosol delivery of both smoking products and carbon monoxide as an indicator of the water-insoluble gas phase of the smoke. Our results indicate that the marijuana research cigarettes burn more rapidly and more efficiently than the reference tobacco cigarette, generating on a total per cigarette or an individual puff basis approximately half the total particulate and carbon monoxide delivery. The more rapid burning rate for marijuana was also reflected by an eight-puff delivery per cigarette, down to a 12 mm butt length, as compared to ten puffs to a 23 mm butt length for tobacco cigarettes

of equivalent unlit length. As expected, no nicotine was demonstrable in marijuana smoke. The nicotine yield from the reference tobacco was similar to published chemical smoke analyses (Benner, 1970). The striking differences in the ratio of the chlorinated hydrocarbon tracer recoverable from the two smoking products is presumably due to the differences in burning characteristics, with more tracer being destroyed during pyrolyzation as well as lost to the sidestream smoke from the marijuana cigarettes.

The aerosols resulting from marijuana and tobacco pyrolyzation in our smoke-generating machine were essentially identical, in spite of the very significant differences in burning characteristics, particulate yield, and carbon monoxide release. Addition of carbon dioxide to the winnowing air in the aerosol centrifuge suppressed Rayleigh-Taylor instability and cloud settling effects at the dilutions used in our exposures. By using two densities of winnowing air, the potential for incorporating any artifacts in the aerosol measurements was very slight. Both aerosols were comparable in nature to those generated by human tobacco smokers (Hatch and Gross, 1964; Hinds, 1978). Thus, it is unlikely, on the basis of our data, that the enhanced tendency of marijuana smoke to coat the conducting passageways of our exposure system is due to any difference in aerosol behavior, as the very slight variation in the results presented for the two products is within the range of analytical error. Furthermore, it is unlikely that there would be any differences in regional deposition of the marijuana and tobacco smoke aerosols within the respiratory system due to particulate size distribution, if the pulmonary physiologic response to both products was identical.

For the bolus of smoke generated from individual puffs, tobacco cigarette smoking in our system provide an exposure level (6.2 μgm/cc) approximately 50% greater than that developed by marijuana smoke (4.1 μgm/cc). When corrected for total number of puffs generated from one marijuana (8 puffs) and one tobacco (10 puffs) cigarette, however, the total amount of marijuana particulate exposure in the delivery channels to the animals was 53% that of the exposure level delivered from tobacco cigarettes. Comparably, the net total amount of gas phase exposure for marijuana, as indicated by the amount of carbon monoxide generated from both products and delivered to the animal exposure channels, also was just over 50% of that delivered from tobacco. In our past experience, smoke particulate retention by the respiratory systems of acutely exposed animals has had a high correlation with carboxyhemoglobin levels, and both intrapulmonary particulate deposition and carboxyhemoglobin concentration have reflected the amount of total particulate matter and carbon dioxide delivered in the smoke exposure racks (Huber *et al.*, 1978c). In that the physical characteristics of the smoke aerosols were essentially identical, a relative difference in deposition and retention of particulate matter within the lungs of the animals exposed to marijuana and tobacco would be anticipated, presuming respiratory volumes and rates, as well as airway resistance to flow, were the same following exposure to the two products. For similar reasons, comparable relative differences in carboxyhemoglobin, reflecting the different exposure levels to carbon monoxide with the two smoking products, also would be anticipated. This was not the case, however. Although, as expected, intrapulmonary particulate retention for tobacco was twice that of marijuana, carboxyhemoglobin levels were essentially identical in the two groups. This implies that respiratory rates and/or volumes, as well as possibly airway resistance, were strikingly different following experimental exposure to marijuana and tobacco. This is not surprising, since marijuana is a potent bronchodilator (Vachon *et al.*, 1973; Tashkin, Shapiro, Frank, 1974; Vachon *et al.*, 1976; Tashkin, Shapiro, Frank, 1976) and tobacco is potentially a pulmonary irritant

and bronchoconstrictor (Costello *et al.*, 1975). The cardiopulmonary effects of marijuana have been reviewed recently (Mahajan, Huber, 1978). Differences in ventilation to perfusion ratios between the two smoke-exposed groups are also likely as marijuana can cause a tachycardia (Hardman, Hosko, 1976) and the nicotine in tobacco a bradycardia (Pradhan *et al.*, 1967).

On the basis of particulate retention, we have developed dose-equivalent extrapolations between our experimental animals and man. Reasonable comparisons can be developed for tobacco. Our data would suggest an animal exposure equivalent to about one pack per day of unfiltered high "tar" tobacco cigarettes for each 10 cigarette exposure cycle, although direct extrapolations cannot be made between animal species without significant reservation (Binns *et al.*, 1976a; Binns *et al.*, 1976b; Binns, 1977). Although comparable extrapolations cannot be made for marijuana because smoke particulate delivery and retention for this agent have not as yet been quantified in man, the dose-equivalence in our studies was considerably less.

Pulmonary antibacterial defenses are comprised of a complex system of interacting components (Green, 1968; Huber, Johanson, LaForce, 1977). As one line of host defenses, particles and organisms landing on the airways are rapidly transported out of the lung by the mucociliary stream. Our data developed following an intrapulmonary challenge of aerosolized phosphorus-labeled staphylococci imply that there was a trend towards less radioisotope clearance from the airways of marijuana-exposed animals than from controls or following exposure to tobacco smoke, perhaps due either to differences in site of deposition or relative differences in toxicity of the two smoking products.

The pulmonary alveolar-macrophage is thought to be the cellular component responsible for bactericidal activity in the lung (Kass, Green, Goldstein, 1966; Huber *et al.*, 1977). Although the thresholds of response were different, exposure to both marijuana and tobacco smoke impaired inactivation of the staphylococal challenge in a dose-dependent manner. Intrapulmonary bactericidal activity was significantly more impaired by marijuana smoke than by tobacco. These differences might be accentuated even further if the exposure conditions were equalized for particulate delivery. Modeling of the response of the host to the staphylococal challenge by a negative exponential decay process permitted calculation of individual rate factors for both the processes of physical clearance and bactericidal activity, as well as for the summation of the two.

Tobacco in low doses did not impair antibacterial defenses. With demonstrable difference in dosimetry and implied differences in the cardiopulmonary physiologic responses, however, it is possible that the exposure of alveolar macrophages deep within the lung to marijuana smoke may be different than to tobacco. Thus, any interpretation of direct comparative biologic effects of the two products on the lung will be difficult both because of the differences in the characteristic of pyrolyzation products and of the apparent differences in the physiologic response of the respiratory system itself.

The mechanisms by which marijuana and tobacco smoke may impair the antibacterial defenses of the host are not known. In our previous reports, a water-soluble gas phase component of fresh smoke from both products (Huber *et al.*, 1975), as well as from other smoking products (Huber *et al.*, 1978c; Huber *et al.*, 1977c; Shea *et al.*, 1978), was cytotoxic in a dose-dependent manner *in vitro* to alveolar macrophages recovered by bronchopulmonary lavage from normal animals. Additional studies indicated that this cytotoxin in marijuana smoke was present in equivalent amounts in THC-extracted marijuana placebo cigarettes and could not be demonstrated *in vitro* by administration of purified THC alone

to intact animals or to tissue culture bioassays, respectively (Huber *et al.*, 1978b). Furthermore, acute exposure of the intact animals to the gas phase of marijuana smoke alone significantly impaired pulmonary antibacterial defenses (Huber *et al.*, 1978c). Green and coworkers have implied, from studies involving only tobacco smoke, that a water soluble gas phase component impairs phagocytosis of bacteria by alveolar macrophages *in vitro*, perhaps by interaction of acrolein or acetaldehyde with sulfhydryl-containing enzymes in the glycolytic pathway (Green, Carolyn, 1967; Green, 1968b; Green, Powell, Morris, 1971; Powell, Green, 1972). Similar emphasis on the cytotoxicity of the gas phase of marijuana smoke has been made by others (Leuchtenberger, Leuchtenberger, Rotter, 1973, Leuchtenberger, Leuchtenberger, Schneider, 1973).

Marijuana and its psychoactive cannabinoid derivatives have several potential therapeutic applications, including use as a bronchodilator in asthma, use as an antiemetic adjunct to cancer chemotherapy, use as a pain reliever and sedative, in the management of glaucoma, and utilization in other more specific pharmacologic actions (Vachon *et al.*, 1976; Vachon *et al.*, 1973; Tashkin *et al.*, 1976; Tashkin *et al.*, 1974; Tashkin *et al.*, 1973; Braude and Szara, 1976; Beaconsfield, Ginsburg, Rainsburg, 1973). When it is consumed by smoking and the portal of entry for the drug is the lung, however, marijuana may have several acute and chronic adverse effects (Maugh, 1974; Nahas *et al.*, 1974; Henderson, Tennant, Guerry, 1972). On the basis of comparative studies in man, it has been implied that marijuana may be considerably more toxic to the lung than tobacco (Tashkin *et al.*, 1976; Vachon, 1976). Our data would support that hypothesis. Any acceptable therapeutic application of this agent must, therefore, consider the smoke in marijuana smoking (Vachon, 1976).

In conclusion, the animal model described herein can be used effectively to evaluate the experimental effects of marijuana and tobacco smoke on the lung. It is difficult to make biologic comparisons, however, as the dosimetry cannot be precisely equated between the two products, in part because of their opposing physiologic effects on the lung.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge with appreciation the contributions of Ms. Meg Turner Cooney, Ms. Mollee Share, and Mr. Jerome Reicher in the preparation of the manuscript and Mr. Carlton Parkinson, Mr. Bethester Butler and their staff for their help in the experimental exposure of animals.

REFERENCES

1. Beaconsfield, P., Ginsburg, J. and Rainsburg, R., Therapeutic potential of marijuana. *N. Engl. J. Med.*, **289**,1315 (1973).
2. Benner, J. F., Tentative summary of leaf and smoke analysis of University of Kentucky reference and alkaloid series cigarettes. Proceedings of the Tobacco and Health Conference, Conference Report #2, p. 30. University of Kentucky, Lexington, Kentucky, 1970.
3. Binns, R., Beven, J. L., Wilton, L. V. and Lugton, W. G. D., Inhalation toxicity studies on cigarette smoke. II. Tobacco smoke inhalation dosimetry studies on small laboratory animals. *Toxicology*, **6**,197–206 (1976).
4. Binns, R., Beven, J. L., Wilton, L. V. and Lugton, W. G. D., Inhalation toxicity studies on cigarette smoke. III. Tobacco smoke inhalation dosimetry study on rats. *Toxicology*, **6**,207–217 (1976).
5. Binns, R., Inhalation toxicity studies on cigarette smoke. IV. Expression of the dose of smoke particulate material applied to the lungs of experimental animals. *Toxicology*, **7**,189–195 (1977).

6. Braude, M. C., Szara, S., Editors. "Pharmacology of Marijuana: A Monograph of the National Institute on Drug Abuse," Volumes I and II. Raven Press, New York, 1976.
7. Davies, P., Sornberger, G. C. and Huber, G. L., The stereology of pulmonary alveolar macrophages after prolonged experimental exposure to tobacco smoke. *Lab. Invest.*, **37**,297–306 (1977).
8. Davies, P., Sornberger, G. C., Engel, E. E. and Huber, G. L., Stereology of lavaged populations of alveolar macrophages. *Exp. Molecular Path.*, in press.
9. Drath, D. B., Harper, A., Gharibian, J., Karnovsky, M. and Huber, G. L., The effect of tobacco smoke on the metabolism and function of rat alveolar macrophages. *J. Cell. Physio.*, **95**,105–113 (1978).
10. Gaoni, Y. and Mechoulam, R., Isolation structure and partial synthesis of an active constituent of hashish. *J. Amer. Chem. Soc.*, **86**,1646–1647 (1964).
11. Green, G. M., Pulmonary clearance of infectious agents. *Ann. Rev. Med.*, **19**,315–336 (1968).
12. Green, G. M., Cigarette smoke: protection of alveolar macrophages by glutathione and cysteine. *Science*, **162**,810–811 (1968).
13. Green, G. M., Powell, G. M. and Morris, T. G., Specific chemical enzyme mediation of tobacco smoke toxicity for pulmonary alveolar macrophages (PAM). *J. Clin. Invest.*, **50**,40a (1971).
14. Green, G. M. and Carolyn, D., The depressant effect of cigarette smoke on the *in vitro* antibacterial activity of alveolar macrophages. *N. Engl. J. Med.*, **276**,421–427 (1967).
15. Green, G. M. and Goldstein, E., A method for quantitating intrapulmonary bacterial inactivation in individual animals. *J. Lab. Clin. Med.*, **68**,669–677 (1966).
16. Goldstein, E., Green, G. M., Seamans, C., The effect of acidosis on pulmonary bactericidal function. *J. Lab. Clin. Med.*, **75**,912–923 (1970).
17. Guerin, M. R. and Nettesheim, P., Summary Report: Oak Ridge National Tobacco Research Program. pp. 34–44. National Technical Information Service, Springfield, Virginia, 1975.
18. Hardman, H. F. and Hosko, M. J., An overview of the cardiovascular-autonomic actions of cannabis. In "Pharmacology of Marijuana," Braude, M. C. and Szara, S., Editors; pp. 231–238. Raven Press, New York, 1976.
19. Hatch, T. F. and Gross, T., "Pulmonary deposition and retention of inhaled particles," pp. 1–192. Academic Press, New York, 1964.
20. Henderson, R. L., Tennant, F. S. Jr. and Guerry, R., Respiratory manifestation of hashish smoking. *Arch. Otolaryng.*, **95**,248–251 (1972).
21. Hinds, W. C., Size characteristics of cigarette smoke. *Amer. Ind. Hyg. J.*, **39**,48–54 (1978).
22. Huber, G. L., Shea, J. W., Hinds, W. C., Pochay, V. E., Weker, R. R., First, M. W. and Sornberger, G. C., The gas phase of marijuana smoke and intrapulmonary bactericidal defenses. *Bull. Europ. Physiopath. Resp.*, submitted, 1978.
23. Huber, G. L., Sornberger, G. C., Pereira, W., Pochay, V. E. and Hinds, W. C., Marijuana, tetrahydrocannabinol and pulmonary antibacterial defenses. *Chest*, submitted, 1978.
24. Huber, G. L., Mahajan, V. K., Shea, J. W., Cutting, M. E. and Sornberger, G. C., Comparative evaluation of the acute biologic effects of smoke from synthetic smoking materials and natural tobacco on alveolar macrophage bactericidal function. *Thorax*, submitted, 1978.
25. Huber, G. L., Pochay, V. E., Mahajan, V. K., McCarthy, C. R., Hinds, W. C., Davies, P., Drath, D. B. and Sornberger, G. C., The effect of chronic exposure to tobacco smoke on the antibacterial defenses of the lung. *Bull. Europ. Physiopath. Resp.*, **13**,145–156 (1977).
26. Huber, G. L., Johanson, W. G. Jr. and LaForce, F. M., Experimental models of pulmonary antimicrobial defenses. In "Respiratory Defense Mechanisms," Part II, Brain, J. D., Proctor, D. F. and Reid, L. M., Editors. "Lung Biology in Health and Disease," Lenfant, C., Executive Editor. pp. 983–1022. Marcel Dekker, New York-Basel, 1977.
27. Huber, G. L., Sornberger, G. C., Mahajan, V. K., Cutting M. E. and McCarthy, C. R., Impairment of alveolar macrophage bactericidal function by cigar smoke. *Bull. Europ. Physiopath. Resp.*, **13**,513–521 (1977).
28. Huber, G. L., Simmons, G. A., McCarthy, C. R., Cutting, M. B., Laguarda, R. and Pereira, W., Depressant effect of marijuana smoke on antibactericidal activity of pulmonary alveolar macrophages. *Chest*, **68**,769–773 (1975).
29. Hunter, R. B., Tobacco substitutes and additives in tobacco products: their testing and marketing in the United Kingdom. First Report of the Independent Scientific Committee on Smoking and Health, Great Britain, 1975.
30. Kass, E. H., Green, G. M. and Goldstein, E., Mechanisms of antibacterial action in the respiratory system. *Bact. Rev.*, **30**,480–495 (1966).
31. Keith, C. H. and Derrick, J. C., Measurement of the particle size distribution and concentration of cigarette smoke by the "conifuge." *J. Colloid Sci.*, **15**,340–356 (1960).
32. LaForce, F. M., Mullane, J. F., Boehme, R. F., Kelly, W. J. and Huber, G. L., The effect of pulmonary edema on antibacterial defenses of the lung. *J. Lab. Clin. Med.*, **82**,634–638 (1973).
33. Laurenzi, G., Berman, L., First, M. W. and Kass, E. H., A quantitative study of the deposition and clearance of bacteria in the murine lung. *J. Clin. Invest.*, **43**,759–768 (1964).

34. LEUCHTENBERGER, C., LEUCHTENBERGER, R. and RITTER, U. Effects of marijuana and tobacco on DNA and chromosomal complement in human lung explants. *Nature*, **242**,403–404 (1973).
35. LEUCHTENBERGER, C., LEUCHTENBERGER, R. and SCHNEIDER, A., Effects of marijuana and tobacco smoke on human lung physiology. *Nature*, **241**,137–139 (1973).
36. LEWIS, C. I., MCGEADY, J. C., TONG, H. S., SCHULTZ, F. J. and SPEARS, A. W., Cigarette smoke tracers: Gas chromatographic analysis of decachlorobiphenyl. *Amer. Rev. Resp. Dis.*, **108**,367–370 (1973).
37. LEWIS, C. I., MCGEADY, J. C., WAGNER, J. R., SCHULTZ, F. J. and SPEARS, A. W., Dichlorobenzophenone as a non-radioactive tracer for cigarette smoke-gas chromatographic analysis of tracer. *Amer. Rev. Resp. Dis.*, **106**,480–484 (1972).
38. LYERLY, L. A. and GREENE, G. H., Determination of nicotine in low nicotine tobacco. *Beitr. Tobakforsch.*, **8**,359–361 (1976).
39. MAHAJAN, V. K. and HUBER, G. L., Marijuana's effects on the cardiopulmonary system. Parts I & II. *Primary Cardiology*, **4**,54–59, 59–63 (1978).
40. MAUGH, T. H., Marijuana: The grass may no longer be greener. *Science*, **185**,683–685 (1974).
41. NAHAS, G. G., SUCIU-FOCA, N., ARMAND, J. P. and MORISHIMA, A., Inhibition of cellular mediated immunity in marijuana smokers. *Science*, **183**,419–420 (1974).
42. PRADHAN, S. N., BHATTACHARYA, I. C. and ATKINSON, K. S., The effects of intraventricular administration of nicotine on blood pressure and some somatic reflexes. *Ann. N.Y. Acad. Sci.*, **142**,50–56 (1967).
43. POWELL, G. M. and GREEN, G. M., Cigarette smoke: a proposed metabolic lesion in alveolar macrophages. *Biochem. Pharmacol.*, **21**,1785–1798 (1972).
44. SCHULTZ, F. J. and WAGNER, J. R., A thirty port smoking machine for continuous smoke generation. *Beitr. Tobakforsch.*, **8**,53–59 (1975).
45. SHEA, J., MAHAJAN, V. K., SORNBERGER, G. C. and HUBER, G. L., Effects of synthetic tobacco substitute cigarette smoke on alveolar macrophage bactericidal activity. *Amer. Rev. Resp. Dis.*, **117**,259 (1978).
46. SHEA, J. W. and HUBER, G. L., Experimental tobacco smoke inhalation and *in vitro* macrophage bactericidal function. *Lab. Clin. Med.*, **92**,270–282 (1978).
47. SINCLAIR, D., Optical properties of aerosols. Handbook of Aerosols. Report TID-4500, USAEC, U.S. Government Printing Office, Washington, D.C., 1950.
48. STAHL, W. R., Scaling of respiratory variables in mammals. *J. Appl. Physiol.*, **22**,453–460 (1967).
49. TASHKIN, D. P., SHAPIRO, B. J., LEE, Y. E. and HARPER, C. E., Subacute effects of heavy marijuana smoking on pulmonary function in healthy men. *N. Engl. J. Med.*, **294**,125–129 (1976).
50. TASHKIN, D. P., SHAPIRO, B. J. and FRANK, I. M., Acute effects of marijuana on airway dynamics in spontaneous and experimentally induced bronchial asthma. In "Pharmacology of Marijuana," Braude, M. C. and Szara, S., Editors. pp. 785–801. Raven Press, New York, 1976.
51. TASHKIN, D. P., SHAPIRO, B. J. and FRANK, I. M., Acute effects of smoked marijuana and oral Δ-9-tetrahydrocannabinol on specific airway conductance in asthmatic subjects. *Amer. Rev. Resp. Dis.*, **109**,420–428 (1974).
52. TASHKIN, D. P., SHAPIRO, B. J. and FRANK, I. M., Acute pulmonary physiological effects of smoked marijuana and oral Δ-9-tetrahydrocannabinol in healthy young men. *N. Engl. J. Med.*, **389**,336, 1973.
53. VACHON, L., The smoke in marijuana smoking. *N. Engl. J. Med.*, **294**,160 (1976).
54. VACHON, L., MIKUS, P., MORRISSEY, M. W., FITZGERALD, M. and GAENSLER, E., Bronchial effect of marijuana smoke in asthma. In "Pharmacology of Marijuana." Braude, M. C. and Szara, S., Editors. pp. 777–784. Raven Press, New York, 1976.
55. VACHON, L., FITZGERALD, M., SOLLIDAY, N. H., GOULD, I. A. and GAENSLER, E. A., Single dose effect of marijuana smoke. Bronchial dynamics and respiratory-center sensitivity in normal subjects. *N. Engl. J. Med.*, **288**,985–989 (1973).
56. WAGNER, J., THAGGARD, N. A. and THOMPSON, W. C., Determination of nicotine contained on Cambridge Filter Pads. Proceedings 31st Tobacco Chemists Research Conference, Greensboro, North Carolina. 1977.

CANNABINOIDS AND MEMBRANE-BOUND ENZYMES

ALAN MELLORS

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario, N1G 2W1, Canada

Summary. Membrane-bound acyltransferases in mouse spleen lymphocytes and brain synaptosomes can be inhibited by submicromolar concentrations of Δ^9-THC *in vitro* and by single doses of Δ^9-THC administered *in vivo*. The extent of inhibition correlates with the psychoactive specificity of the cannabinoid. Molar volume correlations indicate that the specificity of the inhibition is not determined by the partition coefficient or the ability of the cannabinoid to protect erythrocytes against hypotonic hemolysis.

INTRODUCTION

THE strongly hydrophobic nature of the cannabinoids sets them apart from many other classes of psychoactive drugs. Their high partition coefficients for non-aqueous : aqueous distribution and their marked insolubility in aqueous systems indicate that the cannabinoids will reach concentrations in the biophase that are several orders of magnitude greater than their aqueous concentration. Some of the pharmacological effects of cannabinoids can be ascribed to their affinity for biological membranes. However the relative lipophilicity of these compounds does not appear to adequately explain the wide range of psychoactivity within the cannabinoid family. The suggestion that cannabinoids exert their psychoactive potency in a manner analogous to anesthetics has been a valuable spur to research in this area[28]. Any explanation of the mechanism of cannabinoid psychoactivity must account for the high degree of specificity observed. Many biochemical and cellular responses to cannabinoids have been reported in recent years, and it is not always clear which events are unspecific, that is, caused by many large lipophilic molecules, and which events are specific to psychoactive cannabinoids. Among the events which are clearly unspecific are membrane lysis or fluidization, as seen for lysosomes[31, 3] mitochondria[20] plasma membranes and synaptosomes[14]. Many cytotoxic effects are probably unspecific, especially the inhibition of function in lymphocytes[27] erythrocytes[30] macrophages[4] and numerous cell culture systems[16]. These effects while unrelated to the specific neuronal responses may nevertheless be important as therapeutic tools as for example in the reduction of intraocular pressure in glaucoma[13], and in immunosuppression[27]. Unspecific effects may also be a significant factor in producing a variety of symptoms which have been reported in humans who are chronic users of cannabis.

There have been few reports of specific actions of cannabinoids at the biochemical or cellular level. The psychoactive cannabinoid Δ^9-tetrahydrocannabinol (Δ^9-THC) at 10^{-6} M *in vitro* stimulates the uptake of serotonin by mouse brain synaptosomes whereas psychoinactive cannabidiol (CBD) has no effect at this concentration[14]. A number of membrane-bound enzymes are known to be inhibited by cannabinoids *in vitro* and are listed in Table 1. We have studied one of these enzymes, acyl coenzyme A : lysophosphatidyl choline acyltransferase (LPC acyltransferase, E.C. 2.3.1.23) from mouse splenic lymphocytes, and from mouse brain synaptosomes. The enzyme is inhibited by submicromolar levels of Δ^9-THC and is specifically inhibited by psychoactive cannabinoids *in vivo* and *in vitro*[9]. We have used molar volume correlations to predict the inhibition of the membrane-bound enzyme that could be expected from the anesthetic action of psychoactive cannabinoids and find that the observed inhibition is several orders of magnitude greater than that predicted for the anesthetic action. We interpret these results to indicate that psychoactive cannabinoids have biochemical effects which are consistent with a specific stereochemical interaction with the membrane and which are inconsistent with a simple anesthetic mode of action.

TABLE 1. SOME MEMBRANE ENZYMES INHIBITED *in vitro* BY Δ^9-THC.

Enzyme	Membrane	Ki	Reference
ATPase	Plasma; monkey fibroblasts	> 0.5 mM	(16)
Adenyl cyclase	Plasma; mouse neuroblastoma	5 mM	(16)
(Na^+-K^+) ATPase	Synaptosomes; rat brain	5–10 μM	(14)
(Mg^{2+}) ATPase	Synaptosomes; rat brain	25–30 μM	(14)
(Na^+-K^+) ATPase	Microsomes; rat ileum	0.3 μM	(18)
(Na^+-K^+) ATPase	Plasma; rat erythrocytes	> 20 μM	(4)
NADH dehydrogenase	Mitochondria; rat brain, heart	0.2 μM	(1)
LPC acyltransferase	Plasma; mouse lymphocytes	0.3 μM	(10)
	Synaptosomes; mouse brain	0.3 μM	(17)
LPA acyltransferase	Plasma; mouse lymphocytes	6 μM	(8)

MATERIALS AND METHODS

ISOLATION OF MOUSE SPLENIC LYMPHOCYTES

Spleens were removed from 25 g male inbred white mice and gently homogenized in 20 ml Hank's balanced salt solution (BSS). The homogenate was passed through a short column of cotton which was washed with a further 10 ml BSS. The eluted cells were centrifuged in a clinical centrifuge (5,000 g.min) and the pellet was cleared of intact erythrocytes by the method of Boyle[2]. The resultant pellet was resuspended, either in medium RPMI 1640 or in Hank's minimal essential medium (MEM), the latter being a more suitable agent for the acyltransferase assay. Cell concentration was adjusted to 10^7 per ml, based on cell counts and viabilities measured by the Trypan blue exclusion technique. For incubations of lymphocytes in excess of 3 h the medium was supplemented with 10% fetal calf serum.

^{14}C-ACETATE INCORPORATION INTO LYMPHOCYTES

To 10^7 lymphocytes in 1 ml BSS was added 50 μl BSS containing 10 μg concanavalin A (ConA) and the mixture was incubated for 30 min at 37°C. Controls received no ConA. After this preincubation 50 μl of an aqueous solution containing 25 nmoles and 1.4 μCuries ^{14}C-acetate was added, together with 50 μl 10 μM Δ^9-THC in 10% BSA. Another group of controls received ^{14}C-acetate and 50 μl 10% BSA. All groups were incubated at 37°C for 1, 2, 8 or 22 h; and cells were killed at the end of this period by addition of 2 μl cold 5% trichloracetic acid. Following clinical centrifugation (10,000 g.min), lipids were separated by thin layer chromatography on silica gel 7 G (Baker Chemical Company) developed with chloroform : methanol : water (65 : 25 : 4). Radioactivity was determined by liquid scintillation counting, after comparison of separated lipids with known standards.

ISOLATION OF MOUSE BRAIN SYNAPTOSOMES

Synaptosomes were prepared from mouse brain according to Scheme 1 of Cotman[5]. Preparations were stored at ten times the desired final concentration in 0.1 M NaH_2PO_4-Na_2HPO_4 buffer pH 7.4 at $-7°$. Lower storage temperatures substantially decreased enzyme activity. Synaptosomes were diluted in 0.095 M phosphate buffer (pH: 7.4) to give less than 1.0 mg protein per ml solution and aliquots of 1 ml were used to measure LPC acyltransferase activity as described below.

IN VIVO EFFECT OF Δ^9-THC ON MOUSE BRAIN SYNAPTOSOMES AND MOUSE SPLEEN LYMPHOCYTES

Groups of five Swift-Webster male mice, each weighing 35 g, were given Δ^9-THC in 0.05 ml ethanol by tail vein injection at dose levels of 15; 25; 30; 50; 70 mg per kg. Control groups of five animals were injected with 0.05 ml ethanol only and showed no effects compared with uninjected control groups. Mice were sacrificed two hours after injection of Δ^9-THC, since our preliminary studies showed maximum ^{14}C-Δ^9-THC uptake into the lymphocyte membrane at this time. The spleens from each group of mice were removed, pooled and homogenized in Hank's balanced salt solution (BSS) using a Teflon glass homogenizer. The suspension was filtered through a small amount (0.3–0.4 g) of glass wool to remove debris. The filtrate was layered on Ficoll-Paque and lymphocytes were separated by centrifugation at 440 × g for 45 minutes. The interface was removed, washed in BSS twice and resuspended in Hank's minimal essential medium (MEM). Cell viability was determined using the Trypan blue exclusion test. After measuring cell viability, LPC acyltransferase was assayed and 36 hour cell cultures were initiated in order to determine DNA synthesis. In the concanavalin A experiments mice were injected intravenously with 100 μg per mouse concanavalin A (Con A) 3 days before sacrifice. Lymphocytes were separated according to Folch & Waksman[7]. Further assays were carried out as described below.

LPC ACYLTRANSFERASE ASSAY

Lymphocyte or synaptosome suspensions (1 ml) were preincubated in triplicate at 37°C for 20 min. One hundred nmoles of oleoyl-CoA and two hundred nmoles ^{32}P-lysophosphatidylcholine in a volume of 0.1 ml were added to the 1 ml suspensions and incubation continued for a further 15 minutes after which it was stopped with 3 ml cold 5% trichloroacetic acid. Samples were centrifuged for 20 min at 1000 × g and the pellet was subjected to lipid extraction with $CHCl_3 : CH_3OH : H_2O$ (1 : 1 : 0.4, v/v/v). Separation of lipids was carried out on silica gel 7 G (0.25 mm thick) in $CHCl_3 : CH_3OH : H_2O$ (65 : 25 : 4, v/v/v). The individual fractions were located by iodine vapor staining, scraped and counted in a liquid scintillation counter.

LYSOPHOSPHATIDIC ACID (LPA) ACYLTRANSFERASE ASSAY

Lymphocyte preparations containing 10^7 cells per ml MEM were preincubated in duplicates at 37°C for 25 min. with Δ^9-THC at concentrations of 0.1, 0.5, 1, 10, 100 μM in 0.05 ml dimethylsulphoxide (DMSO). The substrates, 200 nmoles of lysophosphatidic acid and 100 nmoles of ^{14}C-oleoyl-CoA per vial were added at the end of preincubation and incubation with the substrates was carried out for 15 min. Previous work had shown that under these conditions the production of phosphatidic acid was first order with respect to enzyme concentration. The reaction was stopped and ^{14}C-phosphatidic acid was isolated and counted by methods essentially similar to those described above for phosphatidyl choline.

CALCIUM UPTAKE

Mouse spleen lymphocytes, 10^7 cells per ml per vial were preincubated for 30 min at 37°C in the presence or absence of Con A (20 μg per vial) and containing different concentrations of Δ^9-THC in 0.05 ml DMSO. Assays were done in duplicate and controls contained 0.05 ml DMSO. After the preincubation 1 μC $^{45}Ca^{2+}$ (specific activity 6.84 μC per mg) was added to each vial and the incubation was resumed for 15 min. The treated cells were collected by centrifugation at 1000 × g for 20 min at 4°C and washed twice in BSS. The washed cell pellet was digested with 0.5 ml NCS tissue solubilizer at 50°C for 2 hours, cooled and acidified with 0.05 ml glacial acetic acid to reduce chemiluminescence. After adding 10 ml Anderson's scintillation fluid (25% v/v Triton X-114, 0.3% w/v 2,5-diphenyloxazole in xylene) the digests were counted in a Beckman LS-255 scintillation counter.

DNA SYNTHESIS

The incorporation of ^{3}H-thymidine into DNA was measured as described previously[17].

AH_{50} DETERMINATION

The concentration of a drug that will give 50% protection of erythrocytes against hemolysis (AH_{50}) was obtained from the literature[32] or was determined on equine erythrocytes according to the method of Machleidt *et al.*[19]. Equine values were corrected by the experimentally determined factor of 0.36 in order to correspond with the literature data on human erythrocytes. The AH_{50} for the lipid-soluble cannabinoids were obtained as follows. The cannabinoids (100 mg/ml ethanol) were dried to an oily film under nitrogen in silanized glass test tubes. One ml of an erythrocyte suspension which had been prepared according to Machleidt *et al.*[19] and at a hematocrit of 85–95 was added to each tube and vortexed 2 minutes. The cells were further incubated in a shaking water bath 1.5 hours at 37°C. The erythrocyte suspension was diluted to a hematocrit of 5–7 with 0.9% saline in 15 mM Tris-HCl pH 7.0, and 0.1 ml added to 2.5 ml 0.45% saline in 15 mM Tris-HCl pH 7.0. After swirling on a vortex shaker for 2 min, the cells were sedimented in a clinical centrifuge (1,500 g-min) and the absorbance of the supernatant determined using a Beckman DB-GT spectrophotometer at 540 nm.

CHARACTERISTIC VOLUMES

The characteristic volumes ($m^3\ mole^{-1}$) are estimates of the actual molar volumes (i.e. the molar volumes at the absolute zero) and are obtained using the method of McGowan[24] by division of the calculated parachors in c.g.s. units by 2.835×10^6. Details of the characteristic volume (V_x) relationships are given in the Discussion section.

MATERIALS

^{14}C-Acetate (53 mCuries per m mole) and ^{32}P-phosphoric acid (carrier free) were obtained from New England Nuclear Inc. ^{32}P-Lysophosphatidylcholine was prepared from ^{32}P-phosphatidylcholine using *Crotalus adamanteus* venom[12, 34]. ^{32}P-Phosphatidylcholine was obtained from rats injected with ^{32}P-phosphate[11]. Acyl coenzyme A (CoA) derivatives and other lipids were purchased from Sigma Chemical Company. Cannabinoids were kindly provided by Health and Welfare Canada. For the acetate incorporation studies, cannabinoids were dispersed in 10% (w/v) bovine serum albumin (BSA) solution, while for LPC acyltransferase experiments cannabinoids and other lipids were administered in dimethylsulfoxide (DMSO) such that 50 μl of solution contained the required amount of lipid. Appropriate controls were performed containing BSA and DMSO solutions respectively. Media, biological extracts and salt solutions were obtained from Grand Island Biological Co., N.Y.

RESULTS

LIPID METABOLISM IN LYMPHOCYTES

During studies of the effect of Δ^9-THC on early events in mouse spleen lymphocyte transformation it was observed that ^{14}C-choline incorporation into phospholipids was

substantially inhibited by Δ^9-THC. Subsequent studies showed that ^{14}C-acetate incorporation into glycerolipids including major phospholipids and triglycerides were similarly inhibited by 10 μM Δ^9-THC[9]. While phosphatidylinositol (PI) showed the most inhibition for ^{14}C-acetate incorporation, this is a minor lipid and slight disturbances of the precursor incorporation into major components are known to be associated with major changes in PI turnover levels[26]. It became clear that the decreased turnover of glycerolipids was not confined to lymphocytes stimulated to transform by concanavalin A but was also true for basal rates of lipid synthesis in unstimulated cells.

Turnover studies cannot distinguish between changes in rates of synthesis or of degradation, and so specific steps in phospholipid metabolism were then examined for sensitivity to cannabinoids. Using ^{32}P-phosphatidylcholine (PC) changes in phospholipase activity in lymphocytes incubated with micromolar levels of Δ^9-THC were sought. No changes were observed but it became apparent that the major phospholipid interconversion observed in these cells was the acylation of lysophosphatidylcholine (LPC) to form PC and that the enzyme responsible, LPC acyltransferase, was sensitive to cannabinoids both in transformed and in unstimulated lymphocytes. Table 2 shows the sensitivity of the lymphocyte LPC acyltransferase enzyme to a variety of cannabinoids over a wide range of concentration. Only Δ^9-THC is substantially inhibitory at concentrations below 10^{-5}M, and its concentration for half-maximal inhibition (K_i) is 0.35 μM. Other cannabinoids inhibited the LPC acyltransferase activity when present in the concentration range 10^{-4}–10^{-3} M and inhibition ranged from 60–80% at the higher concentration. Of the cannabinoids tested on lymphocytes only Δ^9-THC and Δ^8-THC have psychoactivity, the former being the most potent[15]. Thus it is interesting that these two compounds showed the most inhibition and that the K_i for Δ^8-THC (13 μM) was intermediate between the values for the more psychoactive Δ^9-THC and the psycho-inactive cannabinoids.

TABLE 2. THE INHIBITION OF MOUSE SPLEEN LYMPHOCYTE AND MOUSE BRAIN SYNAPTOSOME LYSOPHOSPHATIDYLCHOLINE ACYLTRANSFERASE BY CANNABINOIDS *in vitro*.

Cannabinoid	K_i (M)	
	Lymphocytes	Synaptosomes
Δ^9-THC	3.5×10^{-7}	3.0×10^{-7}
Δ^8-THC	1.3×10^{-5}	5.8×10^{-5}
Synhexyl-Δ^{6a}-THC	n.d.*	2.5×10^{-4}
11-hydroxy-Δ^9-THC	n.d.*	8.5×10^{-5}
Cannabinol	2.0×10^{-4}	5.5×10^{-4}
Cannabigerol	1.9×10^{-4}	2.6×10^{-4}

* not determined.

Our preliminary results had shown that the incorporation of precursors into phospholipids and triglycerides was inhibited by 10 μM Δ^9-THC. The inhibition of LPC acyltransferse could only explain the lowered turnover of phosphatidylcholine, and not decreased turnover of triglycerides and other phospholipids[9]. There is however another acyltransferase activity on the main route of synthesis of triglycerides and phospholipids. This is lysophosphatidate (LPA) acyltransferase which acylates LPA to form phosphatidate (PA),

a precursor of phospholipids and triglycerides. If LPA acyltransferase is similarly inhibited by Δ^9-THC this would give rise to decreases in both phospholipid and triglyceride turnover. We have measured the effect of Δ^9-THC on LPA acyltransferase in unstimulated mouse spleen lymphocytes and compared the inhibition of this enzyme with that of LPC acyltransferase. We have concomitantly measured in these lymphocyte preparations the effect of Δ^9-THC on basal levels of ^{3}H-thymidine uptake and basal levels of Ca^{2+} entry. These latter processes are both associated with blastogenesis and their inhibition in unstimulated lymphocytes by Δ^9-THC might be expected to have relevance to parallel studies on blastogenesis inhibition by Δ^9-THC. Table 3 shows the relative effects of Δ^9-THC on these biochemical events in basal lymphocyte metabolism. It is clear that there is inhibition of LPA acyltransferase by Δ^9-THC but that the inhibition is not nearly as marked as that for LPC acyltransferase.

TABLE 3. THE INHIBITION BY Δ^9-THC OF LPA ACYLTRANSFERASE COMPARED TO THAT OF LPC ACYLTRANSFERASE, AND EFFECTS ON Ca^{2+} UPTAKE AND THYMIDINE INCORPORATION IN MOUSE SPLEEN LYMPHOCYTES *in vitro.*

	K_i(M)
LPA acyltransferase	6×10^{-6}
LPC acyltransferase	3×10^{-7}
Ca^{2+} uptake	3×10^{-7}
Thymidine incorporation	2×10^{-7}

Inhibition of LPA acyltransferase at 10 μM Δ^9-THC is enough to account for the observed decreased turnover of phospholipids and triglycerides at this concentration. The lymphocyte LPC and LPA acyltransferase activities have never been separated and it was not clear whether lymphocytes possess a single acyltransferase capable of acting on LPC and LPA. Our data suggest that there are two distinct enzymes with differing sensitivities for Δ^9-THC. The use of cannabinoids as a tool in lipid metabolism studies appears to be a promising technique in an area where few metabolic inhibitors are available. While it is difficult to estimate the concentration of Δ^9-THC needed for half-maximal inhibition (K_i) of LPA acyltransferase, this would appear to be about 6 μM. This difficulty arises because Δ^9-THC is relatively insoluble at the higher concentrations needed to give maximal inhibition of the LPA acyltransferase. The concentration-dependence of the inhibition is clearly distinct from the inhibition pattern seen for LPA acyltransferase, for which the K_i is 0.35 μM, about 20-fold lower than that for LPA enzyme. This marked difference could be used to distinguish other events which may be associated with one of these lipid-metabolizing enzymes. For example, as shown in Table 2 calcium entry into this lymphocyte system is inhibited by Δ^9-THC at a concentration for half-maximal inhibition of 0.3 μM. Preliminary results in our laboratory indicate that Ca^{2+} entry into lymphocytes during concanavalin-induced blastogenesis is similarly inhibited by Δ^9-THC. It has been shown by many workers that blastogenesis, monitored by thymidine incorporation into lymphocytes following mitogen treatment, is inhibited by Δ^9-THC[(27)] with a range of values reported for the K_i, depending on the amount of serum protein present in the system. We have established that LPC acyltransferase is inhibited similarly by Δ^9-THC in both stimulated and unstimulated lymphocytes. In the experiments we describe here we

measured the inhibition of ^{3}H-thymidine incorporation into the unstimulated mouse spleen lymphocytes (Table 3) and observed a similar inhibition to that seen for lymphocytes undergoing blastogenesis, with a K_i of 0.2 μM which falls close to the level of Δ^9-THC, this also being a level that gives half maximal inhibition of LPC acyltransferase and Ca^{2+} entry into these cells. The conclusions that we have drawn from these experiments are that the processes which are inhibited by Δ^9-THC during blastogenesis of mouse spleen lymphocytes are similarly inhibited in basal metabolism of these cells. The inhibition of LPC acyltransferase, Ca^{2+} entry and DNA synthesis show similar sensitivities to Δ^9-THC whereas LPA acyltransferase inhibition occurs at higher Δ^9-THC concentrations.

LIPID METABOLISM IN SYNAPTOSOMES

Because the psychoactive specificity of cannabinoids is exerted at the neuronal level we have sought evidence that the phenomena we have seen in lymphocyte membranes might also be demonstrated in nervous tissue. We chose mouse brain synaptosomes as a tissue because these preparations have been widely used in studies of the biochemistry of neurotransmitters. These vesicles contain membrane-bound enzymes including LPC acyltransferase, and phospholipid metabolism in these membranous structures has been implicated in the process of neurotransmitter release[33]. We have seen the effect of cannabinoids and other lipophilic substances on the mouse brain synaptosomal LPC acyltransferase activity *in vitro* and we have also measured effects of Δ^9-THC given *in vivo* on this enzyme.

As shown in Figs. 1 and 2 a large number of lipophilic drugs can inhibit mouse brain synaptosomal LPC acyltransferase *in vitro* at concentrations consistent with their anesthetic potency. These compounds have an inhibitory potency (K_i) proportional to their ability to expand erythrocyte membranes (AH_{50}). However the psychoactive cannabinoids

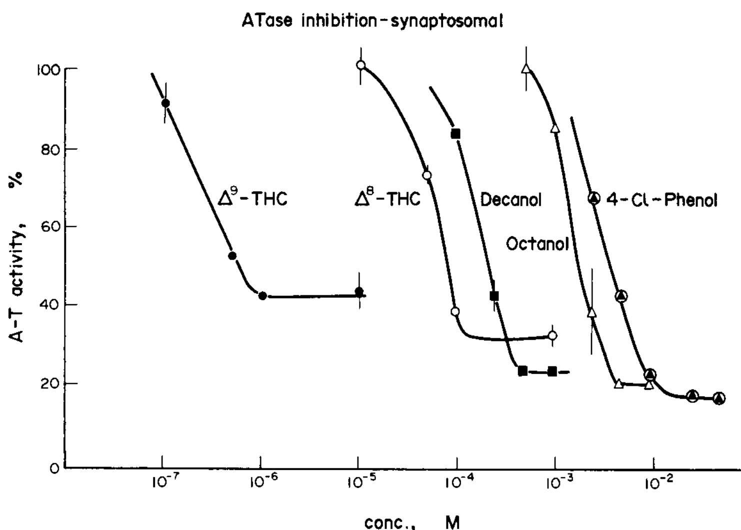


FIG. 1. The inhibition of mouse brain synaptosomal lysophosphatidylcholine acyltransferase (AT) by cannabinoids and anesthetics *in vitro*. Values are expressed as percentages of controls and are given as means ± standard deviations.

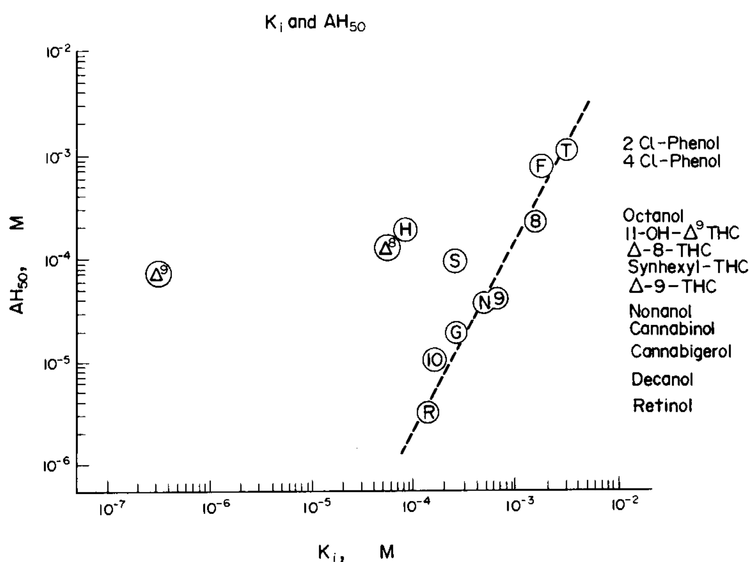


FIG. 2. The inhibition of mouse brain synaptosomal lysophosphatidylcholine acyltransferase (K_i values) compared to the antihemolysis potency (AH_{50}) for cannabinoids and other lipophilic compounds. The symbols are: T, 2-chlorophenol; F, 4-chlorophenol; 8, octanol; H, 11-hydroxy-Δ^9-THC; S, synhexyl Δ^{6a}-THC; Δ^8, Δ^8-THC; Δ^9, Δ^9-THC; 9, nonanol; N, cannabinol; G cannabigerol; 10, decanol; R, retinol.

have an inhibitory potency which is much greater than that for non-psychoactive cannabinoids, and which bears some relation to their relative psychoactivities. Thus Δ^9-THC is about 10^3 times more inhibitory than cannabinol while Δ^8-THC and 11-hydroxy-Δ^9-THC are about ten times more inhibitory than cannabinol. The slightly psychoactive synhexyl Δ^{6a}-THC falls closer to the "anesthetic" line whereas cannabinol and cannabigerol which are not psychoactive fall on the line giving inhibition consistent only with their membrane-expanding properties.

EFFECTS OF Δ^9-THC ADMINISTERED *in vivo*

The effects of Δ^9-THC on splenic lymphocytes and on brain synaptosomes *in vitro* have also been observed following the administration of single doses of Δ^9-THC to mice *in vivo*. Using concanavalin A also given *in vivo* to stimulate splenic lymphocyte blastogenesis and LPC acyltransferase activity, we then monitored the effect of single doses of Δ^9-THC on these increased activities. Preliminary experiments using ^{14}C-Δ^9-THC showed maximum uptake of label by spleen and lymphocytes two hours after the injection of the cannabinoid, and so in subsequent experiments mice were sacrificed two hours after a tail vein injection of Δ^9-THC at does levels of 15–70 mg/kg. Splenic lymphocytes and brain synaptosome preparations were prepared from these treated animals and from controls. The treated animals showed decreases in lymphocyte blastogenesis, lymphocyte LPC acyltransferase activity and brain synaptosomal LPC acyltransferase activity. At the 15 mg/kg level of cannabinoid *in vivo* the incorporation ^3H-thymidine by mouse splenic lymphocytes was inhibited 17% compared to sham-injected controls, inhibition of LPC acyltransferase in these cells was 6% and inhibition of brain synaptosomal LPC acyltransferase was 6%.

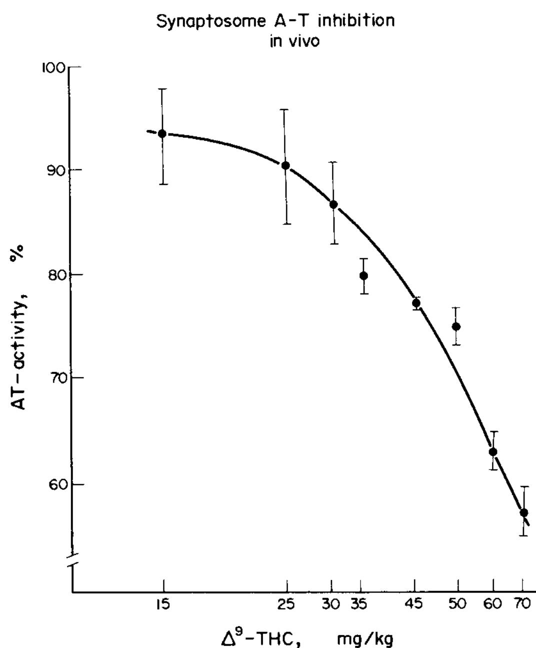


FIG. 3. Inhibition of lysophosphatidylcholine acyltransferase (AT) in synaptosomes from mice given single doses of Δ^9-THC *in vivo*. Values are expressed as percentages of controls and are given as means ± standard deviations.

At the 50 mg/kg dose level these three inhibitions were 60% for ^{3}H-thymidine incorporation, 25% for lymphocyte LPC acyltransferase inhibition and 25% for synaptosomal LPC acyltransferase inhibition. While the single doses required to show these inhibitions are large, compared to psychoactive doses it appears from the dose response curve (Fig. 3) that some inhibition of the synaptosomal LPC acyltransferase could be expected at an *in vivo* psychoactive dose level of 1 mg/kg. By analogy with the levels of LPC acyltransferase inhibition by Δ^9-THC added *in vitro* we estimate that the dose range 15–50 mg/kg *in vivo* produces an effective Δ^9-THC concentration equivalent to 0.04–0.20 μM *in vitro* in lymphocytes and synaptosomes.

DISCUSSION

MOLAR VOLUME CORRELATIONS

In attempting to determine the nature of the cannabinoid interaction with membrane enzymes we have had to consider the question of whether the inhibitions we observe arise solely as a result of the expansion of the lipid phase of the membrane by an anesthetic mechanism or whether there is a more structurally-specific mechanism. While we have used the anti-hemolysis assay (AH_{50} values) as a measure of the effectiveness of a lipid in expanding membranes and thereby exerting anesthetic action, this correlation may not be valid for very large lipids, though it is known to hold for a wide range of smaller hydro-

phobic anesthetics[32]. We have used molar volume relationships to predict the AH_{50} values and the corresponding K_i values for the inhibition of synaptosomal LPC acyltransferase. The use of molar volume relationships to predict partition coefficients was proposed by McGowan[23] and he has applied the equations to physical toxicity[22, 21, 25]. Anesthesia and narcosis are well known examples of physical toxicity and are related closely to the molar volume of the drug concerned. The equation which has been used[23] to predict partition coefficients is

$$\log_{10} x = -kV_x + E_B \qquad (1)$$

where x is the partition coefficient of a drug; V_x is an estimate of the molar volume, obtained by the addition of factors; k is a constant and equals 36,000 moles m^{-3}, as a first approximation, for all non-aqueous phases; and E_B is an interaction term, constant for a given chemical grouping and derived for a variety of physical toxicities[23, 25].

Equation (1) has been useful in correlating and predicting physical toxicities such as anesthetic potency. It can also be used to predict for a dose of the drug that gives a certain toxicity, the ratio of the concentration (C_t) of the drug in the aqueous phase, to the concentration (C_B) in the membrane of biophase, by substituting C_t/C_B for x in Eq. (1). A plot of $-\log_{10} C_t$ against 36,000 $V_x + E_B$ should give a line of slope 45°, and the intercept of this line when 36,000 $V_x + E_B$ is zero is equal to $-\log_{10} C_B$. The relationships of Eq. (1) however are only strictly applicable to systems containing negligible proportions of non-aqueous phase, and the equations must be modified for cases such as most biological systems which contain appreciable hydrophobic phases. Modifications have been derived for cases where drugs are injected into animals[25]. Recently a modification has been derived (McGowan, Greenberg and Mellors, manuscript submitted) which enables the comparison of biphasic systems containing differing amounts of non-aqueous phase, such as the erythrocyte stabilization system and the membrane-bound LPC acyltransferase systems of lymphocytes and synaptosomes. This derivation results in equation 2 which is especially useful for large hydrophobic drugs such as cannabinoids.

$$C_t = A + B \times 10^{-36,000 V_x + E_B} \qquad (2)$$

where A and B are constants for the system and the ratio $A \div B$ is equal to the (volume of non-aqueous phase in the system) $\div$ (volume of aqueous phase in the system).

Equation (2) has been used to correlate the data for the expansion of erythrocyte membranes by hydrophobic molecules using the AH_{50} values plotted in Fig. 2. The equation has the form

$$AH_{50} = 5.3 \times 10^{-6} + 0.60 \times 10^{-36,000}V_x + E_B$$

For compounds whose anesthetic potency increases with molar volume the points on this curve fall close to a line of 45° but for hydrophobic drugs larger than dodecanol there is a limiting value of the AH_{50} corresponding to the constant A which has a value of 5×10^{-6} moles per litre in this system. From the value of A and the value of intercept B it is possible to calculate the amount of non-aqueous phase in the system used to determine AH_{50} values. This is given by the ratio $A \div B$ or $(5.3 \times 10^{-6}) \div 0.60$ which is equal to 8.8×10^{-6}, so that in a litre of the erythrocyte suspension we estimate there is less than 100 μlitres of membrane or other non-aqueous phase. The important feature of this correlation for cannabinoid studies is that the AH_{50} values obtained experimentally for cannabinoids are consistent with the limiting value of the AH_{50} given by all large hydro-

phobic molecules. In particular Δ^9-THC and other psychoactive cannabinoids do not give a membrane expansion greater than that predicted from the molar volume, or greater than that given by non-psychoactive cannabinoids.

In contrast, the same Eq. (2) can be used to correlate the K_i values for the inhibition of LPC acyltransferase using those K_i values plotted in Fig. 2. The substituted equation has the form

$$K_i = 2 \times 10^{-4} + 10^{-36,000 V_x + E_B}$$

Again smaller hydrophobic compounds whose anesthetic potencies increase with molar volume show inhibition of the enzyme in direct proportion to $(V_x + E_B)$, with points on a line of slope 45°. Larger compounds such as retinol and non-psychoactive cannabinoids fall close to a limiting value for the K_i corresponding to constant A which has a value of 2×10^{-4} moles per litre in the synaptosome system. The value of B in this system is 1.0 and hence $A \div B = 2 \times 10^{-4}$, which again is a small value for the ratio of the volume of biophase to the volume of water, but which, at 30 times the value of this ratio in the erythrocytes, is consistent with the much larger concentration of cellular material used in the enzyme assay compared to that in the erythrocyte test system. The major conclusion however from this correlation is that Δ^9-THC, Δ^8-THC and 11-hydroxy Δ^9-THC give K_i values which are considerably lower than the lower limit of 2×10^{-4} M which is predicted by their molar volume values. The non-psychoactive cannabinoids give inhibition of the synaptosomal enzyme which is consistent with the theoretical limit for anesthetics.

The nature of the specific interaction between Δ^9-THC and the membrane-bound enzyme is still a matter of speculation. The lack of correlation between membrane expansion and inhibition make it unlikely that the specificity resides in an interaction of a chiral molecule with the lipid phase alone as proposed in the partial anesthetic theory of action. It remains possible however that the anti-hemolysis assay is inadequate for the measurement of membrane fluidity changes induced by large hydrophobic molecules. The molar volume theory of anesthetic action cannot adequately explain the marked differences in potency between isomers having the same partition coefficients, as for example in the marked anesthetic potency of Alphaxalone (3α-hydroxy-5α-pregnane-11,20-dione), whose 3β-hydroxy isomer is without significant anesthetic activity[29]. These reservations about the validity of the molar volume hypothesis of anesthetic action should not detract however from the usefulness of molar volume correlations in assessing the mechanisms of action of large hydrophobic molecules. The equations described here and elsewhere provide a useful test for physical toxicity mechanisms and deserve wide application in areas where the hydrophobicity of a drug is a substantial contribution to its mode of action.

CONCLUSIONS

Cannabinoids cause membrane changes consistent with their large hydrophobic molecular nature and many biochemical and pharmacological manifestations of these changes have been observed including cytotoxicity, inhibition of cell transformation and impaired metabolism. Our studies indicate that lipid metabolism in lymphocytes and in brain synaptosomes is impaired, following both the *in vivo* and *in vitro* administration of Δ^9-THC. In lymphocytes *in vitro* both basal lipid metabolism and mitogen-induced activities are inhibited to the same degree. It appears that calcium uptake, thymidine incorpora-

tion and LPC acyltransferase activities have a similar sensitivity to Δ^9-THC, whereas LPA acyltransferase an enzyme on the path of glycerolipid synthesis is less sensitive to inhibition by Δ^9-THC.

The inhibition of LPC acyltransferase by Δ^9-THC that we have observed *in vivo* and *in vitro* in lymphocytes and in brain synaptosomes is specific for psychoactive cannabinoids and is not given by non-psychoactive cannabinoids. This appears to be the first correlation of psychoactivity with an enzymic event. Molar volume correlations indicate that the inhibition is much greater than that predicted for the anesthetic action of similar large hydrophobic molecules and that it is not explicable in terms of the partition coefficients, water solubilities or molar volumes of the cannabinoids. The inhibition is apparently due to a more specific interaction than that associated with the perturbation of the membrane lipid bilayer and is probably due to a specific stereochemical interaction with a hydrophobic region of the membrane or enzyme.

ACKNOWLEDGEMENT

This investigation was supported in part by Canada Health and Welfare, the Medical Research Council of Canada and the National Research Council of Canada. The author is indebted to Dr. J. C. McGowan, Dr. L. A. Huszar and especially Mr. J. H. Greenberg for their interest and assistance in these studies.

Sommaire. Les acyltransferases membranaires provenant, chez la souris, de lymphocytes de la rate et de synaptosomes du cerveau peuvent être inhibèes *in vitro* par Δ^9-THC a des concentrations inferieures a 1 μM et aussi *in vivo* suite a des injections simples de Δ^9-THC. Le degrè d'inhibition correspond a la specificité psychoactive du cannabinoide. Les corrélations ètablies sur la base du volume molaire indiquent que la spècificité d'inhibition n'est pas déterminée par le coefficient de partition du cannabinoide ni par sa capacité a protéger les érythrocytes de l'hèmolyse hypotonique.

REFERENCES

1. BARTOVA, A. and BIRMINGHAM, M. K., Effect of Δ^9-tetrahydrocannabinol on mitochondrial NADH-oxidase activity. *J. Biol. Chem.* **251**,5002–5006 (1976).
2. BOYLE, W., An extension of the ^{51}Cr-release assay for the estimation of mouse cytotoxins. *Transplantation* **6**,761–764 (1968).
3. BRITTON, R. S. and MELLORS, A., Lysis of rat liver lysosomes *in vitro* by Δ^9-tetrahydrocannabinol. *Biochem. Pharmacol.* **23**,1342–1343 (1974).
4. CHARI-BITRON, A., Effect of Δ^1-tetrahydrocannabinol on red blood cell membranes and on alveolar macrophages. "Marihuana: Chemistry, Biochemistry and Cellular Effects." (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 273–281, 1976.
5. COTMAN, C. W., Isolation of synaptosomal and synaptic plasma membrane fractions. *Methods in Enzymology* (S. Fleischer, L. Packer, eds.). Academic Press, N.Y. **31**,445–452 (1974).
6. DEL CASTILLO, J., ANDERSON, M. and RUBOTTOM, G. M., Marihuana, absinthe and the central nervous system. *Nature* **253**,365–366 (1975).
7. FOLCH, H. and WAKSMAN, B., Splenic suppressor cell. 1. Activity of thymus-dependent adherent cells. *J. Immunol.* **113**,127–139 (1974).
8. GREENBERG, J. H., MCGOWAN, J. C. and MELLORS, A., Synaptosomal and lymphocyte acyltransferases: specificity of inhibition by cannabinoids. *Proc. Canad. Federation Biol. Soc.* **21**, Abstract 419 (1978).
9. GREENBERG, J. H. and MELLORS, A., Specific inhibition of an acyltransferase by Δ^9-tetrahydrocannabinol. *Biochem. Pharmac.* **27**,329–333 (1978).
10. GREENBERG, J. H., SAUNDERS, M. E. and MELLORS, A., Inhibition of a lymphocyte membrane enzyme by Δ^9-tetrahydrocannabinol *in vitro*. *Science N.Y.* **197**,475–477 (1977).

11. HAMBREY, P. N. and MELLORS, A., Cardiolipin degradation by rat liver lysosomes. *Biochem. Biophys. Res. Commun.* **62**,939–945 (1975).
12. HAVERKATE, F. and VAN DEENEN, L. L. M., Isolation and chemical characterization of phosphatidylglycerol from spinach leaves. *Biochim. Biophys. Acta* **106**,78–92 (1965).
13. HEPLER, R. S. and FRANK, I. R., Marihuana smoking and intraocular pressure. *J. Amer. Med. Ass.* **217**,1392 (1971).
14. HERSHKOVITZ, M., GOLDMAN, R. and RAZ, A., Effects of cannabinoids on neurotransmitter uptake, ATPase activity and morphology of mouse brain synaptosomes. *Biochem. Pharmac.* **26**,1327–1331 (1977).
15. HOLLISTER, L. E., Structure-activity relationships in man of cannabis constituents, and homologs and metabolites of Δ^9-tetrahydrocannabinol. *Pharmacology* **11**,3–11 (1974).
16. HUOT, J., Cellular and biochemical alterations induced *in vitro* by Δ^1-tetrahydrocannabinol: Effects on cell proliferation, nucleic acids, plasma cell membrane ATPase and adenylate cyclase. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 313–327, 1976.
17. HUSZAR, L. A., GREENBERG, J. H. and MELLORS, A., Effects of Δ^9-tetrahydrocannabinol on lymphocyte and synaptosomal lysophosphatidylcholine acyltransferase *in vivo*. *Mol. Pharmac.* **13**,1086–1091 (1977).
18. LAURENT, B. and ROY, P. E., Alteration of membrane integrity by Δ^1-tetrahydrocannabinol. *Int. J. Clin. Pharmac.* **12**,261–266 (1975).
19. MACHLEIDT, H., ROTH, S. and SEEMAN, P., The hydrophobic expansion of erythrocyte membranes by the phenol anaesthetics *Biochim. Biophys. Acta* **255**,178–189 (1972).
20. MAHONEY, J. M. and HARRIS, R. A., Effect of Δ^9-tetrahydrocannabinol on mitochondrial process. *Biochem. Pharmacol* **21**,1217–1226 (1972).
21. MCGOWAN, J. C., ATKINSON, P. N. and RUDDLE, L. H., The physical toxicity of chemicals. V. Interaction terms for solubilities and partition coefficients. *J. Appl. Chem.* **16**,99–102 (1966).
22. MCGOWAN, J. C., The physical toxicity of chemicals. III. Systematic treatment of physical toxicity in aqueous solutions. *J. Appl. Chem.* **2**,651–658 (1952).
23. MCGOWAN, J. C., The physical toxicity of chemicals. IV. Solubilities, partition coefficients and physical toxicities. *J. Appl. Chem.* **4**,41–47 (1954).
24. MCGOWAN, J. C., Molecular volumes and structural chemistry. *Recl. Trav. Chim. Pay-Bas* **75**,193–208 (1956).
25. MCGOWAN, J. C., Activity of physically toxic compounds in solution. *Physico-Chemical and Biophysical Factors affecting the Activity of Pesticides*. Monograph 29 of the Society of Chemical Industry (London), pp. 141–158, 1968.
26. MICHELL, R., Inositol phospholipids and cell surface receptor function. *Biochim Biophys. Acta* **415**,81–147 (1975).
27. NAHAS, G. G., SUCIU-FOCA, N., ARMAND, J.-P. and MORISHIMA, A., Inhibition of cellular mediated immunity of Marihuana smokers. *Science* **183**,419–420 (1974).
28. PATON, W. D. M., PERTWEE, R. G. and TEMPLE, D. M., The general pharmacology of cannabinoids. *Cannabis and its Derivatives: Pharmacology and Experimental Psychology* (W. D. M. Paton and J. Crown, eds.) Oxford University Press, London, pp. 50–75, 1972.
29. PHILLIPS, G. H., Structure-activity relationships in steroidal anaesthetics. *J. Steroid Biochem.* **6**,607–613 (1975).
30. RAZ, A., SCHURR, A. and LIVNE, A., The interaction of hashish components with human erythrocytes. *Biochim. Biophys. Acta* **274**,269–271 (1972).
31. RAZ, A., SCHURR, A., LIVNE, A. and GOLDMAN, R., Effect of hashish compounds on rat liver lysosomes *in vitro*. *Biochem. Pharmacol.* **22**,3129–3131 (1973).
32. ROTH, S. and SEEMAN, P., The membrane concentrations of neutral and positive anesthetics (alcohols, chlorpromazine, morphine) fit the Meyer-Overton rule of anesthesia; negative narcotics do not. *Biochim. Biophys. Acta* **255**,207–219 (1972).
33. TRIFARÓ, J. M., Phospholipid metabolism and adrenal medullary activity. 1. The effect of acetylcholine on tissue uptake and incorporation of orthophosphate-[^{32}P] into nucleotides and phospholipids of bovine adrenal medulla. *Mol. Pharmac.* **5**,382–393 (1969).
34. WELLS, M. A. and HANAHAN, D. J., Phospholipase A from *Crotalus adamanteus* venom. *Methods in Enzymology* (P. D. Boyer ed.) Academic Press, New York, Vol. **14**, pp. 178–184, 1969.

THE EFFECTS OF CANNABINOIDS ON RAT BRAIN SYNAPTOSOMES

WILLIAM L. DEWEY, MRINAL K. PODDAR and KENNETH M. JOHNSON

Department of Pharmacology Medical College of Virginia Richmond, VA 23298

Summary. The effect of cannabinoids was studied on uptake and release of ^{3}[H]-labelled serotonin, dopamine (DA) and norepinephrine (NE) by synaptosomes prepared from rat whole forebrain, hypothalamus or corpus striatum.

Δ^9-THC inhibited uptake and facilitated release of ^{3}[H]-serotonin by forebrain synaptosomes, the release being accompanied by increase of ^{3}[H]-indoleacetic acid in the medium. Over a series of cannabinoids, there was no correlation between inhibition of uptake and behavioral effect. Δ^9-THC also inhibited tryptophan and choline uptake.

Δ^9 and Δ^8-THC at low concentrations stimulated uptake of ^{3}[H]-DA and NE by hypothalamic and striated synaptosomes, and inhibited at high concentrations. Cannabinol and cannabidiol displayed only inhibitory effects at higher concentrations. In tests on release of these amines, Δ^9-THC had a biphasic effect, but cannabinol and cannabidiol only facilitated. The biphasic effects of Δ^9- and Δ^8-THC may therefore provide a molecular basis for biphasic behavioral effects. The metabolite 11-OH-Δ^9-THC differed in its action from Δ^9-THC both on hypothalamic and striated synaptosomes.

INTRODUCTION

PSYCHOACTIVE cannabinoids, like most drugs that alter behavior, have effects on the neurotransmitter functions of the brain. However, a clear picture has not emerged as to the primary effect of these interesting compounds on brain neurotransmitter systems. A thorough review of the literature[1] has shown that Δ^9-THC generally causes no changes in norepinephrine or dopamine levels but in many experiments caused an increase in the synthesis rates of these catecholamines. The effect of Δ^9-THC on serotonin levels was less consistent. The level of serotonin increased in some experiments, decreased in others and in yet a third group of reports there was no change in rodent brain level of serotonin following Δ^9-THC. Three reports exist on the effect of Δ^9-THC on the synthesis or turnover of serotonin in the brain. There was no change in two and a 50 percent reduction in the third report. Usually relatively large doses of Δ^9-THC were used in these experiments but lower doses were generally found to be inactive.

The lack of consistency of the results of the experiments thus far carried out on the effects of the cannabinoids on brain neurotransmitter systems indicated that the effects of these drugs on a more simplified experimental system might be more useful in determining their cellular mechanism of psychotomimetic action. We and others have studied the effects of the natural occurring cannabinoids and a number of synthetic analogues on rat

brain synaptosomes. It is evident from these studies that the cannabinoids alter the function of both neuronal and synaptic vesicular membranes.

METHODS

L-LEUCINE-4,5-[^{3}H](N), (31.5 Ci/mmole); *dl*-norepinephrine D-bitartrate 7-[^{3}H](N), (0.4 Ci/mmole), and 5-hydroxy-indoleacetic-carboxyl[^{14}C]-acid (22.1 mCi/mmole) were obtained from New England Nuclear; 5-HT[^{3}H](G)-creatinine sulphate (500 mCi/mmole) was obtained from Amersham/Searle.

Adult male Sprague-Dawley rats (150–200g) were used in each experiment. Rats were decapitated at the same time each day and the brains were removed rapidly, blotted, chilled and either weighed (for whole brain synaptosome studies) or dissected on ice. The hypothalamus and corpus striatum were dissected in certain experiments by making a transverse section at the level of the optic chiasma which delineated the anterior part of the hypothalamus and passed through the anterior commissure. The hypothalamus was dissected by using the anterior commissure as a horizontal reference and the line between the mammillary bodies in the posterior hypothalamus as the caudal limit. The corpus striatum was dissected with the external walls of the lateral ventricle as the internal limits and the corpus collosum as the external limits.

Synaptosomal uptake of [^{3}H]-radioactive dopamine (DA) tryptophan (try), choline (Ch) or norepinephrine (NE) were measured by the method of Coyle and Snyder (1969). Although some slight modifications existed in certain experiments, the procedure was essentially as follows: the tissue was weighed and homogenized with a Thomas grinding vessel and Teflon pestle in 9 volumes of ice-cold 0.32M sucrose through which 5% CO_2 and 95% O_2 had been bubbled for 15 min. The homogenate was centrifuged at 100 g for 10 min. at 0–4°C. After gently stirring the supernatant to obtain a uniform suspension of synaptosomes, an aliquot of 0.1 ml was added to a flask containing 3.8 ml of Krebs-Henseleit bicarbonate medium (pH 7.4) with glucose (11 mM), half strength calcium (1.3 mM), ascorbic acid (0.2 mg/ml), disodium EDTA (0.05 mg/ml), and pargyline (125 μM), an inhibitor of monoamine oxidase. Fifty microliters of the cannabinoid (final concentration of 5×10^{-9} to 10^{-4} M) or its vehicle, [saline containing 1% polyvinyl pyrrolidone (PVP)], was added to the reaction flasks. Following the 10 min. preliminary incubation at 37°C under 5% CO_2 and 95% O_2 in a Dubnoff metabolic shaker, the radioactive DA, try, Ch or NE (50 μl) was added (50 nM) and the incubation was continued for 5 min., a time at which the uptake of these agents in this system was still linear. The reaction was terminated by adding 4 ml of ice-cold 0.9% NaCl and placing the flasks in an ice bath. The samples were transferred to cold centrifuge tubes and centrifuged at 20,000 g for 20 min. at 0–4°C. The supernatant was discarded and the pellet was washed by suspension in ice-cold 0.9% NaCl and centrifuged at 20,000 g for 20 min. After the surface of the pellet was rinsed with saline, the pellet was solubilized in 0.7 ml NCS tissue solubilizer (Amersham/Searle). The radioactivity of a 0.5 ml aliquot (0.5 ml) was then estimated by liquid scintillation spectrometry using scintillation fluid containing 5 g of 2,5-diphenyloxazole and 100 mg of 1,4-*bis*[2-(4-methyl-5-phenyloxazolyl)]benzene per litre of toluene.

For the release experiments, a synaptosomal suspension was allowed to equilibrate for 10 min. without cannabinoids or PVP vehicle. The [^{3}H]-amine (DA or NE) was then

added and the incubation was continued for 15 min. The reaction was terminated with cold buffer and the reaction mixture was treated in exactly the same manner as described for the uptake experiments. After the second centrifugation at 20,000 g, the pellet was resuspended in 3.95 ml of modified Krebs-Henseleit buffer, pH 7.4. Cannabinoids (10^{-8} to 10^{-4} M) or PVP vehicle (50 μl) was then added and the incubation continued for 10 min. The reaction was terminated with 4 ml of ice cold modified Krebs-Henseleit buffer containing 5 mM EGTA and then centrifuged at 20,000 g for 2 min. One ml of the supernatant was added to a vial and the radioactivity was estimated by liquid scintillation spectroscopy as described in the uptake experiments.

Statistical analysis were performed using Student's *t*-test (two-tailed) or one-way analysis of variance to test for overall significance of effect (with subsequent individual comparisons using Dunnett's *t*-test).

RESULTS AND DISCUSSION

The data presented in Table 1 show that Δ^9-THC inhibited the uptake and facilitated the release of [^{3}H]-serotonin from rat forebrain synaptosomes. These data which have

TABLE 1. THE EFFECTS OF Δ^9-THC ON THE SYNAPTOSOMAL UPTAKE AND RELEASE OF [^{3}H]-SEROTONIN[a]

	Inhibition of uptake (5 min)	Release (20 min)
ED-50	39 μM	322 μM
95% confidence limits	(31–50) μM	(132–795) μM

a. 37 nM [^{3}H]-Serotonin used in each experiment

been reported in more detail[3] suggest that the effects of Δ^9-THC are not limited to either the axonal or synaptic vesicle membrane. These data suggest that the effects of Δ^9-THC are more potent at the axonal membrane in causing inhibition of reuptake of serotonin than at the synaptic vesicle membrane in facilitating release. The confidence limits of the slopes of the dose response curves overlapped prohibiting a clear cut delineation of a difference of potency in these effects of Δ^9-THC[3]. The data presented in Table 2 show that the faci-

TABLE 2. EFFECT OF Δ^9-THC ON THE LEVELS OF RADIOLABELLED SEROTONIN AND INDOLE ACETIC ACIDS IN THE MEDIUM

Concentration of Δ^9-THC (μM)	dpm/ml medium	
	[^{3}H]-Serotonin[a]	[^{3}H]-Indole acetic acids
Vehicle	1947 ± 57	3787 ± 163
20	1672 ± 184	3602 ± 222
55	2053 ± 193	3912 ± 26
100	1781 ± 252	4644 ± 193[b]

a. 1.7×10^{-7} M used.
b. Significantly different from control at $p < 0.01$.

litated release of serotonin is probably at the vesicle rather than from the synaptosome since the increase in radioactivity in the medium is indole acetic acid rather than serotonin. The hypothesis is that the serotonin released from the vesicle is metabolized to the indole acids by monoamine oxidase which is an intracellular enzyme.

The inhibitory effect of Δ^9-THC on the synaptosomal uptake of serotonin was confirmed and extended to include a number of its analogues and metabolites[(4)]. No correlation emerged between potency in inhibiting the uptake of serotonin and the effect of these cannabinoids on behavior of animals or man. Δ^9-THC was shown to be a potent inhibitor of the uptake of the precursors tryptophan and choline into rat brain synaptosomes[(5)]. Collectively, the data from our laboratory presented above and that published by other workers suggest that cannabinoids decrease uptake of a number of substances probably at the neuronal level and facilitate the release of neurotransmitters due to an effect on the membranes of synaptic vesicles. The rest of the data presented in this paper were generated in an attempt to investigate more completely these two active sites of cannabinoid activity and to determine if a correlation exists between the potency of the cannabinoids for either of these two effects and their potency in producing behavioral alterations.

We studied the effects of the naturally occurring cannabinoids Δ^9-THC, cannabinol and cannabidiol as well as Δ^8-THC and the initial metabolite of Δ^9-THC (11-OH-Δ^9-THC) on the uptake and release of [^{3}H]DA and [^{3}H]NE from a synaptosomal enriched homogenate of rat hypothalamus and corpus striatum (see Methods). The data presented in Table 3 show that Δ^9-THC had a biphasic effect on the uptake of both [^{3}H]DA and [^{3}H]NE into synaptosomes from both brain areas. Δ^8-THC produced effects on the uptake of these neurotransmitters which were very similar to those presented in Table 3 for Δ^9-THC. Cannabinol and cannabidiol on the other hand, presented a different profile of activity in these test systems. The effects of cannabinol on the uptake of [^{3}H]DA and [^{3}H]NE are presented in Table 4 as a prototype example of the effects of these two cannabinoids. Neither cannabinol nor cannabidiol caused an increase in the uptake of either [^{3}H]DA or [^{3}H]NE at any concentration tested. These two cannabinoids also differ from Δ^8- and Δ^9-THC in that they have considerably less behavioral effects in both laboratory animals and man.

TABLE 3. EFFECT OF Δ^9-THC ON THE UPTAKE OF [^{3}H]DA AND [^{3}H]NE INTO HYPOTHALAMIC AND CORPUS STRIATAL SYNAPTOSOMES

Concentration of Δ^9-THC (M)	DA		NE	
	H.[a]	C.S.[a]	H.	C.S.
1×10^{-8}	N.C.[b]	N.C.	N.C.	N.C.
5×10^{-8}	$\uparrow p < 0.05$[c]	$\uparrow p < 0.05$	N.C.	N.C.
1×10^{-7}	$\uparrow p < 0.01$	$\uparrow p < 0.005$	$\uparrow p < 0.005$	$p < 0.005$
2×10^{-7}	N.C.	$\uparrow p < 0.005$	$\uparrow p < 0.05$	N.C.
1×10^{-6}	N.C.	N.C.	N.C.	N.C.
1×10^{-5}	$\downarrow p < 0.05$[d]	$\downarrow p < 0.01$	$\downarrow p < 0.005$	$\downarrow p < 0.01$
1×10^{-4}	$\downarrow p < 0.001$	$\downarrow p < 0.001$	$\downarrow p < 0.001$	$\downarrow p < 0.001$

a. H = Hypothalamus, C.S. = corpus striatum
b. N.C. = no change from control
c. Significant increase over control at $p < 0.05$
d. Significant decrease from control at $p < 0.05$

TABLE 4. EFFECT OF CANNABINOL ON THE UPTAKE OF [^{3}H]DA AND [^{3}H]NE INTO HYPOTHALAMIC AND CORPUS STRIATAL SYNAPTOSOMES.

Concentration of CBN (M)	DA		NE	
	H.[a]	C.S.[a]	H.[a]	C.S.[a]
5×10^{-9}	N.C.[b]	N.C.	N.C.	N.C.
1×10^{-8}	N.C.	N.C.	N.C.	N.C.
5×10^{-8}	N.C.	N.C.	N.C.	N.C.
1×10^{-7}	N.C.	N.C.	N.C.	N.C.
1×10^{-6}	$\downarrow p < 0.05$[c]	$\downarrow p < 0.05$	N.C.	N.C.
1×10^{-5}	$\downarrow p < 0.005$	$\downarrow p < 0.001$	N.C.	$\downarrow p < 0.001$

a. H = hypothalamus, C.S. = corpus striatum
b. N.C. = no change from control
c. Significant decrease from control at $p < 0.05$

The data presented in Tables 3 and 4 clearly show that cannabinoids alter the function of the axonal membranes of the hypothalamus and corpus striatum areas rich in noradrenergic and dopaminergic neurons, respectively. The data presented in Table 5 show that Δ^9-THC also has a biphasic effect on the synaptic vesicle membrane as evidenced by alterations in the release of radioactivity from synaptosomes of the hypothalamus and corpus striatum preloaded with [^{3}H]DA and [^{3}H]NE.

TABLE 5. THE EFFECT OF Δ^9-THC ON THE RELEASE OF RADIOACTIVITY FROM SYNAPTOSOMES PRELOADED WITH [^{3}H]DA OR [^{3}H]NE.

Concentration of Δ^9-THC (M)	[^{3}H]DA		[^{3}H]NE	
	H[a]	C.S.[a]	H[a]	C.S.[a]
10^{-7}	N.C.[b]	$\downarrow p < 0.05$	$\downarrow p < 0.05$	$\downarrow p < 0.05$
10^{-5}	N.C.	$\uparrow p < 0.005$	N.C.	N.C.
10^{-4}	$\uparrow p < 0.005$[c]	$\uparrow p < 0.001$	$\uparrow p < 0.00\%$	$\uparrow p < 0.001$

a. H = hypothalamus, C.S. = corpus striatum
b. N.C. = no change from control
c. Significant increase over control at $p < 0.005$

Once again cannabinol and cannabidiol did not have a biphasic effect on the release of either DA or NE from the hypothalamus or corpus striatum. They caused a pronounced facilitation of the release of both neurotransmitters in each brain region.

These biphasic effects of Δ^8 and Δ^9-THC may supply a molecular explanation for a number of other biphasic effects produced by these compounds. They produce tremors at low doses in mice and sedation which is manifested as prostration at very high doses. Cannabinol and cannabidiol do not have these biphasic effects on overt behavior. Low doses increase and high doses decrease response rate [6, 7, 8]. Ho and his colleagues[9] reported that low doses of Δ^9-THC increased brain NE and decreased serotonin levels while high doses had the opposite effects on these neurotransmitters. The levels of cyclic AMP have been reported to be decreased at high doses and increased at low doses[10]. A

more detailed discussion of other biphasic effects of cannabinoids is presented in our detailed presentation of these results[11].

An additional aspect of these studies that is worthy of mention is the pronounced differences between the actions of 11-OH-Δ^9-THC and Δ^9-THC on the uptake and release of DA and NE in these brain regions. It has been proposed for some time that Δ^9-THC is converted to 11-OH-Δ^9-THC and some have suggested that the parent compound had minimal activity, if any at all. The data presented in Table 6 contrasts the effects of Δ^9-THC and its metabolite 11-OH-Δ^9-THC on the release of DA and NE from the hypothalamus. These data show that Δ^9-THC and 11-OH-Δ^9-THC have major differences on the release of DA and NE from synaptosomes. There were also significant differences between the two compounds on the uptake of the neurotransmitters. The metabolite did not produce the typical biphasic effect described above for Δ^8 and Δ^9-THC. A more detailed presentation of the effects of 11-OH-Δ^9-THC on this system is being prepared for publication.

TABLE 6. EFFECTS OF Δ^9-THC AND 11-OH-Δ^9-THC ON THE RELEASE OF DA AND NE FROM HYPOTHALAMIC SYNAPTOSOMES.

	Hypothalamus		Corpus Striatum	
Conc (M)	Δ^9-THC	11-OH-Δ^9-THC	Δ^9-THC	11-OH-Δ^9-THC
5×10^{-9}	—[a]	N.C.[b]	—	$\uparrow p < 0.05$
1×10^{-8}	—	N.C.	—	$\uparrow p < 0.05$
1×10^{-7}	N.C.	N.C.	$\downarrow p < 0.05$	$\uparrow p < 0.01$
1×10^{-6}	—	N.C.	—	$\uparrow p < 0.01$
1×10^{-5}	N.C.	N.C.	N.C.	$\uparrow p < 0.001$
1×10^{-5}	$\uparrow p < 0.001$[c]	$\downarrow p < 0.001$	$\uparrow p < 0.001$	$\uparrow p < 0.001$

a. — = not tested
b. N.C. = no change
c. Significantly greater than control at $p < 0.001$

If this biphasic effect shown by the psychoactive cannabinoids, Δ^8 and Δ^9-THC at the molecular level reflects a cellular mechanism of action for the other biphasic effects reported for Δ^9-THC, then the lack of biphasic action by 11-OH-Δ^9-THC on this system supports our previous contention that Δ^9-THC itself rather than the metabolite might have effects on the brain.

ACKNOWLEDGEMENT

These studies and the preparation of this manuscript were supported by USPHS grants DA-00490, DA-00326 and DA-01647.

REFERENCES

1. HARRIS, L. S., DEWEY, W. L. and RAZDAN, R., Cannabis: Its Chemistry, Pharmacology and Toxicology. In "Drug Addiction" II, W. R. Martin, ed. *Handb. Exp. Pharm.* **45**,371–429 (1977).
2. COYLE, J. T. and SNYDER, S. H., Catecholamines uptake by synaptosomes in homogenates of rat brain: Stereospecificity in different brain areas. *J. Pharmacol. Exp. Ther.* **170**,221–231 (1969).

3. Johnson, K. M., Ho, B. T. and Dewey, W. L., Effects of Δ^9-tetrahydrocannabinol on neurotransmitter accumulation and release mechanisms in rat forebrain synaptosomes. *Life Sci.* **19**,347–356 (1976).
4. Johnson, K. M., Dewey, W. L. and Harris, L. S., Some structural requirements for inhibition of high affinity synaptosomal serotonin uptake by cannabinoids. *Mol. Pharmacol.* **12**,345–352 (1976).
5. Johnson, K. M. and Dewey, W. L., Effects of Δ^9-THC on the synaptosomal uptake of ^{3}H-tryptophan and ^{3}H-choline. *Pharmacol.* (in press).
6. Ferraro, D. P., Grilly, D. M. and Lynch, W. C., Effect of marihuana extract on operant behavior of chimpanzees. *Psychopharmacologia* **22**,333–351 (1971).
7. Davis, W., Moreton, J., King, W. and Pace, S. H., Marihuana on locomotor activity: Biphasic effect and tolerance development. *Res. Comm. Chem. Path. Pharmacol.* **3**,29–35 (1972).
8. Grishman, M. G. and Ferraro, D. P., Biphasic effects of Δ^9-tetrahydrocannabinol on variable interval schedule performance in rats. *Psychopharmacologia* **27**,163–169 (1972).
9. Ho, B. T., Taylor, D. V., Fritchie, G. E., Englert, L. F. and McIsaac, W. M., Neuropharmacological study of Δ^9- and Δ^8-tetrahydrocannabinol in monkeys and mice. *Brain Res.* **38**,163–170 (1972).
10. Dolby, T. W. and Kleinsmith, L. J., Effects of Δ^9-tetrahydrocannabinol on the levels of cyclic AMP in mouse brain. *Biochem. Pharmacol.* **23**,1817–1825 (1974).
11. Poddar, M. K., Dewey, W. L. and Harris, L. S., Effects of cannabinoids on catecholamine uptake and release in hypothalamic and striatal synaptosomes. *J. Pharmacol. Exp. Ther.* (submitted for publication).

THE EFFECT OF *IN VIVO* TREATMENT WITH $(-)\Delta^1$-TETRAHYDROCANNABINOL, AND OTHER PSYCHOACTIVE DRUGS ON THE *IN VITRO* UPTAKE OF BIOGENIC AMINES

MOSHE HERSHKOWITZ

Department of Isotope Research, The Weizmann Institute of Science, Rehovot, Israel.

Summary. Since cannabinoids inhibit the uptake of biogenic amines into synaptosomes *in vitro*, the effects of chronic and acute $(-)\Delta^1$-tetrahydrocannabinol [$(-)\Delta^1$-THC] administration on the uptake of ^{3}H-dopamine (^{3}H-DA) into cortical and striatal synaptosomes from mouse brain were tested in the present study. To test whether the effects of $(-)\Delta^1$-THC on ^{3}H-DA uptake are specific, $(-)\Delta^1$-THC was compared to $(+)\Delta^6$-THC administration.

The uptake of ^{3}H-DA into cortical synaptosomes increased in mice pretreated acutely with $(-)\Delta^1$-THC or cannabidiol (CBD) but not in mice pretreated with $(+)\Delta^6$-THC. The ^{3}H-DA uptake was no longer increased after chronic (1 week) treatment with $(-)\Delta^1$-THC. The effect of *in vivo* treatment with $(-)\Delta^1$-THC on the *in vitro* uptake was not specific to ^{3}H-DA, but enhanced also the uptake of ^{3}H-norepinephrine (^{3}H-NE), ^{3}H-5-hydroxytryptamine (^{3}H-5HT), and ^{3}H-γ-aminobutyric acid (^{3}H-GABA) into cortical or whole brain synaptosomes. Intravenous administration of dopamine or norepinephrine receptor antagonists, i.e. chlorpromazine, haloperidol or propanolol, enhanced the uptake of ^{3}H-DA, and ^{3}H-NE respectively into cortical synaptosomes. Pretreatment with uptake inhibitors such as the tricyclic antidepressants, amphetamine, or with the monoamine oxidase inhibitor, pargyline, caused an inhibition of the uptake of ^{3}H-DA or ^{3}H-NE into the isolated synaptosomes. It is suggested that *in vivo* $(-)\Delta^1$-THC does not act on uptake mechanism but on receptors.

INTRODUCTION

Δ^1-TETRAHYDROCANNABINOL (Δ^1-THC), the main psychoactive component of hashish[1], as well as other cannabinoids, including nonpsychoactive derivatives, have been reported to exert an inhibitory effect on the uptake of certain neurotransmitters into isolated nerve endings from brain[2–6]. Cannabidiol (CBD), the major nonpsychoactive constituent of hashish[7] was found to be more effective than Δ^1-THC in inhibiting the uptake, and as potent as the Δ^1-THC in inhibiting the activity of Na^+-K^+-ATPase and Mg^{++}-ATPase, and in damaging the nerve ending membrane at concentrations higher than 2×10^{-5} M[6]. In an attempt to characterize the effect of Δ^1-THC on the central nervous system *in vivo* the content of several biogenic amines were investigated in animals under the effect of Δ^1-THC. Serotonin content of the brain has been reported to be increased under the effect of Δ^1-THC[8, 9], catecholamine content of the brain to be decreased[8].

Recently, it has been reported that chronic injection of Δ^1-THC increased the content of

γ-aminobutyric acid (GABA) in rats cerebellum, as well as increasing ^{3}H-GABA uptake into cerebellar synaptosomes[10].

Reports on the choline high affinity uptake into isolated synaptosomes indicated that various treatments which alter the activity of cholinergic neurons *in vivo* also cause a parallel change in the high affinity choline uptake *in vitro*[11, 12].

In the present study the actions of acute and chronic treatment with Δ^1-THC, CBD and catecholamine related drugs on the high affinity uptake of biogenic amines into isolated synaptosomes from different mouse brain regions is investigated in order to determine the effect of Δ^1-THC injected to mice on the biogenic amine activity in the brain.

METHODS

Adult male Balb/c mice (20–25 g) were used in all experiments. Drugs were administered into the tail vein; the injection volume did not exceed 0.3 ml. Cannabinoid compounds and chlorimipramine were dissolved in 0.5% Tween-80 in saline. Other drugs, including the water soluble THC, were dissolved in saline. At the appropriate time, the animals were decapitated, the brain rapidly removed to a dish of ice and the cerebral cortices and caudate nucleus were dissected for homogenization and preparation of the crude synaptosomal fraction.

The brain tissue was homogenized in 20 volumes of 0.32 M sucrose. The homogenate was centrifuged at 1000 × g for 10 minutes. The resulting supernatant fraction, S_1 was utilized for the uptake. The uptake of radioactive amines was carried out according to the method described in many previous reports[13, 6]. Portions (0.1 ml) of S_1 fraction were added to test tubes containing 0.9 ml of Krebs Ringer phosphate buffer, pH 7.4 containing: 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 10 mM glucose, 25 mM $NaHCO_3$, 10 mM Na_2EDTA, 0.2 mg/ml ascorbic acid, and 0.1 mM pargyline, pregassed with 95% O_2 5% CO_2. After 5 min. of preincubation at 37°C in a shaking bath under 95% O_2, 5% CO_2 atmosphere 0.05–0.1 μM of the tritiated neurotransmitter was added to each flask (0.2–0.5 μ Ci) and incubation was continued for an additional 5 min. in the same conditions as the preincubation. The uptake was terminated by transferring the tubes from the shaking bath to ice-water bath, and 1.0 ml of ice cold saline containing 10^{-5} M of the appropriate neurotransmitter was added to each tube. The test tubes were centrifuged at 42000 × g and aliquots of the supernatants were taken for radioactivity measurements. The pellets were washed and dissolved in 1.0 ml of 1% sodium dodecyl sulfate (SDS). Ten ml of toluene-triton scintillation liquid were added to the samples and their radioactivity assessed in a Packard 3385 liquid scintillation spectrometer. Radioactivity associated with crude synaptosome suspensions incubated with radioactive labelled neurotransmitters at 0°C was used to correct for nonspecific absorption. The radioactivity taken by the synaptosomes was followed by ascending chromatography on fluorescent silica gel coated plates with *n*-butanol: acetic acid, water (25 : 4 : 10) as the developing solvent.

Chlorpromazine was obtained from Taro Co. (Haifa, Israel), Δ^1-THC and cannabidiol from Makor Chemicals (Jerusalem, Israel), (+)Δ^6-THC and SP-111 were kindly donated by Prof. R. Mechoulam and Dr. Marq Segal, respectively. [^{3}H] serotonin (2.72 Ci/mmole), [^{3}H]γ-aminobutyric acid (36.7 Ci/mmole) were obtained from New England Nuclear

Corp., L-7-[^{3}H]-norepinephrine (10.9 Ci/mmole) and [^{3}H] dopamine (2.3 Ci/mmole) from the Radiochemical Centre (Amersham, England); other drugs were purchased from Sigma Co.

RESULTS

Of the several constituents of marijuana injected into the tail vein of mice, Δ^1-THC was the most potent in its effect on the uptake of ^{3}H-DA into cortical synaptosomes. Thus, although injections of either Δ^1-THC or CBD enhanced, in a dose dependent manner, the uptake of ^{3}H-DA into cortical synaptosomes prepared 30 minutes later, the effect of Δ^1-THC was much greater (Fig. 1). Similarly, at one hour after injection, the uptake of

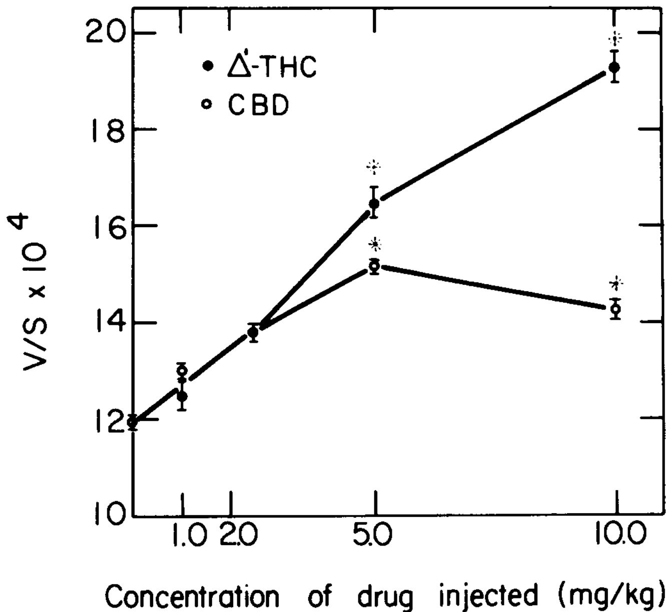


FIG. 1. Dose response curves for the effect of $(-)\Delta^1$-THC and CBD on the uptake of ^{3}H-DA into cortical synaptosomes. Mice were injected into their tail vein with the drug dissolved in Tween saline vehicle. Thirty minutes after injections crude synaptosomes were prepared from cortices, and the uptake analyses were performed as described in legend to Table 1. The values are ^{3}H-DA dpm in synaptosomes per mg protein, per 5 min, per ^{3}H-DA dpm in the incubation medium. The values are means of at least 10 animals $\pm$ S.D. Those significantly different from vehicle treated mice are indicated with an asterisk (Student t test, $p < 0.01$).

^{3}H-DA into cortical synaptosomes was enhanced significantly, in a decreasing order of potency, by Δ^1-THC, SP-111 (a psychoactive water soluble ester of Δ^1-THC) and CBD (Table 1). Interestingly, the addition of CBD to the injection of Δ^1-THC produced a greater effect on ^{3}H-DA uptake than Δ^1-THC alone ($t = 6.34$; $n = 32$; $p < .001$; Table 1).

The effect of THC is stereospecific. Table 1 indicates that $(+)\Delta^6$-THC, the nonpsychoactive stereoisomer of $(-)\Delta^1$-THC, had no effect on the uptake of ^{3}H-DA into cortical synaptosomes prepared from mice 1 hour after injection with the drug.

TABLE 1. EFFECT OF PRETREATMENT WITH DIFFERENT CANNABINOIDS ON THE HIGH AFFINITY UPTAKE OF [^{3}H] DA INTO CORTICAL SYNAPTOSOMES.

Treatment	Net uptake (p moles DA/mg protein /5 min ± S.D.) (n)	Percent change over control vehicle
Control saline	2.40 ± 0.12 (10)	
Control vehicle	2.50 ± 0.22 (24)	
(−)Δ^1-THC	3.70 ± 0.35 (24)	48[a]
(+)Δ^6-THC	2.40 ± 0.30 (10)	−4
SP-111	3.00 ± 0.34 (10)	20[a]
CBD	2.75 ± 0.28 (24)	10[b]
(−)Δ^1-THC + CBD	4.50 ± 0.36 (12)	80[a]

Mice were injected into their tail vein with 10 mg/kg of the drug dissolved in tween saline vehicle. After one hour, crude synaptosomes were prepared from cortices, and incubated with 10^{-7} M dopamine, 0.4 μCi ^{3}H-dopamine at 37°C for 5 min. After preincubation of 5 min the reaction was stopped in cold, and synaptosomes were collected by centrifugation.

a. Level of significance is $p < 0.01$ using a two tailed Student t test.
b. $p < 0.05$.

The effect of Δ^1-THC on the ^{3}H-DA uptake is specific to the brain region from which the synaptosomes were prepared. Thus, the effect on the uptake of ^{3}H-DA is not only significantly greater in the cortex than in the striatum but also lasts longer (Fig. 2). Furthermore, chronic treatment with THC induces tolerance to the enhancing effect of this substance on the uptake of ^{3}H-DA into cortical but not striatal synaptosomes. In particular, after 1 week of treatment with 5 mg/kg of Δ^1-THC (i.p.), an intravenous injection of 10 mg/kg of Δ^1-THC no longer potentiated the uptake of ^{3}H-DA into synaptosomes prepared from cerebral cortex 1 hour after injection. In contrast, the uptake into striatal synaptosomes remained unchanged (Table 2).

Pretreatment with Δ^1-THC does not potentiate the uptake only of DA into cortical synaptosomes. Table 3 indicates that the uptake of NE, 5HT and GABA were also en-

TABLE 2. EFFECT OF PRETREATMENT WITH Δ^1-THC ON THE HIGH AFFINITY UPTAKE OF DOPAMINE INTO SYNAPTOSOMES.

Treatment	Cerebral Cortex		% change	Striatum		% change
	Control (n)	Δ^1-THC (n)		control (n)	Δ^1-THC (n)	
Acute	2.40 ± 0.35 (8)[a]	3.32 ± 0.36 (8)	38[b]	5.90 ± 0.50 (6)	6.12 ± 0.47 (7)	3
Chronic	2.55 ± 0.35 (8)	2.55 ± 0.32 (8)	0	6.42 ± 0.24 (7)	5.97 ± 0.90 (7)	−7

Mice were injected into their tail vein with 10 mg/kg Δ^1-THC, one hour before sacrifice. Crude synaptosomes from cerebral cortex or striatum were incubated with 10^{-7} M dopamine, 0.4 μCi ^{3}H-dopamine, at 37°C for 5 min after preincubation of 5 min. The reaction was stopped in cold and synaptosomes collected by centrifugation. The mice treated chronically were injected daily intraperitoneally with 5 mg kg^{-1} of (−)Δ^1THC during 7 consecutive days. The last injection (10 mg (−)Δ^1-THC kg^{-1}) was administered into the tail vein.

a. Uptake values (pmoles dopamine/mg protein/5 min) are mean ± S.D.
b. Level of significance is $p < 0.01$ using a two-tailed t test.

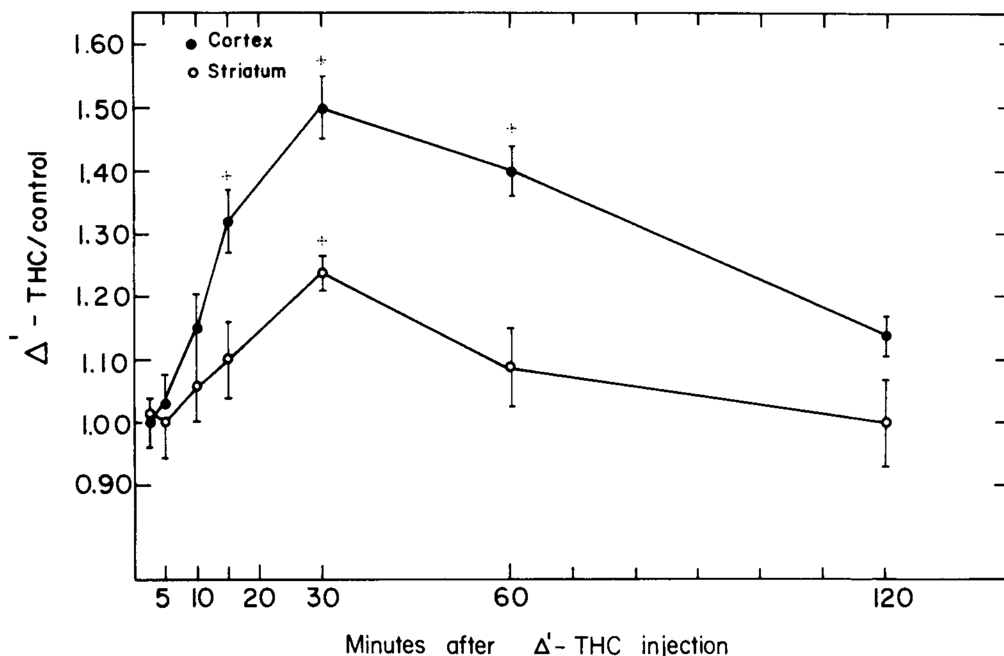


FIG. 2. Time course of the effect of (−)Δ^1-THC on the uptake of ^{3}H-DA into cortical and striatal synaptosomes. Mice were killed at different times after the injection of drug (10 mg Kg^{-1}, i.v.) and the synaptosomes were treated as described in legend to Table 1 and in the Method section. The values are the ratio of the ^{3}H-DA uptake of Δ^1-THC treated mice to the uptake values of vehicle control mice. The values are mean of at least 8 animals ± S.D. Those significantly different from vehicle treated mice are indicated with an asterisk (Student t test, $p < 0.01$).

hanced by Δ^1-THC, with the greatest effect being on GABA. Pretreatment with THC does not induce a nonspecific increase in uptake since the uptake of ^{3}H-Leucine was not affected (Table 3). Similarly, pretreatment with CBD had no significant effect on the *in vitro* uptake of NE, 5HT and GABA (Table 3).

TABLE 3. EFFECT OF PRETREATMENT WITH Δ^1-THC OR CBD ON HIGH AFFINITY UPTAKE OF TRITIATED NE, 5-HT, GABA, AND LEUCINE INTO CORTICAL SYNAPTOSOMES.

Neuro-transmitter	Control (n)	(−)Δ^1-THC (n)	CBD (n)	Δ^1-THC / control	CBD / control
NE	0.73 ± 0.07 (8)[a]	0.90 ± 0.04 (8)	0.70 ± 0.06 (5)	1.23[b]	0.96
5-HT	7.50 ± 0.46 (7)	9.80 ± 1.25 (8)	8.30 ± 1.05 (7)	1.31[b]	1.11
GABA	1.74 ± 0.24 (7)	3.15 ± 0.27 (7)	2.05 ± 0.26 (5)	1.81[b]	1.18
Leucine	5.00 ± 0.42 (5)	5.12 ± 0.36 (5)	—	1.02	—

Δ^1-THC and CBD administrations to animals is described in legend to Table 1. Crude synaptosomes from cerebral cortex were incubated with 10^{-7} M NE, 0.5 μCi [^{3}H]-NE or with 2.5 × 10^{-7} M 5-HT, 2 μCi [^{3}H]-5-HT. The high affinity uptake of GABA and leucine was performed with crude synaptosomes obtained from the whole brain, with 5.10^{-8} M GABA, 2 μCi [^{3}H]-GABA, and 10^{-6} M leucine, 2 μCi [^{3}H]-leucine.

a. pmoles/mg prot./5 min values are mean ± S.D.
b. $p < 0.01$ using two-tailed t test.

In order to compare Δ^1-THC with other drugs known to affect biogenic amines, I tested the effect of *in vivo* pretreatment with several such compounds on the *in vitro* uptake of DA and NE into cortical and striatal synaptosomes (Table 4).

Pretreatment with DA receptors antagonist, CPZ, or with the NE receptor antagonist, propanolol, significantly enhanced the uptake of DA and NE respectively. Haloperidol did not affect the uptake of DA. Pretreatment with the MAO inhibitor, pargyline or with the catecholamine uptake inhibitors; DMI, chlorimipramine, or amphetamine, inhibited the *in vitro* uptake of DA and NE into cortical synaptosomes. Pretreatment with amphetamine inhibited the uptake of DA into striatal synaptosomes more than into cortical synaptosomes. It also affected the uptake of NE more than of DA (Table 4).

TABLE 4. EFFECT OF *IN VIVO* TREATMENT WITH DIFFERENT DRUGS ON THE *IN VITRO* UPTAKE OF DOPAMINE AND NOREPINEPHRINE INTO CORTICAL AND STRIATAL SYNAPTOSOMES.

	Cortex		Striatum
Drug (mg/Kg)	DA Drug/control (n)	NE Drug/control (n)	DA Drug/control (n)
Chloropromazine (5)	1.60[a] (12)		
Haloperidol (1)	1.12 (12)		
Propanolol (1)		1.50[a] (12)	
Pargyline (10)	0.85[b] (12)		
Amphetamine (5)	0.85[b] (16)	0.75[a] (10)	0.80[a] (12)
Amphetamine (10)	0.72[a] (15)	0.47[b] (10)	0.42[a] (12)
Chlorimipramine (5)	0.63[a] (12)		
Desmethylimipramine (5)		0.65[a] (10)	

Schedule of drug injections and the method of analysis uptake of DA and NE is described in Table 1 legend and method section.

a. $p < 0.01$ by "t" test, two tails.
b. $p < 0.05$.

DISCUSSION

The *in vivo* treatment with $(-)\Delta^1$-THC, the psychoactive constituent of marihuana, had affected the *in vitro* uptake of biogenic amines into mice cortical synaptosomes. My results are different from the *in vitro* studies on the effect of cannabinoids on the uptake of biogenic amines in that I found that the effect of $(-)\Delta^1$-THC on uptake, is stimulatory while in the *in vitro* studies it was found to be inhibitory[4, 6]. Another new finding that emerged from the present study is the specific effect of Δ^1-THC on DA uptake in the *in vivo-in vitro* system when compared to the ineffectiveness of its nonpsychoactive stereoisomer, $(+)\Delta^6$-THC (Table 1).

The change in a neurotransmitter uptake *in vitro* after a pretreatment with a drug *in vivo*, may reflect a change in the activity of the appropriate neurons *in vivo*. This change may be due to interaction of the drug with enzymes regulating the biogenic amines turnover such as MAO, and indeed pretreatment with pargyline one hour before sacrifice caused a significant inhibition of uptake into cortical synaptosomes (Table 4). Pretreatment with drugs known to be inhibitors of amine uptake such as chlorimipramine and desmethylimi-

pramine, also caused inhibition of the *in vitro* uptake of DA and NE, respectively, into cortical synaptosomes (Table 4). Similarly, it was reported recently that cocaine, a potent NE uptake inhibitor, inhibits the uptake of NE into synaptosomes that were obtained from animals pretreated with cocaine[17]. Pretreatment with amphetamine, a catecholamine releaser, also caused an inhibition in the uptake of NE into cortical synaptosomes and of DA into cortical and striatal synaptosomes (Table 4). The common action of these drugs is that they increase the amount of the neurotransmitters in the synaptic cleft and by that lead to an increase in the activity of the postsynaptic neurones. The increase in the activity of these neurones, by a negative feedback mechanism, will inhibit the production of the neurotransmitter in the appropriate presynaptic terminal and as a consequence the uptake of that neurotransmitter will be decreased.

On the other hand, drugs which inhibit the activity of postsynaptic neurones by blocking the postsynaptic receptors, cause an increase in the turnover of the appropriate neurotransmitters[18], its release, and in consequence lead to an increase in the uptake of the neurotransmitter into the presynaptic terminals. Indeed, pretreatment with DA and NE receptors antagonists, CPZ, haloperidol and propanolol yielded an increase in the uptake of DA and NE, respectively. Although the effect of haloperidol did not reach statistical significance, this may be due to an effect of haloperidol on release of DA[19] that may have antagonized the postsynaptic receptor effect.

It appears, therefore, that the effects of THC on the uptake of biogenic amines parallels the effect of pretreatment with receptor antagonists on *in vitro* uptake, i.e. an enhancement of uptake of DA and other biogenic amines into cerebral cortex.

The $(-)\Delta^1$-THC effects on the reuptake of DA parallel the effect of THC on several behaviors. The effects of THC on spontaneous activity, catatonia, hypothermia, analgesia, and inhibition of rope climbing are dose dependent, as is the effect on DA uptake (Fig. 1); the duration of these effects correlates well with the duration of the effect on DA uptake (Fig. 2). SP-111 is less potent than $(-)\Delta^1$-THC in affecting behavior[20], and is also less potent in affecting DA uptake (Table 1). CBD potentiates, the depressant effects of $(-)\Delta^1$-THC[21] and it also potentiates the THC effect on DA uptake (Table 1). Tolerance develops to many behavioral effects of Δ^1-THC[22], and it developed also to the enhancing effect of THC on DA uptake into cortical synaptosomes (Table 2). From the present study, it is not clear which behavioral effect of THC is related to the increase in uptake of biogenic amines, but the behavioral effect ought to be potentiated by CBD, develop tolerance, appear in a few minutes after the i.v. administration of THC, and last about 2 hours. Perhaps the inhibitory effect of THC on spontaneous activity seems to be the behavior that fits best the above demands.

The effect of pretreatment with THC on uptake may be the result of an antagonistic interaction between THC and one of the biogenic amine receptors, most probably DA receptor. This suggestion is based on the following findings: (1) the effect is stereospecific (Table 1); (2) it is restricted to neurotransmitters only (Table 3); (3) it is similar to the effect of receptor antagonists on uptake (Table 4); and, (4) it shows brain regional specificity (Fig. 2, Table 2). Stereospecificity is a typical character of receptor binding of neurotransmitters and hormones. Table 3 indicates that the uptake of GABA was affected most by Δ^1-THC treatment; many GABAergic neurones in the brain are inhibited by dopaminergic neurones, therefore, any antagonistic activity on DA receptors will increase GABA turnover and uptake. The preferential effect of the Δ^1-THC treatment on the uptake of DA into cerebral over striatal synaptosomes is due perhaps to a differential distribution of

two different DA receptor populations in the cortex and striatum[23], and one of them may interact preferentially with Δ^1-THC. However, to have more direct indications on receptor-THC interaction needs study of the effect of Δ^1-THC on the biogenic amines induced activity of adenylate cyclase, and the binding of $(-)\Delta^1$-THC to biogenic amine receptors. From published studies of Dolby and Kleinsmith[24] and unpublished results from our laboratory, it appears that there is a specific effect of Δ^1-THC injected into mice on the basal activity of adenylate cyclase, and on the DA induced adenylate cyclase, respectively. In a preliminary study in our laboratory it was observed that $(-)\Delta^1$-THC interacts specifically with DA receptors; the implication of these observations on the nature of THC effect on brain biogenic amines is now under investigation.

ACKNOWLEDGEMENTS

The expert assistance of Tirza Raz and Milayna Subar and the helpful discussion of Dr. Henry Szechtman is gratefully acknowledged. I thank Prof. R. Mechoulam for the generous gift of $(+)\Delta^6$-THC and Dr. Marq Segal for SP-111A.

REFERENCES

1. Mechoulam, R., *Science*, **168**,1159–1166 (1970).
2. Sofia, R. D., Ertel, R. J., Dixit, B. N. and Barry, H. III, *Eur. J. Pharmacol.* **16**,257–259 (1971).
3. Howes, J. and Osgood, P., *Neuropharmacology*, **13**,1109–1114 (1974).
4. Banerjee, S. P., Snyder, S. H. and Mechoulam, R., *J. Pharmacol. Exp. Ther.* **194**,74–81.
5. Johnson, K. M., Dewey, W. L. and Harris, L. S., *Molec. Pharmacol.* **12**,345–352 (1976).
6. Hershkowitz, M., Goldman, R. and Raz, A., *Biochem. Pharmacol.* **26**,1327–1331.
7. Grlic, L., *Bull. Narcotics*, **14**,37–46 (1962).
8. Holtzman, D., Lovell, R. A., Yaffe, J. H. and Freedman, D. K., *Science* **163**,1464–1467 (1969).
9. Sofia, R. D., Dixit, B. N. and Barry, H. III, *Life Sci.* **10**,425–436 (1971).
10. Edery, H. and Gottesfeld, Z., *Brit. J. Pharmacol.* **54**,406–408 (1975).
11. Atweh, S., Simon, J. R. and Kuhar, M. J., *Life Sci.* **17**,1535–1544 (1976).
12. Simon, J. R., Atweh, S. and Kuhar, M. H., *J. Neurochem.* **26**,909–922 (1976).
13. Coyle, J. T. and Snyder, S. H., *J. Pharmacol. Exp. Ther.* **170**,221–231 (1969).
14. Karler, R., Cely, W. and Turkanis, S. A., *Life Sci.*, **13**,1527–1531 (1973).
15. Consroe, P. and Wolkin, A., *J. Pharmacol. Exp. Ther.* **201**,26–32 (1977).
16. Gilbert, J. C., Pertwee, R. G. and Wyllie, M. G., *Br. J. Pharmacol.* **59**,599–601 (1977).
17. Hadfield, M. G., Mott, D. E. W. and Ismay, J. A., in the 7th International Congress of Pharmacology, Paris, 1978, Abstracts p. 767.
18. Sedvall, G., in Handbook of Psychopharmacology, Vol. 6, p. 127, Plenum, New York (1975).
19. Seeman, P. and Lee, T., *J. Pharmacol. exp. Ther.* **190**,131–140 (1974).
20. Dykstra, L. A., McMillan, D. E. and Harris, L. S., *Pharmacol. Biochem. Behav.* **3**,29–32 (1975).
21. Karniol, I. G. and Carlini, E. A., *Psychopharmacol.* **33**,53–70 (1973).
22. Paton, W. D. M., *Ann. Rev. Pharmacol.* **15**,191–220 (1975).
23. Cools, A. R. and Van Rossum, J. M., *Psychopharmacol.* **45**,243–254 (1976).
24. Dolby, T. W. and Kleinsmith, L. J., *Canadian J. Physiol. Pharmacol.* **55**,934–942 (1977).

LONG TERM EFFECTS OF DELTA-9-TETRAHYDROCANNABINOL IN MICE

J. SZEPSENWOL, J. FLETCHER, G. L. MURISON
and E. TORO-GOYCO

Department of Biological Sciences, Florida International University, Miami, FL. and Department of Biochemistry, University of Puerto Rico School of Medicine, San Juan, Puerto Rico.

Summary. Delta-9-tetrahydrocannabinol administered weekly by subcutaneous injection in the amount of 20 μg in 0.05 ml of sesame oil, unlike estrogen did not interfere with the normal development of mice of the C57 B1/6 and BALB/c strains, nor with their reproduction. It caused, however, a high mortality rate among the offspring of these mice. This was particularly high among the mice of the C57 B1/6 strain and was apparently due to an inhibitory effect of THC upon the milk secretion by the mammary gland, since newborn C57 B1/6 mice which had no milk in their stomachs the day after birth survived and developed normally when foster nursed by lactating BALB/c females. Four of 200 BALB/c THC-treated mice developed fibrosarcomas at the point of injection of the drug. Of 46 C57 B1/6 THC-treated mice, one developed a mammary adenocarcinoma. Of the 32 BALB/c females receiving injections of sesame oil, eight developed mammary adenocarcinomas. Most of the other mice of both groups (THC-treated and sesame oil-treated) are still alive and may develop more tumors. All of the above tumors have been transplanted to other mice and appeared to grow most rapidly in THC-treated mice (particularly the fibrosarcomas). The transplanted mammary tumors seem to be slightly inhibited in their growth by THC-treated mice, yet the number of metastases in the lungs in these mice is considerably greater than in sesame oil-treated ones and in controls. On the basis of the results obtained so far, it is thought that sesame oil has an estrogenic effect; it causes mammary carcinogenesis by increasing the production of LH and LTH. THC appears to have an antiestrogenic effect, influencing a decrease in LH and LTH, which is the cause of defective secretion of milk by the mammary glands and the high mortality of the offspring, particularly of the C57 B1/6 mice. In addition, THC appears to have a carcinogenic effect by stimulating development of mesenchymal tumors. Its effect upon parenchymal tumors may be inhibitory. THC in large doses is cytotoxic in tissue cultures. Estrogen causes important changes in the cellular composition of the adenopituitary and consequent tumorigenesis, while THC does not seem to cause any major microscopic changes in the above gland. The subject is still being studied.

INTRODUCTION AND HISTORY

THE main purpose of the present investigation was to find out whether delta-9-tetrahydrocannabinol (THC), the psychoactive ingredient of marihuana, has an estrogenic effect, as postulated by many investigators.

It has been reputed that individuals who are chronic marihuana smokers develop gynecomastia[1, 2, 3]; their serum testosterone levels and sperm counts are decreased[4]. In rodents receiving marihuana extract testicular size and spermatogenesis diminish[5], while

in ovariectomised female rats the uterine size increases[6]. Other investigators found that chronic or acute treatment with THC lowered plasma LH and testosterone levels in male rates, reduced the pituitary response to LH-RH[7] and suppressed prostate growth[8]. In agreement with previous investigators, Goldstein, *et al.*[9] found that following chronic administration of THC or cannabidiol there is a significant reduction in testicular weight of rats and specific depression of an esterase isozyme in the interstitial tissue of the testis[10, 11].

On the other hand, it was reported[12] that oral administration of cannabis resin (containing 50% of THC, 19% of cannabidiol and 14% of cannabinol) causes a decrease in uterine weight in ovariectomised adult female rats and in normal prepuberal female rats. The above investigators also found that cannabis extract administered subcutaneously to female rats, 21 days old, causes a decrease in the water and glycogen contents of the uterus. Similar results were obtained following the administration of cannabis extract to rats pretreated with estradiol benzoate[12]. Fujimoto *et al.* also reported at this symposium (p. 441) that THC or cannabis extracts decrease uterine and ovarian weight in rats. These effects are rather antiestrogenic than estrogenic.

To be estrogenic THC would have to interact with intracellular estrogenic receptors before eliciting an estrogenic response at the nuclear level. According to Rawitch *et al.*[13] it has a weak estrogenic activity by binding to estrogen receptors of the uterine cytosol. On the other hand Smith *et al.*[14], as well as Okey and Bondy[15] found that there is no binding of THC to estrogen receptors.

Other observations of interest are the effect of marihuana upon mating activity of rodents, fertility, embryonic development and growth of the newborn. Miras[16] who used very high doses of marihuana extract, reported reduced fertility. Other investigators[17, 18], using similar doses, 5 mg/kg, did not observe any reduction in mating activity nor any impaired fertility. They did, however, mention an increased mortality of the pups following a subcutaneous injection of 25 mg/kg of delta-9-tetrahydrocannabinol to the nursing dams[17] which was attributed to the toxic effect of the drug. The teratogenic effect of THC obtained by some investigators[18] could not be verified by others who used as high doses of THC as 50 mg/kg in rats.

LONG TERM OF ESTROGEN IN MICE

Mice of the C57 B1 receiving biweekly subcutaneous injections of 10 μg of delestrogen (a slow acting estrogen), beginning at the age of 5 to 7 days, are completely sterile. In the adenopituitary the acidophile cells decrease from 57% to 30% in mice 14 to 54 weeks old, while the basophile cells decrease only slightly. Moreover, greater than 30% of the mice develop pituitary tumors which are either chromophobe adenomas of the pars distalis or intermediate lobe tumors[19]. Similar results have been obtained with mice of the T.M. strain receiving estradiol dipropionate via the digestive tube, except that in this case the mice became sterile after giving birth to one litter. In their adenopituitary both the acidophile and basophile cells decreased significantly and 16% of the mice developed pituitary tumors[20]. We would like to stress the fact that the females of this group which gave birth to one litter nursed their offspring normally. In other words, estrogen did not interfere with the secretory activity of the mammary glands, while delta-9-tetrahydrocannabinol, as it will be shown in the following, inhibits the secretion of milk.

In the present study an attempt was made to compare and contrast the effect upon mice of THC (which has supposedly an estrogenic effect) with that of estrogen.

MATERIALS AND METHODS

Mice of two strains, BALB/c and C57 B1/6 were used in the present experiments. They were divided into three groups. The mice of Group 1 received biweekly subcutaneous injections of 10 micrograms of delestrogen in 0.05 ml of sesame oil. The mice of Group 2 received 20 micrograms of delta-9-tetrahydrocannabinol in 0.05 ml of sesame oil once a week also by subcutaneous injection. The mice of Group 3 received only the vehicle, 0.05 ml of sesame oil once a week. The first and second injections, at the age of 3 to 5 and 10 to 12 days, were done in the interscapular region, while the subsequent injections were in the right groin. For microscopic studies tissues were fixed in Bouin's fluid, embedded in paraffin, sectioned serially and stained with hematoxylin and eosin. A modification of Glenner and Lillie's technique[21, 22] was used for the staining of sections of the pituitary. With this technique the granules in the acidophile cells stain red, those in the basophile cells blue-black, while the chromophobe cells are either agranular or contain grey to pink granules. The cells of the adenopituitary were counted using Chalkley's method[23].

RESULTS

The delestrogen treated mice of both strains (BALB/c and C57 B1/6) of Group 1 were limited in their growth. The average weight of these mice at the age of 6 months to one year was 22 grams, as compared to 30–35 grams of the controls. Moreover, as it was demonstrated previously[19] all of the delestrogen-treated mice remained sterile. No mature follicles nor corpora lutea could be found in the ovaries of the females. The testes in the males were atrophic and contained no spermatozoa.

One of the BALB/c female of this group developed a mammary tumor. It was killed at the age of 254 days. The tumor, which was a carcinosarcoma, was transplanted subcutaneously to other mice (estrogen-treated, THC-treated and controls). Growth was most rapid in estrogen-treated and least rapid in controls. The epithelial cells which were present in the original tumor disappeared from second and third generation tumors. This seems to be also true for the tissue cultures, prepared from the above tumor, from which the epithelial cells present originally, disappeared and in their place giant cells were found.

THE EFFECT OF DELTA-9-TETRAHYDROCANNABINOL

The mice of Group 2, receiving injections of delta-9-tetrahydrocannabinol (THC) unlike those of Group 1, were not inhibited in their growth, nor in their reproduction. The number of offspring per litter varied greatly: in some cases it was limited to 4 or 5, while in others it was as large as 10 to 12 offspring. The most striking, however, was the high neonatal mortality rate among the newborn of the THC-treated females. Of the two strains used, the BALB/c and the C57 B1/6, the offspring of the latter were the most affected. Of these only one litter out of 10 survived. Neonatal malformation[18], rarely seen, could not

FIG. 1. C57 B1/6 newborn mice, offspring of a THC-treated female, having no milk in their stomachs the first day after birth (to the left) foster nursed by a lactating BALB/c female (to the right).

be the cause of death of the newborn. Offspring of THC-treated C57 B1/6 mice which had no milk in their stomach the first day after birth (which usually die of starvation) foster nursed by lactating females of the BALB/c strain, survived and developed normally (Fig. 1). It was also found that another way of saving the offspring of a THC-treated C57 B1/6 female is to inject the latter with sesame oil instead of THC during the last week of pregnancy and immediately after the delivery.

Another finding, which greatly surprised us, is the development of malignant tumors by the THC-treated mice. As shown in Table 1, of a total of 200 BALB/c mice of this group, 4 mice (3 females and 1 male) have already developed rapidly growing fibrosarcomas at the

TABLE 1. TUMORS IN MICE OF THE BALB/c STRAIN RECEIVING INJECTIONS OF SESAME OIL, OF SESAME OIL + THC AND OF ESTROGEN.

	Number of		Incidence of fibrosarcoma	Incidence of mammary cancer	Incidence of other tumors
	Males	Females			
Controls	150	180	0	0	1* (0.03%)
Delestrogen	20	26	0	1 (3.8%)	0
Sesame oil	28	32	0	8 (25%)	0
Delta-9-THC	100	100	4 (2%)	0	0

* Lymphosarcoma.

points of injection of THC. In one female (183 days old) and in one male (214 days old) the tumors had their origin in the interscapular region where the mice received their first two injections of the drug. In the other two females (410 and 445 days old) the fibrosarcomas developed in the right groin, the place where they received most of the THC injections (Fig. 2 and 3).

Fragments of every one of the above fibrosarcomas of 1 mm^3 were transplanted subcutaneously (with the use of 13 gauge needle) to control BALB/c mice, to mice receiving injections of delestrogen, of sesame oil and to THC-treated ones. The take was 100% in the mice of the four groups, but growth was most rapid in the THC-treated mice, as seen by the size of the tumors and by the relative number of mitotic figures.

Tissue from the fibrosarcoma of the second generation have been cultivated *in vitro* in Dulbecco MEM + 10% fetal calf serum where growth was found to be equally fast whether the transplant was removed from a control mouse or from a THC-treated one. Such cultures, each containing approximately 10^6 cells, were equally divided and injected subcutaneously, one half to a control mouse and the other half to a THC-treated one. In the latter the tumor developing from the tissue culture cells could be detected one week after innoculation and at 21 days it extended over half of the body of the mouse, while in the control it was only slightly noticeable (Fig. 4). Twenty four days after inoculation the THC-treated mouse became moribund and had to be sacrificed. The control mouse outlived the THC-treated one by 20 days during which it developed an enormous tumor.

Besides the four mice with fibrosarcoma just described, three more THC-treated mice of Group 2 are now starting to develop tumors at the right groin, but since so far the nature of these neoplasms could not be determined, they are not listed in Table 1.

FIG. 2. THC-treated BALB/c female 183 days old (left) and male 214 days old (right) with primary fibrosarcomas originating from the interscapular region, the point of the first two injections of the drug.

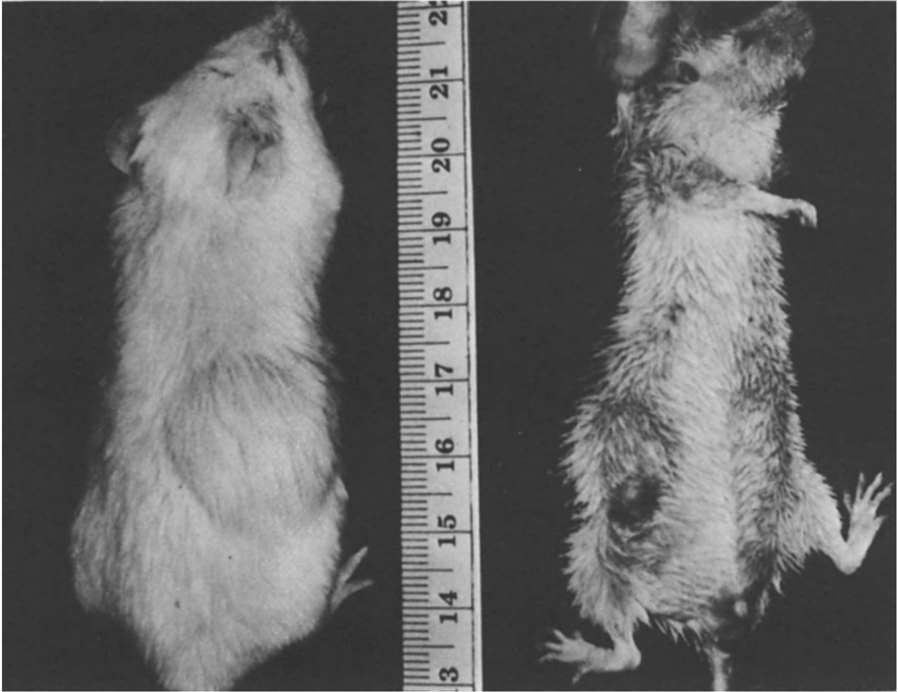


FIG. 3. THC-treated BALB/c females 410 and 445 days old with primary fibrosarcomas originating from the right groin, the region where the mice received most of the injections.

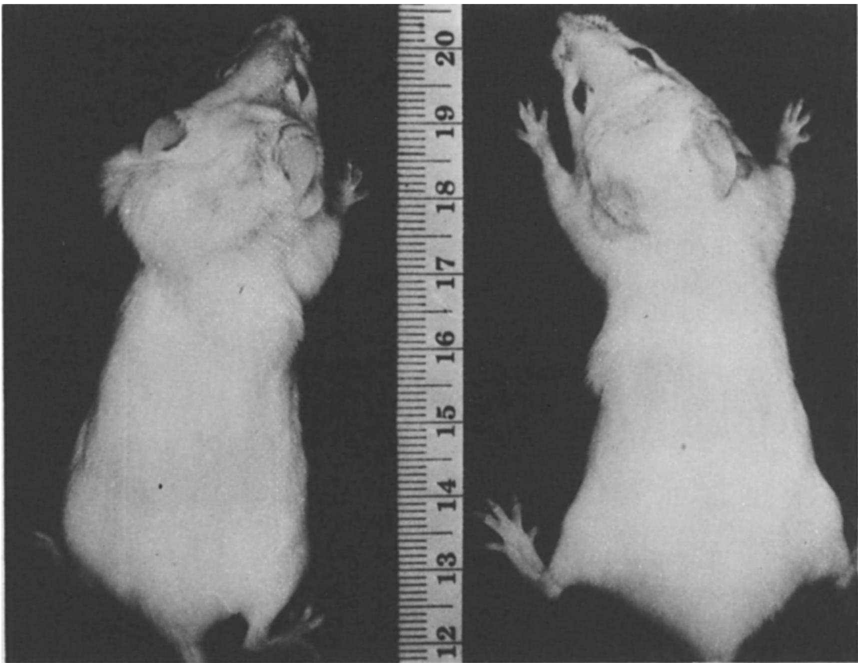


FIG. 4. A THC-treated BALB/c mouse (left) and a control (right) 19 days after having been injected subcutaneously with an equal number of fibrosarcoma cells cultivated *in vitro*. The THC-treated one has a very large tumor, while in the control the tumor is slightly noticeable.

Of the initial 46 C57 B1/6 mice of Group 2, receiving injections of THC, one female 248 days old, developed a mammary adenocarcinoma. The mouse reproduced, but did not nurse her offspring. The tumor developed close to the right groin. Some encapsulated oil vesicles were found close to the tumor but not in contact with it. Upon microscopic examination the tumor was found to be very rich in fibrous tissue separating the lobules of epithelial cells (as seen in fibroadenomas). The reason it was diagnosed as an adenocarcinoma is because of the metastatic nodules found in the lungs. The cells of this tumor grew equally well in control as in THC-treated mice. They are also growing well in cultures *in vitro*.

THE EFFECT OF SESAME OIL

The BALB/c mice of Group 3, receiving weekly subcutaneous injections of 0.05 ml of sesame oil, like those of Group 2, developed and reproduced normally. Moreover, the offspring of these mice, unlike those of the THC-treated ones, had plenty of milk in their stomach almost as soon as they were born and they developed normally, without any need of foster nursing.

Of the 60 BALB/c mice, receiving injections of sesame oil, none developed, so far, any fibrosarcoma, while of the 32 females of this group, eight already developed mammary adenocarcinomas at an age varying from 213 to 334 days. There was no relation between the points of injection of the sesame oil and the origin of the mammary cancer. Most of the mice had three to four tumors developing simultaneously. The neoplasms were typical adenocarcinomas of Type A and B of Dunn's classification[24]. Unlike the mammary cancer, developed by the C57 B1/6 THC-treated female, these tumors were poor in connective tissue stroma. Moreover, multiple metastases of the tumor were found in mediastinum and the lungs. Another characteristic of these mice with mammary cancer is the size of their ovaries, containing follicles in various stages of development, atretic follicles and above all extra large corpora lutea.

The incidence of mammary cancer among the sesame oil-treated BALB/c females is, so far, close to 25 %. An incidence which may eventually change since of the original 32 females, 20 are still alive. Of the control breeding BALB/c females, which did not receive any injections of sesame oil, so far, none developed mammary cancer, yet some of them are already close to two years of age. Similarly, none of the control C57 B1/6 breeding females, which are also of the same age as the BALB/c mice, developed breast cancer. As for the females of this strain receiving injections of sesame oil, they have not reached, as yet, the age for mammary carcinogenesis.

THE EFFECT OF ESTROGEN, OF LIPIDS AND OF THC UPON THE ADENOPITUITARY

It was found that in the BALB/c mice, like in those of the C57 B1, delestrogen as well as a diet supplemented with egg yolk (rich in lipids) cause a significant decrease in the relative number of acidophile cells in the adenopituitary (see Table 2). From the preliminary results of the present study it appears that in the BALB/c mice receiving injections of THC, sesame oil and delestrogen there is similarly an important decrease in the acidophile cells in the pars distalis of the pituitary.

TABLE 2. AVERAGE OF ACIDOPHILE CELLS IN THE ADENOPITUITARY OF BALB/c MICE MAINTAINED ON PURINA CHOW SUPPLEMENTED WITH EGG YOLK AND OF MICE RECEIVING MONTHLY INJECTIONS OF DELESTROGEN.

	Control	Egg yolk	Delestrogen
Mean	45.99%	40.02%	42.15%
t		3.84	2.52

Average of acidophile cells in the adenopituitary of BALB/c mice receiving weekly injections of THC, sesame oil and biweekly injections of delestrogen

	Control	THC	Sesame oil	Delestrogen
Mean	45.0%	32.1%	31.9%	28.0%

As shown in Table 2 the decrease in the relative number of the acidophile cells in the THC-treated mice is the same as in those receiving injections of sesame oil. Moreover, as shown in Figs. 5 and 6, while in mice receiving injections of delestrogen the pituitary becomes enlarged (in the BABL/c mice) or tumorous (in the C57 Bl/c mice), there is no sign of any changes in the size of the gland in THC-treated ones.

THE EFFECT OF THC UPON CELLS OF MALIGNANT TUMORS IN TISSUE CULTURES

The effect of delta-9-tetrahydrocannabinol upon cultures of hepatome, mammary adenocarcinoma and fibrosarcoma is being studied. So far, it was found that in the dose of 50 μg per ml of culture medium a small number of cells survive. The cytotoxicity appears to be dose related. An effect was noticed with a concentration of 5 μg/1 ml of culture medium.

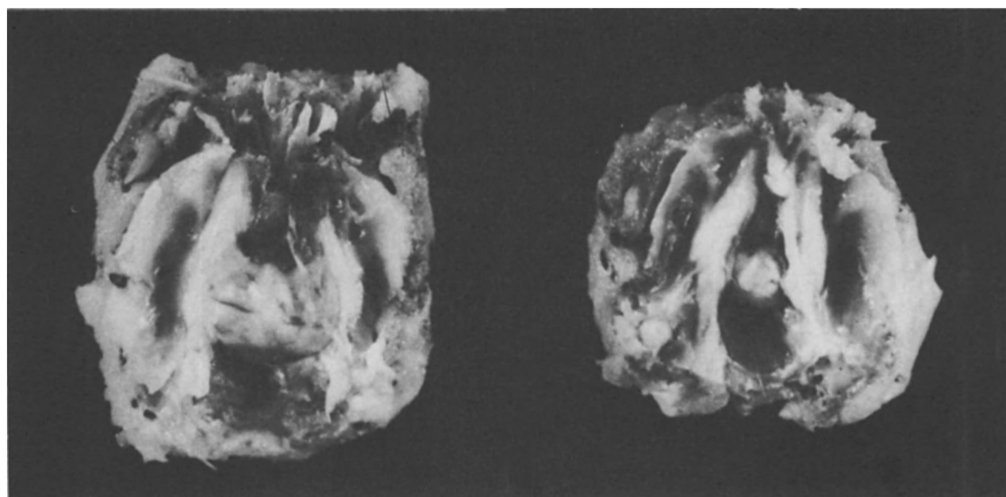


FIG. 5. Pituitaries of estrogen-treated mice. The one on the left is of a C57 B1/6 262 days old with a large tumor. The one on the right is of a BALB/c mouse 428 days old in which the pituitary is considerably enlarged.

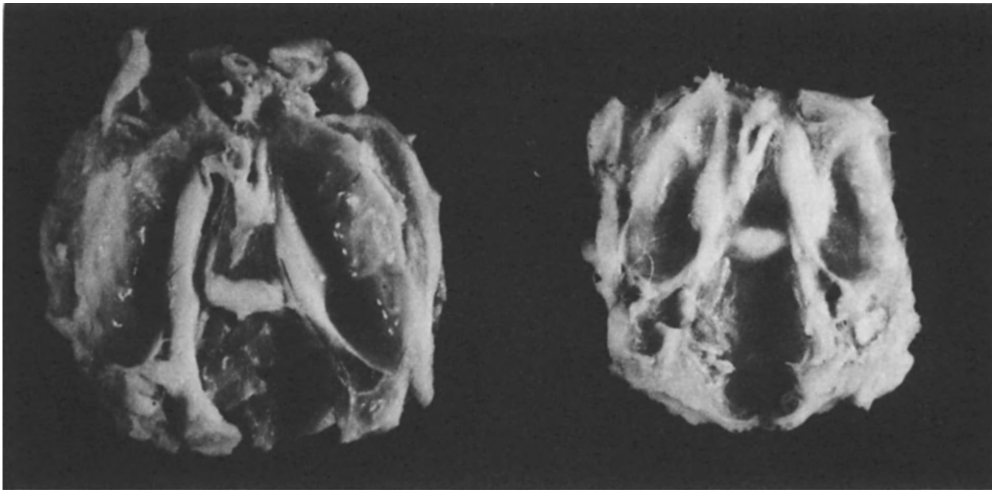


FIG. 6. Pituitaries of THC-treated mice. The one on the left is of a C57 Bl/6 mouse and the one on the right is of a BALB/c mouse. In both of them, which are 450 days old, the pituitaries are of normal size.

DISCUSSION

The effect of delta-9-tetrahydrocannabinol upon mice of the BALB/c and C57 Bl/6 strains, in the dose used, differs radically from that of estrogen. Unlike the latter, which inhibits to some extent growth of the animal and causes complete sterility, THC in the dose used did not interfere with growth nor with the reproduction of mice.

According to some investigators[18, 25] large doses of THC, administered to the females on certain days of pregnancy, cause abnormalities in the newborn. In the present study no such malformations were observed. The high mortality rate among the offspring of the THC-treated C57 Bl/6 females was caused simply by starvation. The newborns which had no milk in their stomach, the day after birth, survived and developed normally when foster nursed by a lactating BALB/c female.

The inhibitory effect upon the secretory activity of the mammary glands, which is more pronounced in the C57 Bl/6 than in the BALB/c mice, could not be attributed to the sesame oil (the vehicle used for the THC) since the offspring of mice receiving injections of the latter developed normally; they did not have to be foster nursed. The mechanism by which THC affects the mammary glands is not entirely clear. According to Chakravarty *et al.*[26] in pregnant rats submitted to acute THC treatment there is a decrease in the circulating prolactin and luteinizing hormone. It still remains to be seen whether the same is true for mice chronically treated with rather small doses of THC.

As to the fibrosarcomas developed by four of the BALB/c THC-treated mice, the question can be raised whether they were caused by the THC or by the sesame oil (the vehicle of the THC). The same question was raised previously in connection with tumors arising at the site of injection of estrogens dissolved in oil, since occasionally such tumors developed in animals administered sesame oil. It was consequently thought that the physical presence of oil repeatedly injected at weekly intervals may be responsible for the carcinogenic reaction. Gardner[27] mentions the case of one animal in his colony which

developed a fibrosarcoma around a pellet of one part of stilbestrol and three parts of cholesterol (the tumor incidence in this case was less than 0.1 %). According to Hieger[28] cholesterol is carcinogenic; it produces tumors at the site of injection.

In the present experiments the only mice to develop fibrosarcoma are the THC-treated ones of the BALB/c strain. None of the controls of the same strain, nor any of the mice receiving injections of sesame oil or of delestrogen (the vehicle of which was also sesame oil), developed such neoplasms. It is true that the sesame oil-treated mice developed mammary adenocarcinomas, but the latter did not develop at the points of injection, since several glands, at a distance from the points of injection, became tumorous at the same time. The tumorigenesis in this case was caused by some other mechanism as it will be shown in the following.

It appears thus that delta-9-tetrahydrocannabinol, besides having a local subcutaneous effect at the site of injection, has a general growth promoting effect; otherwise it would be hard to explain the fact that fragments of a fibrosarcoma of equal size transplanted subcutaneously grew faster in THC-treated mice than in controls, in sesame oil-treated mice and in estrogen-treated ones. The difference between THC-treated mice and controls was even greater when they were inoculated with an equal number of fibrosarcoma cells cultivated *in vitro*. In the THC-treated mouse the tumor developing from these cells appeared considerably earlier than in the control and the time of survival of the mouse shorter by 20 days than that of the latter.

Some investigators have found, contrary to our results, that THC retards growth of transplanted Lewis lung adenocarcinoma, increasing the time of survival of mice[29]. One consequently wonders whether the difference in the results is due to the types of tumors used (in the present experiments mesenchymal tumors produced by the THC-treated mice were used, while the above investigators transplanted a parenchymal tumor of a different origin) or to a difference in the dose of THC and its mode of administration. High doses of THC, without any doubt, are cytotoxic.

The development of mammary cancer by one estrogen-treated BALB/c female and by eight mice of the same strain receiving injections of sesame oil, although not directly related to the subject of this study (namely the effect of THC) is of considerable interest, particularly that none of the THC-treated mice of the same strain developed such tumors. Unlike the fibrosarcomas, which developed in the THC-treated mice at the site of injection of the delta-9-tetrahydrocannabinol, the mammary adenocarcinomas did not develop at the site of injection of the sesame oil.

It was demonstrated previously by one of the authors[30] that mice maintained on a diet supplemented with egg yolk, egg lipids or cholesterol and lard, develop a high incidence of mammary adenocarcinoma. The ovaries of these mice were usually large. In the present experiments the females which developed mammary cancer had large ovaries due to the extra large corpora lutea. The development of the latter could not have taken place in the absence of luteinizing hormone and prolactin, which is also luteotrophic. Both of these hormones are primary factors in mammary carcinogenesis.

It is thought consequently that oil administered by subcutaneous injections in relatively small quantities, like fat administered per os, stimulates the production of luteinizing hormone (LH), and prolactin (LTH) probably in larger quantities than normally. These in turn cause the development of mammary cancer. Delta-9-tetrahydrocannabinol, which according to Chakravarty *et al.*[26] causes a decrease in the circulating LH and LTH would have an inhibitory effect upon mammary carcinogenesis. Actually, none of the THC-

treated BALB/c females developed mammary cancer. The development by one of the THC-treated C57 B1/6 mice of a mammary cancer remains, for the time being, without an explanation since so far we were unable to determine the effect of sesame oil in the mice of this strain. Increase by THC of tumors in mice has been described[31].

REFERENCES

1. HARMON, J. and ALIAPOULIOS, M. A., Gynecomastia in Marihuana Users. *N. Engl. J. Med.* **287**,936 (1972).
2. HARMON, J. and ALIAPOULIOS, M. A., Marihuana Induced Gynecomastia: Clinical and Laboratory Experience. *Srg. Forum* **24**,423–425 (1974).
3. HARMON, J., LOCKE, D., ALIAPOULIOS, M. A. and MACINDOL, J. H., The Interference with Testicular Development of Delta-9-Tetrahydrocannabinol. *Srg. Forum* **27**,350–352 (1976).
4. KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. and TORO, G. Depression of Plasma Testosterone Level After Chronic Intensive Marihuana Use. *N. Engl. J. Med.* **290**,872–874 (1974).
5. DIXIT, V. P., SHARMA, V. N. and LOHIYA, N. K., The Effect of Chronically Administered Cannabis Extract on the Testicular Function in Mice. *Eur. J. Pharmacol.* **26**,111–114, 1974.
6. SOLOMON, J., COCCHIA, M. A., GRAY, R., SHATTUCK, D. and VOSSMER, A. Uterotrophic Effect of Delta-9-Tetrahydrocannabinol in Ovarectomised Rats, *Science* **192**,559–561 (1976).
7. SYMONS, A. M., TEALE, J. D. and MARKS, V., Effect of Delta-9-Tetrahydrocannabinol on the Hypothalamic-Pituitary-Gonadal System in the Maturing Male Rats. *Proc. Soc. for Exp. Endocrinology* **68**,43–44.
8. COLLU, R., LETARTE, J., LEBOEUF, G. and DUCHARME, J. R., Endocrine Effects of Chronic Administration of Psychactive Drug to Prepubescent Male Rats, I: Delta-9-THC. *Life Sciences* **16**,533 (1975).
9. GOLDSTEIN, H., HARCELRODE, J. and NYQUIST, S. E., Effect of Chronic Administration of Delta-9-Tetrahydrocannabinol and Cannabidiol on Rat Testicular Esterase Isozymes. *Life Sciences* **20**,951–954 (1977).
10. OKEY, A. B. and TRUANT, G. S., Cannabis Demasculinizes Rats But Is Not Estrogenic. *Life Sciences* **17**,1113 (1975).
11. OKEY, A. B. and BONDY, G. P., Is Delta-9-Tetrahydrocannabinol Estrogenic? *Science* **195**,904–905 (1977).
12. CHAKRAVARTY, I. and GHOSH, J. J., Effect of Cannabis Extract on Uterine Glycogen Metabolism in Prepuberal Rats under Normal and Estradiol-Treated Conditions. *Biochemical Pharmacology* **26**,859–862 (1977).
13. RAWITCH, A. B., SCHULTZ, G. S., EBNER, K. E. and VARDARIS, R. M., Competition of Delta-9-Tetrahydrocannabinol with Estrogen in Rat Uterine Estrogen Receptors. *Science* **197**,1189–1191 (1977).
14. SMITH, R. G., SMITH, C. G., BESCH, N. and BESCH, P. K., Is the Mechanism of Inhibition of Gonadotropin Release by Delta-9-Tetrahydrocannabinol Receptor Mediated? *The Physiologist*, Vol. 20, No. 4, p. 89 (1977).
15. OKEY, A. B. and BONDY, G. P., Delta-9-Tetrahydrocannabinol and 17 β-Estradiol Bind to Different Macromolecules in Estrogen Target Tissues. *Science*, **200**,312–314 (1978).
16. MIRAS, C. J., In "Hashish. Its Chemistry and Pharmacology" (Wolstenholme, G. E. W. and Knight, J., Eds.), pp. 37–52, Little Brown, Boston, 1965.
17. BORGEN, L. A., DAVIS, W. M. and PACE, H. B., Effect of Synthetic Delta-9-Tetrahydrocannabinol on Pregnancy and Offspring in the Rat. *Toxicol. Appl. Pharmacol.* **20**,480–486, 1971.
18. HALEY, S. L., WRIGHT, P. L., PLANK, J. B., KEPLINGER, M. L., BRAUDE, M. C. and CALANDRA, J. C., The Effect of Natural and Synthetic Delta-9-Tetrahydrocannabinol on Fetal Development. *Toxicol. Pharmacol.* **25**,450 (1973).
19. SZEPSENWOL, J. and YDRACH, A. A., Cellular Changes in the Pituitary Leading Toward Tumorigenesis in Mice of the C57 B1 Strain Treated with Estrogen. *La Cellule* **68**,59–60 (1969).
20. BOSCHETTI, N. V., Effect of Corn Oil and Estradiol Dipropionate Administered Per Os upon the Hypophysis and Reproductivity of Mice of the T.M. Strain. *Ann. D'Histochimie* **21**,335–343 (1976).
21. GLENNER, G. G. and LILLIE, R. D., A Rhodocyan Technique for Staining Anterior Pituitary, *Stain Technology* **32**,187–190 (1957).
22. SZEPSENWOL, J., ROMAN, A. and SANTIAGO, J., Changes in the Pituitary of Mice on Diets Supplemented with Egg Yolk, with Extracts of Eggs or with Cholesterol. *Proc. Soc. Exp. Biol. and Med.* **122**,1284–1286.
23. CHALKLEY, H. W., Method for Quantitative Morphologic Analysis of Tissue. *J. Nat. Cancer Inst.* **4**,47–53 (1943).
24. DUNN THALMA, B., "Morphology of Mammary Tumors in Mice. Physiopathology of Cancer." Hoeber and Harper, pp. 38–84, 1959.
25. MANTILLA-PLATA, B., CLEVE, G. L. and HARBISON, R. D., Delta-9-Tetrahydrocannabinol-Induced Changes in Prenatal Growth and Development of Mice. *Toxicology and Applied Pharmacology* **33**,330–340 (1975).
26. CHAKRAVARTY, I., SHETH, A. R. and GHOSH, J. J., Effect of Acute Delta-9-Tetrahydrocannabinol Treatment on Serum Luteinizing Hormone and Prolactin Levels in Adult Female Rats. *Biochemical Pharmacology*.

27. Gardner, W. U., Pfeiffer, C. A. and Trentin, J. J., Hormonal Factors in Experimental Carcinogenesis. In "The Physiopathology of Cancer," Edited by Homburger, F., Hoeber and Hafner, 1959.
28. Hieger, L. "Carcinogenesis." Academic Press, London and New York, 1961.
29. Munson, A. E., Harris, L. S., Friedman, M. A., Dewey, W. L. and Carchman, R. A., Antineoplastic Activity of Cannabinoids. *J. Nat. Cancer Inst.* **55**,597–602 (1975).
30. Szepsenwol, J., Carcinogenic Effect of Egg White, Egg Yolk and Lipids in Mice. *Proc. Soc. Exp. Biol. and Med.* **112**,1073–1076 (1963). Carcinogenic Effect of Ether Extract of Whole Egg, Alcohol Extract of Egg Yolk and Powdered Egg Free of the Ether Extractable Part in Mice. *Proc. Soc. Exp. Biol. and Med.* **116**,1136–1139 (1964).
31. Szepsenwol, J., Fletcher, J. and Toro-Goyco, E. Tumors in mice receiving subcutaneous injections of Δ^9-tetrahydrocannabinol. *Fed. Proc.* **38** (1979).

HYPOPLOID METAPHASES IN CULTURED LYMPHOCYTES OF MARIHUANA SMOKERS

AKIRA MORISHIMA, RICHARD T. HENRICH,
JAYASHREE JAYARAMAN and GABRIEL G. NAHAS

Departments of Pediatrics and Anesthesiology, College of Physicians & Surgeons, Columbia University, New York, New York 10032 U.S.A.

Summary. In a previous study we reported an increased incidence of hypoploid metaphases in cultured lymphocytes of marihuana smokers. In these studies hypoploid metaphases were defined as metaphase nuclei with a chromosomal complement of less than 30 chromosomes. The purpose of the present study was to examine the cytogenetic effect of " heavy " marihuana smoking under a controlled environment.

Five male subjects with prior histories of chronic marihuana smoking were used in this study. Subjects were initially kept in a drug-free environment. At the end of this period the subjects were encouraged to consume as many cigarettes as they could tolerate. The daily average was 14 cigarettes per day. Following the smoking period all subjects were deprived of marihuana for a period of over 18 days.

Heparinized blood was obtained from all subjects before, during and after smoking. Metaphase and anaphase preparations were made from these samples.

The incidence of hypoploid cells was significantly higher in all subjects during the smoking period. The incidence was also higher in samples obtained after the smoking period when compared with samples from before the smoking period. No increase in chromosome breakages or segregational errors of chromosomes in the anaphase preparations was found in any of the samples obtained.

The increased incidence of hypoploid metaphases observed during heavy marihuana smoking in a controlled environment confirms our previous observation. These observations would suggest a causal relationship between moderate to heavy marihuana smoking and induction of hypoploid cells.

INCREASED incidence of hypoploid metaphases in cultured lymphocytes of marihuana smokers was reported previously[9]. In this study, the frequency of cells containing less than 30 chromosomes per metaphase plate was examined. In the 3-day cultures of lymphocytes obtained from 10 "moderate" users of marihuana as defined by the National Commission on Marihuana[10], the incidence of such hypoploid nuclei was significantly greater than that found in the paired controls. When delta-9-tetrahydrocannabinol (THC) or olivetol, which has a molecular structure corresponding to the C-ring of cannabinoids, was added to the culture medium of lymphocytes obtained from a healthy adult control, induction of hypoploid nuclei was observed[9].

Increased frequency of chromosomal breakages have been reported in metaphase preparations of lymphocytes obtained from marihuana smokers by Stenchever and his colleagues[11], although other workers have failed to observe such abnormalities[4, 5]. In heavy hashish users, structural alterations of chromosomes and numerical changes of

chromosomal complement, as well as chromosomal breakages have been observed[6]. *In vitro* induction of segregational errors of chromosomes (SEC) by THC and olivetol has been observed in anaphase and telophase preparations of human lymphocyte cultures[1, 8]. The types of SEC induced included anaphase and telophase bridge formations, anaphase lags and unequal segregations of chromosomes in bipolar divisions. In addition, olivetol induced multipolar segregations. Induction of SEC by marihuana smoke has been observed also in cultured human lung cells[2, 3].

The purpose of the present study was to further examine the cytogenetic effect of "heavy" marihuana smoking under a controlled environment.

MATERIALS AND METHODS

Five volunteers with histories of chronic marihuana smoking during the immediate past were admitted to the Research Unit of New York Psychiatric Institute. They ranged in age from 22 to 32 (median age 23), and had histories of smoking at least 10 marihuana cigarettes per week for a minimum of 6 years. Seven healthy students and laboratory workers who had no history of using marihuana served as the controls. They ranged in age from 21 to 23 years (median age 22). Users of tobacco and/or alcohol (maximum of three times per week) were evenly distributed among the smokers and the controls. None of the smokers admitted to use of other drugs of abuse. Only males were chosen for the study in order to avoid the possible effects of estrogens on the mitotic rates[7]. Detailed histories were obtained from the smokers upon admission, and they were subjected to routine physical and laboratory examinations. No evidence of unusual exposure to radiation or viral infections were obtained. There was no history of recent infectious disease. They were, then, placed on the study protocol for various biochemical, physiological and psychological investigations, the cytogenetic study constituting one of many studies.

During the first 32 days of hospitalization, subject T.P. was kept in a drug-free environment, and the remaining 4 subjects were maintained in a similar environment for 22 days. On the 33rd day, subject T.P. was allowed to smoke as many marihuana cigarettes as he desired for a period of 28 days. During this period he smoked a total of 375 marihuana cigarettes (supplied by NIDA), weighing 0.9 g each and containing 2.0% THC. His daily average was 13.4 cigarettes per day. The remaining 4 subjects were allowed to smoke 2 marihuana cigarettes on the 23rd day. The allowance of cigarettes was increased daily, thereafter, until the 31st day when they were encouraged to consume as many cigarettes as they could tolerate. The smoking period extended over 29 days for these subjects. Their total consumption ranged from 339 to 443 cigarettes during this period, for a daily average of 15.3, 15.3, 11.7 and 14.6 cigarettes per day for subjects M.H., K.S., A.G., and S.S. respectively. The marihuana cigarettes they consumed weighed 0.9 g each and contained 2.1% THC. Following the smoking period, all subjects were deprived of marihuana for a period of 18 days for T.P. and 23 days for the other 4 subjects. Blood specimens for the period before smoking were obtained on the 12th day of hospitalization, on the 15th and 25th day of smoking, and on the 8th and 18th day after deprivation of marihuana cigarettes in subject T.P. For other subjects specimens were obtained on the 15th and 19th day of hospitalization, 22nd and 25th day of smoking, and on the 10th day after cessation of smoking (Table 1). However, subject S.S. refused the sampling during the period before smoking. The total numbers of marihuana cigarettes consumed at the time of the blood

TABLE 1. SCHEDULE OF BLOOD SAMPLING, BEFORE, DURING AND AFTER THE SMOKING PERIOD OF MARIHUANA CIGARETTES.

Subjects	Before smoking		During smoking			After smoking
	No. of days free of M.C. when tested	Total No. of days free of M.C.	No. of days on M.C. when tested	Total No. of M.C. consumed when tested	Total No. of days on M.C.	No. of days off M.C. when tested
T.P.	12	32	15	154	28	8
			25	320		18
M.H.	15	22	22	280	29	10
	19		25	349		
K.S.	15	22	22	280	29	10
	19		25	339		
A.G.	15	22	22	214	29	10
	19		25	257		
S.S.	—	22	22	275	29	10
			25	333		

M.C. = Marihuana cigarettes.

sampling are listed in Table 1. Approximately 80% of each marihuana cigarette was smoked by the subjects, leaving butts of less than 0.2 g each.

Heparinized blood of 20 ml was obtained from all subjects at each sampling period. A similar sample was obtained from control subjects on the same day. Plasma containing leukocytes was separated by gravity sedimentation at room temperature. Quadruplicate cultures of each sample were prepared in the manner previously described[9]. After 72 hours of incubation at 37°C cells were harvested. Metaphase preparations were made by the methods previously reported[9]. Anaphase and telophase preparations were prepared without exposing the cells to a hypotonic solution[1, 8].

In the metaphase preparations all metaphase nuclei, and in the anaphase and telophase preparations all mitotic cells in a predetermined area of the slides were subjected to photomicrography. The number of chromosomes per each metaphase was determined and tabulated in a manner previously reported[9]. Since the chromosome number per complement cannot be determined with confidence in all metaphase nuclei when over 31 chromosomes are present, cells containing more than 31 chromosomes were considered as "normal" and pooled for tabulation. The mitotic cells in the anaphase and telophase preparations were classified as normal or abnormal, and the type of SEC was determined in accordance with the classification system developed in this laboratory[1]. All observations were carried out by 3 independent investigators in a double-blind fashion.

RESULTS

The incidence of hypoploid cells was similar among 2 samples when more than 1 sample was obtained from a subject within the same period of hospitalization. Therefore, those data were pooled. Similarly, little fluctuation in the incidence of hypoploid cells was observed among different control subjects and among various sampling periods. The frequency of "normal" metaphases containing more than 31 chromosomes was 80.51% ± 6.995 (S.D.) among the control samples. The incidence of "normal" metaphases was

significantly lower in 4 of the 5 subjects during the smoking period when compared to controls (Table 2). Further, the incidence of "normal" metaphase was lower during the smoking period in all subjects when compared with the data obtained from both the before and after smoking periods of each subject. This decrease, and therefore, the increase in the incidence of hypoploid cells containing less than 30 chromosomes per metaphase was statistically significant when subjected to a paired t-test ($p < 0.05$). No significant increase in hypoploid cells was observed in samples obtained during the periods before or after smoking when compared with the controls. However, the incidence was higher in samples obtained after the smoking period when compared with samples obtained before the smoking period. This difference was significant ($p < 0.05$) in the 4 subjects who could be paired.

TABLE 2. PERCENTAGE OF METAPHASES WITH VARYING NUMBER OF CHROMOSOMES IN LYMPHOCYTES OBTAINED FROM INDIVIDUALS SUBJECTED TO MARIHUANA SMOKING.

	No. of chromosomes per metaphase								
	Before smoking			During smoking			After smoking		
Subjects	1–30	> 31	No. of metaphases	1–30	> 31	No. of metaphases	1–30	> 31	No. of metaphases
T.P.	13.8	86.3	400	29.6	70.4	324	29.3	70.7	345
M.H.	9.9	90.1	156	36.3	63.8*	160	19.0	81.0	124
K.S.	11.4	88.6	176	34.4	65.6*	250	15.3	84.7	150
A.G.	16.2	83.8	167	34.5	65.5*	184	22.0	78.0	116
S.S.	—	—	—	43.5	56.5*	252	17.7	82.3	99

Control > 31 chromosomes per metaphase.
$n = 7$, $\bar{x} = 80.51\% \pm 6.995$ (S.D.).
* $p < 0.05$.

The incidence of chromosomal breakages, including chromatid breaks, was examined in 20 suitable metaphases with 46 chromosomes from each sample. No increase in chromosome breakages was found in any of the samples obtained from marihuana smokers. The only abnormality found was chromatid breaks which occurred in less than 1% of the cells of both the smoking subjects and the controls.

SEC was examined in a total of 1,383 cells obtained from marihuana smokers. The incidence of SEC was 1.20% before smoking, 2.01% during smoking and 1.28% after smoking in these subjects. Their incidence was similar to that found among the controls in whom the incidence was 1.15% ± 1.662 (S.D.) in 1,104 cells examined.

DISCUSSION

The increased incidence of hypoploid metaphases observed during heavy marihuana smoking confirms our previous observation[9]. It is of interest that the incidence of hypoploid cells returned to normal while abstaining from marihuana smoking. This observation strongly suggests the causal relationship between heavy marihuana smoking and induction of hypoploid cells. The higher incidence of hypoploid cells found in samples

obtained after the smoking period than that found before the smoking period may be explained by the shorter period of abstinence from marihuana cigarettes. It appears that more than 10 days of abstinence may be necessary for the incidence of hypoploid cells to return to the normal base line.

Examination of hypoploid cells, defined as a marked decrease of chromosomal complement per metaphase nucleus, has not been applied widely to studies on effects of chemical agents. Although THC and olivetol are capable of induction of hypoploid cells *in vitro*[9], it is not known whether such an effect is unique to cannabinoids and related compounds. Miras and his co-workers have reported the presence of hyperploid and hypoploid cells in cultured lymphocytes of hashish users[6]. However, it is not clear from their description whether hypoploid cells observed represented severe numerical loss of chromosomes or mere hypodiploid cells with few missing chromosomes.

The failure to observe increased incidence of chromosomal breakages in heavy marihuana smokers is in agreement with the observations of some investigators[4, 5] but is discordant with others[6, 11]. However, the lack of increase in anaphase or telophase bridge formations observed in the studies on SEC tends to support the absence of chromosomal breakages in our subjects. These bridge formations would be expected to produce chromosomal breakages in the succeeding cell generations, if the cell can survive the damage.

Induction of SEC by THC *in vitro*[1], provides a possible explanation for the underlying mechanisms leading to increased incidence of hypoploid cells. Unequal segregation of chromosomes in bipolar divisions induced by THC, and multipolar divisions induced by olivetol[8] could produce hypoploid metaphases in the succeeding cell generation. However, these *in vitro* observations could not be confirmed directly in heavy marihuana smokers. Thus, the mechanisms leading to induction of hypoploid cells in marihuana smoking requires further exploration.

ACKNOWLEDGEMENT

This work was supported by a grant from the National Institute on Drug Abuse (5-RO1-DA-01476).

REFERENCES

1. Henrich, R. T., Nogawa, T. and Morishima, A., Segregational errors of chromosomes induced by delta-9-tetrahydrocannabinol in normal human lymphocytes. *Mutation Res.* (Submitted).
2. Leuchtenberger, C., Leuchtenberger, R. and Ritter, U., Effects of marijuana and tobacco smoke on DNA and chromosomal complement in human lung explants. *Nature* **242**,403–404 (1973).
3. Leuchtenberger, C., Leuchtenberger, R. and Schneider, A., Effects of marijuana and tobacco smoke on human lung physiology. *Nature* **241**,137–139 (1973).
4. Martin, P. A., Thorburn, M. J. and Bryant, S. A., *In vivo* and *in vitro* studies of the cytogenetic effects of cannabis sativa in rats and men. *Teratol.* **9**,81–86 (1973).
5. Matsuyama, S. S., Yen, Fu-Sen, Jarvik, L. F., Sparkes, R. S., Fu, Tsu-Ken, Fisher, H., Reccius, N. and Frank, I. M., Marihuana exposure *in vivo* and human lymphocyte chromosomes. *Mutation Res.* **48**,255–266 (1977).
6. Miras, C. J., Kyrkou, K. A. and Markidou, S. G., Chromosomal abnormalities in heavy hashish users. ST/SOA/SER. S/56, United Nations Secretariat, pp. 1–13, 1978.
7. Morishima, A. and Henrich, R. T., Lymphocyte transformation and oral contraceptives. *Lancet* **2**,646 (1974).

8. MORISHIMA, A., HENRICH, R. T., JOU, S. and NAHAS, G. G., Errors of chromosome segregation induced by olivetol, a compound with the structure of C-ring common to cannabinoids: Formation of bridges and multipolar divisions. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, W. D. M. Paton, and J. E. Idanpaan-Heikkila eds.) Springer-Verlag, New York, 265–271, 1976.
9. MORISHIMA, A., MILSTEIN, M., HENRICH, R. T. and NAHAS, G. G., Effects of marihuana smoking, cannabinoids, and olivetol on replication of human lymphocytes: Formation of micronuclei. "The Pharmacology of Marihuana." (M. C. Braude and S. Szara eds.) Raven Press, New York, vol. 2, 711–722, 1976.
10. National Commission on Marihuana and Drug Abuse, Marihuana: A signal of misunderstanding. First Report. Washington, D.C.: U.S. Government Printing Office (1972).
11. STENCHEVER, M. A., KUNYSZ, T. J. and ALLEN, M. A., Chromosome breakage in users of marihuana. *Am. J. Obstet. Gynecol.* **118**,106–113 (1974).

OBSERVATIONS IN CHRONIC HASHISH USERS: NUCLEAR ABERRATIONS IN BLOOD AND SPERM AND ABNORMAL ACROSOMES IN SPERMATOZOA

MARIETTA R. ISSIDORIDES
Department of Psychiatry, University of Athens, Greece

INTRODUCTION

A POPULATION of 47 chronic cannabis users and 40 matched controls, extensively studied by a battery of medical, neurophysiological, psychophysiological, psychological and psychiatric tests[1], has served as a source of material for the study of cellular effects of chronic cannabis use in man[2]. Although findings from the clinical tests failed to distinguish users from non-users on most of the above investigated parameters[1], findings from the cellular studies were quite unique in clearly indicating repercussions of the drug on a particular metabolic pathway in two cell populations as functionally diverse as peripheral blood lymphocytes and ejaculated spermatozoa[2]. Specifically, the light and electron cytochemical methods used showed a reduction of arginine-rich histones in most of the nuclei of both cell types. This reduction seemed counterbalanced by increased amounts of lysine-rich fractions. While in somatic cells lysine-rich histone H1 functions as a condensing factor of the chromatin repressing genomic expression[3], in germ cells of male eutherian mammals H1, along with other somatic histones, is shed during spermiogenesis and substituted by newly synthesized arginine-rich protamine[4], which achieves total condensation of the chromatin and repression of the sperm genome[5]. Clearly, the similarly altered histone ratio in lymphocytes and sperm of chronic users has entirely different implications for the functional capacity of each cell type. Namely, the somatic cell becomes over-repressed by the relative increase of H1 in the nucleus and should, theoretically, under-respond to antigenic stimuli[6]. The germ cell, on the other hand, remains arrested in its maturation by the low content of protamine, which is essential—due to its high thiol content—for the formation of S-S cross links[7] that endow the sperm chromatin with stability and resistance and the sperm tail with motility[5].

Functional repercussions, predictable from the low levels of arginine-rich proteins in the two cell types, have been substantiated by the findings of inhibited cellular-mediated immunity[8] and of decreased motility and total sperm count[9] in heavy marihuana users. Supporting evidence for the causal relation of low protamine content to sperm functional

impairment derives from electrophoretic studies of nucleoproteins in terminal germ cells from infertile, oligospermic patients, which showed that in these cells no protamines are detectable, but somatic histones exclusively[10]. It thus becomes apparent that chronic cannabis use may mimic this so-called "idopathic spermatidic arrest" by inducing the specific metabolic aberrations which characterize this pathological condition.

A single unifying mechanism, by which cannabis exerts its many actions in a variety of tissues, may not be a biological prerequisite. Data, however, relevant to such an eventuality exist with respect to histones[11, 12] and justify the search for additional parameters, in cells of chronic users, that might indicate perturbations of a common metabolic denominator.

It is widely accepted that interruption of transcription, either by natural means or with inhibitors, is probably sufficient to induce conformational changes in chromatin leading to condensation[13]. In view of this and of the mounting evidence that THC inhibits transcription in a variety of cellular systems[14], the present study was initiated. It explores material obtained from the same chronic hashish users and controls as in the previous report[2], but with new cytochemical methods that probe chromatin ultrastructure[15] and are designed to differentiate condensed from dispersed chromatin in the interphase nucleus with enhanced contrast.

MATERIAL AND METHODS

The selection criteria of chronic hashish users and controls as well as the collection procedures of blood samples and spermatozoa have been described elsewhere[2]. All samples were coded and processed blind until completion of the observations.

Cell pellets were prepared for electron microscopy by a new method[15], which includes glutaraldehyde fixation, potassium permanganate postfixation, phosphotungstic acid-hematoxylin (PTAH) block-staining, and which clearly demarcates condensed from dispersed chromatin[16]. Smears from capillary blood were fixed in methanol and stained with PTAH-Neutral red[17] as well as with the standard Giemsa method[18]. The study of blood smears included material from 34 chronic hashish users and 18 controls, while the study of leukocytes in the electron microscope was limited to 10 of the users and 10 age-matched controls. The sperm samples included those previously studied for arginine-rich proteins.

RESULTS AND DISCUSSION

PART I

State of the Chromatin in Blood Cells

In routine Giemsa preparations of peripheral blood smears from control subjects (Fig. 1) nuclei were stained dark purple in a pink cytoplasm. In Giemsa preparations of chronic users "gaps" in staining were observed in most of the nuclei. One or more lobes (Figs. 2a, 2b) of a polymorph nucleus, as well as portions of a lobe (Figs. 2b, 2c) remained white, i.e. completely unstained. Similar gaps in staining were also observed in the nuclei of

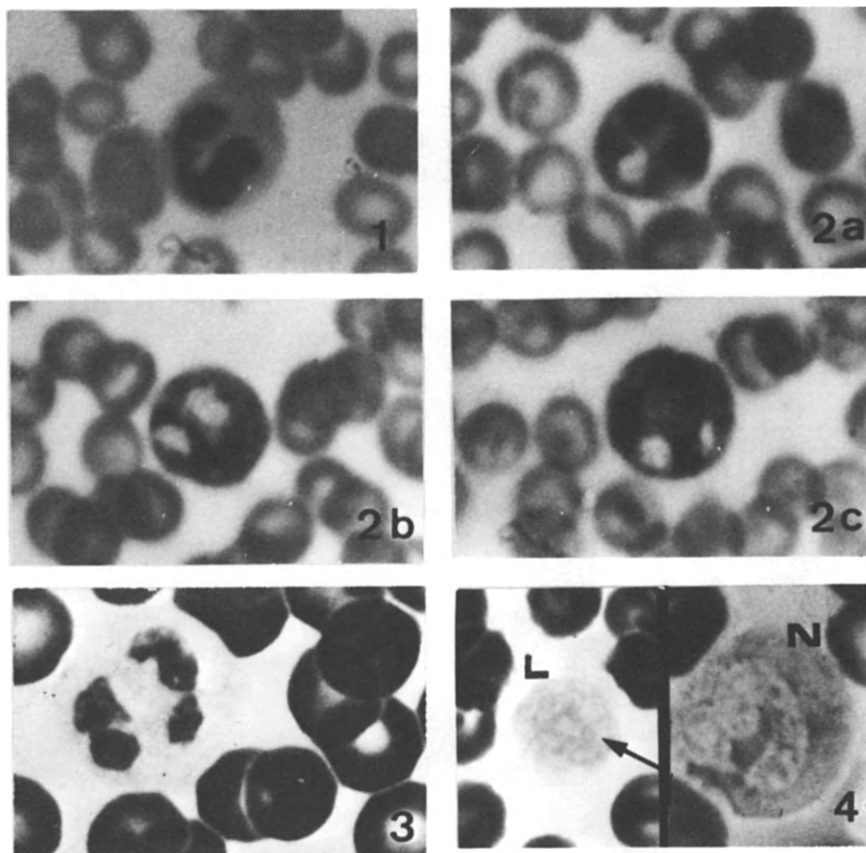


FIG. 1. Neutrophil of control subject showing uniformly stained nucleus. Giemsa.

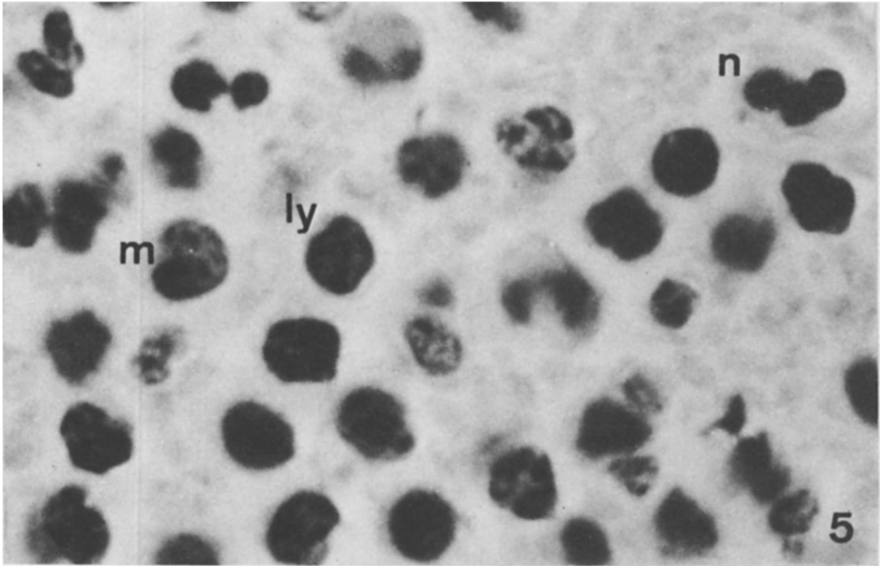
FIGS. 2a,b,c. Neutrophils of three different users showing "gaps" in nuclear staining. Giemsa.

FIG. 3. Control subject. Neutrophil showing darkly stained histones of the nucleus in a clear cytoplasm. PTAH-Neutral red.

FIG. 4. Chronic hashish user. Lymphocyte (L) and neutrophil (N) showing totally unstained heterochromatin in their nuclei. Counterstained cytoplasm denotes moderate acidity. PTAH-Neutral red.

lymphocytes. In order to determine whether the unstained areas represented disintegrated parts of the chromatin or nuceoprotein loci inaccessible to the small thiazin molecule, duplicate smears were stained with PTAH-Neutral red and compared to the Giemsa preparations of the same user. It was found that PTAH, a large anionic molecule which forms precipitates with histones[19] stained the entire nuclear area in control leukocytes (Fig. 3), but failed to stain the nuclei in the cells of the users (Fig. 4). Although unstained, the latter were clearly visible in the reddish cytoplasm and were found to be intact (N and L in Fig. 4). Hence, the Giemsa "gaps" could not be ascribed to missing portions of nuclear material. These findings suggest that an unusual state prevails in the methanol-fixed nuclei of the chronic users and interferes with staining. The most likely explanation in accordance with previous findings[2] is that a high degree of nuclear condensation and possibly additional molecules[20] mask charged groups in the chromatin and prevent dye binding to variable degrees, which depend upon the size and charge of the dye molecules.

With the PTAH-block-staining method for glutaraldehyde/$KMnO_4$ fixed tissue, thick epon sections can be studied directly in the light microscope without further staining and compared to the thin sections of the same block viewed in the electron microscope. An epon thick section of a leukocyte pellet from a control (Fig. 5) shows strong binding of the stain in the nuclei of neutrophils (n), monocytes (m) and the majority of lymphocytes (ly). The similarly prepared section of a leukocyte pellet from a chronic user (Fig. 6) shows



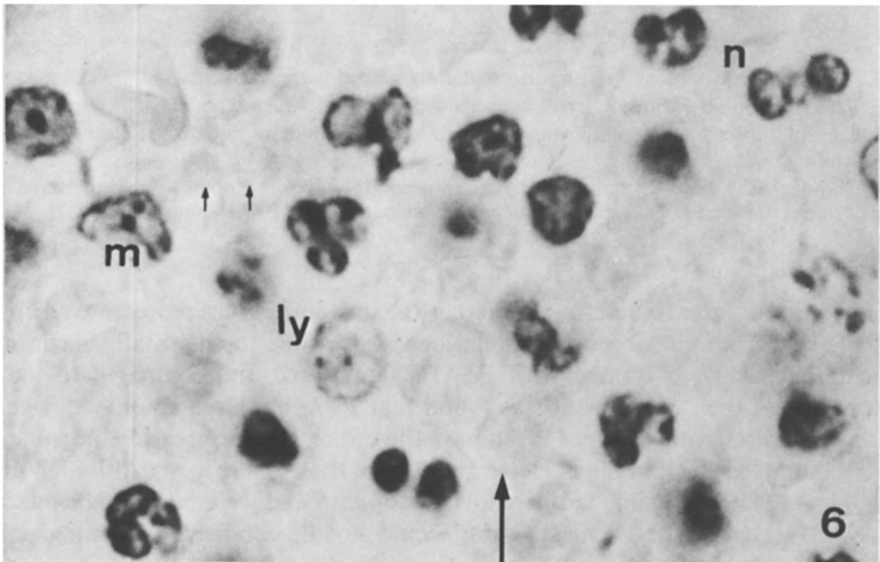


FIG. 5. Epon thick section of leukocyte pellet from control subject. Nuclei of neutrophil (n), lymphocyte (ly) and monocyte (m) are deeply stained indicating penetration of the dye and binding to basic groups. PTAH-block-staining.

FIG. 6. Epon thick section of leukocyte pellet from chronic user. Several nuclei (n, ly, m) show reduced binding of the stain, while others are faintly visible as unstained circular profiles (arrows). PTAH-block-staining.

much reduced binding of the stain in about one half of the nuclei and absence of binding in the other half. These unstained nuclei are faintly visible in the section as circular profiles (Fig. 6, arrows). The underlying ultrastructure, responsible for this differential staining of control and users' leukocytes by PTAH, was studied in the thin sections of the same samples.

As described elsewhere in detail[15, 16] PTAH does not bind to condensed chromatin, which thus remains electron lucent, but imparts density to the fibers of dispersed chromatin. Figure 7 illustrates the normal amounts of heterochromatin masses and euchromatin fibers usually observed in control neutrophils after this method. In the control nuclei the heavily stained zone (z), bordering on the nuclear envelope, as well as the condensed chromatin masses are discontinuous at the nuclear pores allowing for nucleocytoplasmic interactions and macromolecular transfers[21]. In the cells of chronic users (Fig. 8) the above parameters are conspicuously altered. The nuclei have a reduced size. Occasionally, as seen in this micrograph, two lobes appear fused. Chromatin forms a continuous compact mass along the nuclear envelope and large condensations in the center of the lobes. The nuclear sap is sequestered in a small volume and euchromatin fibers are not evident. Zone z is paler and continuous around the periphery of the nucleus. Two observations of potential functional significance are the near absence of pores and the presence of an unstained layer or clear space (Fig. 8, facing arrows) that separates the nucleus from the cytoplasm. All these features, differentiating the nuclei of chronic users from those of controls, are observed as a rule in terminally repressed cells such as mature avian erythrocytes[22], which do not export RNA to the cytoplasm[23], and in transiently repressed cells such as thymocytes under the influence of cortisone[24]. These observations on the ultrastructure of nuclei in chronic cannabis users establish the existence of inappropriate condensation of the chromatin and alterations of the nuclear boundary denoting transcriptional arrest. They are in accord with experimental findings from the *in vitro* application of delta 9-THC in cell cultures[25, 26], i.e. chromatin aggregation and synthesis inhibition, especially of macromolecules. These findings were attributed to the presence of labelled THC in the nucleus[26] and to its probable binding to DNA or nuclear proteins[25]. No evidence is available in favor of—or against—the operation of such a mechanism in our material. On the other hand, chromatin condensation in resting cells and chromosome compaction with mitotic arrest are distinctive features of arginine-free media[35].

PART II

Ultrastructure of the Sperm Head

In a preliminary study of sperm from chronic hashish users[2] the finding that, despite low protamine content, the sperm heads displayed the normal species-specific shape was taken as an indicator of normal reproductive capacity[27]. Currently this notion tends to be discarded as extensive studies have demonstrated that a normally shaped human nucleus may often be reproductively abnormal at the molecular and ultrastructural level[28, 29].

In the present study of sperm from the same chronic users the attempt was made to elucidate this question. PTAH-block-staining was found to be most sensitive for the detection of various states of condensation in the chromatin and in revealing structural details

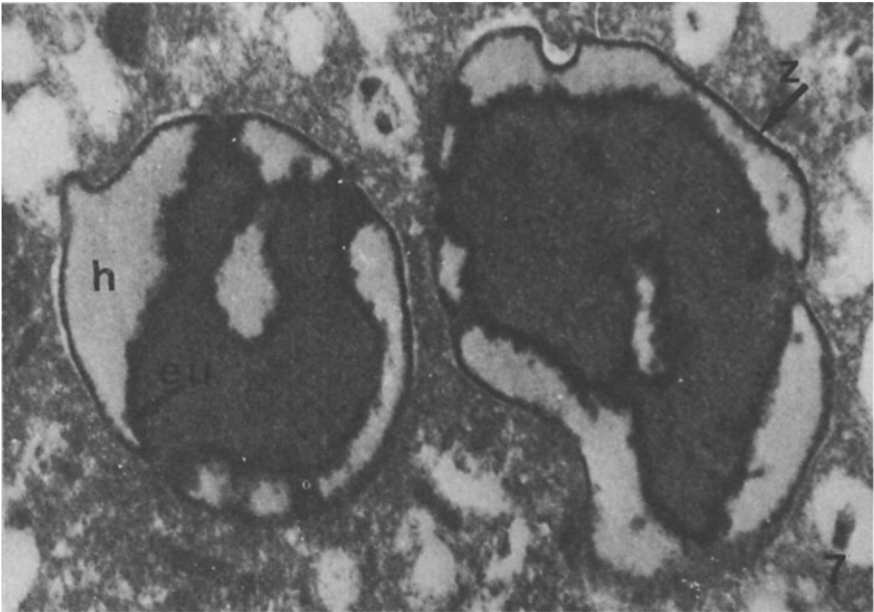


FIG. 7. Electron micrograph of control neutrophil from pellet of Fig. 5. White heterochromatin masses (h) fringed with black euchromatin fibers (eu) have a peripheral localization. Note size of nucleus, large nucleoplasmic space and zone z, the exterior limit of the heterochromatin. PTAH-block-staining.

FIG. 8. Electron micrograph of user's neutrophil from pellet of Fig. 6. Nuclear lobes appear fused and of reduced volume. Ratio of nucleoplasm to heterochromatin is greatly reduced. Euchromatin fibers, if present, are obscured by the densely staining nuclear sap. A continuous clear space (d) surrounds the nucleus and in places (facing arrows) appears bound by a cytoplasmic membrane. PTAH-block-staining.

in the acrosomes. A normal shape of the human sperm nucleus is seen in Fig. 9, which is representative of the majority of mature spermatozoa in the controls. The homogeneous, unstructured, ground-glass appearance of the nucleus and its low electron-density by this method are indicative of total condensation of the chromatin. A typical acrosome covers the anterior two-thirds of the head, showing a distinct region of differentiation in the posterior segment. The nucleus is outlined by a densely stained zone which, possibly, incorporates the nuclear envelope. It is separated from the acrosome by a clear subacrosomal space, which contains traces of filamentous material. The ejaculates of the controls contained the usual variants (small, round, tapered, etc.) of head shapes[30]. Their acrosomes were normal and their chromatin was, as a rule, condensed.

The most prevalent abnormalities of sperm heads in ejaculates of chronic users are illustrated in Figs. 10–12. Observations revealed that acrosomal morphogenesis was greatly affected. Nuclei with normal chromatin condensation (Fig. 10) were capped by a spongy, fuzzy, disorganized layer of acrosomal substance, which covered more than three-fourths of the head length. The clear subacrosomal space was no more evident. It was filled with dense material sequestered between the nuclear envelope and a membrane, which followed closely the contour of the nucleus as a second envelope except caudally where it merged with normal posterior segments. In another population of similarly condensed sperm heads, acrosomes were completely absent. The nuclei were surrounded by a dense layer of short filamentous projections.

The second most prevalent abnormality observed was incomplete condensation of the chromatin in sperm heads of normal size and shape, but lacking acrosomes. In Fig. 11 a ring of condensed chromatin of a homogeneous texture (c) surrounds an oval island of diffuse fibers at the rostral end of the head, while a second larger island of diffuse fibers (f) is surrounded by a mosaic of condensed (grey) and decondensed (black) chromatin fibers (d). A normal acrosome is absent; the nucleus is covered by a layer of dense material, partly membrane-bound in the rostral half of the head and frayed in the caudal. This nucleus illustrates the three states of aggregation of the chromatin (diffuse, dispersed and compact), which were encountered in various proportions in a large number of sperm heads of "normal" shape in the ejaculates of the users. It is to be noted that homogeneous (grey) compact chromatin territories are always demarcated by a thin black line which is obliterated when these separate territories coalesce in the process of nuclear condensation.

Figure 12 illustrates a third type of abnormality, less frequently observed. The size and state of condensation of this nucleus are normally encountered in the testis, in spermatids attached to Sertoli cells. These forms proceed to complete maturation before their release into the lumen of the tubules[31]. Their presence in the ejaculates is strong evidence for the occurrence of spermatidic maturation arrest in chronic users, a condition in accord with their sperm's low protamine content[2, 10]. Round packets (cp) of compact chromatin fill the wider nuclear area. They represent the negative image of the "mosaic of granular texture" described by Bedford *et al.*[28] with the standard EM stains, and which denotes incomplete condensation of the chromatin. The fact that the acrosome, already transformed into a normal organelle, has not established contact with the nucleus (Fig. 12) is further evidence of perturbed maturation concerning membranes. It may also be a clue to the absence of acrosomes in the sperm populations described earlier. It indicates abnormal disparate development since the acrosomal apparatus is closely opposed to the nucleus before the onset of condensation[32].

These observations support previous findings indicating that cannabis inhibits sperm

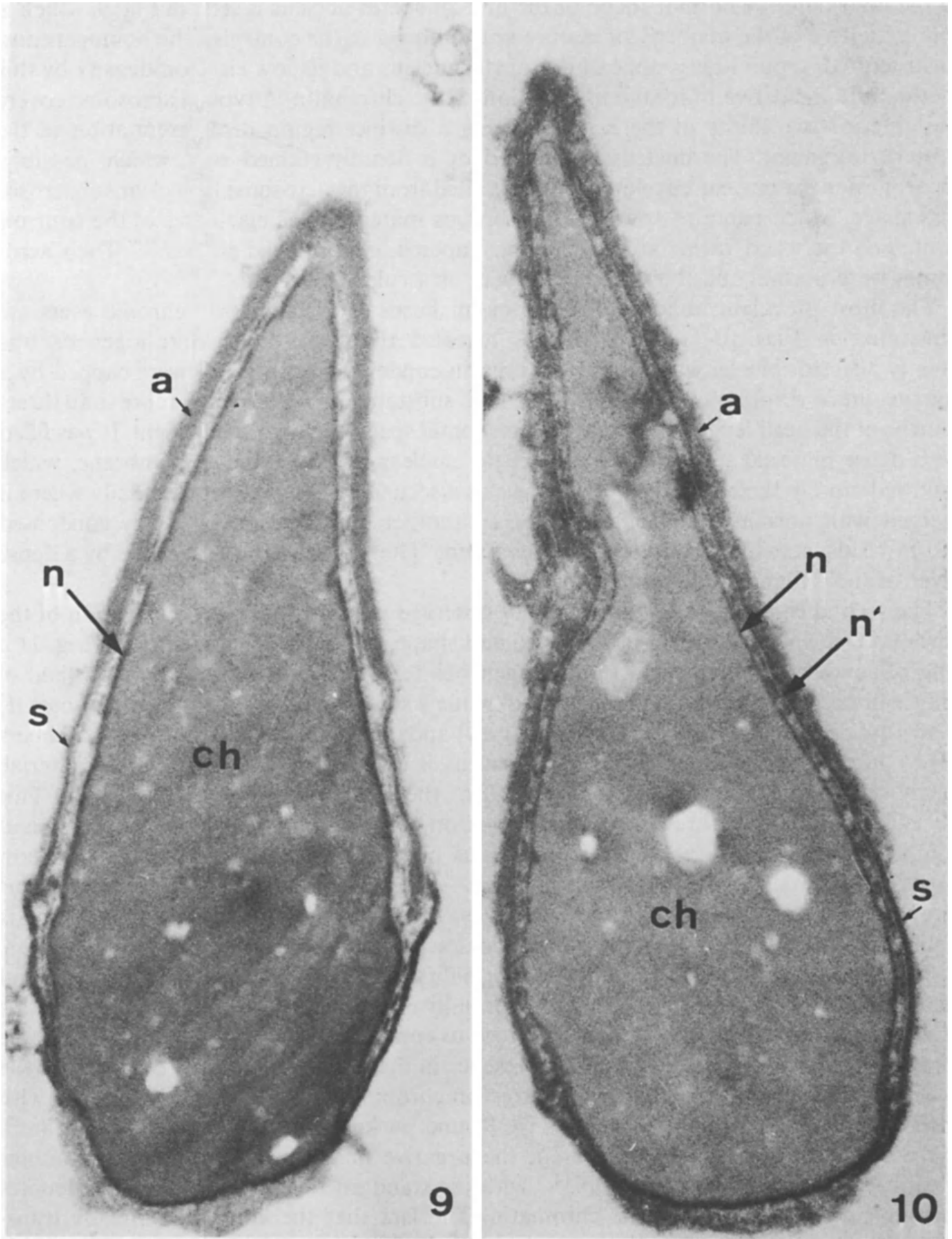
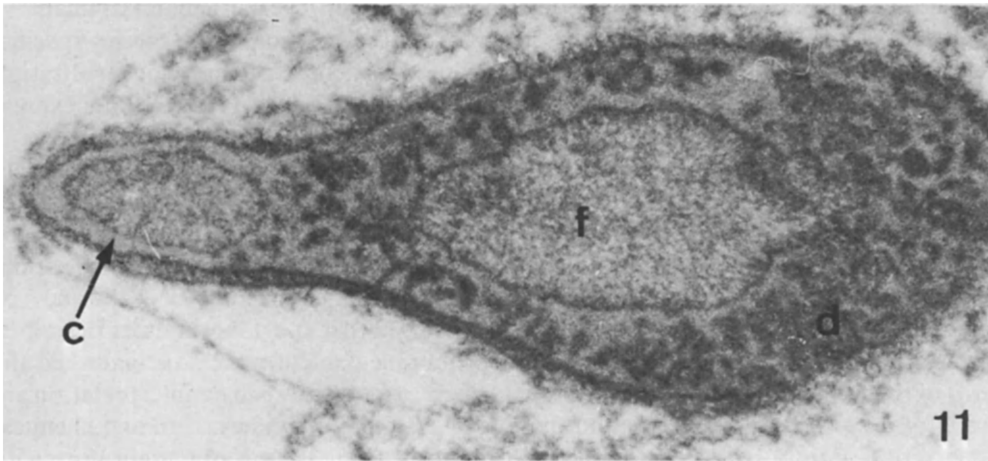


FIG. 9. Control subject. Sperm head with condensed chromatin (ch), dense nuclear envelope (n) and acrosome (a) terminating in a thin posterior segment (s). PTAH-block-staining.

FIG. 10. Chronic hashish user. Sperm head with condensed chromatin (ch), but unusual nuclear coverings. Thin nuclear envelope (n) contoured by second envelope (n′) which may represent the inner acrosomal membrane (?)since it merges with a posterior segment (s). Acrosomal substance (a) spongy and disorganized. PTAH-block-staining.



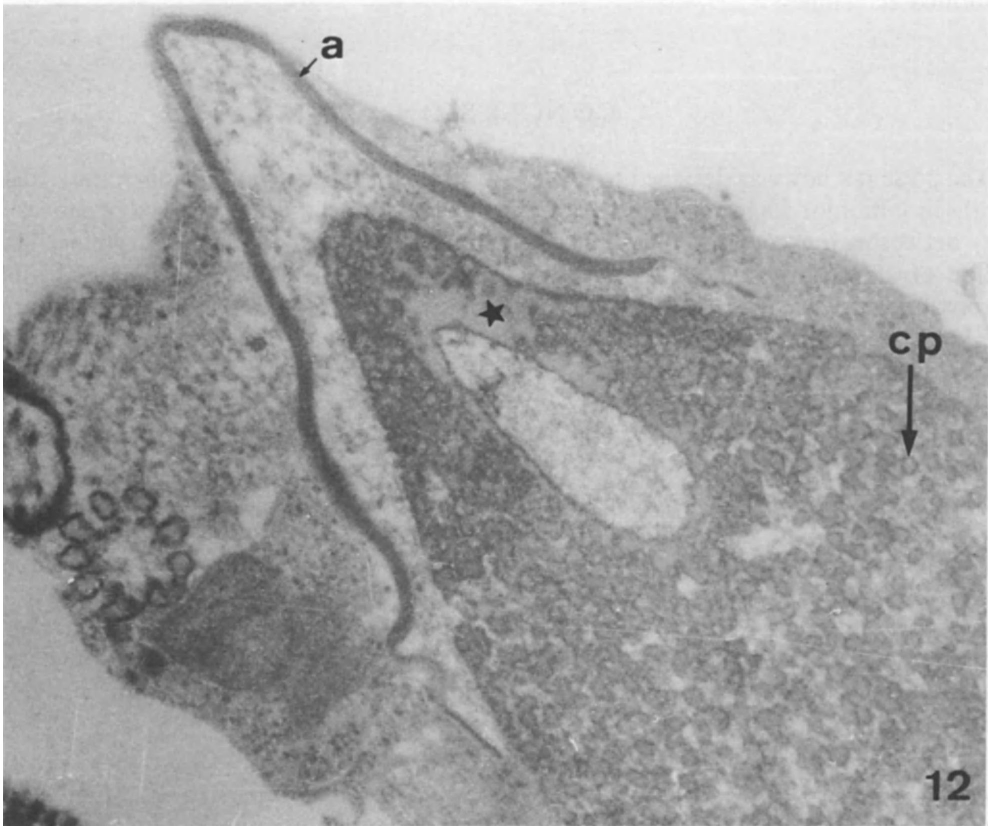


FIG. 11. Chronic cannabis user. Sperm head without acrosome. Nucleus shows three stages of condensation of chromatin: c, condensed; f, diffuse; d, dispersed. PTAH-block-staining.

FIG. 12. Chronic cannabis user. Abnormal immature spermatid showing unattached acrosome (a), small area of condensed chromatin (star) and large number of round uniform condensed chromatin packets distributed in the nuclear area, (cp). Several cytoplasmic organelles are still attached to this exfoliated cell. PTAH-block-staining.

maturation in man[2, 9] and produces deformed acrosomes in experimental animals[33]. The shape of the sperm head and the structure of the acrosome are species-specific, genetically controlled characters, which determine the sperm's capacity for fertilization and normal embryogenesis[32]. The fact that cannabis interferes with their normal expression supports the drug's interaction with the genome[33].

Although the chemical mechanisms involved in acrosome differentiation are largely unknown, those involved in head shaping are known to be related to the properties of arginine-rich proteins[4, 5, 7, 11, 34]. In fact, the first deformation of the sperm nucleus toward its definitive shape is always associated with the onset of chromatin condensation, a process requiring the coordinated synthesis of specific arginine-rich histones or protamines[32, 7]. It is reasonable, therefore, to propose that the abnormalities described here are the natural consequence of the low protamine content which distinguished the sperm of the users from that of the non-users[2]. Consistent with such an interpretation are reports of a correlation between abnormalities of chromatin condensation and chemical evidence of failure of tritiated arginine incorporation in the sperm of mutant mice with deformed acrosomes[11].

CONCLUSION

The present study was designed to explore morphological correlates of macromolecular synthesis inhibition focusing on histones. The results support the hypothesis that cannabis may act through a common metabolic denominator, namely depletion of arginine. This action of the drug would resolve the paradox of *opposite* repercussions of cannabis on chromatin condensation in lymphocytes and sperm, despite the *similar* repercussions on the levels of arginine-rich proteins in both cell populations[2]. Opposite, cell-dependent, effects on chromatin condensation have been found in experimental[35] and hereditary[11] arginine-deficient states.

The cluster of defects in brain, sperm and blood of independent behavioral mutants of a single gene, known to occur in mice[36, 37], allows one to postulate the existence of a shared metabolic coordinating process of selective value. In this context, information on a specific mechanism of action of cannabis in the two peripheral systems may be relevant to the brain and help to focus on the neuronal groups primarily involved by singling out similar chemical modifications.

At the organismic level, immune reactions, antibacterial activity and fertile sperm share with brain mechanisms for adaptive behavior the responsibility for the preservation of self and species. It is not surprising that as a systemic cluster they are targets of behavior-modifying drugs.

ACKNOWLEDGEMENTS

The author expresses her appreciation to Dr. G. D. Pappas and Dr. D. P. Purpura for the use of EM facilities in the Department of Neuroscience, Albert Einstein College of Medicine, New York. The study was supported by the National Research Foundation of Greece, Grant No. 453/135.

REFERENCES

1. STEFANIS, C. N., DORNBUSH, R. and FINK, M., eds., "Hashish: Studies of Long-Term Use," Raven Press, New York (1977).
2. STEFANIS, C. N. and ISSIDORIDES, M. R., Cellular effects of chronic cannabis use in man. In Nahas, G. G. ed. "Marihuana: Chemistry, Biochemistry, and Cellular Effects," Springer-Verlag, New York, pp. 533–550, 1976.
3. LITTAU, V. C., BURDICK, C., ALLFREY, V. G. and MIRSKY, A. E., (1965) The role of histones in the maintenance of chromatin structure, *Proc. Nat. Acad. Sci. USA* **54**,1204–12 (1965).
4. VAUGHN, J. C., The relationship of the "Sphere chromatophile" to the fate of displaced histones following histone transition in rat spermiogenesis. *J. Cell Biol.*, **31**,257–278 (1966).
5. CALVIN, H. I. and BEDFORD, J. M., Formation of disulfide bonds in the nucleus and accessory structures of mammalian spermatozoa during maturation in the epididymis. *J. Reprod. Fert. Suppl.*, **13**,65–75 (1971).
6. BLACK, M. M. and ANSLEY, H. R. (1965) Antigen induced changes in lymphoid cell histones. *J. Cell Biol.*, **26**,797–803 (1971).
7. COELINGH, J. P., ROZIJN, T. H. and MONFOORT, C. H., Isolation and partial characterization of a basic protein from bovine sperm heads. *Biochim. Biophys. Acta* **188**,353–356 (1969).
8. NAHAS, G. G., SUCIU-FOCA, N., ARMAND, J. P. and MORISHIMA, A., Inhibition of cellular-mediated immunity in marihuana smokers, *Science* **183**,419–420 (1974).
9. HEMBREE, W. C., III, ZEIDENBERG, P. and NAHAS, G. G., Marihuana's effect on human gonadal function. In Nahas, G. G., ed. "Marihuana: Chemistry, Biochemistry, and Cellular Effects," Springer-Verlag, New York, pp. 521-532, 1976.
10. SILVESTRONI, L., FRAJESE, G. and FABRIZIO, M., Histones instead of protamines in terminal germ cells of infertile oligospermic men. *Fertil. Steril.* **27**,1428–1437.
11. GODOWICZ, B., Histochemical and autoradiographic studies of abnormal spermatozoa in mice of the imbred strain KE. *Folia Histochem.* **15**,211–215 (1977).
12. KOPECNY, V. and PAVLOK, A., (1975) Autoradiographic study of mouse spermatozoan arginine-rich nuclear protein in fertilization. *J. Exp. Zool.* **191**,85–96 (1977).
13. SINCLAIR, G. D. and BRASCH, K., The reversible action of a-amanitin on nuclear structure and molecular composition. *Exp. Cell Res.* **111**,1–14 (1978).
14. NAHAS, G. G. ed. "Marihuana: Chemistry, Biochemistry, and Cellular Effects," Springer-Verlag, New York, 1976.
15. ISSIDORIDES, M. R., STEFANIS, C. N., VARSOU, E. and KATSORCHIS, T., Altered chromatin ultrastructure in neutrophils of schizophrenics. *Nature* **258**,612–614 (1975).
16. ISSIDORIDES, M. R., ZIOUDROU, C., LYKOURAS, E. and STEFANIS, C. N., Drug-induced changes in chromatin ultrastructure and nuclear basic proteins of the neutrophils of chronic schizophrenics. *Prog. Neuro-Psychopharmac.* **2**,79–85 (1978).
17. ISSIDORIDES, M. R., GARELIS, E. and STEFANIS, C. N., Investigation of nuclear metabolic processes in schizophrenia, (in Greek) *Enkephalos* **10**,115–126 (1973).
18. MIALE, J. B., "Laboratory Medicine: Hematology," C. V. Mosby Company, St. Louis, 1972.
19. TERNER, J. Y., Phosphotungstic acid hematoxylin, reactivity *in vitro*. *J. Histochem. Cytochem.* **14**,345–351 (1966).
20. COMINGS, D. E. and AVELINO, E., Mechanisms of chromosome banding VII. Interaction of methylene blue with DNA in chromatin. *Chromosoma (Berl.)* **51**,365–379 (1975).
21. HARRIS, J. R., The biochemistry and ultrastructure of the nuclear envelope. *Biochim. Biophys. Acta* **515**,55–104 (1978).
22. ISSIDORIDES, M. R. and KATSORCHIS, T., "Black and white" contrast of dispersed and compact chromatin obtained with a new EM method: phosphotungstic acid hematoxylin block-staining. (submitted)
23. DARDICK, I. and SETTERFIELD, G., Volume of condensed chromatin in developing primitive-line erythrocytes of chick. *Exp. Cell Res.* **100**,159–171 (1976).
24. KATSORCHIS, T., Histochemical Studies by Light and Electron Microscopy of Variably Activated Nuclei. Doctorate Thesis (in Greek), Department of Biology, U. of Athens, Athens (1976).
25. HUOT, J., Cellular and biochemical alterations induced *in vitro* by THC. In Nahas, G. G. ed., "Marihuana: Chemistry, Biochemistry, and Cellular Effects," Springer-Verlag, New York, pp. 313-327, 1976.
26. CARCHMAN, R. A., END, D. W., PARKER, M. R. and WARNER, W., Marihuana and cell function. This volume pp. 219-228.
27. STEINBERGER, E., Hormonal control of mammalian spermatogenesis. *Physiol. Rev.* **51**,1–22 (1971).
28. BEDFORD, J. M., BENT, M. J. and CALVIN, H. C., Variations in structural character and stability of nuclear chromatin in morphologically normal human spermatozoa. *J. Reprod. Fert.* **33**,19–29 (1973).
29. GLEDHILL, B. L., GLEDHILL, M. P., RIGLER, R., Jr. and RINGERTZ, N. R., Atypical changes of deoxyribonucleoprotein during spermiogenesis associated with a case of infertility in the bull. *J. Reprod. Fert.* **12**,575 (1966).

30. FREUND, M., Standards for the rating of human sperm morphology: a cooperative study. *Int. J. Fert. Suppl.* **11**,97 (1966).
31. BEDFORD, J. M., CALVIN, H. and COOPER, G. W., The maturation of spermatozoa in the human epididymis. *J. Reprod. Fert., Suppl.* **18**,199–213 (1973).
32. FAWCETT, D. W., ANDERSON, W. A. and PHILLIPS, D. M., Morphogenetic factors influencing the shape of the sperm head. *Dev. Biol.* **26**,220–251 (1971).
33. ZIMMERMAN, A. M., ZIMMERMAN, S. and RAJ, Y., Effects of cannabinoids on spermatogenesis in mice. This volume pp. 407–418.
34. BLOCH, D. P. and HEW, H. Y. C., Schedule of spermatogenesis in the pulmonate snail *Helix aspersa*, with special reference to histone transition. *J. Biophys. Biochem. Cytol.* **7**,515–532 (1960).
35. WEISSFELD, A. S. and ROUSE, H., Arginine deprivation in KB cells II. Characterization of the DNA synthesized during starvation. *J. Cell Biol.* **75**,889–898 (1977).
36. MULLEN, R. J., EICHER, E. M. and SIDMAN, R. L., Purkinje cell degeneration, a new neurological mutation in the mouse. *Proc. Nat. Acad. Sci. USA* **73**,208–212 (1976).
37. HOLLANDER, W. F., BRYAN, J. H. D. and GOWEN, J. W., Pleiotropic effects of a mutant at the P locus from X-irradiated mice. *Genetics* **45**,413–418 (1960).

CANNABINOIDS AND CELLULAR RESPONSES: A SUMMARY

J.-C. JARDILLIER

THE effects of different cannabinoids (psychoactive and non-psychoactive) on several mammalian cells lines are exerted at micromolar concentrations.

1. ACTION AT THE LEVEL OF THE PLASMA MEMBRANE

These effects are numerous and related in most cases to the lipophile nature of THC and of its derivatives. Na^+, K^+ and Mg^{++} ATPases are inhibited *in vitro* with concentration of 10^{-6} M. *In vivo*, tolerance seems to develop to this effect. The inhibitory mechanism is not the same for all ATPases.

Biosynthesis of membrane phospholipids is also altered as a result of acyltransferase inhibition. The membrane action of cannabinoids is also reflected by their inhibitory effect on intracellular incorporation of the precursors of the biosynthesis of macromolecules such as thymidine, uridine and leucine. In the case of thymidine inhibition of uptake may be observed within 15 seconds after exposure to 6.5×10^{-5} M THC.

2. HORMONAL INTERACTIONS AND EFFECTS ON THE NUCLEUS

In vitro, cannabinoids interfere with certain hormone mediated cellular mechanisms: In Leydig cells, they inhibit testosterone synthesis mediated by choriogonadotropic hormone (HCG) or by dibutyryl cyclic AMP. The inhibitory effect of dexamethasone on the biosynthesis of nucleic acids and proteins is potentiated by cannabinoids. There is no effect of THC at the level of the binding between steroid and cytosol receptor. However, THC facilitates the translocation of the hormone receptor complex as indicated by a concentration of nuclear material clearly visible in the electron microscope.

At the level of the nucleus, cannabinoids exert certain specific effects in altering the biosynthesis of chromosomal proteins especially histones and non histones.

3. ACTION ON SPECIALIZED CELLS

The specific functions of certain types of specialized cells may be altered by THC. In neurons, there is a preferential fixation of THC to mitochondria, and a decrease of ribo-

somes fixed to the nuclear membrane; THC produce biphasic alterations in the neurotransmitters dopamine and norepinephrine, which are related to concentration of the drug: stimulating with nanomolar concentrations, inhibiting with micromolar concentrations.

In myocardial cells cultured *in vitro*, THC decreases the frequency of contraction and number of "pace maker" cells. Assorted biochemical changes include alteration of glycolysis and of the activity of several intracellular enzymes.

In micromolar concentrations, which may be reached in chronic consumption, psychoactive and non-psychoactive cannabinoids alter basic cellular functions including structural and functional properties of the genome. These include condensation of the nucleus and inhibition of chromosomal protein synthesis such as histones.

RÉSUMÉ

Les effets des cannabinoides (psychoactifs ou non psychoactifs) sur le metabolisme de nombreux types de cellules (humaines ou animales, transformées ou non) s'exercent à des concentrations micromolaires.

1. ACTION DES CANNABINOIDES AU NIVEAU DE LA MEMBRANE PLASMIQUE

Les effets observés sont très divers et dans la majorité des cas probablement dus au caractère lipophile du THC et de ces dérivés.

Les ATPases Mg^{++}, Na^{+} et K^{+} dépendantes sont inhibées *in vivo* et *in vitro* à des concentrations en THC de l'ordre de 10^{-6} M, le mécanisme d'inhibition n'est pas le même pour toutes ces ATPases.

La biosynthèse des phospholipides membranaires est également altérée en raison d'une inhibition des acyltransferases.

L'action membranaire des cannabinoïdes se traduit aussi par une inhibition de l'incorporation intra-cellulaire de précurseurs de la biosynthèse des macromolécules tels la thymidine, l'uridine et la leucine. Dans le cas de l'incorporation de la thymidine, une inhibition très précoce peut être observée dès la 15è seconde d'incubation en présence de $6{,}5 . 10^{-5}$ M de THC.

2. INTERACTIONS HORMONALES ET EFFETS SUR LE NOYAU

Les cannabinoides interfèrent *in vitro* sur certains métabolismes cellulaires qui font intervenir des hormones.

Les cannabinoïdes s'opposent, dans les cellules de Leydig à la biosynthèse de testostérone induite par l'hormone chorio gonadotrophique (HCG) ou par le dibutyryl-c AMP.

L'effet inhibiteur de la dexaméthasone sur la biosynthèse des acides nucléïques et des protéines est potentialisé par les cannabinoïdes, aucun effet n'est observé au niveau de la liaison stéroïde-récepteur cytosolique mais le THC faciliterait la translocation du complexe

hormone-récepteur ce qui peut être objectivée notamment par une condensation du matériel nucléaire en microscopie électronique.

Au niveau nucléaire, les cannabinoïdes exercent certains effets spécifiques en altérant la biosynthèse de protéines chromosomiques, histones et non-histones.

3. ACTION SUR LES CELLULES SPÉCIALISÉES

Les fonctions spécifiques de certains types de cellules spécialisées peuvent être altérées par le THC.

Dans les cellules nerveuses, on observe une fixation préférentielle du THC sur les mitochondries, une diminution des ribosomes fixés à la membrane nucléaire et, au niveau des neuro-transmetteurs, des interactions complexes avec la dopamine et la norépinéphrine, très sensibles à des variations mineures de la concentration en THC.

Sur des cellules myocardiques cultivées *in vitro*, le THC provoque une diminution de la fréquence des contractions et du nombre des cellules "pace makers". On observe également des modifications de la glycolyse et de l'activité de plusieurs enzymes intra-cellulaires.

A des concentrations micromolaires qui peuvent être attein tes au cours de la consommation chronique du cannabis, les cannabinoides psychoactifs et non-psychoactifs, altèrent les fonctions cellulaires fondamentales, y compris celles du génôme. Celles-çi se manifestent par une condensation du noyau et une inhibition de la synthèse des protéines chromosomiques comme les histones.

EFFECTS OF CANNABIS ON SEX HORMONES AND TESTICULAR ENZYMES OF THE RODENT

JACK HARCLERODE, S. E. NYQUIST, BARRY NAZAR, and DONALD LOWE

Department of Biology, Bucknell University, Lewisburg, PA 17837, U.S.A.

Abstract. The effect of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) on testosterone production and testicular enzymes was examined in rat testes. Acute doses of THC and CBD at 10 mg/kg depressed testosterone formation in testis microsomes. Chronic THC, 2 mg/kg, but not CBD, evoked the most dramatic decrease in testicular enzyme activity. 2 mg delta-9-THC/kg depressed two marker proteins used to assess rat testicular cell function: gamma-glutamyl transpeptidase (GTP), used as a marker of Sertoli cell plasma membranes, and cytochrome P-450, a microsomal component used to assess interstitial cell function. After two days of treatment, both were significantly depressed. No changes were observed in sorbitol dehydrogenase activity or beta-glucuronidase activity. Supplementation with gonadotropins of THC treated rats led to a significant restoration of the GTP and P-450 levels indicating that the lower gonadotropin levels in the plasma of THC treated animals is mediating the effect of THC in those animals. Both LH and FSH were more potent in overcoming the effects of THC than either hormone alone. It is suggested that the reduction of testosterone synthesis in testes of THC treated rats may be the result of the reduction in P-450 content.

Summary. Treatment of rats with 2 mg Δ^9-tetrahydrocannabinol (THC)/kg of body weight caused a depression of testosterone production by testicular microsomes and a reduction in testicular weight after nine days of treatment. These changes are correlated with a reduction in testicular microsomal cytochrome P-450, a marker of interstitial cells, and testicular γ-glutamyl transpeptidase (GTP), a marker of Sertoli cell plasma membranes. THC had no effect on sorbitol dehydrogenase used as a marker of pachytene spermatocytes, or β-glucuronidase activities, a crude marker of interstitial cells. Cytochrome P-450 and γ-GTP, as well as testicular weights, were restored most effectively in THC treated rats by injections of both follicle stimulating hormone and luteinizing hormone. It is suggested that the reduction of testosterone synthesis in testes of THC treated rats may be the result of its effect on the hypothalamo-hypophyseal area which reduces gonadotropin levels, causing a reduced interstitial cell microsomal cytochrome P-450 content needed for the synthesis of testosterone.

INTRODUCTION

IN RECENT years interest has mounted concerning the role of various constituents of marijuana in male reproductive physiology. Kolodny *et al.*[1] reported reduced plasma testosterone, oligospermia and impotence in 20 heterosexual men who had used marijuana at least four days a week for a minimum of six months. Mendelson *et al.*[2] were not able to corroborate the initial findings of Kolodny in twenty-seven male subjects in a controlled research ward setting. No changes were found for plasma testosterone levels after twenty-

one days of two marijuana cigarettes per day. In a second more extensive study, with more than seventy male chronic marijuana users studied for a thirty-one day period, Mendelson showed that plasma testosterone levels for all subjects at all times were within normal limits. These data were supported by Cushman[3]. However, Kolodny *et al.*[4] showed that male volunteers given five marijuana cigarettes daily had depressed testosterone levels and after four weeks of use testosterone continued to fall with continued use. Cohen[5] showed reduced plasma testosterone in twenty subjects two to three hours after marijuana use, and a progressive decline in testosterone levels after four weeks of smoking to 60% of the non-smoking base line. One week of abstinence from marijuana allowed return of plasma testosterone levels of 84% of non-smoking levels.

Animal investigations have shown fairly consistent alterations in gonadal functioning with marijuana treatment. Smith *et al.*[6] found a consistent and significant decrease in serum testosterone concentration following acute doses of THC in rhesus monkeys. The average decrease during the first twenty-four hours was 65% and testosterone levels returned to normal during the following three day period. List *et al.*[7] found significant depression of testosterone formation by rat testis microsomes following both acute and chronic doses of either THC or cannabidiol (CBD). The *in vitro* studies by Dalterio *et al.*[8] found that addition of either THC or CBD to an incubation medium containing decapsulated mouse testes caused a significant reduction in accumulation of testosterone in the media. Schwarz *et al.*[9] showed a rapid reduction of testicular microsomal P-450, a marker for interstitial cells. This work also demonstrated a significant reduction of gamma-glutamyl transpeptidase (GTP), a marker of Sertoli cell membranes. Schwarz and his co-workers suggest that the reduction of testosterone synthesis in the testis of THC treated rats may be the result of reduction in cytochrome P-450 content. This is supported by Goldstein *et al.*[10] who reported that THC caused a specific depression of a major esterase isoenzyme located in the interstitial tissue of the rat testis. Esterase levels in testicular tissues have been shown to parallel that of steroidogenic activity[11].

THC has been shown to have effects on the weight and morphology of the testis. Dixit *et al.*[12] found regression of Leydig cell tissue and reduction of testicular weight in mice treated chronically with cannabis extract. Also, Vyas *et al.*[13] showed chronic doses of THC administered to pigeons caused decreased gross testicular weight but that Leydig cells showed proliferation.

The findings of testicular alterations following the use of marijuana suggest possible changes in the levels of the pituitary gonadotropins, LH and FSH. In 1975 Kolodny reported a significant reduction of plasma LH in human subjects after four weeks of chronic treatment with THC and reduction of plasma FSH after eight weeks. This is in contrast to an earlier study by Cushman[3] which had reported normal levels of FSH and LH in male marijuana smokers. Several studies with laboratory animals have supported Kolodny's findings of reduced LH following THC administration. Collu *et al.*[14] and Smith *et al.*[6] found reduced LH levels in rats and rhesus monkeys respectively following acute doses of THC. Symons *et al.*[15] found lower plasma LH after both chronic and acute treatment with THC and reduced pituitary response with hypothalamic LH releasing factor in the rat. The effect of THC on FSH remains unestablished for Kolodny's report of reduced FSH was not observed in a similar experiment by Hembree *et al.*[16] nor in Collu's study of THC treated rats.

In summary, acute and chronic marijuana use appears to lower the testicular production of testosterone probably by acting through the hypothalamohypophyseal axis. This reduc-

tion in androgen correlates with an interference with spermatogenesis and a decrease in secondary sexual characteristics. None of the studies reported here have shown whether THC causes testicular defects by acting directly on the testis or whether the changes observed in testicular function, such as decreased testosterone production and decreased enzyme content, are due to THC's effect on the hypothalamo-hypophyseal axis through lowered gonadotropins.

The studies reviewed here have examined the effect that THC has on androgen biosynthesis, cytochrome P-450 content, and selected testicular marker enzymes. Cytochrome P-450 is located exclusively in interstitial cells[17]. The three marker enzymes that were examined were gamma-glutamyl transpeptidase (GTP), a component of Sertoli cell plasma membranes and believed to be involved in amino acid exchange of glutamyl peptides[18]; sorbitol dehydrogenase (SDH), a marker of pachytene spermatocytes and a cytoplasmic enzyme which catalyses the $NADH_2$-dependent interconversion of fructose and sorbitol[19]; and beta-glucuronidase, concentrated in the interstitial cells[20]. Also, the effect of administering supplementary gonadotropins, LH, FSH, and LH and FSH in combination, along with THC on the same testicular enzyme was examined.

METHODS

Male Wistar rats weighing 200–350 gm were injected intraperitoneally with 2 mg/kg or 10 mg/kg of THC, CBD, or a vehicle containing 10% propylene glycol, 1% polysorbate (Tween-80), and 89% physiological saline as indicated. Animals were fed Purina Lab Chow and water ad libitum and subjected to a 12 hr light-dark schedule. All animals were sacrificed in the middle of the light cycle 24 hrs after the final injection treatment of each experiment. Protein determinations were conducted by the method of Lowry *et al.*[21].

The formation of testosterone was assayed by a modification of the method of Menard and Purvis[22]. The microsomal CO hemoprotein (cytochrome P-450 + P-420) was measured with the Cary-14 spectrophotometer using a reduced-carbon monoxide versus reduced difference spectrum as described by Omura and Sato[23]. GTP assays were performed according to the procedure of Glenner *et al.*[24], using N-(γ-L-glutamyl)-β-naphthylamide as substrate. SDH activities were determined by an assay which measured changes in the concentration of $NADH_2$, a coenzyme in the SDH catalyzed reduction of fructose to sorbitol[25]. Beta-glucuronidase assays were performed according to the method of Talalay *et al.*[20]. Details of individual experiments are given in figure legends.

RESULTS

ANDROGEN BIOSYNTHESIS

Acute doses of THC or CBD at 10 mg/kg significantly depressed testosterone formation in testis microsomes. Repeated THC administration evoked the most dramatic decrease in enzyme activity (Fig. 1). However, chronic THC, 2 mg/kg, but not CBD, evoked a more marked decrease in testicular enzyme activity.

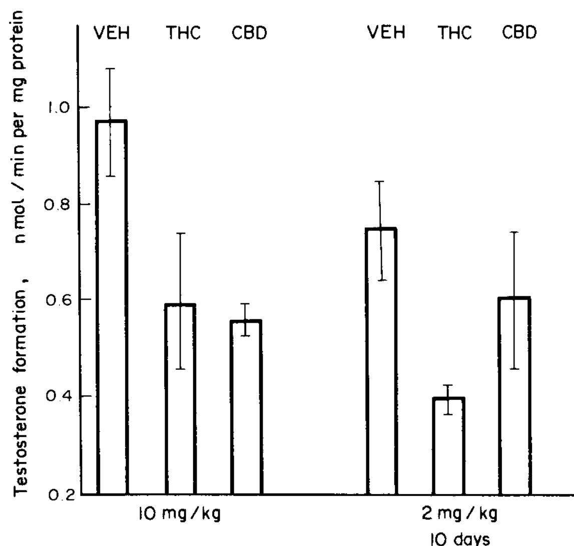


FIG. 1. Androgen production *in vitro* by microsomal fractions of rat testes. Each datum represents the mean ±S.D. for three experiments. (From List, Nazar, Nyquist and Harclerode)

THC AND TESTICULAR ENZYMES

A substantial reduction of the microsomal cytochrome P-450 and its breakdown product cytochrome P-420 was observed in THC treated rats (Fig. 2). When expressed relative to total microsomal protein, the concentration of microsomal P-450 declined in THC treated rats to a level of about 70% ($p < 0.05$) that of the vehicle treated rats after two days and 60% ($p < 0.05$) after nine days. The total microsomal P-450 content was also corresondingly reduced ($p < 0.05$).

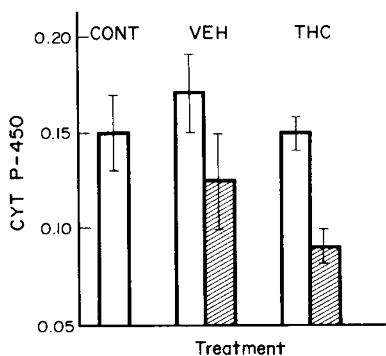


FIG. 2. The effect of THC treatment on rat testes microsomal P-450 content. P-450 content is expressed as the sum of P-450 and P-420. Chronic dosages of 2 mg THC/kg body weight were administered daily. There were four animals in each experimental group except control, which had three. Experiments were repeated twice. Values are expressed in nmoles/mg protein ± Standard Error of the Mean. Open blocks are controls and two-day values, crosshatched are nine-day values. (From Schwarz, Harclerode and Nyquist)

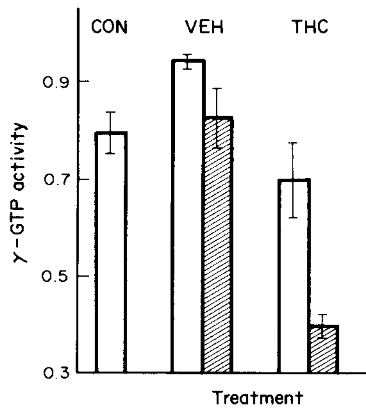


Fig. 3. The effect of THC treatment on rat testes gamma glutamyl transpeptidase. Chronic dosages of 2 mg THC/kg body weight were administered daily. There were four animals in each experimental group except control, which had three. Experiments were repeated twice. Values are expressed in nmoles naphthylamine/min./mg protein ± Standard Error of the Mean. Open blocks are controls and two-day values, crosshatched are nine-day values. (From Schwarz, Harclerode and Nyquist)

The GTP activity (Fig. 3) in THC treated rats was significantly reduced after two days of THC administration and further reduced after 9 days to a level of approximately 60% ($p < 0.05$). Statistically insignificant reductions in SDH levels of THC treated rats were noted after 2 and 9 days of THC administration (Fig. 4). Beta-glucuronidase activities showed no significant differences between THC and vehicle rats (Fig. 5).

THC AND GONADOTROPINS EFFECT ON BODY AND TESTICULAR WEIGHT

No significant differences between the average growth rates of the various groups of rats were noticed following either two or nine days of THC or THC and gonadotropin admin-

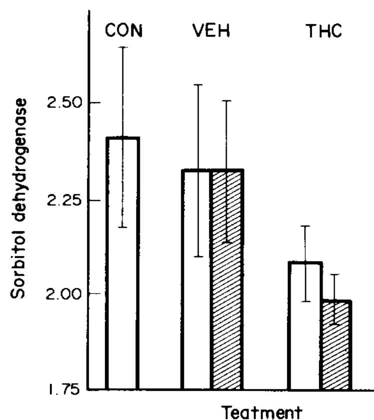


Fig. 4. Effect of THC treatment on rat testes sorbitol dehydrogenase activity. Chronic dosages of 2 mg THC/kg body weight were administered daily. There were four animals in each experimental group, except control, which had three. The experiments were repeated twice. Values are expressed in nmoles $NADH_2$/min./mg protein ± Standard Error of the Mean. (From Schwarz, Harclerode and Nyquist)

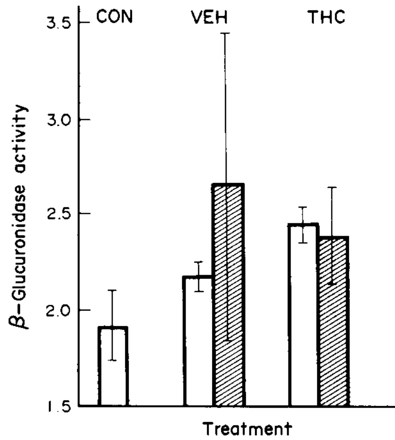


Fig. 5. Effect of THC treatment on rat testes beta-glucuronidase activity. Chronic dosages of 2 mg THC/kg body weight were administered daily. There were four animals in each experimental group except control, which had three. Experiments were repeated twice. Values are expressed in nmoles phenolphthalein/min./mg protein ± Standard Error of the Mean. (From Schwarz, Harclerode and Nyquist)

istration. A significant ($p < 0.05$) decline in testis weight expressed on a per 100 g body weight basis was found to occur after 9 days in THC treated rats (Fig. 6). After 9 days administration of either gonadotropin was sufficient ($p < 0.05$) to relieve this depression. Injections of both gonadotropins were more effective in this regard than either hormone alone and raised testicular weights above those of the control animals ($p < 0.01$).

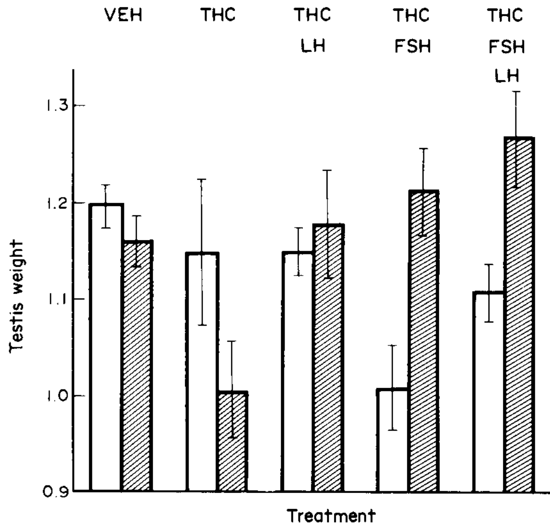


Fig. 6. Effect of THC and gonadotropin on rat testes weight. Weights shown are those found after chronic daily administration of 2 mg THC/kg body weight and twice daily doses of 20 μg LH/kg and 90 μg FSH/kg body weight where indicated. Values are expressed as gm testes weight/100 gm body weight ± Standard Error of the Mean. Each group consisted of 6 experimental animals. Values are expressed as means ± Standard Error. Open blocks are after two days of treatment; crosshatched blocks are after nine days of treatment.

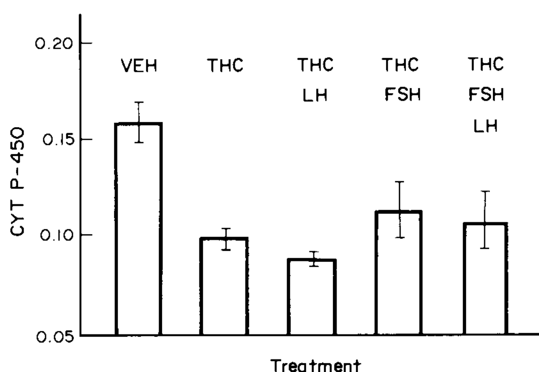


FIG. 7. Effect of THC and gonadotropins on rat testes microsomal P-450 concentration. Values shown are after chronic daily administration of 2 mg THC/kg body weight for nine days and twice daily doses of 20 μg LH/kg and 90 μg FSH/kg body weight where indicated. Each group consisted of three animals. Values are expressed as nmoles/mg protein ± Standard Error.

THC AND GONADOTROPIN EFFECT ON TESTICULAR ENZYMES

Cytochrome P-450 content was reduced in THC treated animals to about 70% of the level that was found in the vehicle control group ($p < 0.05$) after nine days of THC treatment when expressed on a per mg protein basis (Fig. 7). Treatment with either gonadotropin alone or both gonadotropins in combination did not restore cytochrome P-450 levels to vehicle control levels. When the data were examined in terms of the total cytochrome P-450 in the testes, THC still significantly depressed the values below vehicle control; however, treatment with either gonadotropin raised content to non-significant levels, and treatment with both hormones restored cytochrome P-450 content to vehicle control levels (Fig. 8).

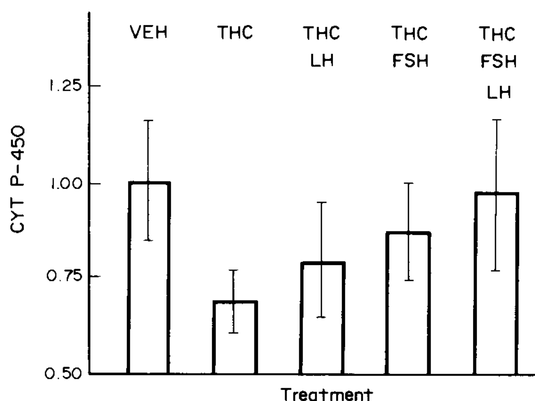


FIG. 8. Effect of THC and gonadotropins on total rat testes microsomal P-450 content. Values shown are after chronic daily administration of 2 mg THC/kg body weight for nine days and twice daily doses of 20 μg LH/kg and 90 μg FSH/kg body weight where indicated. Each group consisted of three animals. Values are expressed as moles/total testes ± Standard Error.

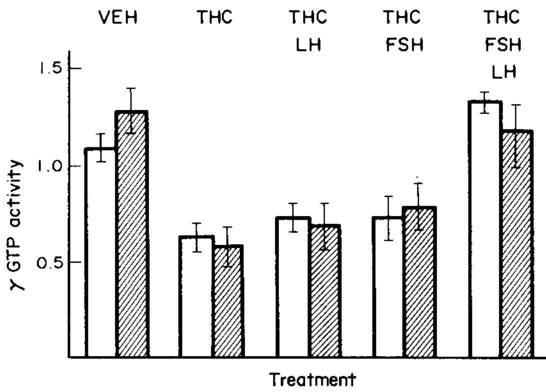


FIG. 9. Effect of THC and gonadotropins on rat testes gamma glutamyl transpeptidase. Values shown are after chronic daily administration of 2 mg THC/kg body weight for two or nine days and twice daily doses of 20 μg LH/kg and 90 μg FSH/kg body weight where indicated. Each group consisted of three animals. Experiments were repeated twice. Values are expressed as nmoles naphthylamine/min./mg protein ± Standard Error.

The GTP activity (Fig. 9) was found to fall consistently in THC treated rats to approximately 60% of that in the vehicle control rats by the second day of treatment and to approximately 40% by the ninth day ($p < 0.01$). Supplementation of both hormones was found to maintain the activity of the enzyme at both two and nine days ($p < 0.01$) while administration of either one alone did not significantly raise the activity of THC treated rats. When the data are expressed on the basis of the total testis, rats which received THC and LH were noticed to have suffered significant ($p < 0.05$) losses in GTP activity between the second and ninth day. Also by day nine their GTP activity was significantly less than the THC and FSH treated animals ($p < 0.01$) (Fig. 10). THC treated rats which received both FSH and LH for either two or nine days had statistically the same levels of GTP in the testes as vehicle control testes.

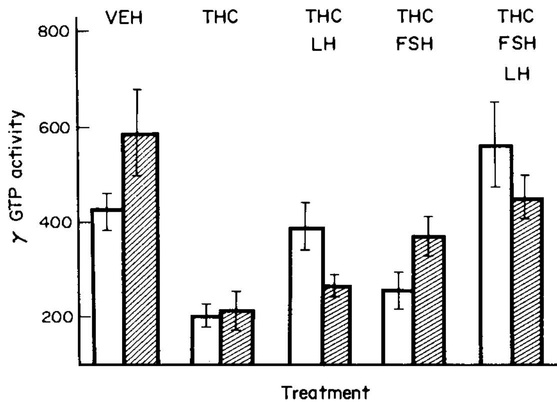


FIG. 10. Effect of THC and gonadotropins on rat testes gamma glutamyl transpeptidase. Values shown are after chronic daily administration of 2 mg THC/kg body weight for two or nine days and twice daily doses of 20 μg LH/kg and 90 μg FSH/kg body weight where indicated. Each group consisted of three animals. Experiments were repeated twice. Values are expressed as nmoles naphthylamine/min./mg total testes ± Standard Error.

DISCUSSION

The studies discussed here have demonstrated that chronic treatment of rats with THC causes a marked reduction in testicular microsomal production of testosterone which is accompanied by a decrease in testicular weight. Correlated with these changes are a reduction in microsomal cytochrome P-450 and GTP. Treatment of THC treated rats with both gonadotropins was able to restore normal testicular weight, microsomal P-450 and GTP activities.

The correlation between the testosterone biosynthetic activities and the cytochrome P-450 levels suggests a possible mechanism for the THC mediated decline in testosterone synthesis by testis microsomes. There are apparently two cytochrome P-450 pools involved in the synthesis of testosterone by the Leydig cell, e.g. mitochondrial and microsomal. Both mitochondrial and microsomal fractions of the Leydig cells are involved in the biosynthetic pathway of testosterone synthesis; however, Purvis *et al.*[26] believed that the rate limiting step in testosterone synthesis involves a cytochrome P-450-dependent cholesterol side chain cleavage enzyme complex localized in the mitochondria. They were able to estimate the half-life of mitochondrial P-450 to be only one or two days and to be under the control of pituitary gonadotropins. It is suggested that THC may be acting to lower testosterone through its action on either mitochondrial or microsomal P-450.

Menard and Purvis[27] believe that plasma levels of LH also control the microsomal cytochrome P-450 content in chick testis, since microsomal P-450 declines after hypophysectomy with an estimated half-life of 3.3 days (Purvis *et al.*)[28]. Purvis *et al.*[26] have shown that two important enzymes involved in testosterone synthesis, 17α-hydroxylase and C_{17}-C_{20}-lyase, are located on the microsomes and contain cytochrome P-450 as their active site. These enzymes also respond to plasma gonadotropin levels[28]. This correlates well with our observations that microsomal cytochrome P-450 is reduced after two days of THC injection in rats and is probably caused by a reduction in plasma LH levels. Moreover, the reduced microsomal P-450 probably accounts for some of the reduction in testosterone synthesis or secretion. Thus, our data suggest that THC causes a reduction in plasma testosterone by lowering LH-induced cytochrome P-450 content of the Leydig cell with a subsequent effect on cytochrome P-450-dependent enzymes.

Our findings of relatively similar β-glucuronidase activity in THC and vehicle treated rats suggest that the effect of THC on the Leydig cell is probably a selective effect on testicular microsomal cytochrome P-450 rather than a major reduction in Leydig cell number or size. In addition, since no significant changes were observed in sorbitol dehydrogenase activity, a component of pachytene spermatocytes, it may be inferred that no significant decrease in that developmental stage of the germ cell line has occurred.

Kolodny *et al.*[29] and Collu *et al.*[14] believe that changes in FSH after THC administration do not become apparent until some time after changes in plasma LH and testosterone become apparent. Care should be exerted when interpreting data or extrapolating from human to rat data. For example, differential fluctuations in the levels of circulating LH and FSH are frequently observed in the humans; however, in the rat dissociated changes in LH and FSH levels seldom occur under a wide variety of circumstances[30]. In addition, some reports have suggested that both FSH and LH may be synthesized and secreted by a single cell type in the rat[31]. Other evidence from a variety of systems suggests two separate cellular origins[32]. Also in all systems examined release of LH-RF from the hypothalamus stimulates release of both gonadotropins.

Means *et al.*[33] report that although FSH has a major role in stimulating the development of the immature Sertoli cell, other hormones, particularly testosterone, have a strong influence on mature Sertoli cell function. Testosterone stimulates the Sertoli cell to produce androgen binding protein (ABP)[33, 34]. In addition, testosterone receptors exist in Sertoli cell nuclei and mitochondria, and testosterone is needed to maintain spermatogenesis in hypophysectomized rats, further strengthening its role in maintaining Sertoli activity.

Our findings of reduced GTP activity in THC treated rats may have been due to a lower level of testosterone synthesis and secretion. In essence, then, THC would cause lower LH levels, which in turn would cause a reduction in testosterone synthesis by reducing the microsomal cytochrome P-450 content of interstitial cells. These lowered testosterone levels would result in decreased Sertoli cell function as reflected in reduced GTP activity. Alternatively a reduction in FSH stimulation of the Sertoli cell could possibly bring about similar changes. Which, if either, of these two alternatives exists has not been conclusively established.

It is also evident from our data that THC is not acting directly on the testis to cause a reduction of cytochrome P-450 and GTP activity. Rather, THC probably has its effect directly on the hypothalamo-hypophyseal axis to reduce gonadotropin as is seen in the experiment where THC was given with gonadotropins and normal testicular weight and cytochrome P-450 and GTP activities were maintained. Unanswered, of course, is the question of whether THC effects a reduction in circulatory gonadotropin levels by acting on the pituitary directly to cause reduced gonadotropin secretion, or on the hypothalamus directly to inhibit the formation or release of the gonadotropin-releasing factor. Another experiment involving THC given with gonadotropin-releasing factor would be helpful in clarifying this issue.

Résumé. Nous avons observé (après neuf jours de traitement), dans des rats traités au THC dans la proportion de 2 mg. au kilogramme de poids corporel, une dépression de la production du testosterone par les microsomes testiculaires et une diminution du poids des testicules. Ces changements correspondent à une diminution du cytochrome P-450 dans les microsomes testiculaires (cytochrome P-450 étant un indice de cellules intersticielles), et du GTP testiculaire (indice des membranes du plasma de la cellule Sertoli). THC n'a eu aucun effet sur le sorbitol dehydrogenase employé comme indice spermatocytes pachytènes, ni sur l'action de β-glucuronidase, indice approximatif de cellules intersticielles. Le cytochrome P-450 et GTP, aussi bien que le poids testiculaire initial ont été pleinement rendus aux rats qu'on avait traités au THC en leur injectant une hormone pour stimuler les follicules et une hormone luteinisante. Nous estimons que la diminution de la synthèse du testosterone dans les testicules des rats traités au THC serait le résultat de l'effet de THC sur la région hypothalamo-hypophyséale, c'est-à-dire la réduction du niveau de gonadotropin, ce qui a pour résultat la diminution de la quantité du cytochrome P-450 contenue dans les microsomes des cellules intersticielles, diminution nécessaire à la synthèse du testosterone.

REFERENCES

1. Kolodny, R. C., Masters, W. H., Kolodner, R. M. and Toro, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *N. Engl. J. Med.* **290**(16),872 (1974).
2. Mendelson, J. H., Kuehnle, J., Ellingboe, J. and Babor, T. F., Plasma testosterone levels before, during and after chronic marihuana smoking. *N. Engl. J. Med.* **291**(20),1051 (1974).
3. Cushman, P., Jr., Plasma testosterone levels in healthy male marijuana smokers. *Am. J. Drug. Alcohol Abuse* **2**(2),269 (1975).
4. Kolodny, R. C., Presentation at the International Academy of Sex Research, St. Louis, Missouri (1975).

5. COHEN, S., The 94-day cannabis study. *Ann. N.Y. Acad. Sci.* **282**,211 (1976).
6. SMITH, C. G., MOORE, C. E., BESCH, N. F. and BESCH, P. K., The effect of marijuana (delta-9-tetrahydrocannabinol) on the secretion of sex hormones in the mature Rhesus monkey. *Clin. Chem.* **22**(7),1120 (1976).
7. LIST, A., NAZAR, B., NYQUIST, S. and HARCLERODE, J., The effects of delta-9-tetrahydrocannabinol and cannabidiol on the metabolism of gonadal steroids in the rat. *Drug. Metab. Dispos.* **5**(3),268 (1977).
8. DALTERIO, S., BARTKE, A. and BURSTEIN, S., Cannabinoids inhibit testosterone secretion by mouse testes *in vitro*. *Science* **196**(4297),1472 (1977).
9. SCHWARZ, S., HARCLERODE, J. and NYQUIST, S. E., Effects of Δ^9-tetrahydrocannabinol administration on marker proteins of rat testicular cells. *Life Sciences* **22**,7 (1978).
10. GOLDSTEIN, H., HARCLERODE, J. and NYQUIST, S. E., Effects of chronic administration of delta-9-tetrahydrocannabinol and cannabidiol on rat testicular esterase isozymes. *Life Sciences* **20**(6),951 (1977).
11. NEIMI, M., HARKONEN, N. and KOKKO, A., Localization and identification of testicular esterases in the rat. *J. Histochem. Cytochem.* **10**,186 (1962).
12. DIXIT, V. P., SHARMA, V. N. and LOHIYA, N. K., The effect of chronically administered cannabis extract on the testicular function of mice. *Eur. J. of Pharmacol.* **26**,111 (1974).
13. VYAS, D. K. and SINGH, R., Effect of cannabis and opium on the testis of the pigeon *Columba livia* Gmelin. *Indian J. Exp. Biol.* **14**(1),22 (1976).
14. COLLU, R., LETARTE, J., LEBOEUF, G. and DUCHARME, J. R., Endocrine effects of chronic administration of psychoactive drugs to prepuberal male rats. I: Δ^9-tetrahydrocannabinol. *Life Sciences* **16**,533 (1974).
15. SYMONS, A. M., TEALE, J. D. and MARKS, V., Proceedings: Effect of delta-9-tetrahydrocannabinol on the hypothalamic-pituitary-gonadal system in the maturing male rat. *J. Endocrinol.* **68**(3),43P (1976).
16. HEMBREE, W. C., III, ZEIDENBERG, P. and NAHAS, G. G., Marihuana's effects on human gonadal function. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (Nahas, G. G., *et al.*, ed.). Springer-Verlag, New York, 1976.
17. MENARD, R., LATIF, S. and PURVIS, J., The intracellular localization of cytochrome P-450-dependent enzymes in the rat testis. *Endocrinology* **98**,1587 (1975).
18. HODGEN, G. and SHERINS, R., Enzyme as markers of testicular growth and development in rat. *Endocrinology* **93**,985 (1973).
19. MILLS, N. and MEANS, A., SDH in rat testes: Change of activity during development, after hypophysectomy, and following gonadotrophic hormone administration. *Endocrinology* **91**,147 (1972).
20. TALALAY, P., FISHMAN, W. H. and HUGGINS, C., Chromogenic substrates. II. Phenolphthalein glucuronic acid as substrate for the assay of glucuronidase activity. *J. Biol. Chem.* **166**,757 (1946).
21. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J., Protein measurements with Folin phenol reagent. *J. Biol. Chem.* **193**,265 (1951).
22. MENARD, R. and PURVIS, J., Studies of cytochrome P-450 in testis microsomes. *Arch. Biochem. Biophys.* **154**,8 (1973).
23. OMURA, T. and SATO, R., The carbon monoxide-binding pigments of liver microsomes. *J. Biol. Chem.* **239**,2370 (1964).
24. GLENNER, G., FOLK, J. and MCMILLAN, P., Histochemical demonstration of gamma-glutamyl transpeptidase activity. *J. Histochem. and Cytochem.* **10**,481 (1962).
25. Sigma Technical Bulletin No. 50-UV.
26. PURVIS, J., CANICK, J., HOLOGGITAS, J. and LATIF, S., Control of cytochrome P-450 in rat testis mitochondria by human chorionic gonadotrophin. *Arch. Biochem. Biophys.* **159**,32 (1973).
27. MENARD, R. and PURVIS, J., Stimulation of the levels of cytochrome P-450 and 17α-hydroxylase in chick testis microsomes by pituitary hormones. *Endocrinology* **91**,105 (1972).
28. PURVIS, J., CANICK, J., LATIF, S., ROSENBAUM, J., HOLOGGITAS, J. and MENARD, R., Lifetime of microsomal cytochrome P-450 and steroidogenic enzymes in rat testis as influenced by human chorionic gonadotrophin. *Arch. Biochem. Biophys.* **159**,39 (1973).
29. KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. and TORO, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *New Eng. J. Med.* **290**,872 (1974).
30. BAKER, H. W. G., BREMNER, W. J., BURGER, H. G., DE KRETSER, D. M., DULMANIS, A., EDDIE, L. W., HUDSON, B., KEOGH, E. J., LEE, V. W. K. and RENNE, G. C., Testicular control of follicle-stimulating hormone secretion. *Recent Progress in Hormone Research* **32**,429 (1976).
31. HERBERT, D., Immunocytochemical evidence that LH and FSH are present in the same cell type in the Rhesus monkey pituitary gland. *Endocrinology* **98**,1554 (1976).
32. KUROSUMI, K., Identification of the LH and FSH gonadotrophs in rat anterior pituitary gland. *Arch. Histol. Japan* **29**,329 (1968).
33. MEANS, A., FAKUNDING, J., HUCKINS, C., TINDALL, D. and VITALE, R., Follicle stimulating hormone, the Sertoli cell and spermatogenesis. *Recent Progress in Hormone Research* **32**,477 (1976).
34. TINDALL, D. and MEANS, A., Concerning the hormonal regulations of androgen binding protein in rat testis. *Endocrinology* **99**,809 (1976).

EFFECTS OF CANNABINOIDS ON SPERMATOGENESIS IN MICE

ARTHUR M. ZIMMERMAN

Department of Zoology, University of Toronto, Toronto, Ontario, Canada

SELMA ZIMMERMAN

Division of Natural Science, Glendon College, York University, Toronto, Ontario, Canada

and

A. YESODA RAJ

Department of Zoology, University of Toronto, Toronto, Ontario, Canada

Abstract. Sperm morphology was investigated in hybrid mice of genotype (C57BL × C3H)F_1 following treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN) and cannabidiol (CBD). Mice were treated for 5 consecutive days and 35 days after the last intraperitoneal injection the epididymal sperm were scored in the light microscope and assessed in the scanning electron microscope. The Δ^9 THC (5 and 10 mg/kg) and CBN (10 and 25 mg/kg) treated mice had a statistically higher incidence of abnormal sperm than the vehicle (dimethylsulfoxide) treated controls. Normal sperm have a smooth kidney-shaped head with a prominent hook; abnormal sperm have shapes which displayed heads without hooks, banana shaped heads, amorphous heads and folded heads.

Cytogenetic assessment of primary spermatocytes obtained from mice treated with CBN (10 mg/kg), CBD (10 mg/kg) and Δ^9-THC (10 mg/kg) for 5 days showed an increase in the number of ring and chain translocations when cells were assessed 16 days after the last dose. The incidence of translocations in the cannabinoid treated animals was 3–5 fold greater than the vehicle controls (DMSO). The significance of the results is discussed in terms of the potential genetic effects of the cannabinoids.

I. INTRODUCTION

THE action of marihuana has been more clearly defined since 1970, when purified Δ^9-tetrahydrocannabinol (Δ^9-THC also designated Δ^1-THC) became available for investigation (cf. reviews 2,27). Various reports in the literature suggest that marihuana and Δ^9-THC affect growth, development and reproduction. Our laboratory is interested in determining the influence of marihuana on spermatogenesis. In such an analysis, it is fruitful to use various cannabinoids in order to determine whether they differ from one another in their effect on spermatogenesis. Spermatogenesis is a complicated process in which the differentiation of sperm is controlled by an intricate neurohormonal mechanism. Thus in analyzing marihuana action on sperm differentiation, it is important to consider whether marihuana acts directly on the sperm-forming cells via interference with genetic and/or cellular mechanisms or indirectly by affecting the neuro-endocrine functions which

regulate spermatogenesis. In view of the complex interaction between spermatogenesis and testosterone levels as well as the contribution of the sperm cell to fetal development, it is pertinent to briefly review the literature with respect to the effects of marihuana on testosterone levels, fetal development and spermatogenesis.

A. INFLUENCE OF CANNABINOIDS ON TESTOSTERONE

Conflicting reports in the literature make it difficult to ascertain whether cannabinoids affect plasma-testosterone levels in man. Kolodny and coworkers report a depression of plasma testosterone after chronic[20] or acute[21] marihuana use. In contrast to these reports, Mendelson *et al.*,[26] and Schaefer *et al.*,[32] were unable to demonstrate a depression of plasma testosterone. It is possible, however, as Kolodny and coworkers[21] have pointed out, that the discrepancies may be explained by methodological differences in the investigations. Recent studies by Hembree *et al.*[16] show that in a hospital setting, daily heavy use of marihuana is not associated with a decrease in serum testosterone levels.

Reports of testosterone levels in rodents suggest that Δ^9-THC depresses testosterone levels[9, 34]. *In vitro* studies also show an inhibitory effect of cannabinoids on testosterone[4]. In addition, cannabinoids decrease the weight of accessory sex glands in mice and rats[5, 6, 7, 29].

B. TERATOGENIC EFFECTS OF THC

Teratogenic effects of Δ^9-THC are also difficult to evaluate since various routes of administration, varying times of treatment during gestation, and different dosages have been used. In rabbits, Δ^9-THC treatment induced eventrations and hairlip[11]. In mice, high dosages of Δ^9-THC caused exencephaly and cleft palate[18]. Using more moderate dosages of Δ^9-THC, Mantilla-Plata and coworkers reported induction of cleft palate in Swiss Webster mice[24, 25]. Moreover, teratogenic effects induced by Δ^9-THC were potentiated by treatment with phenobarbital[13]. Teratogenic effects of tetrahydrocannabinol were also demonstrated in Zebrafish[35]. However, other investigators did not observe teratogenic effects in hamsters, rabbits, mice and rats following treatment with marihuana or specific cannabinoid extracts[10, 12, 19, 30, 31].

C. EFFECTS OF MARIHUANA ON SPERMATOGENESIS

Cannabinoids induced disruption of spermatogenesis in mice and was accompanied by Leydig cell degeneration[7]. In humans, marihuana induced a reduction of sperm concentration and total sperm count, however, there was no indication of abnormal sperm morphology[16].

It is difficult to ascertain the mechanism of the effect of marihuana, however, it should be emphasized that a simple dose-response relationship between sperm reduction and sperm production has not been established and is unlikely in view of the multifactorial nature of the system[16].

II. SPERM MORPHOLOGY AND MUTAGENICITY

Spermatogenesis is a complex process involving germ cell differentiation, the result of which is a highly structured, species-specific cell, called a spermatozoon. Early investigations showed that the incidence of abnormal sperm morphology increases following ionizing radiation[23, 28]. Recently, quantitative measurements of radiation dosage revealed that the incidence of abnormalities is radiation dose-dependent and the frequency of abnormal sperm morphology is a function of the specific strain of mice irradiated[3].

Sperm morphology appears to be under genetic control, however, the mechanism by which abnormal sperm are produced has not been elucidated. It is interesting to note that F_1 and F_2 progeny of irradiated male mice have an elevated incidence of abnormal sperm and, in specific individuals the high proportion of abnormal sperm are found for several generations[17]. One possible explanation for induction of abnormal sperm is directly induced genetic damage to spermatogenic cells[37, 39].

Wyrobek *et al.*[39] investigated the possibility that abnormal sperm morphology resulted from chromosomal abnormalities, i.e. whether chromosomal translocation in the diploid germ cell, or chromosomal imbalance (aneuploidy) within the haploid cell (spermatid) caused sperm head abnormalities. The investigators measured the incidence of abnormal sperm in mice, heterozygous and homozygous for 24 various reciprocal and Robertsonian translocations. Diploid cells of these mice contain translocated chromosomes, and a predictable proportion of the gametes carry translocated chromosomes. The investigators report that the levels of sperm head shape abnormalities were not related to the presence of translocated chromosomes in the germ cells or chromosome aneuploidy in the spermatid cells.

During the last several years more than 60 chemicals have been tested on mice, and sperm morphology has been assessed[15, 37, 38]. The sperm of hybrid male mice of genotype (C57BL × C3H)F_1 were examined at 1, 4 and 10 weeks after the mice received 5 consecutive daily intraperitonial injections of various chemical agents. The fraction of sperm with abnormal shape in the treated mice was compared to that of non-treated controls. Among the agents investigated, approximately 45 of them were considered mutagenic in mammalian cells *in vivo*, that is, agents known to cause heritable mutations, dominant lethal mutations, somatic mutations and chromosomal aberrations. The sperm assay method gave a success rate of 67% for the known mutagens as compared with a 64% success rate seen with the Ames *Salmonella* assay for the same panel of agents. It must be emphasized that depending upon the choice of agents tested, the success rate may vary.

It is evident that no single test will identify all potential mutagenic agents, however, by using a battery of tests, such as the *Salmonella* test, micronuclei assay and sperm abnormality assay, it is possible to increase the successful identification of potentially deleterious agents. Employing both the sperm abnormality assay and the *Salmonella* assay, Heddle and Bruce[15] report a success rate of 89% for the panel of 61 compounds they investigated.

III. INFLUENCE OF CANNABINOIDS: SPERM MORPHOLOGY AND CYTOGENETICS

In view of the reports which suggest that cannabinoids affect spermatogenesis and cause teratogenic effects we initiated a programme to investigate the effects of specific cannabin-

oids on sperm differentiation. We wish to report on two aspects of this study, namely: (1) the consequences on sperm morphology when germinal cells, in the primary spermatocyte stage, were exposed to cannabinoids[40] and (2) a cytogenetic study of primary spermatocytes which were exposed to cannabinoids during the spermatogonium stage.

A. GENERAL METHODOLOGY

1. *Sperm cell preparation*

The procedure for evaluation of sperm morphology was essentially that of Wyrobek and Bruce[37, 38]. Young hybrid male mice genotype (C57BL × C3H)F_1 from Bio Breeding Laboratories of Canada Ltd. were obtained at 11–14 weeks of age. The animals received 5 daily intraperitoneal injections of a specific cannabinoid, mitomycin C or vehicle. Thirty-five days after the last treatment, the cauda epididymis was removed from the test animal and a sperm suspension was prepared. The epididymal sperm were assayed for sperm abnormality. Sperm scored, at this time, were assumed to have been in the early primary spermatocyte stage, at the time of drug treatment. Cells were prepared for light microscopy or scanning electron microscopy. For light microscopy, the sperm suspension was stained with 1% Eosin Y in aqueous solution. The smears were prepared and 800 sperm from each animals were scored under 400 × magnification. The slides were scored using a double blind procedure. For scanning electron microscopy, epididymal sperm were fixed in 2.5% gluteraldehyde in phosphate buffered saline (PBS) pH 7.3 for a minimum of 30 minutes. Following fixation the cells were washed by centrifugation three times with PBS and three times with glass distilled water. The cells were frozen with liquid nitrogen, dehydrated in a Speedivac-Pearse tissue drier Model I (Edwards High Vacuum Ltd., Sussex, England) and coated with a thin layer of gold. The specimens were viewed in the Cambridge S180 Scanning Electron Microscope at an accelerating voltage of 30 KV.

2. *Cannabinoids*

The cannabinoids investigated were Δ^9-tetrahydrocannabinol (5 and 10 mg/kg), cannabinol (10 and 25 mg/kg) and cannabidiol (10 and 25 mg/kg). Mitomycin C (0.6 mg/kg) was used as a positive control. Since all agents were dissolved in dimethylsulphoxide, a vehicle control group was established in which the animals received 0.1 ml dimethylsulphoxide. Higher concentrations of THC, CBN and CBD were also tested. However, due to the toxicity of the cannabinoids there was a high mortality and no animals survived the 35 day recovery period (Table 1). In general, Δ^9- and Δ^8-THC were the most toxic of the cannabinoids investigated and none of the animals survived a dosage of 25 mg/kg. CBD was the least toxic since 60% of the animals survived at 25 mg/kg. The order of toxicity in the cannabinoid series was Δ^9- and Δ^8-THC > CBN > CBD.

3. *Cytogenetic evaluation*

Cannabinoids were administered to mice as previously described for 5 days. The testes were excised from the test animal 16 days after the last treatment at which time primary

TABLE 1. PERCENT OF ABNORMAL SPERM FOLLOWING TREATMENT.

Treatment	Number of animals		% survived	Averages	± SE	P. value*
	Initial	Survivors				
Control	15	14	93	1.54	0.11	.095
DMSO 0.1 ml/animal	15	15	100	1.86	0.17	—
Mit. C 0.6 mg/kg	20	9	45	9.22	1.08	.000‡
Δ^9-THC						
10 mg/kg	24	11	46	5.26	1.58	.003‡
5 mg/kg	30	24	80	3.82	0.81	.003‡
CBN						
25 mg/kg	31	4	13	8.63	3.40	.049‡
10 mg/kg	27	15	56	3.26	0.69	.045‡
CBD						
25 mg/kg	23	14	64	2.51	0.37	.057
10 mg/kg	21	12	57	2.40	0.62	.334
Mit. C 2.4 mg/kg	6	0				
Δ^9-THC 300 mg/kg	4	0				
100 mg/kg	4	0				
75 mg/kg	10	0				
50 mg/kg	10	0				
25 mg/kg	10	0				
CBN 300 mg/kg	4	0				
100 mg/kg	4	0				
50 mg/kg	7	0				
CBD 300 mg/kg	4	0				
100 mg/kg	4	0				
50 mg/kg	7	0				
Δ^8-THC 300 mg/kg	4	0				
100 mg/kg	4	0				
25 mg/kg	4	0				

* derived from *t*-test comparison to DMSO
‡ significant at the 5% level

spermatocytes were evaluated for aberrations. Testes were rinsed in 2.2% sodium citrate and the tunica was removed. The tubules were gently squeezed on a glass plate in a drop of 2.2% sodium citrate solution. The cells were washed by centrifugation and placed into 1% sodium citrate. Methanol/acetic acid (3 : 1 v/v) was used as a fixative. The fixative solution was replaced 2 times; cells were placed on slides, air dried and stained with aceto-orcein[1].

B. SPERM MORPHOLOGY ASSAY

The percentage of abnormal sperm in the (C57BL × C3H)F_1 strain of mice is consistently low and the mean frequency of abnormalities as reported by Wyrobek and Bruce[37] is 1.8% with 90% interval for the population extending from 1.2 to 3.4. Zimmerman *et al.*[40], report non-treated control values ($n = 14$) of 1.54 ± 0.11 SE for the average incidence of abnormal sperm. Individual values are considered positive when the treated group exceeds the control group by 1%[38]. The individual values for sperm abnormality

are summarized in Table 1 and each value is compared statistically to the DMSO series where the average value was 1.86. Thus an agent is considered to display a positive response in the sperm assay when the sperm abnormalities exceed 3% (a guide line).

Mitomycin C, the positive control, elicited a sperm abnormality of over 9%. Δ^9-THC at 5 and 10 mg/kg gave values of 3.82 and 5.26%, respectively. These values were statistically significant at the 5% level when compared to the DMSO controls. CBN at 10 and 25 mg/kg produced values of 3.26 and 8.63% respectively; these were also significantly different from the DMSO values at the 5% level. In the CBD series, however, the frequency of abnormally shaped sperm was not significantly different from the DMSO controls, at either the low (10 mg/kg) or high (25 mg/kg) dose.

The data is plotted in Fig. 1 where it can be seen that the results of THC and CBN at both low and high dosages are considered positive according to the criteria proposed by Wyrobek and Bruce[38]. CBD values, although slightly higher than the DMSO controls, do not exceed the 3% guide line, and statistically are not different from the DMSO controls.

The strong positive effect recorded with 25 mg/kg CBN may result from the fact that only four of the 31 mice treated survived the 35 day recovery period. This factor probably contributed to the strong positive response. However, this is not the only explanation since more than 50% of the animals survived at the lower dose of CBN (10 mg/kg), yet the incidence of abnormal sperm is statistically greater than the DMSO controls.

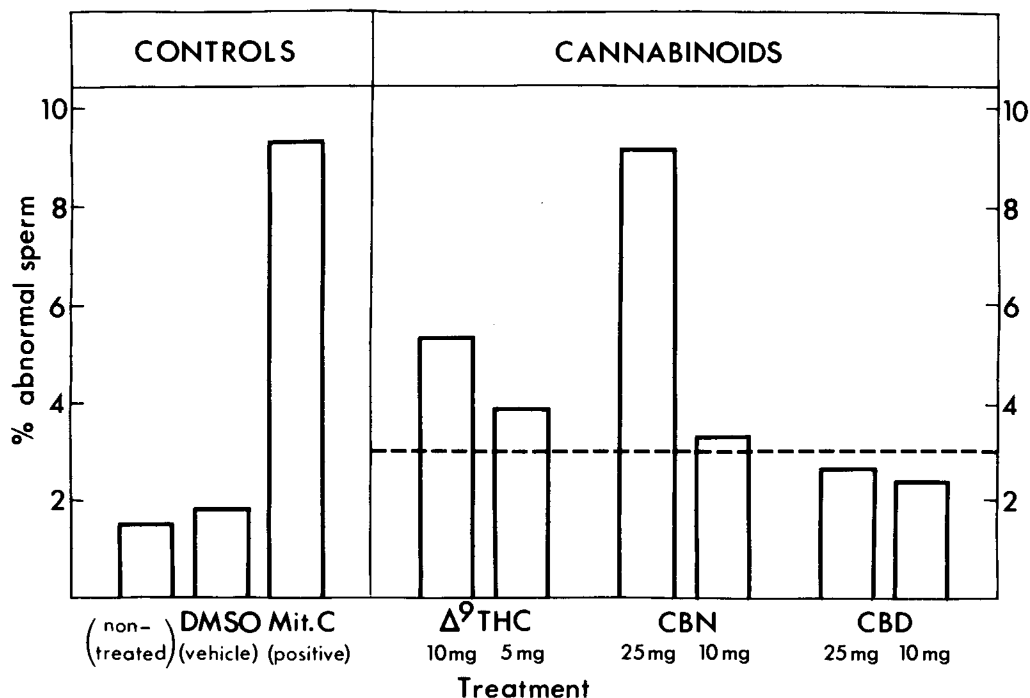


FIG. 1. The effects of cannabinoids on sperm morphology. The mice in each series were treated for 5 days with one of the cannabinoids, vehicle (DMSO) or mitomycin C. The dosage shown below each cannabinoid is expressed in mg/kg. Each value represents the percent of abnormal sperm observed 35 days after the last treatment. The dashed line at the 3% level represents the demarcation line above which induced abnormalities are considered to be deleterious. (From the work of Zimmerman *et al.*,[40].)

C. SPERM MORPHOLOGY AS VISUALIZED IN THE SCANNING ELECTRON MICROSCOPE

The shape of murine sperm from non-treated normal and drug treated animals was evaluated in the scanning electron microscope[40]. Representative normal sperm are illustrated in Fig. 2a,b. The normal sperm has a kidney shaped head with a prominent curved hook on the top. Several different abnormal sperm were identified; they included heads without hooks, banana shaped heads, amorphous heads and folded heads. In Fig. 2c–f representative sperm from animals receiving 10 mg/kg of Δ^9-THC are shown.

D. CYTOGENETICS

In order to further ascertain the effects of cannabinoids on spermatogenesis a cytogenetic evaluation was initiated. Mice were treated for 5 consecutive days with a specific

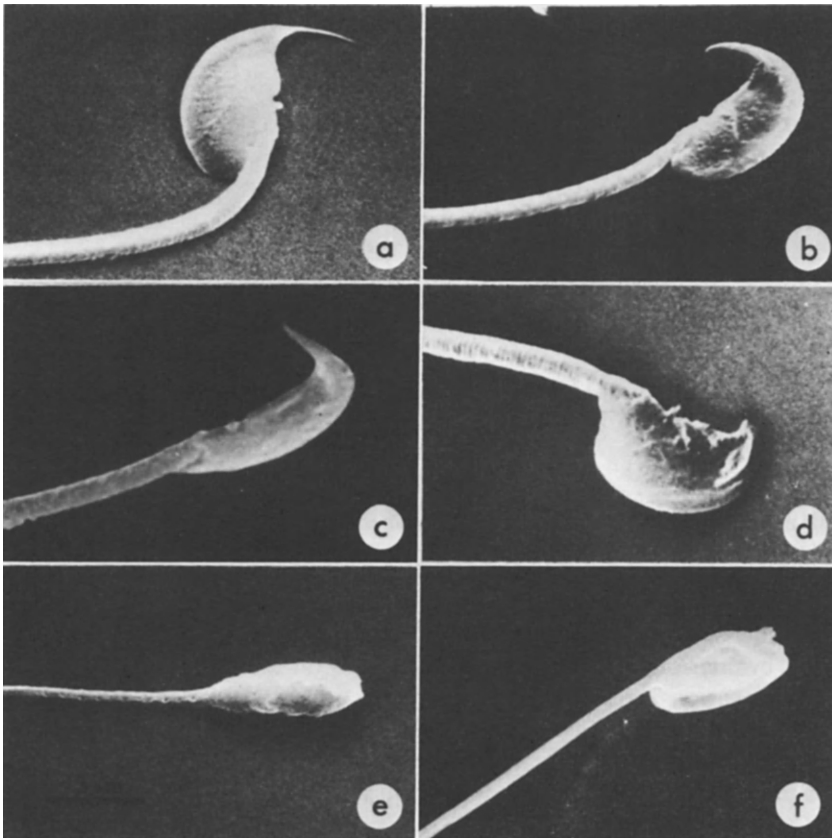


FIG. 2. Scanning electron micrographs of epididymal sperm obtained from control and Δ^9-THC treated mice. In the normal sperm (a and b) the most characteristic feature is the hook which protrudes from the head. Abnormal sperm comprised between 3.8 and 5.2% of population from THC treated mice. The abnormal shaped sperm head included (c) banana shaped heads, (d) sperm heads without hooks and (e) amorphous heads. Folded heads (f) were occasionally observed but were not included in the scoring for abnormal sperm since it is possible that these shapes may be fixation artifacts. (From the work of Zimmerman *et al.*,[40].)

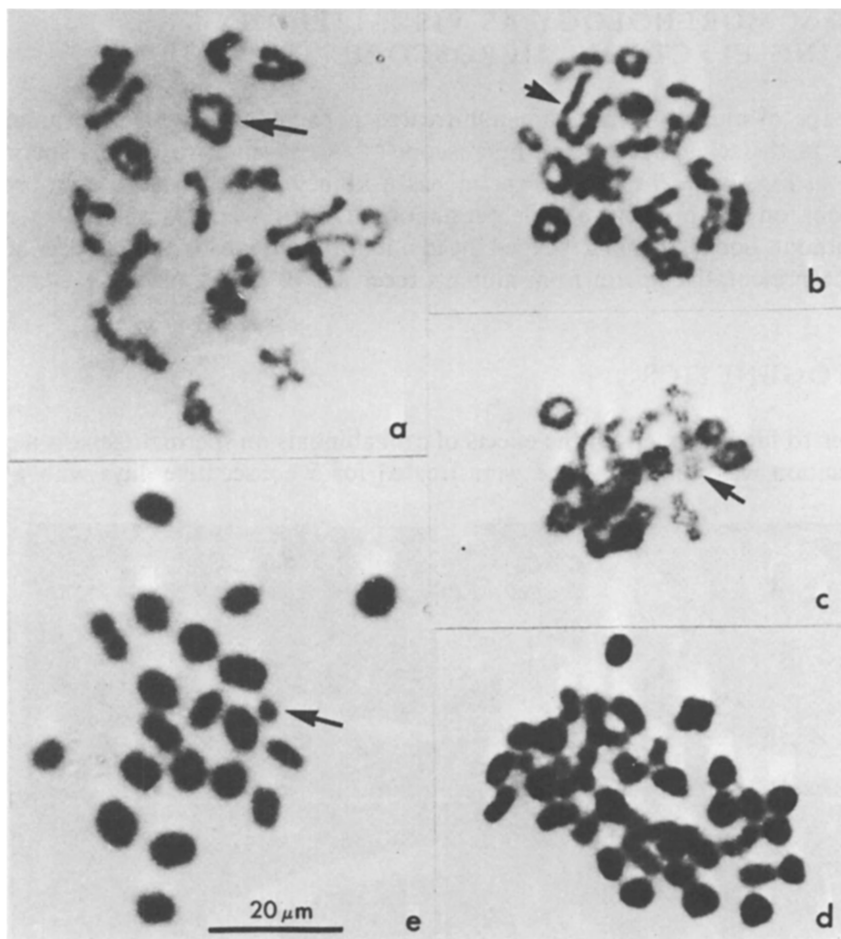


FIG. 3. Photomicrographs of primary spermatocyte at diakinesis illustrating ring and chain translocations and early metaphase chromosomes showing aneuploidy and broken segments induced by Δ^9-THC. Mice were treated for 5 days with 10 mg/kg of Δ^9-THC and the primary spermatocytes were assessed 16 days after the last treatment. (a) Translocation ring of 4 chromosomes and 18 bivalents. (b) Translocation chain of 4 chromosomes. (c) Translocation chain of 3 chromosomes. (d) Aneuploid cell showing 30 bivalents. (e) Broken chromosome segment and 20 bivalents.

cannabinoid and 16 days after the last treatment the primary spermatocytes were prepared for cytological evaluation. The prophase meiotic cells (at diakinesis) were scored for ring and chain translocations, chromosome breaks, deletions and aneuploidy. Cells evaluated at this time were assumed to have been in the spermatogonium state (2–3 weeks earlier) when the cannabinoids were administered to the animals (cf. Ref. 8).

The average number of ring and chain translocations in non-treated and DMSO controls were 0.87 and 1.22 per 100 cells analyzed, respectively. In the mitomycin C-treated mice the value was 6.73; the percentage of translocations in the Δ^9-THC, CBN and CBD cells were 5.74, 4.95 and 6.48, respectively. In general, the incidence of *translocations* in the cannabinoid treated animals was 3–5 fold greater than the DMSO controls. The *total number* of abnormalities in the cannabinoid treated animals was approximately twice as great as in the control animals (Table 2, Fig. 3); there was no appreciable difference in the

TABLE 2. CYTOGENETIC ANALYSIS OF PRIMARY SPERMATOCYTES*

Drug	Animal	Total No. cells analyzed	Translocations ring & chain	Chromosome breaks	Aneuploidy	Percentages	
						Translocations	Total abnormalities
Control	1	164	2	3	3	1.20	4.87
	2	152	1	4	4	0.65	5.92
	3	130	1	4	4	0.76	6.92
						0.87	5.90 avg.
DMSO	1	156	3	6	4	1.92	8.33
	2	187	1	4	3	0.53	4.27
						1.22	6.30 avg.
Mit. C (0.6 mg/kg)	1	127	6	2	—	4.72	6.29
	2	183	16	6	5	8.74	14.75
						6.73	11.74 avg.
CBN (10 mg/kg)	1	226	10	12	8	4.42	13.27
	2	146	8	2	2	5.47	8.21
						4.95	10.72 avg.
THC (10 mg/kg)	1	199	17	6‡		8.54	11.55
	2	122	7	5	5	5.73	13.93
	3	135	3	4	5	2.96	8.88
						5.74	11.45 avg.
CBD (10 mg/kg)	1	121	11	7	—	9.09	14.8
	2	198	7	6	4	3.53	8.58
	3	161	11	5	3	6.83	11.80
						6.48	11.72 avg.

* 5 days treatment, analyses 16 days after last dose
‡ Chromosome deletions

observed number of chromosome breaks, deletions and aneuploidy between the different treatments. In summary, the major aberrations induced by the cannabinoids were ring and chain translocations.

IV. CONCLUDING REMARKS

In general these experiments demonstrate an increased incidence of abnormally shaped sperm heads in Δ^9-THC and CBN treated hybrid mice (C57BL × C3H)F_1. However, the exact mechanism for induction of the abnormal sperm has not been established. In the present experiments, the cytogenetic evidence indicates that cells exposed to cannabinoids in the spermatogonia stage show an increase in the number of ring and chain translocations when observed in the primary spermatocyte stage. The relationship of these translocations to sperm morphology remains unclear. Further evidence of chromosome breakage following cannabinoid treatment may be seen from the work of Zimmerman and Raj (unpublished) in our laboratory, who found that Δ^9-THC, CBN and CBD gave a positive response in the micronucleus test system (cf. Refs. 14, 36). The Wyrobek *et al.*[39] study refutes a direct relationship between the presence of a translocation and/or aneuploidy condition in cells undergoing spermatogenesis and the formation of an abnormally shaped sperm head. Nevertheless, their experiments do not rule out the possibility that translocations involving chromosomal segments other than those observed may be more directly involved in the development of sperm with normal head shape. It is of interest to note that, in our studies, CBD treatment (10 mg/kg) resulted in the highest percentage of translocations but was least effective in inducing sperm head abnormalities.

Alternative hypotheses which may be proposed for the induction of abnormal sperm are point mutation or modified gene function which may affect protein structure or cell metabolism. The current investigation does not allow us to conclude that these cannabinoids are mutagenic. Reports from our laboratory[41] and those of Legator and coworkers[22, 33] suggest that Δ^9-THC is not mutagenic. We reported negative findings using a battery of tests, including the Ames *Salmonella* assay and tests with fibroblasts from normal and *Xeroderma* patients in which chromosome aberrations, sister chromatid exchanges and unscheduled DNA synthesis were assessed. Legator and coworkers[22, 33] also reported a failure to detect mutagenic effects of Δ^9-tetrahydrocannabinol using the Ames *Salmonella* test, micronucleus assay, dominant lethal test and host-mediated assay.

The apparent contradictory reports from our own laboratory and other laboratories requires us to be cautious in the interpretation of our results. It may be that the specific strain of mice (C57BL × C3H)F_1 used in these experiments metabolize cannabinoids to intermediary compounds and that these metabolites interfere directly with the germ cells resulting in identifiable meiotic aberrations (translocations) and sperm head aberrations. It is also possible that the cannabinoids and/or their metabolites induce sperm head abnormalities by interfering with spermiogenesis. In conclusion we wish to emphasize the need for additional information concerning the effect of cannabinoids and their metabolites on cytogenetic structure and sperm morphology. We also propose that the effect of these drugs on protein synthesis and cell metabolism be studied in cells involved in spermatogenesis in order to ascertain biochemical alterations which may play a role in sperm formation.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Ms. Helen Laurence for her expert technical assistance and their thanks to Mr. Eric Y. C. Lin for his assistance with the Scanning Electron Microscope.

This investigation was supported by a grant from National Research Council (to A.M.Z.) and a Minor Research Grant from Glendon College, York University (to S.Z.).

REFERENCES

1. ADLER, I. D., Comparative cytogenetic study after H treatment of mice spermatogonia with Mitomycin C. *Mutat. Res.* **23**,369–371 (1974).
2. BRAUDE, M. C. and SZARA, S., eds., "Pharmacology of Marihuana." Vol. 1 and 2. Raven Press, New York, 1976.
3. BRUCE, W. R., FURRER, R. and WYROBEK, A. J., Abnormalities in the shape of murine sperm after acute testicular X irradiation. *Mutat. Res.* **23**,381–386 (1974).
4. DALTERIO, S., BARTKE, A. and BURSTEIN, S., Cannabinoids inhibit testosterone secretion by mouse testes *in vitro*. *Science* **196**,1472–1473 (1977).
5. COLLU, R., LETARTE, J., LEBOEUF, G. and DUCHARME, J. R., Endocrine effects of chronic administration of psychoactive drugs to prepuberal male rats. 1. Δ^9-tetrahydrocannabinol. *Life Sci.* **16**,533–542 (1975).
6. DIXIT, V. P. and LOHIYA, N. K., Effects of cannabis extract in the response of accessory sex organs of adult male mice to testosterone. *Indian J. Physiol. Pharmacol.* **19**,98–100 (1975).
7. DIXIT, V. P., SHARMA, V. N. and LOHIYA, N. K., The effect of chronically administered cannabis extract on the testicular function of mice. *Eur. J. Pharmacol.* **26**,111–114 (1974).
8. EHLING, U. H., Differential spermatogenic response of mice to the induction of mutations by antineoplastic drugs. *Mutat. Res.* **26**,285–295 (1974).
9. ESBER, H. J., ROSENKRANTZ, H. and BOGDEN, A. E., Assessment of the effect of Δ^9-tetrahydrocannabinol on testicular and thyroid hormone levels in rats. *Fed. Proc.* **35**,727 (1976).
10. FLEISCHMAN, R. W., HAYDEN, D. W., ROSENKRANTZ, H. and BRAUDE, M. C., Teratologic evaluation of Δ^9-tetrahydrocannabinol in mice, including a review of the literature. *Teratology* **12**,47–50 (1975).
11. FOURNIER, E., ROSENBERG, E., HARDY, N. and NAHAS, G. G., Teratologic effects of cannabis extracts in rabbits: a preliminary study. "Marihuana Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 457–468, 1976.
12. HALEY, S. L., WRIGHT, P. L., PLANK, J. B., KEPLINGER, M. L., BRAUDE, M. C. and CALANDRA, J. C., The effect of natural and synthetic Δ^9-tetrahydrocannabinol on fetal development. *Toxicol. Appl. Pharmacol.* **25**,450 (abstract) (1973).
13. HARBISON, R. D., MANTILLA-PLATA, B. and LUBIN, S. J., Alteration of Δ^9-tetrahydrocannabinol induced teratogenicity by stimulation and inhibition of its metabolism. *J. Pharmacol. Exp. Ther.* **202**,455–465 (1977).
14. HEDDLE, J. A., A rapid *in vivo* test for chromosomal damage. *Mutat. Res.* **18**,187–190 (1973).
15. HEDDLE, J. A. and BRUCE, W. R., On the use of multiple assays for mutagenicity, especially the micronucleus, *Salmonella*, and sperm abnormality assays. "Progress in Genetic Toxicology." (D. Scott, B. A. Bridges and F. H. Sobels, eds.). Elsevier/North-Holland Biomedical Press, pp. 265–274, 1977.
16. HEMBREE, W. C., ZEIDENBERG, P. and NAHAS, G. G., Marihuana's effects on human gonadal function. "Marihuana, Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 521–532, 1976.
17. HUGENHOLTZ, A. P. and BRUCE, W. R., Induction and transmission of elevated levels of abnormality shaped murine sperm. *Can. J. Genet. Cytol.* (in press).
18. JONEJA, M. G., A study of teratological effects of intravenous, subcutaneous and intragastric administration of Δ^9-tetrahydrocannabinol in mice. *Toxicol. Appl. Pharmacol.* **36**,151–162 (1976).
19. JONEJA, M. G., Effects of Δ^9-tetrahydrocannabinol on hamster fetuses. *J. Toxicol. and Environ. Health* **2**,1031–1040 (1977).
20. KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. and TORO, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *N. Engl. J. Med.* **290**,872–874 (1974).
21. KOLODNY, R. C., LESSIN, P., TORO, G., MASTERS, W. H. and COHEN, S., Depression of plasma testosterone with acute marihuana administration. "Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, pp. 217–225, 1976.

22. LEGATOR, M. S., WEBER, E., CONNOR, T. and STOECKEL, M., Failure to detect mutagenic effects of Δ^9-tetrahydrocannabinol in the dominant lethal test host-mediated assay, blood urine studies and cytogenetic evaluation with mice. "Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, pp. 699–709, 1976.
23. MACLEOD, J., HOTCHKISS, R. S. and SITTERSON, B. W., Recovery of male fertility after sterilization by nuclear radiation. *J. Am. Ass.* **187**,637–641 (1964).
24. MANTILLA-PLATA, B. and HARBISON, R., Alteration of Δ^9-tetrahydrocannabinol induced prenatal toxicity by phenobarbital and SKF-525A. "Marihuana, Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, pp. 469–480, 1976.
25. MANTILLA-PLATA, B., CLEWE, G. L. and HARBISON, R. D., Δ^9-tetrahydrocannabinol-induced changes in prenatal growth and development of mice. *Toxicol. Appl. Pharmacol.* **33**,333–340 (1975).
26. MENDELSON, J. H., KUEHNLE, J., ELLINGBOE, J. and BABOR, T. F., Plasma testosterone levels before, during and after chronic marihuana smoking. *N. Engl. J. Med.* **291**,1051–1055 (1974).
27. NAHAS, G. G., ed., "Marihuana Chemistry, Biochemistry and Cellular Effects." Springer-Verlag, New York, 1976.
28. OAKBERG, E. F. and DIMINNO, R. L., X-ray sensitivity of primary spermatocytes of the mouse. *Intern. J. Radiation. Biol.* **2**,196–209 (1960).
29. OKEY, A. B. and TRUANT, G. S., Cannabis demasculinizes but is not estrogenic. *Life Sci.* **17**,1113–1118 (1975).
30. PACE, H. B., DAVIS, W. M. and BORGEN, L. A., Teratogenesis and marihuana. *Ann. N.Y. Acad. Sci.* **191**,123–131 (1971).
31. ROSENKRANTZ, H. and BRAUDE, M. C., Comparative chronic toxicities of Δ^9-THC administered by inhalation or orally in rats. "Pharmacology of Marihuana." (M. C. Braude and S. Szara, ed.). Raven Press, New York, pp. 571–584, 1976.
32. SCHAEFER, C. F., GUNN, C. G. and DUBOWSKI, K. M., Normal plasma testosterone concentrations after marihuana smoking. *N. Engl. J. Med.* **292**,867–868 (1975).
33. STOECKEL, M., WEBER, E., CONNOR, T. and LEGATOR, M. S., Failure to detect mutagenic effects of Δ^9-tetrahydrocannabinol in *in vitro* and *in vivo* studies with mice. *Mutat. Res.* **31**,313–314 (1975).
34. SYMONS, A. M., TEALE, J. D. and MARKS, V., Effect of Δ^9-tetrahydrocannabinol on the hypothalamic-pituitary-gonadal system in the maturing male rat. *J. Endocrinol.* **68**,43P-44P (abstract) (1976).
35. THOMAS, R. J., The toxicologic and teratologic effects of Δ^9-tetrahydrocannabinol in the Zebrafish embryo. *Toxicol. Appl. Pharmacol.* **32**,184–190 (1975).
36. VON LEDEBUR, M. and SCHMID, W., The micronucleus test. Methodological aspects. *Mutat. Res.* **19**,109–117 (1973).
37. WYROBEK, A. J. and BRUCE, W. R., Chemical induction of sperm abnormalities in mice. *Proc. Nat. Acad. Sci. U.S.A.* **72**(11),4425–4429 (1975).
38. WYROBEK, A. J. and BRUCE, W. R., The induction of sperm shape abnormalities in mice and humans. "Chemical Mutagens." (A. Hollaender and de Serres, eds.). Plenum Press, New York (in press).
39. WYROBEK, A. J., HEDDLE, J. A. and BRUCE, W. R., Chromosomal abnormalities and the morphology of mouse sperm heads. *Can. J. Genet. Cytol.* **17**,675–681 (1975).
40. ZIMMERMAN, A. M., BRUCE, W. R. and ZIMMERMAN, S., Effects of cannabinoids on sperm morphology. *Pharmacol.* in press, 1979.
41. ZIMMERMAN, A. M., STICH, H. and SAN, R., Non-mutagenic action of cannabinoids *in vitro*. *Pharmacol.* **16**,333–343 (1978).

EFFECTS OF MARIHUANA INHALATION ON SPERMATOGENESIS OF THE RAT

HOSEA F. S. HUANG, GABRIEL G. NAHAS, and WYLIE C. HEMBREE III

Departments of Medicine, Obstetrics and Gynecology, and Anesthesiology, Columbia University College of Physicians and Surgeons, New York, 10032

Summary. The effect of marihuana smoking on spermatogenesis was studied in normal mature rats and in post vitamin A treated, vitamin A deficient rats. A specific decrease in epididymal sperm was noted only after inhalation of marihuana smoke for a period of 75 days at 16 puffs/day. In the absence of tissue and urine cannabinoid levels, no direct comparison to human use can be made, although the amount of THC, as calculated by Rosenkrantz, is comparable to that used in humans. Spermiogenesis may be specifically affected by marihuana smoking as indicated by increased dissociation of sperm head and tail and by the histological changes observed in seminiferous tubule. Marihuana exposure had little effect upon DNA specific activity after H^3-thymidine administration, although a modest decrease in DNA synthesis, accompanied by a total decrease in germ cell number, cannot be excluded by this experimental design. Elevations in serum FSH levels following marihuana exposure may indicate a specific and sensitive effect upon Sertoli cell function.

INTRODUCTION

THE effect of cannabinoids on male gonadal function has been reported frequently. We have found that marihuana smoking in human volunteers resulted in a reduction in sperm count (Hembree *et al.*, 1976) and an increase of abnormal sperm morphology (Huang *et al.*, 1978a.) Although other labs have reported that marihuana smoking caused reduction of serum testosterone (Kolodny *et al.*, 1974), we found no significant change of serum testosterone in our patients (Hembree *et al.*, 1976). The time course of the decline in sperm count suggests that spermiogenesis might be a target for marihuana effects. In rodents, treatment with cannabis extracts has been reported to cause spermatogenic arrest, abnormal spermatogenic cell association and reduction in sperm production (Dixit *et al.*, 1974; Fujimoto *et al.*, 1978). However, the specificity and mechanism of cannabinoid action upon male gonadal function has not been clarified. In an attempt to investigate the specific effects of marihuana smoking on spermatogenesis of the rat, a series of experiments was undertaken using a special device, a Walton Smoke Exposure Machine, to deliver marihuana smoke to animals in amounts approximating those in human consumption.

MATERIALS AND METHODS

Animal: Normal mature male Sprague-Dawley rats (250 gm) were caged in an airconditioned, light controlled animal room, given *ad lib.* water and commercial rat pellet. Vitamin

A deficient rats were prepared by raising 21-day-old weanling male Sprague Dawley rats on a vitamin A deficient diet (Huang and Hembree, 1978). Spermatogenesis of vitamin A deficient rats (VAD) was reinitiated by oral feeding of 1 mg vitamin A and maintained by a return to a normal diet.

Cannabinoid material: Marihuana cigarettes, containing approximately 1.75% Δ^9-THC and 0.027% cannabinol, and marihuana placebo cigarettes were supplied by the National Institute on Drug Abuse. All cannabinoid material was stored at 5°C and was maintained at 60% humidity and 22–23% for 48 hours before being used.

INHALATION PROCEDURE

A Walton smoke exposure machine (Process Instruments Corporation, Brooklyn, N.Y.) with a 3-cigarette capacity was used throughout the experiments. Using this machine, rats in each treatment protocol were exposed each morning either to marihuana placebo smoke or to marihuana smoke. Inhalation dosages of marihuana smoke are expressed as number of puffs per exposure. Rats exposed to 4 puffs and 16 puffs of marihuana smoke inhale approximately 0.4 and 3 mg of Δ^9-THC per kg of body weight according to the calculation method of Rosenkrantz (1976).

TREATMENT PROTOCOL

Normal mature male rats were assigned to one of the following five groups. Group 1, the laboratory control group, received no treatment throughout the experimental period. Group 2, the machine control group, was allowed to go through the machine for 1 cycle daily but received no smoke. Group 3, the placebo control group, was exposed to 16 puffs of marihuana placebo cigarette smoke daily. Group 4, the low inhalation dosage group, was exposed to 4 puffs of marihuana smoke daily. Group 5, the high inhalation dosage group, was exposed to 16 puffs of marihuana smoke daily. Two sets of studies were undertaken in which rats received 30 exposures in 30 days and 75 exposures in 90 days.

Animals in experiments using post vitamin A treated, vitamin A deficient (PVA-VAD) rats were assigned to one of the following groups. Group 1, machine control group, exposed to machine for 1 cycle daily for 5 days before and 10 days after vitamin A feeding. Group 2, placebo control group, exposed to 16 puffs of marihuana placebo cigarette smoke 5 days before and 10 days after vitamin A feeding. Group 3, exposed to 16 puffs of placebo smoke for 5 days before and to 16 puffs of marihuana smoke for 10 days after vitamin A feeding. Group 4, exposed to 16 puffs of marihuana smoke 10 days before and 10 days after vitamin A feeding.

RESULTS

Weight gain was slightly retarded ($P < 0.10$) by 30 high dose marihuana smoke exposures. Although 75 daily exposures to the machine also resulted in reduced growth rate as compared to laboratory control animals, the machine control animals and low dose

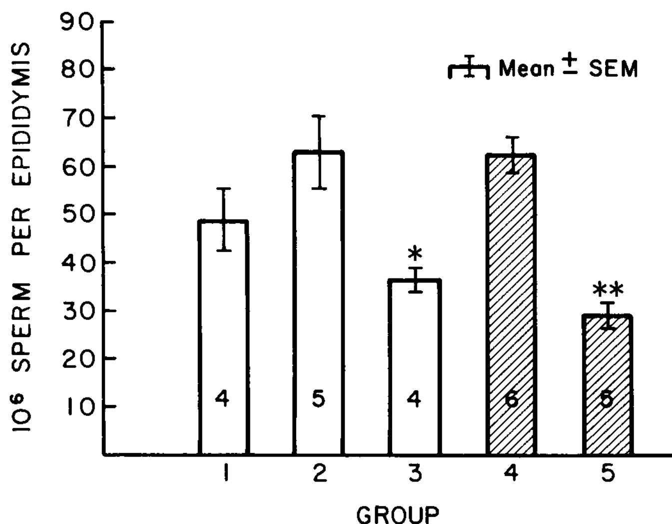


FIG. 1. Effects of 30 daily exposures of marihuana smoke on rat epididymal sperm count, expressed as mean ± SEM x 10^6. Number inside each bar represents the number of animals used. Group 1, laboratory control. Group 2, machine control. Group 3, placebo control. Group 4, 4-puff marihuana. Group 5, 16-puff marihuana. * Significantly different from Group 2 ($P < 0.01$); ** significantly different from Group 1 ($P < 0.05$), Groups 2 & 4 ($P < 0.01$) and Group 3 ($P < 0.10$).

inhalation animals gained significantly more weight than either the placebo control animals or the high dose inhalation animals ($P < 0.05$).

The testis and seminal vesicle of rats in the high inhalation group were significantly smaller ($P < 0.05$) when compared to that of laboratory control, machine control and low inhalation groups.

Animals in Groups 2–5 had a significantly higher adrenal gland weight ($P < 0.05$) than the laboratory control group only after 75 exposures.

The effect of long term exposure to marihuana smoke on testicular function caused a significantly reduced epididymal sperm count. Although exposure to placebo smoke for 30 days also caused a reduction in total epididymal sperm (Fig. 1), the sperm count in the 16-puff marihuana exposure rats was significantly lower ($P < 0.10$) than that of the placebo smoking rats. After 75 exposures of 16 puffs of marihuana smoke, sperm production was significantly affected as shown in Fig. 2. Rats that received either placebo smoke or 4 puffs marihuana smoke also had lower epididymal sperm count as compared to lab control and machine control groups, although the differences were not statistically significant.

A qualitative change in rat sperm was noted in addition to the quantitative change in sperm number. Sperm taken from the cauda epididymidis of rats exposed to 30 daily 16-puff marihuana smoking tended to have a higher incidence of dissociated head and tail (Fig. 3) as compared to sperm taken from rats of other groups. Quantitation of this observation is currently being undertaken.

Histological observation of testicular sections reveals that there were observable changes after 30 daily exposures to 16-puff marihuana smoke. Dramatic changes were noted after 75 daily exposures to marihuana smoke, however. These abnormalities include early release of spermatocytes and spermatids as well as disorganized or incomplete cellular association, shown in Fig. 4. Note the presence of sperm in most of the tubules of

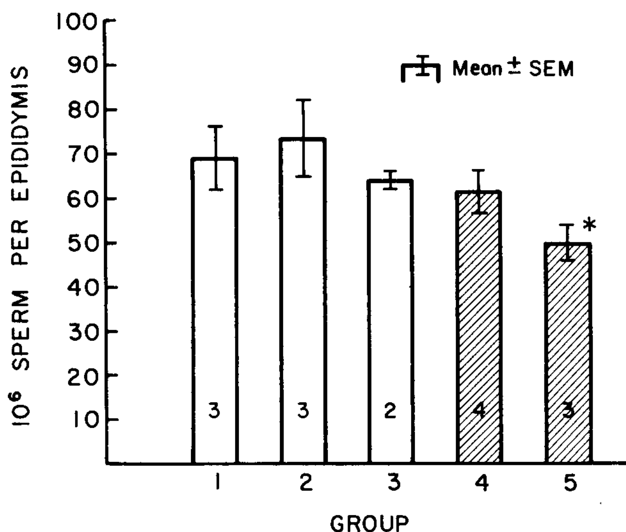


FIG. 2. Effects of 75 daily exposures of marihuana smoke on rat epididymal sperm count, expressed as mean ± SEM × 10^6. Number inside each bar represents the number of animals used. Experimental design as described in Fig. 1. *Significantly different from Groups 1 & 3 ($P < 0.05$) and 2 & 4 ($P < 0.025$).

the 4-puff marihuana smoking rats in spite of tubular disorganization and in contrast to the tubules of the 16-puff marihuana smoking rat.

Twenty-four hours after intratesticular injection of 10 μCi ^{3}H-thymidine, there was no significant change in DNA specific activity of collagenase, DNAase, trypsin treated testicular cells (Bellve *et al.*, 1977) in association with high dose placebo and marihuana smoking. After 75 daily exposures to 4-puff marihuana smoke, however, testicular DNA specific activity increased as compared to machine controls and to laboratory animals.

When PVA-VAD rats model was used, acute marihuana smoking produced a more pronounced effect. Under normal conditions, spermatogenesis of PVA-VAD rats is reinitiated after vitamin A feeding. Pachytene spermatocytes can be seen 10–14 days after vitamin A administration (Huang & Hembree, 1978) (Fig. 5). When VAD rats were exposed to 16-puff marihuana smoke for 10 days before vitamin A administration, and then 10 days after vitamin A treatment, regeneration of spermatogenesis was markedly impaired in 2 of 3 animals. Both degeneration of pachytene spermatocytes and a decreased proliferation of germ cells were observed.

DISCUSSION

Both endocrine and cellular biochemical functions of the testis are directed toward the production of functionally normal male gametes, spermatozoa. Any factor that might alter hormone production or responsiveness, or interfere with cell division or differentiation will be reflected in a decreased efficiency of sperm production. In the rat, in which estimation of sperm production by repeated ejaculation is technically difficult, total epididymal sperm count provides a more accurate index of sperm production. The significant reduction in

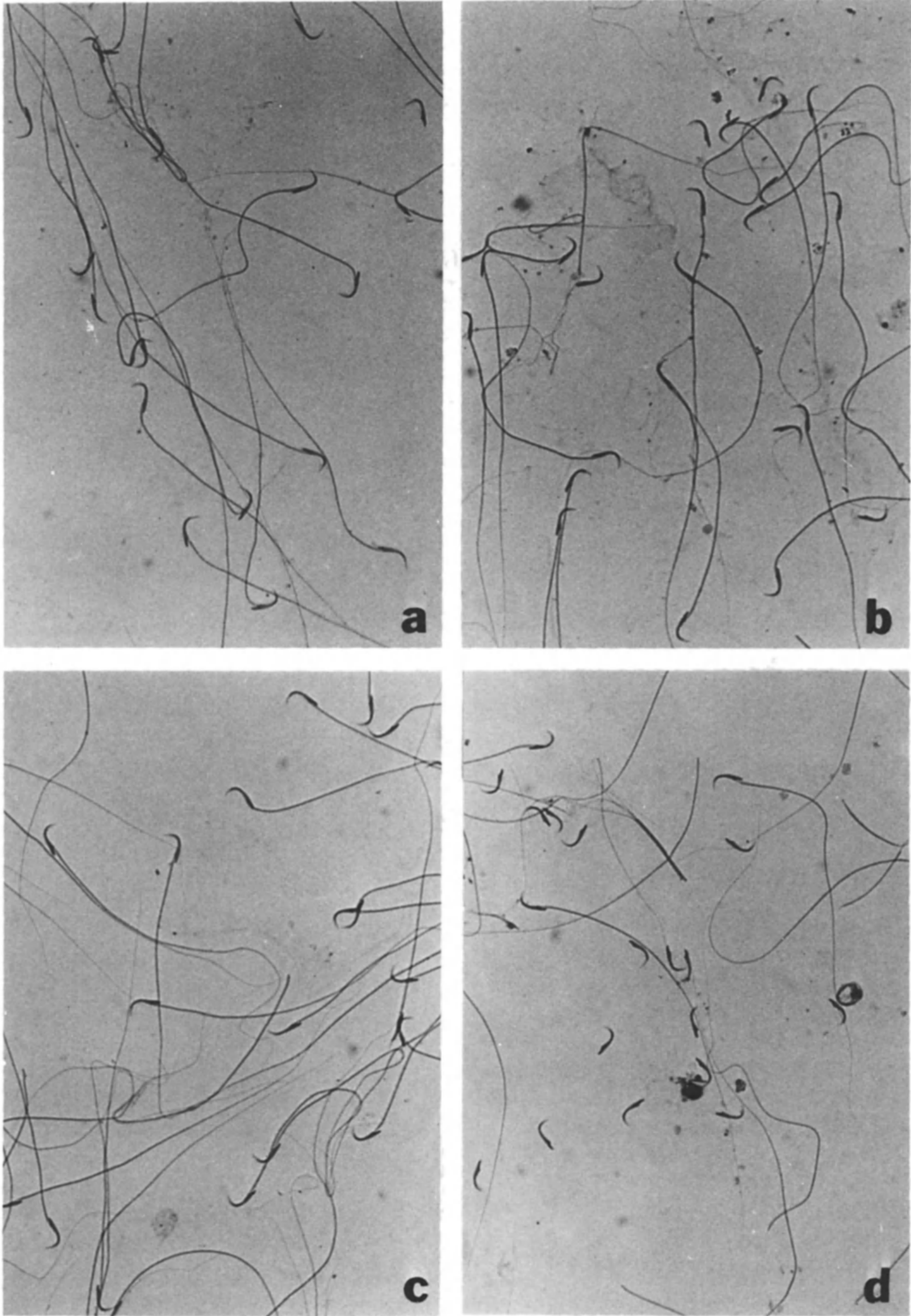


FIG. 3. Rat sperm morphology after 30 daily exposures to (*a*) machine, (*b*) 16 puffs of placebo marihuana smoke, (*c*) 4 puffs of marihuana smoke, (*d*) 16 puffs of marihuana smoke.

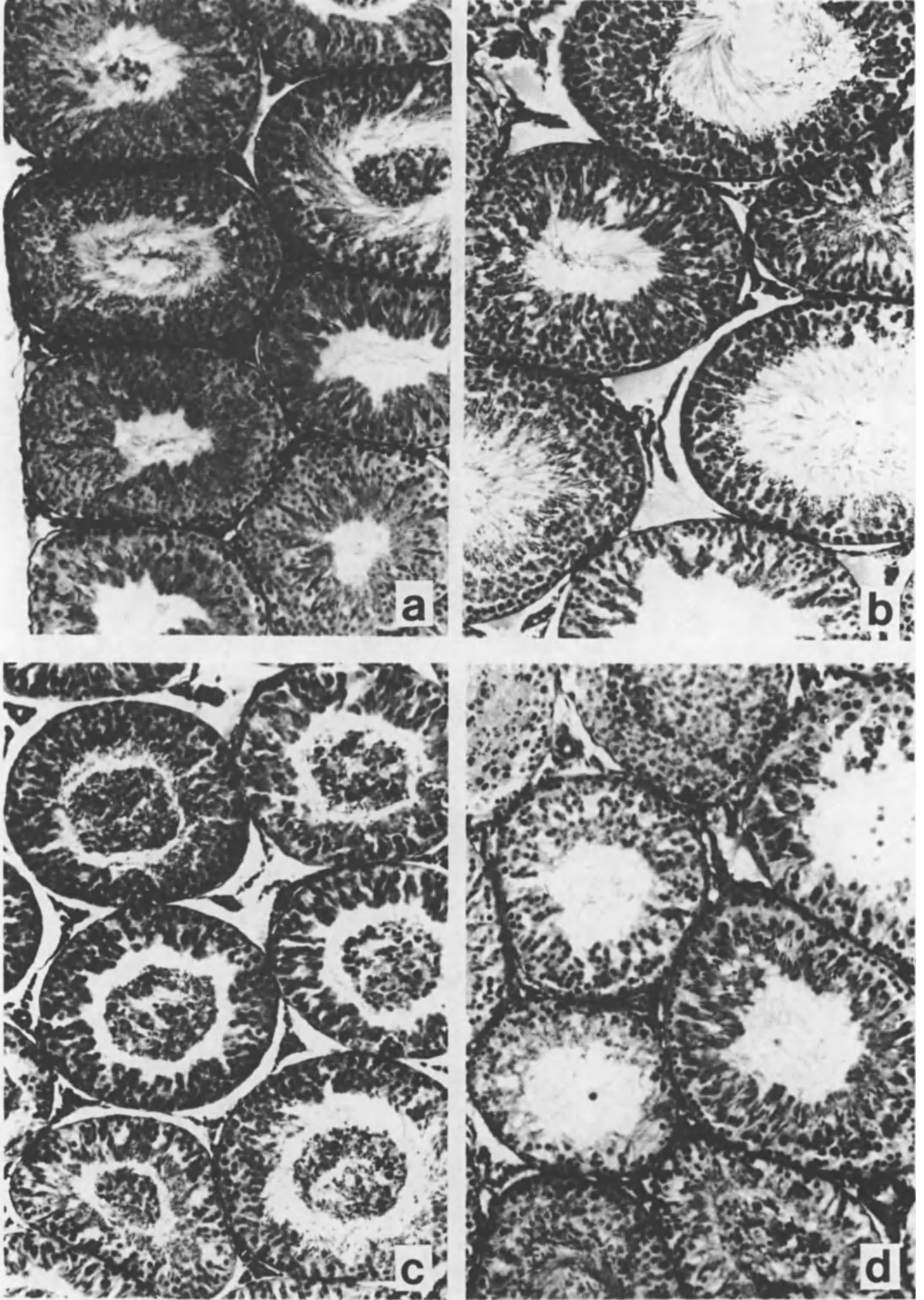


FIG. 4. Photomicrograph of testicular histology from rat after 75 exposures to (*a*) machine, (*b*) 16 puffs of placebo marihuana smoke, (*c*) 4 puffs of marihuana smoke, (*d*) 16 puffs of marihuana smoke.

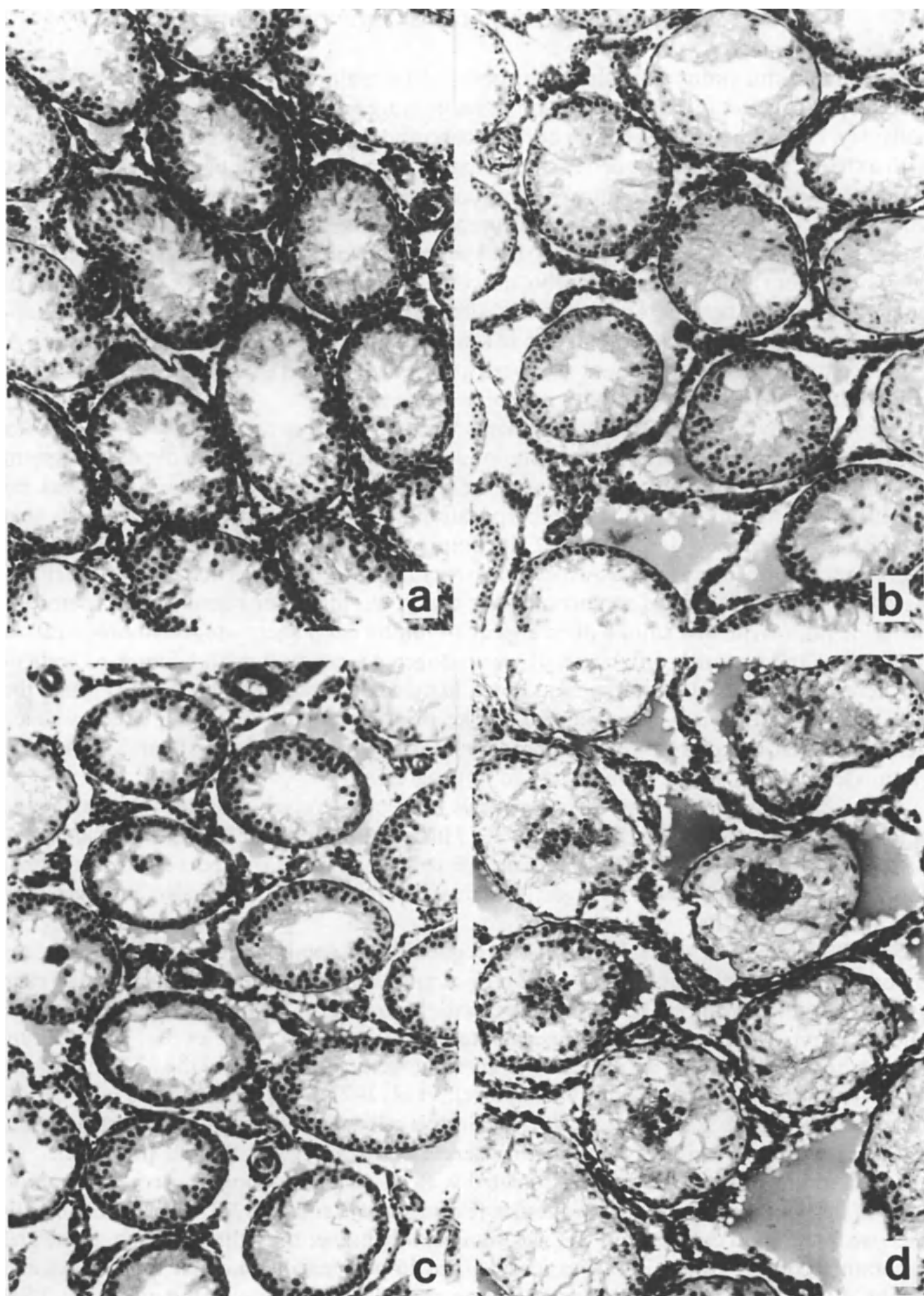


FIG. 5. Photomicrograph of testicular histology from post vitamin A treated Vitamin A deficient rats exposed to (*a*) machine for 5 days before and 10 days after vitamin A treatment (*b*) 16 puffs placebo marihuana smoke for 5 days before and 10 days after vitamin A treatment, (*c*) 16 puffs placebo marihuana smoke for 5 days before, and 16 puffs marihuana smoke for 10 days after vitamin A treatment, (*d*) 16 puffs of marihuana smoke for 10 days before and after vitamin A treatment.

epididymal sperm count after chronic exposure to a high dose of marihuana smoke suggests a specific effect of cannabinoids upon spermatogenesis. After 30 daily exposures of 16 puffs/day, inhalation of smoke from both placebo and marihuana cigarettes was associated with a significant decrease in sperm count. Smaller amounts of marihuana smoking did not alter sperm count. However, after 75 exposures, epididymal count was normal in placebo smoking rats, whereas reduced sperm number was sustained in the 16-puff marihuana smoking animals. These data suggest that spermatogenesis may be adversely affected by components of the marihuana placebo, as well as by marihuana. Further, a specific marihuana effect may become apparent only after prolonged exposure. This finding is supported by abnormal cellular association of seminiferous tubules seen after 75 exposures. A similar reduction in sperm count has been reported in mice (Dixit *et al.*, 1974) and rats (Fujimoto *et al.*, 1978) treated with marihuana extract.

Germ cell DNA specific activity following ^{3}H-thymidine injection was used as an index of spermatogonial proliferation. In spite of a significant reduction in epididymal sperm count after 30 daily exposures to large amounts of marihuana smoke, there was no significant change in ^{3}H-thymidine incorporation into testicular DNA. This suggests that inhibition of germ cell division is not the dominant action of marihuana in the rat. The significance of the increased DNA specific activity after 75 exposures in the 4-puff marihuana group is not certain and requires further study. On the other hand, in the vitamin A deficient rat, marihuana smoke does appear to inhibit early spermatogonial proliferation in response to vitamin A. Inhibition of spermatogenic regeneration and depressed testicular incorporation of ^{3}H-thymidine was noted in marihuana smoking PVA-VAD rats if the VAD rats were exposed to marihuana smoke prior to vitamin A feeding. Studies which define the relationship between spermatogonial proliferation in the VAD and PVA-VAD rat model and that in the normal rat are in progress.

It has been suggested that spermiogenesis may be adversely affected by marihuana smoking in humans (Hembree *et al.*, 1976; Huang *et al.*, 1978). The higher incidence of dissociated sperm heads and tails, incomplete cellular association and release of spermatocytes and early spermatids suggest that marihuana smoking may also interfere with spermiogenesis in rats.

Sertoli cells play a central role in the regulation of spermatogenesis. They form the blood-testis barrier regulating the entry of circulating substances into germ cells; each germ cell maintains direct cellular contact with Sertoli cells throughout spermatogenesis, and they control the final release of sperm into seminiferous tubules (Fawcett, 1975). Serum FSH is frequently used as an indicator of spermatogenic function, and the FSH-inhibiting factor, or "inhibin" (McCullagh, 1932; Setchell, *et al.*, 1977), may be associated with Sertoli cells (Steinberger & Steinberger, 1976), the Sertoli cell-spermatogonial unit (Kruger *et al.*, 1974) or with a certain stage of spermiogenesis (Huang *et al.*, 1978a). In humans, an elevated serum FSH is frequently associated with a severe reduction of sperm production or with degenerating seminiferous tubules (Franchimont *et al.*, 1972). In preliminary studies, we have found that serum FSH was significantly higher than that of the machine and laboratory control groups in rats exposed to the 16-puff marihuana dose for 30 days and to the 4-puff and 16-puff dose for 75 exposures. Placebo exposure did not alter FSH levels. If confirmed, these results indicate that marihuana may alter Sertoli cell or spermatogonial function, prior to or separate from the effect upon sperm production. This effect upon FSH secretion may be more sensitive and specific than other effects previously described.

ACKNOWLEDGEMENT

The authors wish to thank Ms. Lorraine Billig and Diane T. W. Wang for their technical assistance. This study was supported by NIDA grant 5 ROIDA00894.

REFERENCES

1. Bellve, A. R., Cavicchia, J. C., Michelle, C. F., O'brien, D. A., Bhatnagar, Y. M. and Dym, M., Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J. Cell. Biol.* **74**,68–85 (1977).
2. De Kretser, D. M., Burger, H. G. and Hudson, B., the relationship between germinal cells and serum FSH levels in males with infertility. *J. Clin. Endocrin. Metab.* **38**,387–793 (1974).
3. Dixit, V. P., Sharma, V. N. and Lohiya, N. Y., The effect of chronically administered cannabis extract on the testicular function of mice. *Eur. J. Pharmacol.*, **26**,111–114 (1974).
4. Fawcett, D. W., Ultrastructure and function of Sertoli cell. In Handbook of Physiology, Sec. 7, Vol. V, Endocrinology. The Male Reproductive System, D. W. Hamilton and Greep, R. O., eds. *Amer. Physiol. Society*, Washington, D.C. pp. 21–55 (1975).
5. Franchimont, P., Millet, D., Vendesely, E., Letawe, J., Legros, J. J. and Netter, A., Relationship between spermatogenesis and serum gonadotropin levels in azoospermia and oligospermia. *J. Clinc. Endocr. Metab.* **34**,1003–1008 (1972).
6. Franchimont, P., Chari, S., Schellen, A. M. C. M. and Demoulin, A., Relationship between Gonadotrophins, Spermatogenesis and Seminal Plasma. *J. Steroid Biochemistry* **6**,1037–1041 (1975).
7. Fujimoto, G. I., Rosenbaum, R. M., Ziegler, D., Rettura, G. and Morrill, G. A., Effects of marihuana extract given orally on male rat reproduction and gonads. *Ann. Meeting. The Endodrine Soc., 60th*, p. 373 (1978).
8. Hembree, W. C., Zeidenberg, P. and Nahas, G. G., Marihuana effects upon human gonadal function. In "Marihuana chemistry, biochemistry and cellular effects." G. G. Nahas, W. D. M. Paton and Idänpään-Heikkilä, eds., Springer-Verlag, N.Y., pp. 521–532, 1976.
9. Huang, H. F. S., Dyrenfurth, I. and Hembree, W. C., Incomplete feedback regulation of FSH in the Vitamin A deficient rat. *Ann. Meeting. The Endocrine Soc., 60th*, p. 79 (1978a).
10. Huang, H. F. S., Nahas, G. G., Hembree, W. C., Morphological changes of spermatozoa during marihuana induced depression of human spermatogenesis. *Fed. Proc.* **37**(3),739 (1978b).
11. Huang, H. F. S. and Hembree, W. C., Hormonal histological and Biochemical Characteristics of Spermatogenesis (SG) in Vitamin A Deficient (VAD) and in Post-Vitamin A Treated Rats. *Ann. Meeting, Amer. Soc. Andrology, 3rd*, p. 39 (1978).
12. Kolodny, R. C., Masters, W. H., Kolodner, R. M. and Toro, G., Depression of Plasma Testosterone Levels After Chronic Intensive Marihuana Use. *N. Engl. J. Med.* **290**,872 (1974).
13. Krueger, P. M., Hodgen, G. D., Sherins, R. J., New evidence for the role of the Sertoli cell and spermatogonia in feedback control of FSH secretion in male rats. (1974).
14. McCullagh, D. R., Dual endocrine activity of the testis. *Science, N.Y.* **76**,19–20 (1932).
15. Rosenkrantz, H., The immune response and marihuana. In "Marihuana, Chemistry, Biochemistry and Cellular Effects." G. G. Nahas, W. D. M. Paton and Idänpään-Heikkilä, eds., Springer-Verlag, N.Y. pp. 441–456, 1976.
16. Setchell, B. P., Davies, R. V. and Main, S. J., Inhibin. In "The Testis Vol. IV Advances in Physiology, Biochemistry and Function." A. D. Johnson & W. R. Gomes, eds. Academic Press, N.Y. pp. 190–227, 1977.
17. Steinberger, A. and Steinberger, E., Secretion of an FSH-inhibiting factor by cultured Sertoli cells. *Endocrinology* **99**,918–921 (1976).

CHANGES IN HUMAN SPERMATOZOA ASSOCIATED WITH HIGH DOSE MARIHUANA SMOKING

W. C. HEMBREE III, G. G. NAHAS, P. ZEIDENBERG and H. F. S. HUANG

Departments of Medicine, Obstetrics and Gynecology, Psychiatry, and Anesthesiology, Columbia University College of Physicians and Surgeons, New York, 10032

Summary. We have shown that four weeks of high dose marihuana use (8–20 cigarettes/day) in 16 healthy, chronic marihuana smokers was associated with a significant decline in sperm concentration and total sperm count during the fifth and sixth weeks after the first exposure. This was preceded by a decrease in sperm motility and accompanied by a reduction in the number of sperm with normal morphology. In 12 of 16 subjects, a highly significant decrease was noted which was sustained until the end of the study in 11 subjects.

No evidence was obtained suggesting an hormonal mechanism for observed effects. The most likely explanation is a direct cannabinoid effect upon the germinal epithelium during spermiogenesis. Loss of motility response to cyclic AMP and a phosphodiesterase inhibitor, theophylline, in two subjects indicates that the sperm produced following cannabinoid exposure may also have structural or biochemical defects in sperm function. The improvement in sperm motility following cessation of chronic smoking raises some optimism concerning the reversibility of the abnormalities and, if confirmed in additional subjects, gives further credence to the causal relationship between acute marihuana exposure and the abnormalities subsequently observed. No conclusion can be reached regarding the possibility of adverse effects of acute or chronic marihuana use upon human reproduction. On the other hand, it is essential that further studies be undertaken which will examine this possibility.

MARIHUANA smoking in men has been reported to reduce testosterone levels[13], pituitary hormone levels[14] and to be associated with gynecomastia[7]. We previously reported that four weeks of inhalation of large quantities of marihuana in a controlled hospital environment was associated with a highly significant decrease in the concentration of ejaculated sperm and in the total sperm count in three of the four chronic marihuana smokers studied[8]. Because the fall in sperm count was neither preceded by nor associated with a significant fall in serum testosterone, LH and FSH concentrations in these initial studies, it was proposed that cannabinoids may exert a direct effect upon the germinal epithelium, thereby suppressing total sperm production.

Numerous agents are known to exert direct testicular effects. In animals, testosterone alone can maintain normal spermatogenesis[27]. By contrast, X-irradiation[16], antimetabolites[29], perhaps estrogens[1], viruses and the humoral factors associated with an anaphylactic reaction[18] are known to reduce the number of ejaculated sperm by direct action upon the testis. In addition, genito-urinary tract infections can reversibly depress sperm number, motility and morphology[3]. Hypogonadotropism will also result in varying degrees of oligozoospermia. Although "stress" is popularly believed and has been reported to be associated with depression of sperm counts[15], the studies are limited and

poorly controlled. Approximately 10% of otherwise normal adult males have idiopathic oligospermia. Most agents known to alter the number of ejaculated sperm also reduce the extent and quality of sperm motility and the fraction of sperm with normal morphology.

The worldwide prevalence of cannabinoid use and proposed clinical applications make it necessary to determine whether cannabinoids exert any effect(s) upon the male reproductive system and, if so, whether such effect(s) are harmful and/or reversible. The complexity of the male reproductive system requires that animal model systems be developed to study both the morphological and biochemical details of the consequences of treatment with cannabinoids. Studies from this and other laboratories are reported elsewhere in this volume[11].

Studies to determine the effects of marihuana smoking upon human spermatogenesis have been continued in an effort to investigate the consistency of our initial observations and to determine the specificity of the results. Herein, we report data from 12 additional subjects in whom changes in the number of ejaculated sperm were associated with one month of high dose cannabinoid exposure; we describe a simple *in vitro* assay of sperm function and the changes in response to the assay associated with marihuana smoking; and we describe the results using a protocol for examining the *reversibility* of abnormal spermatogenesis in chronic marihuana users.

CLINICAL PROTOCOL

Regular marihuana users, age 18–29, were admitted to the New York State Psychiatric Institute Clinical Research Center for 9–12 weeks during which time all activities inside and outside the hospital were under 24-hour surveillance. Subjects were selected who had no acute or chronic illnesses known to affect reproductive function. Each had a normal physical examination, including normal testicular size and the absence of a varicocele. Because of the observations made in the initial four subjects, only men with total sperm counts greater than 100 million, normal motility and morphology were selected.

Semen specimens were obtained twice weekly by masturbation and analyzed for routine clinical parameters: volume, concentration, percent motility, progression of motility and morphology. The period of prior sexual continence was noted. Once weekly, blood was obtained for testosterone, luteinizing hormone (LH) and follicle stimulating hormone (FSH), a total of five samples drawn at 15-minute intervals. This procedure was utilized to ascertain a range of hormone levels, mindful that sampling could occur during the onset or following one of the frequent episodic secretory bursts of LH or FSH release[23]. After a 3–4 week control period during which there was no smoking, each subject was allowed to smoke *ad lib.* marihuana cigarettes prepared by the National Institute of Drug Abuse and standardized to contain 2% Δ^9-tetrahydrocannabinol. The number of cigarettes varied daily between 6 and 20, averaging 8/day throughout the 4-week smoking period. Subsequently, measurement of each parameter continued during an additional 3–4 week non-smoking period.

A similar protocol is being utilized, in a group of chronic marihuana smokers, to study the effect of cannabinoid withdrawal upon the characteristics of ejaculated sperm. Subjects are examined by one of us (WH) and are screened for multidrug use. Selection is based upon the consistent observation of one or more abnormalities of the semen analysis in chronic smokers who have no apparent cause, e.g. a varicocele, genito-urinary tract infec-

tion, hormone deficiency or excess, or testicular atrophy. Each subject is first studied for two to three months as an outpatient. Although each person who volunteers for the study is motivated in part by his desire to stop using marihuana, only those individuals are selected who indicate that they would continue to smoke if they did not participate. Should anyone desire to stop before completion of the 3-month outpatient protocol, they are urged to do so; they may continue to participate as non-smoking outpatients or are admitted if adequate outpatient control data have been obtained.

During the outpatient phase of the study, semen specimens are submitted every two weeks, five blood samples are drawn for hormone levels and the first-voided morning urine is obtained for total cannabinoids (by radioimmunoassay) and creatinine. Volunteers are then admitted to the General Clinical Research Center of the Presbyterian Hospital for 90 days during which time sperm production is assessed using the same protocol as that employed for the studies of high-dose smoking effects.

Adrenal function is monitored by weekly 24-hour urine samples for 17-ketosteroids and 17-ketogenic steroids, as well as cortisol levels in those samples obtained in weekly blood tests. Subjects may leave the hospital on occasion for less than eight hours and, upon return, blood alcohol levels are measured and urine is obtained for cannabinoids and a drug screen.

Total urinary cannabinoids are measured by radioimmunoassay, utilizing a modification of the method described by Collaborative Research, Inc.[2] and by Dr. Vincent Marks[28]. ^{3}H-Δ^9-tetrahydrocannabinol (11.7 Ci/mMole) was obtained from Collaborative Research, Inc. and purity confirmed by thin layer chromatography[17]. ^{14}C-tetrahydrocannabinol (101 μCi/mg, NIDA) was utilized as a standard to ascertain precise standard solubility, and antisera were furnished by Dr. Marks and purchased from Collaborative Research, Inc. Unbound ^{3}H-Δ^9-THC was precipitated by dextran-coated charcoal and log-linear standard curves were obtained over a range from 0.3 to 5.0 ng/tube. Twenty microliter aliquots of diluted urine were assayed in duplicate at four dilutions. For each urine sample assayed, the cannabinoid concentration was calculated using only the data from aliquots containing THC-equivalent amounts of cannabinoids within the standard range. If non-parallelism was demonstrated, the urine was reassayed.

The response of sperm motility to cyclic nucleotides and to phosphodiesterase inhibitors has been reported[24]. In known fertile donors, we have shown that the increment in motility produced by addition of theophylline, is inversely proportional to the initial motility[9].

In these experiments, semen was diluted in Bavister's solution, control and treatment samples were brought to the concentration of cyclic AMP (10 mM) and theophylline (10 mM) which produces maximal stimulation of motility, each tube was incubated at 37°C in 95% O_2; 5% CO_2 and the percent progressive motility determined at hourly intervals for four to five hours. A typical response is shown in Fig. 1, the rate of decay of motility in the control samples being approximately the same as that of the treated samples after the initial period of stimulation. Cyclic AMP is often associated with an initial fall in motility below control levels (shown here) or with late stimulation. Thus, percent stimulation is calculated from both one-hour and four-hour data. Data are calculated as the fractional motility of the treated sample as compared to that of the control and reported as percents:

$$\%\ \text{motility}_{\text{theo}} / \%\ \text{motility}_{\text{control}} \times 100 = \text{percent motility response}$$

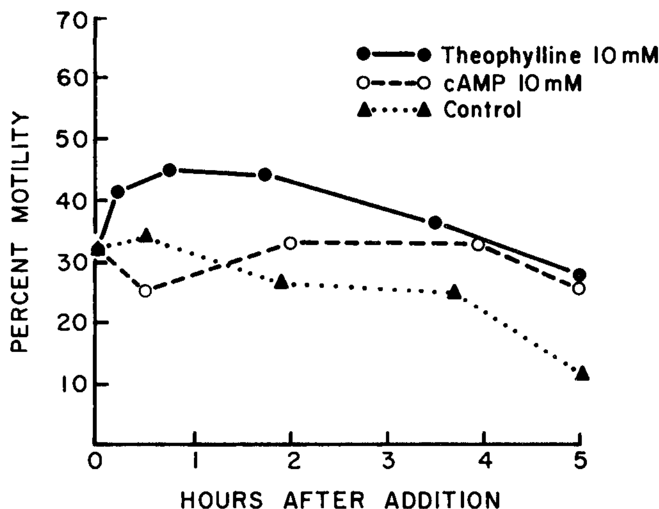


FIG. 1. Response of Sperm Motility to Theophylline and Cyclic AMP. Semen was diluted in Bavister's solution and incubated at 37°C in 95% O_2 and 5% CO_2. Percent motility was determined at hourly intervals. Theophylline final concentration (10 mM) and cAMP final concentration (10 mM) are those yielding maximum stimulation in donors.

RESULTS

SPERM CONCENTRATION

Data from 16 subjects are shown in Fig. 2. Details of semen characteristics from each subject are reported elsewhere[10]. The average sperm concentration during the four-week pre-smoking control period was used to normalize individual data during subsequent weeks in each subject so that direct comparisons could be made between subjects and group statistics could be calculated. For the group, a significant reduction in sperm concentration occurred during the first ($p < 0.001$) and second weeks ($p < 0.01$) of the post-smoking period. No significant change in semen volume occurred in any subject. In 12/16 subjects, a sustained decrease in sperm concentration, below the 95% confidence limits calculated for the control period, was observed after three weeks (5 subjects) or four weeks (2) of smoking, or during the first (3) or second week (2) post-smoking. These decreases were sustained for at least two weeks and remained low until the end of the study in 11 subjects. There was a trend toward normal in the group as a whole during the third and fourth week postsmoking (Fig. 2). There was also a significant decrease in percent mobility and the percent of sperm with normal morphology which occurred during the fourth week of smoking and during the first non-smoking week, respectively.

MOTILITY RESPONSE

Response of sperm motility to theophylline and AMP was studied in two subjects, TP and RS. TP had no significant change in sperm concentration, although sperm motility and morphology decreased during the post-smoking period. Although there was no

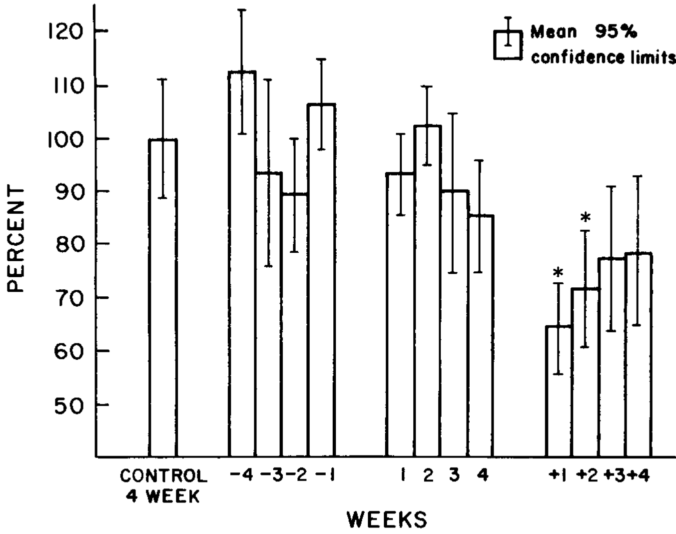


FIG. 2. Marihuana effect upon sperm concentration.—Grouped data. Sperm concentration for each subject is compared by normalizing each value to the sperm concentration during the control period, using the average from the 4-week control period as 100%. Brackets represent 95% confidence limits. Numbers on the abscissa represent weeks of study during the pre-smoking (−4 to −1), smoking (1 to 4) and post-smoking (+1 to +4) periods. Changes were statistically significant (*) at +1 week ($P < 0.001$) and +2 week ($P < 0.01$) post-smoking.

significant decrease in motility, responsiveness to theophylline at 1 hour and 4 hours began to decrease by the second week of smoking (Fig. 3), with a highly significant loss of responsiveness during the smoking ($p < 0.001$) and post-smoking ($p < 0.001$) periods (Fig. 4). Response to cAMP was also markedly diminished during the post-smoking period. Subject RS, who had smoked large quantities of marihuana in the two weeks prior to admission, had a significant and sustained fall in sperm count two weeks after admission. Thus, he was not given additional marihuana and he agreed to remain in the hospital for the 90-day study period. These data are not included in the sixteen subjects. During the first 30 days (pre-smoking), sperm motility remained normal and motility stimulation was

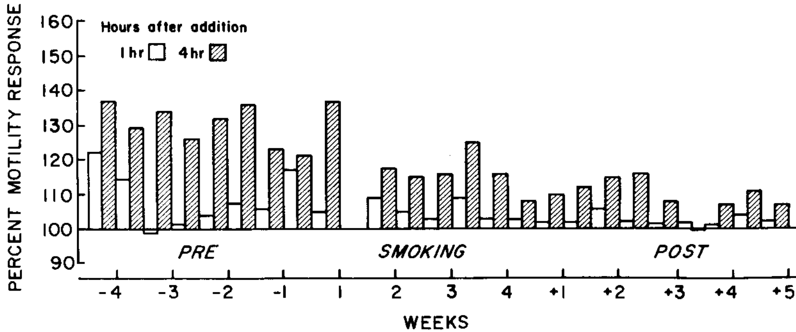


FIG. 3. Sperm Motility Response to Theophylline. Calculation of percent motility response is based upon control percent motility at 1 hour (□) and 4 hours (▨) as 100%. (See Text). Bars represent the responses in individual semen specimens from Subject T.P. Weeks of the study are shown on the abscissa.

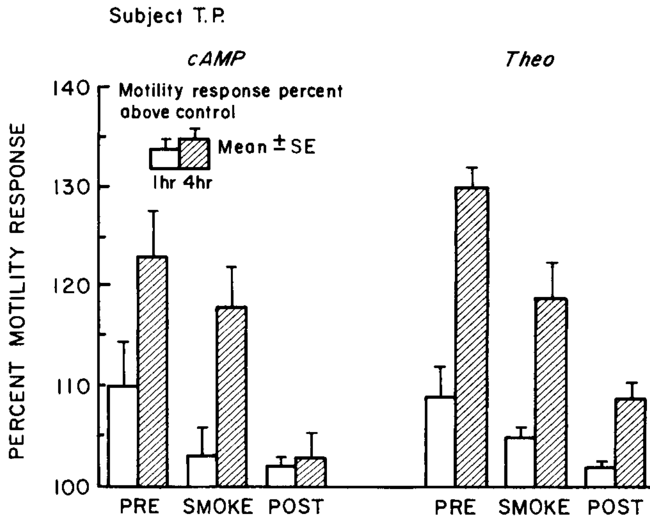


FIG. 4. Motility Response to Cyclic AMP and Theophylline. In subject T.P., the average motility response to cAMP (left) and to theophylline (right) during the pre-smoking control period (PRE), smoking period (SMOKE) and post-smoking period (POST) are shown 1 hour (open bar) and 4 hours (hatched bar). Brackets represented 2 standard errors. 1 hour responses to cAMP are decreased during the smoking and post-smoking periods ($P < 0.001$). Responses to theophylline are also decreased at both 1 hour ($P < 0.01$) and 4 hours ($P < 0.001$).

similar to that observed in TP. Marked variations in motility were observed during the designated smoking and post-smoking periods. During the final 30-day, post-smoking period, motility response both to theophylline and to cyclic AMP was virtually absent. (Fig. 5).

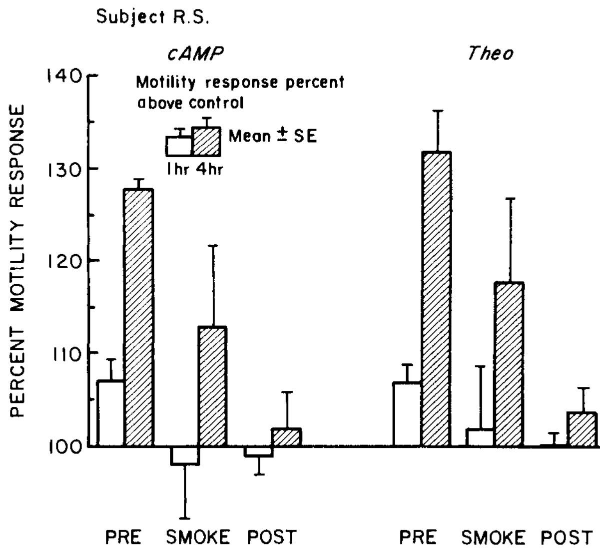


FIG. 5. Motility Response to Cyclic AMP and Theophylline. Subject R.S., as in Fig. 4. No marihuana was smoked during the 12-week study (See Text). Responses to cAMP and theophylline are reduced at 1 hour and 4 hours only during the post-smoking period ($P < 0.001$) (week, 9–12).

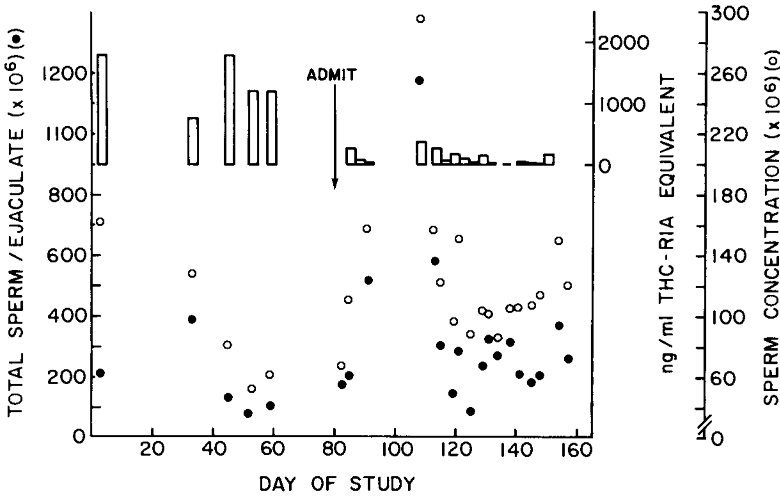


FIG. 6. Sperm Production Following Marihuana Withdrawal. Subject J.P. Total sperm per ejaculate (●) is shown on the left and sperm concentration (○) on the far right. Urinary cannabinoids at ng/ml of THC-radioimmunoassay equivalent are shown by open bars on the near right. Hospitalization and cessation of marihuana began day 80 of the study.

MARIHUANA WITHDRAWAL

Complete data on semen characteristics and urinary cannabinoid levels are available in only one subject. JP, a 24-year-old student who used a Bong to smoke 1–2 marihuana cigarettes/day had urinary cannabinoid levels comparable to subjects smoking 5–10 cigarettes/day. Although sperm concentration and total sperm counts were normal (Fig. 6), percent motility was abnormal (Fig. 7). After admission, urinary cannabinoid levels, declined to the limit of assay detection within five days and remained low, except for four admitted indiscretions of a single cigarette (days 109, 114, 120 and 152). Only one semen specimen, which was obtained after 10 days of sexual continence and following an

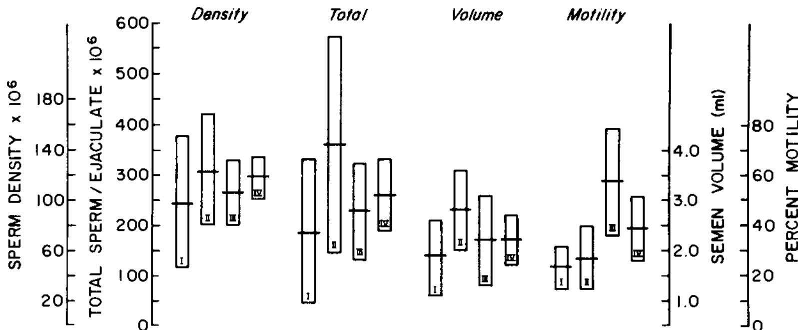


FIG. 7. Changes in Semen Characteristics Following Marihuana Withdrawal. Subject J.P. Open bars represent the 95% confidence limits and horizontal lines the mean sperm density, total sperm per ejaculate, semen volume and percent sperm motility in semen specimens obtained during days 1–60 (I), days 80–115 (II), days 116–140 (III) and days 140–160 (IV) of the study. Sperm motility was significantly greater than control (I) during periods III ($P < 0.01$) and IV ($P < 0.05$). No other significant changes were noted.

8-day emergency pass for personal business, contained a significantly increased sperm density (Day 108). Otherwise, there was no other significant increase in sperm density, total sperm count or semen volume (Fig. 7). However, sperm motility rose to normal levels within 40 days of hospitalization ($p < 0.01$) and remained increased throughout ($p < 0.05$). Analyses of sperm morphology are incomplete.

DISCUSSION

Sperm production is a complex process requiring 74 days within the testicular tubule with an average of 11 days transit time, thereafter, required for sperm to appear in the ejaculate. The variability in the number of sperm per ejaculate in serial specimens has been well documented[25, 6], with the coefficients of variation ranging between 10 and 40%. In serial specimens, only sperm morphology remains constant[19], in the absence of major perturbing events. Thus, in this experimental design in which sperm number is compared before and after marihuana exposure, there must be an average change of 20–80% (depending upon the variance in each sample) if the change is to attain statistical significance. The optimal control period for assessing sperm production and sperm quality is six months, or two spermatogenic cycles, with semen specimens obtained at regular intervals of no less than every two weeks[25]. In this manner, a realistic determination can be made of individual variability while spontaneous trends in sperm production can be excluded. Attempts to insure that the period of sexual continence prior to ejaculation is approximately the same for each analysis will reduce the intrinsic experimental variability. This is especially true if sperm number is low. In our experiments, a period of 36–60 hours of prior sexual continence was requested. Compliance was not always observed, perhaps accounting for the broad fluctuations observed in some subjects.

In the data reported herein, 4–8 specimens were examined during the 4-week control period; the coefficients of variation were in the predictable range. Data from two-week periods were grouped for comparison to the control period and an average decrease in sperm density of 35% was required to achieve statistical significance. The probability that four successive samples would fall below the 95% confidence limits is less than 1%. Thus, the significance of the changes observed in the grouped data was confirmed by linear analyses in 12/16 of the individual subjects.

A limitation in the interpretation of quantitative studies of human spermatogenesis is the fact that sperm number per ejaculate may not directly correlate with sperm production. Rather, sperm count is also a function of smooth muscle contraction of the ductus deferens, ampulla of the vas and seminal vesicle (through the thoracic-lumbar-sympathetic outflow)[12], closure of the posterior bladder neck (sympathetic and parasympathetic) and of clonic contraction of pelvic striated muscle (parasympathetic sacral outflow). In our studies, we cannot exclude the possibility that marihuana smoking results in deficient ampullary and ductus deferens contraction; there would be no alteration in seminal fluid volume. However, if bladder neck closure was incomplete, causing partial retrograde ejaculation, or if prostatic and/or seminal vesicle fluid emission was reduced, there would have been a decrease in semen volume. This was observed in only one subject, although change in seminal fluid volume preceded that in the total sperm count.

Acute and chronic prostatitis or seminal vesiculitis may result in abnormalities of sperm number, motility and morphology[3]. Similarly, acute viral infection can totally abolish

sperm count, presumably due to a transient orchitis[18]. None of our subjects had evidence of male reproductive tract infection and each denied a history of recent illness. It is unlikely that a randomly acquired infectious process could have been responsible for the decline in sperm number which bore a synchronous relationship to the protocol. Only four subjects were hospitalized at a time and the study of the 16 subjects took place over a two-year period.

It must be emphasized that our data do not establish that the observed changes in semen characteristics were caused by effects of inhaled cannabinoids upon the testis. Yet, alternative explanations are either highly unlikely or untestable in the human. Thus, if the data are a reflection of changes in sperm production, it is appropriate to consider possible mechanisms. First, an indirect effect of THC upon spermatogenesis could result from hormone suppression, either at the hypothalamic-pituitary level or at the Leydig cell level. Although no sustained reduction in LH, FSH or testosterone could be demonstrated, this experimental design could not exclude the possibility that intermittent acute suppression is associated with smoking. Whether intermittent gonadotropin suppression causes a reduction in sperm number is not known. However, induction of temporary azoospermia by exogenous testosterone has been utilized as a therapeutic measure in oligospermic individuals[22], the mechanism of suppression being pharmacologic hypogonadotropism. In our experience, administration of 200 mg testosterone enanthate every two weeks causes a sustained suppression of both LH and FSH within one week after the first injection. Reduction of sperm number below the 95% confidence limits was observed in a few patients by six weeks, although the average time required was eight weeks. This is in contrast to the four-week average onset of statistically significant changes following high dose marihuana smoking. Another difference is that hormonally induced azoospermia is associated with either improvement or lack of change in sperm motility and morphology. Thus, our data tend to exclude gonadotropin inhibition, with secondary Leydig cell suppression, as a likely mechanism.

Marihuana has been reported to exert estrogenic effects in rodents and in men[26, 7]. Both THC and marihuana extracts are found to be active agonists in *in vitro* assays of estrogenic activity[21]. Estrogen is known to induce azoospermia via gonadotropin suppression and direct testicular effects, though evidence of neither was noted. Therefore, unless there is selective testicular uptake of marihuana products, direct action upon the germinal epithelium via an estrogen (or anti-androgen) mechanism is unlikely.

Δ^9-THC has been reported to alter membrane stability and to interfere with cell division and metabolism by a non-specific change in membrane permeability[4]. The time of maximum effect noted in these studies, 4–5 weeks after initiation of marihuana smoking, is consistent with an action upon spermiogenesis. This phase of spermatogenesis, which is primarily morphogenetic, is coordinated through the Sertoli cell, which maintains membrane contact with all germ cells. Sertoli cells contain highly complex, membranous subcellular organelles which are contiguous to developing spermatids[5]. Functional or anatomic disruption of Sertoli cell function could reduce sperm number, as well as result in the production of abnormally shaped sperm and/or sperm in which the biochemical and structural machinery for motility is altered. It should be pointed out that the total eight-week study period in this protocol is not sufficient time to observe any changes that might have occurred as a result of decreased spermatogonial division, increased spermatogonial degeneration or inhibition of meiosis. Such effects would have been manifested between 40 and 80 days following marihuana exposure.

Few methods are available to assess the fertilizing capacity of human sperm *in vitro*. Specific anatomical defects in sperm are associated with absence of biochemical properties, e.g. ATP-ase phosphohydrolase deficiency and absence of sperm tail dynein arms in situs inversus[20]. Recently, we have attempted to develop assay systems the responses to which correlate with the fertilizing capacity of sperm. Abnormal responses of sperm motility to the phosphodiesterase inhibitors, theophylline and caffeine have been reported[24]. In this study, we showed progressive loss of responsiveness to both theophylline and to cyclic AMP in two marihuana subjects. It remains to be determined whether this abnormality is characteristic of sperm in chronic marihuana smokers. Although there are no data in humans which suggest that marihuana adversely affects reproduction, it is essential that efforts be made to characterize the abnormalities noted and to assess their potential consequences upon reproductive capacity and fetal development. It is hoped that studies of this type will shed some light on this possibility.

We have begun studies to determine the extent to which abnormalities in ejaculated sperm found in chronic marihuana smokers are reversible. The results of the study reported herein indicate that an improvement in sperm motility occurred 35 days after hospital admission and cessation of marihuana smoking. Sperm morphology and hormone data are not yet complete. Although we can conclude nothing from this single study, it does indicate that an improvement in semen characteristics can occur during hospitalization, unlike the previous studies in which sperm motility either declined or remained unchanged. Such observations, if confirmed, speak against the results as being non-specific effects of hospitalization and further validate the claim of a specific marihuana effect.

ACKNOWLEDGEMENT

This study was supported by NIDA grant 5ROI DA 00894. The collaboration of Drs. Sidney Malitz, Jerome Jaffe and Donald Klein of the New York State Psychiatric Institute is gratefully acknowledged.

REFERENCES

1. ADLER, A., BURGER, H., DAVIS, J., DALMONIS, A., HUDSON, B., SARFATY, G. and STRATTON, M., Carcinoma of prostate: Response of luteinizing hormone and testosterone to oestrogen therapy. *Br. Med. J.* **1**,28 (1968).
2. CHASE, A. R., KELLEY, P. R., TAUNTON-RIGBY, A., JONES, R. T. and HARWOOD, T., Quantitation of Cannabinoids in Biological Fluids by Radioimmunoassay. Research Monograph Series # 7, Cannabinoid Assays in Humans, US HEW Alcohol, Drug Abuse and Mental Health Administration (1976).
3. DERRICK, F. C. and DAHLBERG, B., Male Genital Tract Infections and Sperm Viability in "Human Semen and Fertility Regulation in Men," (E. S. E. Hafez, eds.), C. U. Mosby Company, St. Louis, Mo., p. 389, 1976.
4. DESOÏZE, B., LEGER, C., BANCHEREAU, J. and NAHAS, G. G., Inhibitory effects of diazepam and other psychotropic drugs on blastogenesis of cultured T lymphocytes. *Fed. Proc.* **37**,739 (1978).
5. DYM, M. and CAVICCHIA, J. C., Functional morphology of the testis. *Biol. Reprod.* **18**,1-15 (1978).
6. FREUND, M., Effect of frequency of emission on semen output and on estimate of daily sperm production in man. *J. Reprod. Fertil.* **6**,269 (1963).
7. HARMON, J. and ALIAPOULIOS, M. A., Gynecomastia in marihuana users. *N. England J. Med.* **287**,936 (1972).
8. HEMBREE, W. C., ZEIDENBERG, P. and NAHAS, G. G., Marihuana's Effects on Human Gonadal Function in "Marihuana: Chemistry, Biochemistry, and Cellular Effects," (G. G. Nahas, ed.) Springer-Verlag, New York, 1976.

9. HEMBREE, W. and OVERSTREET, J., Defects in Human Sperm Function in Clinical Infertility. Reduced Zona Pellucida Penetration *in Vitro* in "The Testis in Normal and Infertile Men" (P. Troen and H. R. Nankin, eds.) Raven Press, New York, p. 513, 1977.
10. HEMBREE, W. C., ZEIDENBERG, P., NAHAS, G. G., DYRENFURTH, I. and LINKIE, D., Evidence for a Direct Effect of Marihuana Smoking Upon the Characteristics of Ejaculated Human Spermatozoa. In preparation.
11. HUANG, H. F. S., NAHAS, G. G. and HEMBREE, W. C., Effects of Marihuana Inhalation on Spermatogenesis of the Rat, this volume. pp. 419–427.
12. KEDIA, K. and MARKLAND, C., "Effect of Sympathectomy and Drugs on Ejaculation in Control of Male Fertility" (J. J. Sciarra, C. Markland, J. J. Speidel, eds.) Harper and Row, New York, p. 240, 1975.
13. KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. and TORO, G., Depression of Plasma Testosterone Levels after Chronic Intensive Marihuana Use. *N. Engl. J. Med.* **290**,872 (1974).
14. KOLODNY, R. C., Paper Presented at First Meeting of the International Academy of Sex Research, State University of New York at Stony Brook, 13 September (1975).
15. KREUZ, L. E., ROSE, R. M. and JENNINGS, J. R., Suppression of plasma testosterone levels and psychological stress. *Arch. Gen. Psychiat.* **26**,479 (1972).
16. LACY, D., Comparison of effects produced by high doses of ionizing radiation and oestrogenic hormone on the seminiferous tubules of rat testes in "Effects of Ionizing Radiation on the Reproductive System" (Carlson and Gassner, eds.) Pergamon Press, Oxford, England, p. 103, 1963.
17. LEIGHTY, E. G., Metabolism and Distribution of Cannabinoids in Rats after Different Methods of Administration. *Biochem. Pharmacol.* **22**,1613 (1973).
18. MACLEOD, J., A testicular response during and following a severe allergic reaction. *Fertil. Steril.* **13**,531 (1967).
19. MACLEOD, J. and GOLD, R. Z., The male factor in fertility and infertility. II. Spermatozoon counts in 1000 men of known fertility and in 1000 cases of infertile marriage. *J. Urol.* **66**,436 (1951).
20. PETERSON, H. and REBBE, H., Absence of Arms in the Axoneme of Immobile Human Spermatozoa. *Biol. Reprod.* **12**,541 (1975).
21. RIFKA, S. M., SAUER, M., HAWKS, R. L., CUTLER, G. B. and LORIAUX, D. L., Marihuana as an estrogen. *Ann. Meeting, The Endocrine Soc., 60th*, p. 200 (1978).
22. RAWLEY, M. J. and HELLER, C. G., The testosterone rebound phenomenon in the treatment of male infertility. *Fertil. Steril.* **23**,498.
23. SANTEN, R. J. and BARDIN, C. W., Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms. *J. Clin. Invest.* **52**,2617 (1973).
24. SCHOENFELD, C., AMELAR, R. D. and DUBIN, L., Stimulation of ejaculated human spermatozoa by caffeine. *Fertil. & Steril.* **26**(2),158 (1975).
25. SHERINS, R. J., BRIGHTWELL, D. and STERNTHAL, P. M., Longitudinal Analysis of Semen of Fertile and Infertile Men in "The Testis in Normal and Infertile Men" (P. Troen and H. R. Nankin, eds.) Raven Press, New York, p. 473, 1977.
26. SOLOMON, J., COCCHIA, M. A., GRAY, R., SHATTUCK, D. and VOSSMER, A., Uterotropic effects of delta-9-tetrahydrocannabinol in ovariectomized rats. *Science* **192**,559 (1976).
27. STEINBERGER, E., SWERDLOFF, R. S. and HORTON, R., The Control of Testicular Function. In "Frontier in Reproduction and Fertility Control. A Review of the Reproductive Sciences and Contraceptive Development." (R. O. Greep and M. A. Koblinsky, eds.) The MIT Press, Cambridge, Mass., p. 264, 1977.
28. TEALE, J. D., FORMAN, E. J., KING, L. J., PIALL, E. M. and MARKS, V., The development of a radioimmunoassay for cannabinoids in blood and urine. *J. Pharm. Pharmacol.* **27**,465 (1975).
29. VAN THIEL, D. H., SHERINS, R. J., MYERS, G. H., JR. and DEVITA, V. T., JR. Evidence for a specific seminiferous tubular factor affecting follicle stimulating hormone secretion in man. *J. Clin. Invest.* **51**,1009 (1972).

EFFECTS OF CANNABINOIDS ON REPRODUCTIVE ORGANS IN THE FEMALE FISCHER RAT

G. I. FUJIMOTO, A. B. KOSTELLOW, R. ROSENBAUM
G. A. MORRILL and E. BLOCH

Departments of Biochemistry, Physiology, Pathology and Gynecology and Obstetrics, Albert Einstein College of Medicine, Bronx, New York 10461.

Abstract. We investigated the effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) and crude marihuana extract (CME) given orally on the ovaries, uteri and adrenals in the Fischer rat. Dosages of 1, 5 and 25 mg/kg Δ^9-THC and 3, 15 and 75 mg/kg CME were administered daily for 72 d. Both vehicle-intubated and untreated controls were included. Half the animals were continued another 30 d without treatment (recovery period). After 72 d treatment uterine and ovarian weights were considerably reduced in animals treated with the highest dose of either Δ^9-THC or CME. In animals allowed to recover from treatment for 30 d uteri and ovaries had regained their weights to approximately 85–90% of that of the controls. Based on the above organ weights, on reproductive tract histology and on vaginal smear analysis, we conclude that Δ^9-THC and CME prolong the diestrus stage of the cycle.

INTRODUCTION

CANNABINOIDS have been reported to affect reproductive organs in the female. Chronic treatment with cannabis extract depresses uterine weights in rats given 5 mg/day for 64 d, i.p., (Dixit *et al.*, 1975), in ovariectomized rats given several dosages orally for 15 d (Okey and Truant, 1975) and in mice given 1 mg/d for 64 d, i.p. (Dixit *et al.*, 1975). Reduced ovarian weights and depression of cyclic activity are reported in these studies (Dixit *et al.* 1975).

In contrast, one report has indicated little or no effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) on uterine or ovarian weights in rats after oral administration of up to 50 mg/kg daily for 28 to 180 d (Rosenkrantz *et al.*, 1975). Menstrual cycles in monkeys are not altered by 1 mg/kg daily feeding of Δ^9-THC (Sassenrath and Chapman, 1975) nor estrous cycles in mice by daily oral administration of up to 25 mg/kg crude marihuana extract (CME) or Δ^9-THC (Kostellow *et al.*, 1978). A gain in uterine weight in ovariectomized rats after 14 d of treatment, i.p. has been reported with 2.5 mg/kg Δ^9-THC daily (Solomon *et al.*, 1976).

To try to resolve some of the seeming contradictions reported with rats we have compared the effects of crude marihuana extract and Δ^9-THC given by oral intubation daily at 3 dosage levels for 72 d on uterine, ovarian and adrenal weights in female Fischer rats.

MATERIALS AND METHODS

CANNABINOID PREPARATIONS

Δ^9-Tetrahydrocannabinol (Δ^9-THC, QCD 84923–84924) and crude marihuana extract (CME, QCD 76463) containing 29% Δ^9-THC were provided by the National Institute on Drug Abuse (NIDA).

Δ^9-THC (0.5 g) was dissolved in sesame oil (0.75 g) and to this solution was added with thorough mixing 0.75 g polysorbate 80. This mixture was diluted to 100 ml with H_2O with vigorous shaking to form a suspension of 5 mg Δ^9-THC/ml (high dose). Dilutions were made with aqueous vehicle to 1 mg Δ^9-THC/ml (medium dose) and to 0.2 mg Δ^9-THC/ml (low dose).

CME (1.5 g) was dissolved in 0.75 g sesame oil and mixed with 0.75 g polysorbate 80 (15 mg CME/ml, high dose). Dilutions were made with vehicle to 3 mg/ml (medium dose) and to 0.6 mg/ml (low dose).

ANIMALS AND PROCEDURE

Female Fischer rats were obtained from Charles River Breeding Laboratories. They were 60–70 days old and weighed between 155 and 175 g when received. Each animal was kept in a separate cage and a light-dark cycle, 7 A.M. on, 7 P.M. off, was maintained.

During the first week vaginal smears were observed daily to be sure that the rats were cycling normally. The animals were ranked according to weight, and subdivided into sets containing 8 animals each of similar weights. Animals were then assigned to the eight experimental groups so that each group included one animal from the set of heaviest rats, one from the set of second heaviest, and so on.

The eight experimental groups were as follows: low, medium and high doses of Δ^9-THC, low, medium and high doses of CME, vehicle-treated controls, and untreated controls. Each group contained 20–22 rats.

Cannabinoids were administered daily for 72 days by oral intubation between 9 A.M. and 11 A.M. At the end of the treatment period, half of each group of experimental and control animals was sacrificed, and half was maintained without treatment for an additional 30 days. Animals were weighed weekly. As reported elsewhere (Kostellow, *et al.*), the estrous cycle characteristics were determined during the middle and at the end of treatment.

RESULTS

After 72 days of treatment with Δ^9-THC or CME, the rats were sacrificed and the uteri, ovaries and adrenals weighed and fixed for histological study. Uterine weights were found to be reduced in proportion to the cannabinoid dose. As shown in Table 1, the average uterine weight of rats treated with the highest dose of Δ^9-THC was only 41% of uteri from rats intubated with vehicle alone. Rats treated with the highest dose of CME had an average uterine weight of 42% compared with vehicle treated animals (Table 2). Since animals receiving cannabinoids in the higher dosages did not gain weight at the same rate as the controls (Fig. 1, 2), uterine weights relative to body weight are included (Tables 3 and 4).

TABLE 1. TISSUE WET WEIGHTS FROM FEMALE FISCHER RATS AFTER 72 DAYS INTUBATION WITH Δ^9-THC.

Treatment	mg wet weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	50.5 ± 3.3[b]	64.7 ± 8.7	431 ± 92
Intubated control (10)	51.8 ± 4.2	62.7 ± 7.1	402 ± 84
1.0 mg THC/kg (9)	48.7 ± 3.7	59.0 ± 4.9	379 ± 102
5.0 mg THC/kg (9)	44.4 ± 8.3	50.7 ± 9.8	257 ± 156
25.0 mg THC/kg (10)	47.8 ± 7.4	48.0 ± 10.6	164 ± 70
F	2.07	6.56	10.18
df	4/43	4/43	4/43
P	N.S.[c]	< 0.01	< 0.01

a. Number of animals per group.
b. Standard deviation.
c. Not significant.

TABLE 2. TISSUE WET WEIGHTS FROM FEMALE FISCHER RATS AFTER 72 DAYS INTUBATION WITH CRUDE MARIHUANA EXTRACT (CME).

Treatment	mg wet weight		
	Adrenals	Ovaries	Uterus
None (11)[a]	50.5 ± 3.3[b]	64.7 ± 8.7	431 ± 92
Intubated control (10)	51.8 ± 4.2	62.7 ± 7.1	402 ± 84
3.0 mg CME/kg (10)	49.8 ± 4.1	60.7 ± 6.3	376 ± 100
15.0 mg CME/kg (10)	47.7 ± 10.1	54.9 ± 12.2	325 ± 217
75.0 mg CME/kg (11)	42.0 ± 8.6	40.0 ± 8.9	170 ± 100
F	2.99	11.40	5.62
df	4/45	4/45	4/45
P	< 0.05	< 0.01	< 0.01

a. Number of animals per group.
b. Standard deviation.

TABLE 3. RELATIVE TISSUE WET WEIGHTS FROM FEMALE FISCHER RATS AFTER 72 DAYS INTUBATION WITH Δ^9-THC.

Treatment	mg wet weight/100 g final body weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	26.1 ± 2.4[b]	33.4 ± 4.2	223 ± 48
Intubated control (10)	27.8 ± 2.5	33.6 ± 3.7	215 ± 44
1.0 mg THC/kg (9)	25.1 ± 2.5	30.5 ± 3.6	194 ± 47
5.0 mg THC/kg (9)	24.2 ± 3.8	27.7 ± 4.7	139 ± 80
25.0 mg THC/kg (10)	30.2 ± 5.1	28.3 ± 6.4	101 ± 39
F	4.02	3.09	8.14
df	4/43	4/43	4/42
P	< 0.01	< 0.05	< 0.01

a. Number of animals per group.
b. Standard deviation.

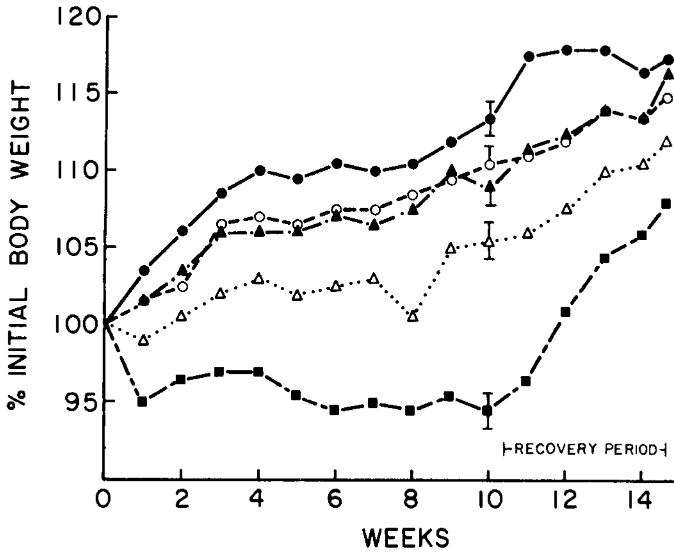


FIG. 1

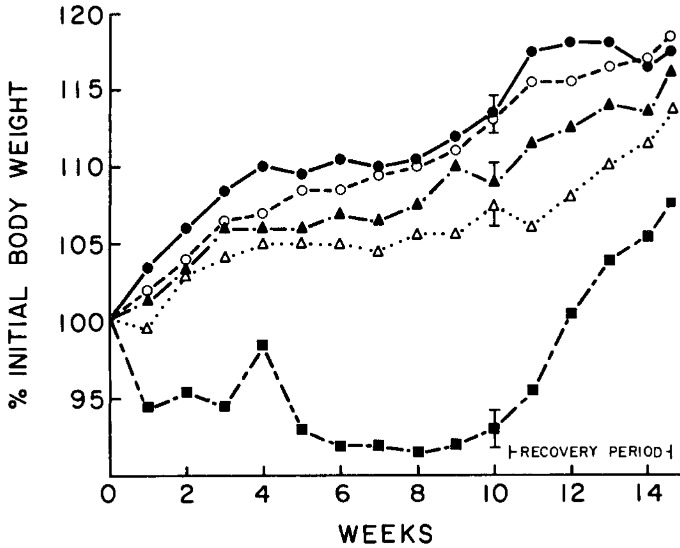


FIG. 2

Body weights of female Fischer rats are plotted over a 72 d treatment period with Δ^9-THC (Fig. 1) and CME (Fig. 2) followed by a 30 d recovery period. Treatment groups are: 25 mg/kg Δ^9-THC or 75 mg/kg CME, ■--■; 5 mg/kg Δ^9-THC or 15 mg/kg CME, △···△; 1 mg/kg Δ^9-THC or 3 mg/kg CME, ○--○; control, intubated with vehicle, ▲—▲; untreated control, ●—●. Vertical bars are ±S.E.M.

Ovarian weights were also lower in animals given cannabinoids. Tables 1–4 show the absolute and relative weights for the eight experimental groups. It can be seen that the highest doses of Δ^9-THC and CME decrease ovarian weights to 77% and 64%, respectively, of the vehicle-intubated controls. Adrenal weights in cannabinoid-treated animals were not consistently significantly different from those of the controls (Tables 1–4).

TABLE 4. RELATIVE TISSUE WET WEIGHTS FROM FEMALE FISCHER RATS AFTER 72 DAYS INTUBATION WITH CME.

Treatment	mg wet weight/100 g final body weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	26.1 ± 2.4[b]	33.4 ± 4.2	223 ± 48
Intubated control (10)	27.8 ± 2.5	33.6 ± 3.7	215 ± 44
3.0 mg CME/kg (10)	25.4 ± 2.0	30.9 ± 3.3	192 ± 51
15.0 mg CME/kg (10)	25.0 ± 3.6	29.0 ± 5.7	137 ± 56
75.0 mg CME/kg (10)	27.5 ± 7.2	25.8 ± 5.1	93.0 ± 36
F	< 1	4.77	11.10
df	4/45	4/45	4/45
P	N.S.[c]	< 0.01	< 0.01

a. Number of animals per group.
b. Standard deviation.
c. Not significant.

Vaginal smears obtained at mid-treatment and at the end of the treatment period indicated increasing irregularity in the estrous cycle, and prolongation of the diestrus phase in cannabinoid-treated animals (Kostellow, *et al.*). Control animals continued to cycle normally.

Sections of uteri and ovaries from animals exposed to high doses of CME and Δ^9-THC were examined and stained by hematoxylin and eosin. Despite the fact that animals entered the experiment with random estrous cycles, the majority of sections studied appeared significantly uniform. Cells of the uterine mucosa were generally columnar and neutrophils were present throughout the stroma. The endometrical glands were collapsed and somewhat atrophic. Vaginal epithelium was thin and neutrophils could be seen in the vaginal stroma as well as in strands of mucus present in the vaginal lumen. Some nucleated cells could also be detected. The ovaries contained large corpora lutea and small, vesicular follicles. These tissues from the majority of animals showed a histologic pattern consistent

TABLE 5. TISSUE WET WEIGHTS FROM FEMALE FISCHER RATS FOLLOWING A 30-DAY RECOVERY PERIOD AFTER 72 DAYS INTUBATION WITH Δ^9-THC.

Treatment	mg wet weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	52.7 ± 4.9[b]	58.7 ± 7.0	546 ± 156
Intubated control (10)	48.8 ± 4.2	57.7 ± 5.8	440 ± 78
1.0 mg THC/kg (10)	52.1 ± 6.4	58.6 ± 4.7	479 ± 116
5.0 mg THC/kg (10)	48.4 ± 6.8	51.7 ± 11.6	360 ± 63
25.0 mg THC/kg (10)	46.4 ± 5.5	50.8 ± 5.2	390 ± 73
F	1.96	2.62	4.56
df	4/45	4/45	4/45
p	N.S.[c]	< 0.05	< 0.01

a. Number of animals per group.
b. Standard deviation.
c. Not significant.

Table 6. Tissue Wet Weights from Female Fischer Rats following a 30-Day Recovery Period after 72 Days Intubation with CME.

Treatment	mg wet weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	52.7 ± 4.9[b]	58.7 ± 7.0	546 ± 156
Intubated control (10)	48.8 ± 4.2	57.7 ± 5.8	440 ± 78
3.0 mg CME/kg (10)	50.1 ± 3.9	60.9 ± 9.0	515 ± 129
15.0 mg CME/kg (8)	45.9 ± 6.6	45.8 ± 9.5	426 ± 168
75.0 mg CME/kg (9)	46.2 ± 2.1	50.9 ± 6.1	371 ± 76
F	3.15	5.43	2.64
df	4/42	4/42	4/42
P	< 0.05	< 0.01	< 0.05

a. Number of animals per group.
b. Standard deviation.

Table 7. Relative Tissue Wet Weights from Female Fischer Rats following a 30-Day Recovery Period after 72 Days Intubation with Δ^9-THC.

Treatment	mg wet weight/100 g final body weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	26.0 ± 2.3[b]	29.0 ± 3.0	271 ± 78
Intubated control (10)	24.9 ± 1.6	29.5 ± 3.1	225 ± 40
1.0 mg THC/kg (10)	26.2 ± 3.4	29.5 ± 2.2	242 ± 61
5.0 mg THC/kg (10)	24.9 ± 2.0	26.4 ± 5.0	187 ± 35
25.0 mg THC/kg (10)	24.9 ± 2.1	27.3 ± 2.1	210 ± 39
F	< 1	1.70	3.22
df	4/45	4/45	4/45
P	N.S.[c]	N.S.	< 0.05

a. Number of animals per group.
b. Standard deviation.
c. Not significant.

Table 8. Relative Tissue Wet Weights from Female Fischer Rats following a 30-Day Recovery Period after 72 Days Intubation with CME.

Treatment	mg wet weight/100 g final body weight		
	Adrenals	Ovaries	Uterus
None (10)[a]	26.0 ± 2.3[b]	29.0 ± 3.0	271 ± 78
Intubated control (10)	24.9 ± 1.6	29.5 ± 3.1	225 ± 40
3.0 mg CME/kg (10)	24.5 ± 2.0	29.8 ± 4.0	252 ± 60
15.0 mg CME/kg (8)	23.6 ± 2.2	23.6 ± 4.4	217 ± 76
75.0 mg CME/kg (9)	25.4 ± 1.5	27.8 ± 2.1	203 ± 33
F	1.81	4.30	1.78
df	4/42	4/42	4/42
P	N.S.[c]	< 0.01	N.S.

a. Number of animals per group.
b. Standard deviation.
c. Not significant.

with the diestrus stage of the cycle. It was not possible to detect differences between animals treated with CME from those treated with Δ^9-THC.

After a 30 d recovery period the animals were sacrificed and tissues excised as described above. As shown in Tables 5 and 6 animals which had received the highest dose of Δ^9-THC or CME now had regained uterine and ovarian weights to within 84 to 89 percent of the vehicle-intubated control group. In terms of relative weights (Tables 7 and 8), both uterine and ovarian weights were now 90–94 percent of the control weights.

DISCUSSION

The uteri of female Fischer rats show a significant reduction in weight following long-term treatment with the cannabinoids Δ^9-THC and CME. The percent of weight loss is roughly proportional to the cannabinoid dose. Histological examination shows that a majority of the animals treated with the highest dose of either cannabinoid preparation appeared to be in the diestrus stage of the cycle. This is consistent with results obtained from vaginal smears (Kostellow, *et al.*).

Thus, three lines of evidence indicate that these organs from the high dose animals are typical of uteri which have had considerably less than the normal amount of hormone stimulation. Either sex hormone production or the response to it appears to be decreased as a result of cannabinoid treatment.

Since ovarian weights in these rats were also depressed in a dose-related manner, ovarian hormone production may have been reduced. The effect on the ovaries suggests that a possible site of Δ^9-THC or CME action may be the hypothalamo-pituitary axis causing suppression of gonadotropin production. The determination of serum levels of gonadotropins would be necessary to establish whether either pituitary or ovarian function was altered by cannabinoid treatment. After a recovery period of 30 d both absolute and relative uterine and ovarian weights were approaching control weights. A longer recovery period would be required to determine whether or not these organs can fully recover.

In summary, the data indicate that long-term treatment with Δ^9-THC and CME lead to a pronounced reduction of hormonal stimulation of the uterus and ovary resulting in a prolongation of the diestrus phase of the uterine reproductive cycle.

ACKNOWLEDGEMENTS

We wish to acknowledge the excellent technical assistance of Ms. J. Kunar and Mr. L. Macedonia. This work was supported by NIDA Contract No. 271-76-3318.

REFERENCES

1. Dixit, V. P., Manjula, A. and Lohiya, N. K., *Endokrinologie* **66**,365–368 (1975).
2. Kostellow, A. B., Bloch, E., Morrill, G. A. and Fujimoto, G. I., *Fed. Proc.* **37**,858 (1978).
3. Kostellow, A. B., Bloch, E., Rosenbaum, R., Morrill, G. A. and Fujimoto, G. I., in preparation.
4. Okey, A. B. and Truant, G. S., *Life Sci.* **17**,1113–1118 (1975).
5. Rosenkrantz, H., Sprague, R. A., Fleishman, R. W. and Braude, M. C., *Toxicol. Appl. Pharmacol.* **32**,399–417 (1975).
6. Sassenrath, E. N. and Chapman, L. F., *Fed. Proc.* **34**,1666 (1975).
7. Solomon, J., Cocchia, M. A., Gray, R., Shattuck, D. and Vossmer, A., *Science* **192**,559–561 (1976).

EFFECT OF Δ⁹-TETRAHYDROCANNABINOL (THC) ON FEMALE REPRODUCTIVE FUNCTION

CAROL GRACE SMITH

Department of Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20014,

MICHAEL TIMOTHY SMITH

Department of Neuropathology, Armed Forces Institute of Pathology, Washington, D.C. 20306,

NORMA F. BESCH, ROY G. SMITH

Reproductive Research Laboratory, Department of Obstetrics and Gynecology, Baylor College of Medicine, Houston, Texas 77030,

and

RICARDO H. ASCH

Department of Obstetrics and Gynecology, University of Texas Health Science Center, San Antonio, Texas 78284

Summary. Single doses of Δ⁹-tetrahydrocannabinol (5.0, 2.5, 1.25, or 0.625 mg/kg) can decrease the levels of both LH and FSH in the ovariectomized Rhesus monkey. This inhibition of gonadotropins (50–88%) lasts for 6–24 hours depending upon the dose of THC. There are no great differences in the response of the two gonadotropins to THC. The inhibition of gonadotropin levels by THC appears to be at the level of the hypothalamus since both LH and FSH are released from the pituitary gland in response to LHRF in the presence of THC.

Daily administration of THC (2.5 mg/kg) during the luteal phase of the menstrual cycle in normal animals produces no consistent changes in progesterone levels or significant shortening in the length of the luteal phase of the cycle. This treatment during the luteal phase does result in the absence of ovulation associated with alterations in the levels of LH and progesterone in the subsequent cycle in these monkeys.

We find no evidence of any direct estrogenic or anti-estrogenic activity of THC: (a) Short-term administration of THC (2.5 mg/kg) causes no stimulatory effects on endometrial histology or vaginal cytology in ovariectomized monkeys; (b) THC does not selectively bind to any cytosol macromolecule in Rhesus monkey uterus; (c) THC, cannabidiol or marihuana extract do not compete with estrogen for estrogen receptors in this tissue; and (d) THC administration does not alter the number of estrogen receptor binding sites in the cytoplasm of target organ cells.

INTRODUCTION

DESPITE the widespread use of marihuana by increasing numbers of young men and women, there are only a few studies of the effects of the drug on reproduction, and most of these studies concern the effects of the drug on the male[6, 7, 23]. Females of some species have been shown to be more responsive to certain of the cannabis effects than males, and yet, less information exists on the effect of marihuana on reproductive hormones in the female. In 1972, Marks[13] reported a depression in LH secretion following THC administration to ovariectomized rats. This effect was dose dependent, and occurred at one hour after THC administration. Nir *et al.*[15] indicated that this suppression of LH in the normal rat was sufficient to inhibit ovulation. Recent studies[1, 2] confirm the observation that THC administration can alter reproductive hormones and inhibit ovulation, but a controversy exists over the mechanism(s) by which these effects are produced. One recent report[28] claims that THC has estrogenic activity, causing stimulation of the growth of reproductive tissues in ovariectomized rats through a direct interaction with estrogen receptors in these tissues. Several other studies[17, 18, 26] indicate that THC has no direct estrogenic activity, nor is it capable of competing with specific estrogen receptors in reproductive target tissues. The role of estrogen receptors in the mechanism of action of THC is currently unresolved.

The present study has examined the effects of THC on the reproductive system of the female Rhesus monkey. The study concerns the effect of the drug on neuroendocrine control of gonadotropin levels, on ovarian function and the menstrual cycle, and on the target organ tissues of the female reproductive system. The results will be divided into three parts as indicated on the diagram on p. 451.

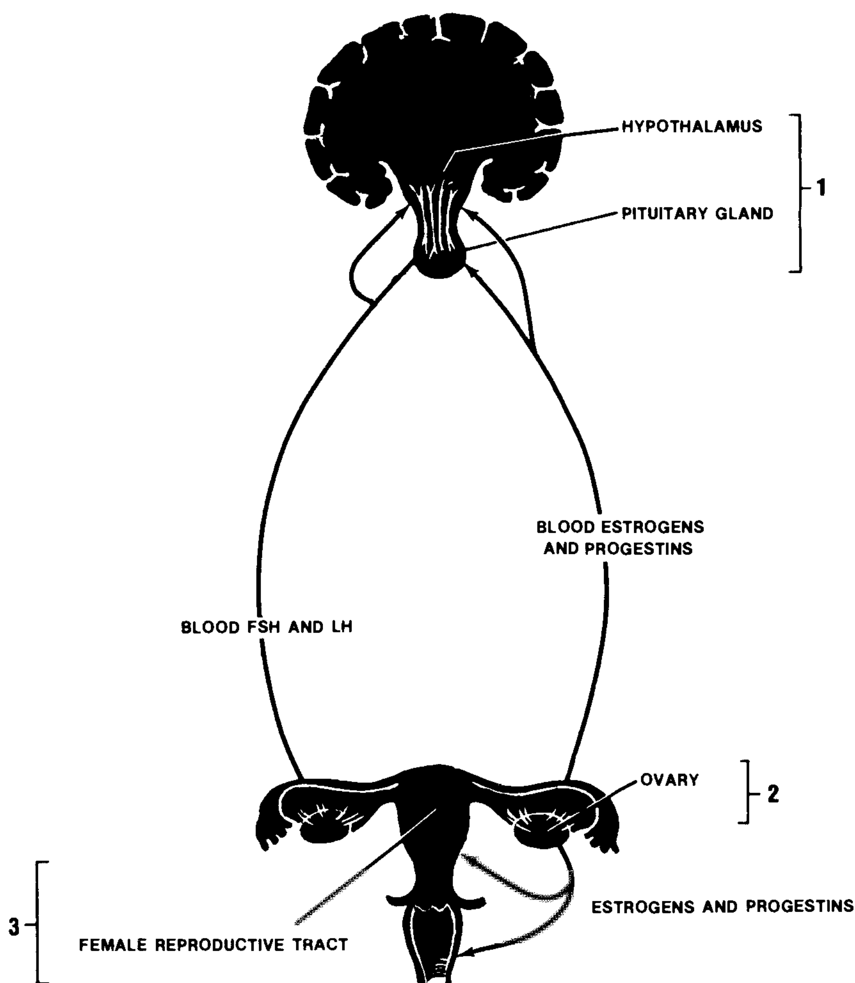
METHODS

RADIOIMMUNOASSAY PROCEDURES

The assay of Rhesus LH, a double-antibody radioimmunoassay, was performed with minor modifications as described by Niswender *et al.*[16]. The constituents of the assay are an antiovine LH serum (GDN No. 15), ovine LH (LER-1056-C2) for iodination, and a Rhesus pituitary standard, LER-1909-2. The five-day assay is performed at 4°C. The standards (400 to 12,800 ng per tube) and unknowns (50 to 200 μl of serum) are incubated with the antisera (1 : 100,000) for one day followed by a one-day incubation with ^{125}I-LH (50,000 c.p.m.) and a three-day incubation with goat anti-rabbit gamma globulin (dilution determined by titer). The results are expressed in terms of nanograms of LER-1909-2 in 100 ml of serum.

The assay for Rhesus FSH is similar to the procedure described by Boorman *et al.*[4] and differed from the LH assay only in the constitutents of the assay. An anti-human FSH serum (NIAMD batch 4) served as the antibody and rat FSH was used for iodination. The same standard was used as for LH but with a different range (200 to 6400 ng per tube).

Both gonadrotropins were iodinated with an enzymatic method[14] which produces a material with high specific activity and with less damaged hormone than obtained with the chloramine-T method. The anti-ovine LH serum, GDN No. 15, was provided by G. D. Niswender. The ovine LH, the Rhesus pituitary standard, the anti-human FSH serum and



1. Studies on the effects of administration of THC on the pituitary-hypothalamic control of gonadotropins.
2. Studies on the direct effects of THC on ovarian function and the menstrual cycle.
3. Studies on the direct effects of THC on the target organ tissues of the female reproductive system.

the rat FSH were provided by the National Pituitary Agency through the Hormone Distribution Office of the National Institute of Arthritis and Metabolic Diseases.

ANIMAL CARE, BLOOD DRAWING, AND PREPARATION OF THC FOR INJECTION

Mature female Rhesus monkeys (*Macaca mulatta*) were caged individually and housed in the Department of Laboratory Animal Medicine primate facilities. Animals were exposed to a 14-hour light, 10-hour dark cycle, and maintained under conditions of 75 ± 2°F and 50% relative humidity. A daily ration of monkey chow was provided *ad libitum* together with fresh fruit and vegetables twice weekly. Water was available *ad*

libitum. Monkeys were checked daily for occurrence of menses. The first day of vaginal bleeding was designated as day 1 of the menstrual cycle. Beginning on day 8, blood samples were taken daily. Estrogen levels during the proliferative phase are about 50 picograms per ml. The levels increase to 130 picograms per ml (estrogen peak) several hours before the LH surge. The LH surge lasts about 50 hours, and ovulation follows the LH surge by 28 to 56 hours.

On the day following the estrogen peak, laparoscopies began and were done daily to visually determine ovulation. Laparoscopy was performed under surgical anesthesia (3–5 mg/kg phencyclidine hydrochloride). Ovulation was verified by visualization of recent ovulation points on the ovary. Plasma progesterone determinations were made 24, 72, and 120 hours after the estrogen peak to further verify that ovulation had occurred.

Bilateral ovariectomy and hysterectomy were performed under phencyclidine hydrochloride anesthesia (3–5 mg/kg) via mid-central subumbilical incision. Ovaries were examined, and complete removal of ovarian tissue confirmed. Aseptic surgical technique was used throughout the procedures.

Blood samples (1.0–3.0 ml) were drawn into syringes by vein puncture. No anesthesia or tranquilizer was required for this procedure. Blood samples were centrifuged, and the serum was stored at −20°C until assayed. Chewable multiple vitamins with iron and minerals were administered daily to avoid depressed hematocrits. Hematology and blood chemistries were evaluated twice yearly.

Δ^9-tetrahydrocannabinol was obtained from the National Institute of Mental Health. The alcohol was evaporated under constant stream of nitrogen gas. The residue was homogenized in Tween 80 and saline. The final concentration was 4% Tween 80 and 96% saline. An intramuscular injection was used to administer the THC or vehicle. In the short-term studies, the site of intramuscular injection was changed daily in order to avoid alterations in drug absorption caused by the effects of vehicle on tissues at the site of injection.

STATISTICAL ANALYSIS OF DATA

The effects of drug treatment on hormone levels in the group of monkeys were analyzed to detect significant changes over time (for example, see Fig. 1). A repeated measures one-way analysis of variance (ANOVA) was used to determine whether significant changes occurred at the time intervals following drug administration. When the one-way ANOVA indicated significant differences across time intervals, Duncan's New Multiple Range Test[8] was used to identify the specific intervals at which significant differences occurred.

Drug effects on individual monkeys were evaluated by comparing post-treatment hormone levels with control levels determined for each animal. A 95% confidence interval was established for each animal by combining 48 separate hormone measurements obtained from vehicle control and baseline series (during which no drug or vehicle was administered). The frequency distributions of these values for each animal approximated a normal distribution, therefore $\bar{x}_i \pm 1.96\sigma$ was used to define each individual monkey's range of normal values.

RESULTS AND DISCUSSION

1. STUDIES ON THE EFFECTS OF THC ADMINISTRATION ON THE PITUITARY-HYPOTHALAMIC CONTROL OF GONADOTROPINS

Five sexually mature Rhesus monkeys with established normal menstrual cycles were ovariectomized. Studies were begun five weeks later. Normal variation in gonadotropin levels in these animals and response to estrogen administration have been previously described[3]. The monkeys were administered a single dose of vehicle or THC. The doses of THC which were chosen for this study included 5.0, 2.5, 1.25, 0.625, and 0.3125 mg/kg. Blood was drawn from each animal 24 hours prior to administration of THC or vehicle. The drug or vehicle was administered at 12:00 h, blood was drawn from each monkey every six hours for the next 24 hours (18:00 h; 24:00 h; 06:00 h; 12:00 h) and then daily at 12:00 h for three more days (see Fig. 1). Since they did not appear to be eating spontaneously for the first several hours following the larger doses of THC, the animals were given fruit as supplemental food after the administration of THC or vehicle.

A summary of the results obtained in the LH study is shown in Figs. 1 and 2. Figure 1 shows the means and standard deviations in LH levels for the group following the administration of vehicle, 2.5 mg/kg or 5.0 mg/kg of THC. Whereas the vehicle caused no significant change in the LH levels, the 2.5 and 5.0 mg/kg doses produced significant depressions in the LH levels at 6 and 12 hours after their respective administration. Figure

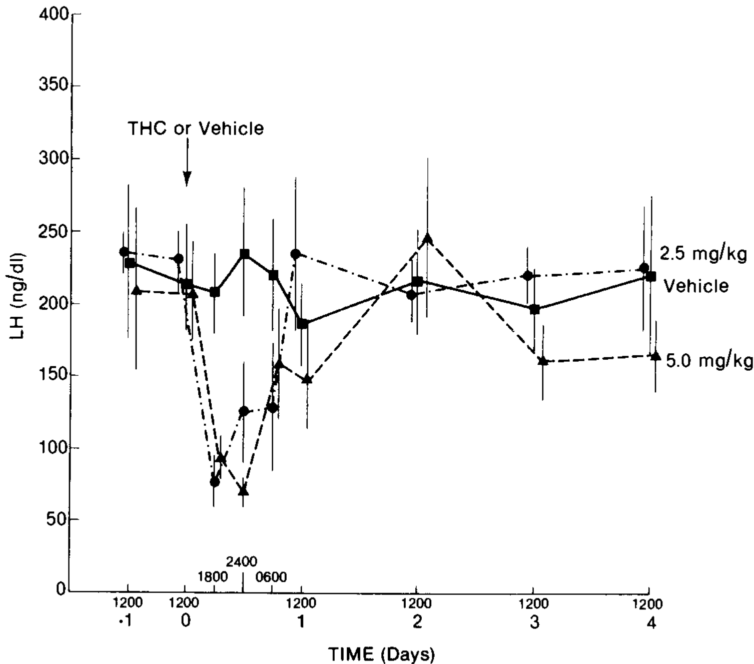


FIG. 1. The effect of vehicle, 5.0 mg/kg, or 2.5 mg/kg of THC on LH levels in ovariectomized Rhesus monkeys. Each point represents the mean and standard deviation for four monkeys. Significant decreases occurred at 6 and 12 hours following THC administration (both doses).

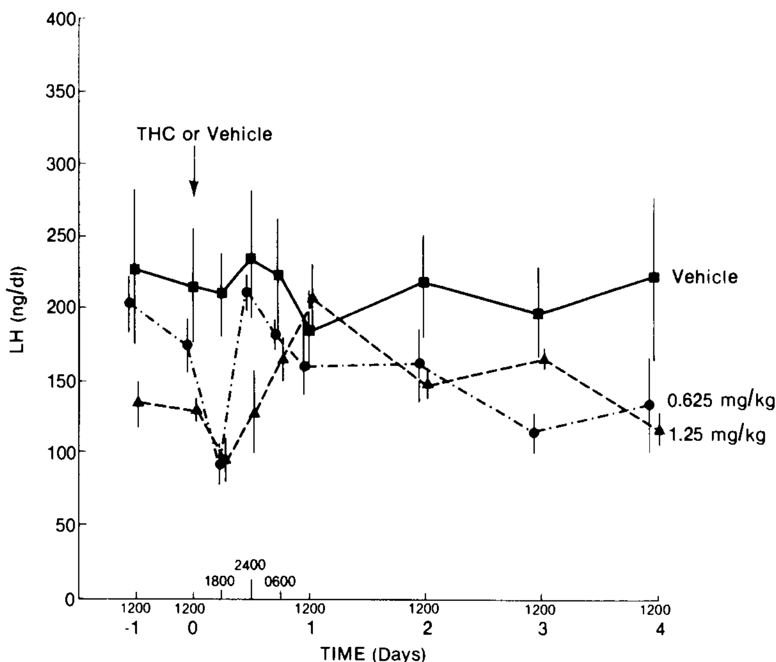


FIG. 2. The effect of vehicle, 1.25 mg/kg, or 0.625 mg/kg of THC on LH levels in ovariectomized Rhesus monkeys. Each point represents the mean and standard deviation for four monkeys. Significant decreases occurred at six hours following THC administration (both doses).

2 shows that two lower doses, 0.625 mg/kg and 1.25 mg/kg THC, produced significant depressions in LH levels at only six hours after administration. The 0.3 mg/kg dose of THC (not shown in figures) caused no significant changes in LH levels at any of the time intervals studied.

The effect of THC on FSH levels is shown in Figs. 3 and 4. Figure 3 shows the means and standard deviations in FSH levels for the group following the administration of vehicle, 2.5 mg/kg or 5.0 mg/kg of THC. The vehicle caused no significant changes in FSH levels. Following the 5.0 mg/kg dose of THC (Fig. 3), a significant depression in FSH level was seen at 6, 12, 18, and 24 hours. The 2.5 mg/kg dose of THC (Fig. 3), produced a significant depression only at 12 hours. Figure 4 shows the results with the 1.25 mg/kg and 0.625 mg/kg doses of THC. Significant changes for these two doses were present only at six hours after drug administration. The 0.3 mg/kg dose of THC (not shown) caused no significant changes in FSH levels at any of the time intervals studied.

A comparison of the effects of the various doses of THC on the levels of LH and the levels of FSH shows no great difference between the two gonadotropins. The average maximum decrease in LH and FSH (not shown in figures) following the 5.0 mg/kg dose of THC was 68% and 56% respectively. The average maximum response following the 2.5 mg/kg dose of THC was 69% and 44% for LH and FSH, respectively. While the average maximum response following the 1.25 or 0.625 mg/kg dose was somewhat smaller than for the higher doses (41% average decrease in LH and 37% average decrease in FSH after the 1.25 mg/kg dose; 44% average decrease in LH and 25% average decrease in FSH after the 0.625 mg/kg dose), the most obvious difference was in the duration of the

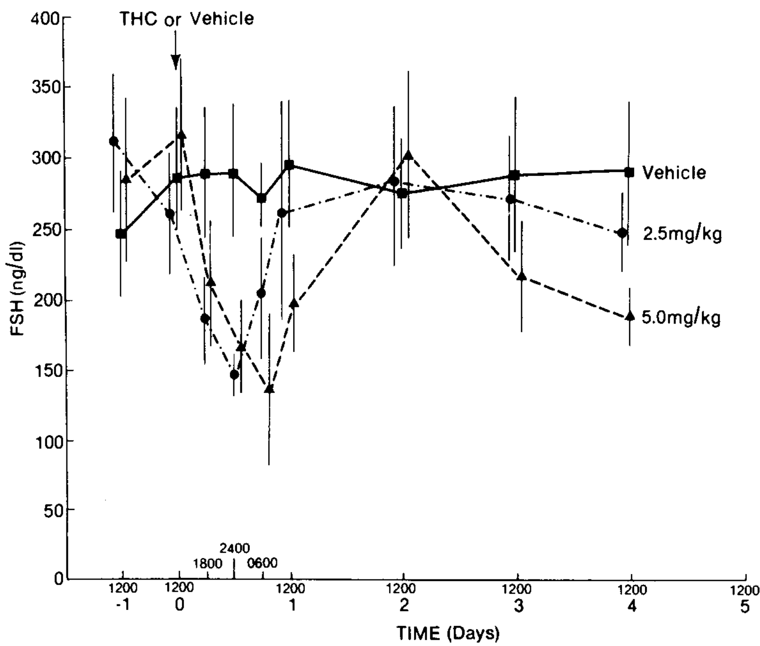


FIG. 3. The effect of vehicle, 5.0 mg/kg or 2.5 mg/kg of THC on the FSH levels in ovariectomized Rhesus monkeys. Each point represents the mean and standard deviation for four monkeys. Significant decreases were observed at 6, 12, 18, and 24 hours following the 5.0 mg/kg dose and at 12 hours following the 2.5 mg/kg dose.

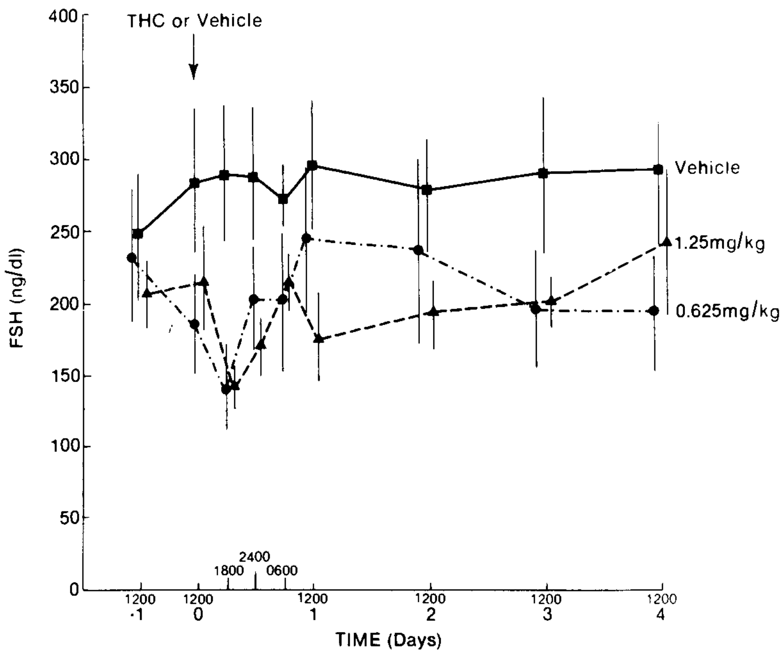


FIG. 4. The effect of vehicle, 1.25 mg/kg, or 0.625 mg/kg of THC on FSH levels in ovariectomized Rhesus monkeys. Each point represents the mean and standard deviation for four monkeys. Significant decreases in FSH levels were seen at 6 hours after THC administration (both doses).

response. The larger doses (5.0 or 2.5 mg/kg) produced significant decreases in both gonadotropins that could be measured at 12–24 hours after drug administration, while the effects of the lower doses (1.25 or 0.625 mg/kg) could be seen only at six hours after THC administration.

Comparison of the time course of the effects of THC on gonadotropin levels in individual animals (not shown) showed no major differences in LH and FSH. The time interval at which the maximum decrease in hormone level occurred was generally the same for FSH and LH. The exception occurred in two monkeys following the 2.5 mg/kg dose. In these two animals, the maximum decrease in FSH level preceded the maximum decrease in LH level.

In order to determine if the effect of THC on gonadotropins was mediated through a direct effect on the pituitary gland, it was necessary to determine if the pituitary gland can respond to gonadotropin releasing factor while under the inhibitory influence of THC. Six hours after administration of a single dose of 2.5 mg/kg of THC, each animal was administered 100 μg of synthetic LH releasing factor (LHRF) (intravenously). Blood was drawn immediately before the administration of synthetic LHRF (0 time) and at 30, 60, 90, and 120 minutes after the releasing factor administration. The six-hour time interval after THC administration was chosen for these studies because the 2.5 mg/kg dose of THC produced statistically significant depression of both LH and FSH for at least 12 hours after THC administration (shown in Figs. 1 and 3).

The results from the releasing factor studies on the mechanism of action of THC are shown in Table 1. The LH and FSH levels are significantly elevated at 30, 60, 90 and 120 minutes following the LHRF when compared to initial levels (0 time). The averages were 101% and 67% for LH and FSH respectively. These increases in LH and FSH levels in response to LHRF can be considered as recovery from the effects of THC, since all of the values for LH and FSH measured from 30 to 120 minutes after LHRF are within the 95% confidence interval for normal values for the individual animals.

In summary, we have shown that THC, in the single dose levels studied, can decrease the levels of both LH and FSH in the ovariectomized Rhesus monkey. The inhibition of gonadotropins lasts for 6–24 hours depending upon the dose of THC, and the effect is reversible. The mechanism appears to be through hypothalamic control of FSH and LH secretion, since the pituitary responds to releasing factor in the presence of THC.

2. STUDIES ON THE DIRECT EFFECTS OF THC ON OVARIAN FUNCTION AND THE MENSTRUAL CYCLE

The inhibitory action of THC on gonadotropin secretion may explain some of the observed reproductive consequences of chronic, intensive marihuana use in young men[9, 10, 12]. The decreases in sex steroid secretion and sperm count and changes in sperm morphology, are thought to be mediated by a reversible hypothalamic or pituitary effect since discontinuation of drug use or administration of HCG has been shown to reverse these effects. The observation that THC inhibits both LH and FSH levels may explain the drug's effect on both sex steroid secretion and sperm production. We are currently investigating the effects of THC on the hormonal control of the menstrual cycle. One might expect that THC could alter sex steroid secretion and block ovulation in the female monkey.

TABLE 1. EFFECT OF SYNTHETIC LUTEINIZING HORMONE RELEASING FACTOR (LHRF) ADMINISTRATION ON LUTEINIZING HORMONE (LH) AND FOLLICLE STIMULATING HORMONE (FSH) LEVELS DEPRESSED BY PRIOR ADMINISTRATION OF DELTA-9-TETRAHYDROCANNABINOL (THC) IN FIVE OVARIECTOMIZED RHESUS MONKEYS.

Time[a]	Luteinizing hormone levels (ng/*dl*)					
	Animal number					$\bar{x} \pm$ SEM
	#1	#2	#3	#4	#5	
0 min	79	78	108	86	83	87 ± 5
30 min	163	155	167	141	109	147 ± 10[b]
60 min	165	160	189	155	121	158 ± 11[b]
90 min	188	146	205	178	130	169 ± 14[b]
120 min	197	109	194	184	120	161 ± 19[b]

Time[a]	Follicle stimulating hormone levels (ng/*dl*)					
	Animal number					$\bar{x} \pm$ SEM
	#1	#2	#3	#4	#5	
0 min	232	80	168	212	73	153 ± 33
30 min	365	112	231	246	94	210 ± 49[b]
60 min	406	141	248	283	119	239 ± 52[b]
90 min	396	145	228	290	121	236 ± 50[b]
120 min	361	140	272	335	111	244 ± 51[b]

a. Time intervals after LHRF administration. THC (2.5 mg/kg) was administered 6 hours before LHRF administration.
b. Values obtained at the 30, 60, 90, 120 minute intervals were significantly elevated compared to 0 time levels for both LH and FSH.

Some preliminary observations have been made concerning the effects of THC on the luteal phase of the menstrual cycle. In these studies, THC was administered to monkeys in which ovulation and corpus luteum formation had been verified by LH surge, elevated progesterone levels, and laparoscopic visualization of the ovaries. In our laboratory, ovulation is determined with an accuracy of ± 12 hours. Progesterone secretion by the corpus luteum during the luteal phase is thought to be maintained by the LH secretion of the pituitary gland; thus, luteal activity can be evaluated by daily post-ovulatory plasma progesterone levels (greater than 4 mg/ml).

Daily administration of THC (2.5 mg/kg) during the luteal phase (example shown in Fig. 5) did not cause consistent changes in the progesterone levels measured each day immediately before the THC administration. Some shortening of the luteal phase was observed in the treated monkeys, but this decrease in cycle length was not significant when compared to standard cycle lengths established for the colony of monkeys.

An important finding in these preliminary studies, has been the absence of ovulation in all animals in the cycle following the THC treatment cycle. These anovulatory cycles are associated with sub-normal levels of LH early in the post-treatment cycle and subnormal levels of progesterone late in the post-treatment cycle. The absence of ovulation in the post-treatment cycle was confirmed by laparoscopy on the days indicated (Fig. 5).

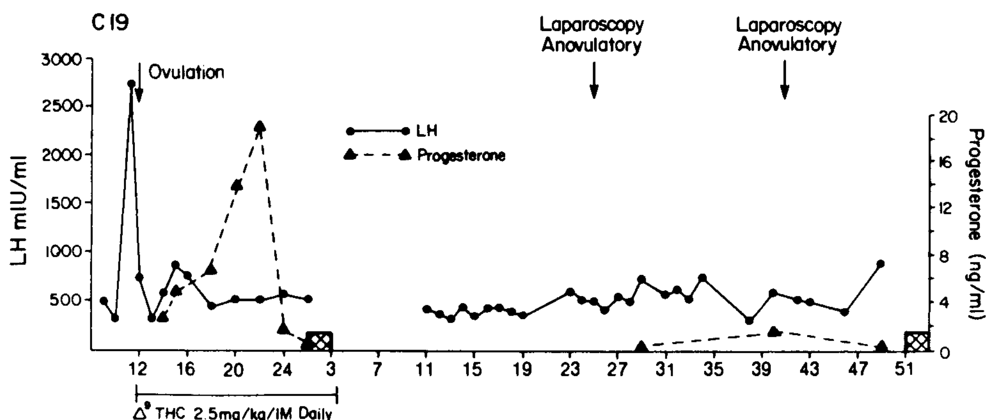


FIG. 5. The effect of Δ^9-THC on the luteal phase of the menstrual cycle of the Rhesus monkey. Daily administration of THC (2.5 mg/kg) does not alter LH or progesterone levels during the luteal phase. Drug treatment does produce an absence of ovulation associated with altered levels of both LH and progesterone in the subsequent cycle. (The first day of vaginal bleeding is designated as day 1 of each cycle and is indicated by cross hatched bars on the figure).

These preliminary results indicate that daily administration of THC (2.5 mg/kg) during the luteal phase of the menstrual cycle can cause disruption of the subsequent cycle and produce an anovulatory cycle. No consistent changes in progesterone levels or length of luteal phases were detected during the treatment cycles indicating that the THC does not induce luteolysis in primates as estrogen administration does. The exact mechanism by which THC produces these changes in the menstrual cycle is not known.

3. STUDIES ON THE DIRECT EFFECTS OF THC ON THE TARGET ORGAN TISSUES OF THE FEMALE REPRODUCTIVE SYSTEM

Proposed mechanisms for the disruption of the reproductive system produced by marihuana include a direct action of THC at the cellular level of the reproductive target organs. Studies by Shoemaker and Harmon indicate that THC may compete with sex steroids for their receptor proteins in the target organ cells(22). The binding of THC to these receptors would either antagonize the trophic effects of steroids in these tissues or produce the same trophic effect as the sex steroid. Solomon *et al.*, have shown a positive trophic effect of THC on reproductive tissues in ovariectomized rats when THC was administered by intraperitoneal injection(28). Their work however, has been criticized by Okey and Bondy who claim that experiments in which THC is administered by intraperitoneal injection give erratic and unreliable results, caused by inflammation of abdominal organs. Considerable disagreement exists regarding the estrogenic properties of THC or of some estrogenic compound, perhaps formed from THC *in vivo*. The present study was designed to settle this dispute and ascertain the direct effect of THC on the reproductive system.

Estrogenic activity and estrogen receptor interactions were studied in the primate rather than in rodents. This is particularly important since it has been shown by Smith *et al.* that steroid receptors from different animals do not necessarily have the same ligand binding specificity[27].

a. In vitro studies of cannabinoids and estrogen receptors

To investigate *in vitro* binding of THC to estrogen receptors in the primate, cytosol was isolated from the uterus, hypothalamus and pituitary gland of Rhesus monkeys which had been ovariectomized 4 days prior to removal of tissues. The tissues were minced and homogenized at 4°C in 4–10 volumes of buffer containing 10 mM tris-HCl (pH 7.4), 1.5 mM EDTA, 12 mM thioglycerol, and 10% glycerol. The homogenate was centrifuged at 1000 g for 15 minutes to remove nuclei and the resulting supernatant was centrifuged at 105,000 g for 90 minutes.

To define the concentration of estradiol necessary to saturate the receptor, Scatchard analyses[21] were performed on the supernatant. A fixed concentration of cytosol was incubated with increasing concentrations of ^{3}H-estradiol of specific activity of 85–96 Ci/mmole (1×10^{-10} M – 4×10^{-9} M) for 18 hours at 4°C in the presence and absence of a 250-fold excess of diethylstilbestrol (DES). DES was used to measure nonspecific binding of ^{3}H-estradiol in cytosol since DES at these concentrations displaces estradiol from its receptor sites rather than from any contaminating serum binding proteins such as sex steroid binding globulin. After incubation of cytosol with ^{3}H-estradiol, and ^{3}H-estradiol with DES, the free ^{3}H-estradiol was removed by charcoal absorption[24]. The specific binding of estradiol was computed by subtracting nonspecific binding from total binding; the specific bound was plotted versus the bound/free ratio. The negative reciprocal of the slope yields the equilibrium dissociation constant (K_D), and the intercept on the abscissa yields the concentration of specific estrogen receptor sites. The concentration of binding sites was expressed in moles/mg protein.

To determine if THC, cannabidiol, or marihuana extract would compete for estrogen receptor sites, competitive binding assays were performed on cytosol using a fixed concentration of ^{3}H-estradiol (2×10^{-9}) with increasing concentrations (0–3.8×10^{-4} M) of THC, cannabidiol, or marihuana extract, and for comparison purposes similar assays were performed using increasing concentrations of DES (0–1.2×10^{-6} M). The assays were incubated for 18 hours at 4°C and the amount of ^{3}H-estradiol bound, at each concentration of competitor, was determined by charcoal absorption assay. The amount of ^{3}H-estradiol bound was plotted versus the log of the concentration of the competitor.

Figure 6 shows a typical curve obtained from the Rhesus monkey estrogen receptor. The curve is biphasic, similar to that observed previously in Scatchard plots obtained from human uterine cytosol and chick oviduct cytosol[25]. By using the method described by Rosenthal to resolve the two binding components[20], the receptor having the highest affinity for estradiol was found to have an equilibrium dissociation constant (K_D) of 1.7×10^{-10} M [$\pm 0.7 \times 10^{-10}$ M; $n = 4$][26]. This K_D is similar to that measured for the human uterine cytoplasmic estrogen receptor[5]. The concentration of binding sites for estradiol in the monkey uterine cytosol was 2.2×10^{-13} moles/mg protein ($\pm 0.4 \times 10^{-13}$ moles/mg protein; $n = 4$). The concentration of ^{3}H-estradiol necessary to preferentially saturate the high affinity estrogen receptor was 2×10^{-9} M. This concentration was used

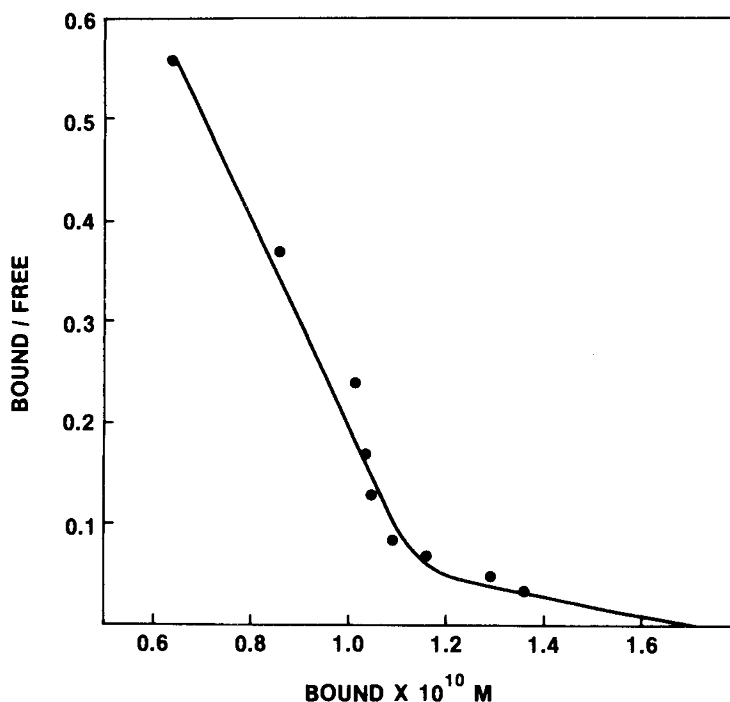


FIG. 6. Scatchard analysis of the specific binding of estradiol (concentration range of 1×10^{-10} M to 4×10^{-9} M) in uterine cytosol from the ovariectomized Rhesus monkey. The assay tubes were incubated for 18 hours at 4°C and bound ^{3}H-estradiol separated from free-charcoal absorption assay. Specific binding of ^{3}H-estradiol was determined by subtracting the nonspecific binding (obtained using similar concentrations of ^{3}H-estradiol incubated in the presence of excess unlabeled diethylstilbesterol) from total binding. Specifically bound estradiol was plotted versus the bound/free ratio. The mean K_D of 4 determinations calculated from the reciprocal of the slope was 1.7×10^{-10} M ($\pm$ 0.7×10^{-10} M). The concentration of estrogen specific binding sites was 2.2×10^{-13} moles/mg protein ($\pm$ 0.4×10^{-13} moles/mg protein).

in the subsequent competitive binding studies to confirm that the ^{3}H-estradiol binding was specific for estradiol and DES.

Figure 7 shows a typical curve obtained for Rhesus monkey uterine cytosol. The figure shows very clearly that DES competes significantly for estrogen receptor sites at a concentration as low as 1.4×10^{-9} M (0.2 ng). THC does not compete even at a concentration of 3.8×10^{-6} M (600 ng). Shown in Table 2 are the results of studies with cannabidiol, marihuana extract and THC using three other uterine specimens at different protein concentrations along with the results obtained using human uterine tissue. The slopes of thr DES competition curves from data in Table 2 are different from that shown in Fig. 7. This difference, however, is not surprising since the cytosol protein concentrations were different. Although the cytosol protein concentration was changed, THC, cannabidiol, and marihuana extract still did not compete with ^{3}H-estradiol for the monkey or human estrogen receptor.

The presence of an estrogen receptor similar to that found in uterine cytosol was detected in pituitary cytosol. Competition studies showed that THC, cannabidiol, and marihuana extract did not displace ^{3}H-estradiol from the pituitary estrogen receptor. Estrogen receptors were not detectable in hypothalamic cytosol, but this does not preclude

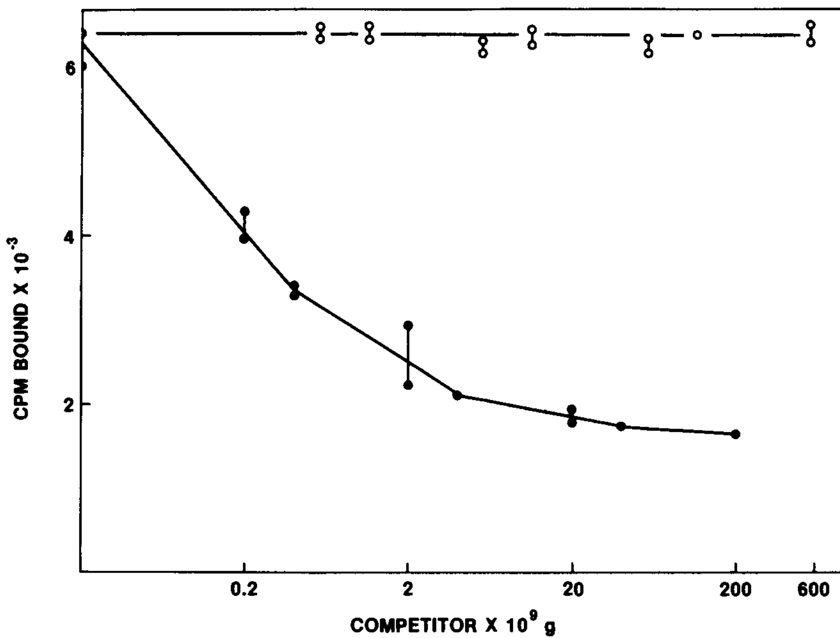


FIG. 7. Competitive binding assay using cytosol from ovariectomized Rhesus monkey uterus. Cytosol (2 ng/ml protein concentration) was added to solutions containing 2×10^{-9} M ^{3}H-estradiol with either DES (0–1.2×10^{-6} M) —●——●—; THC (0–3.8×10^{-6} M) —○——○—. The solutions were incubated at 4°C for 18 hours before measuring the amount of ^{3}H-estradiol bound to macromolecules using a charcoal absorption assay. The error bars denote the within-assay variation of data obtained from one animal.

TABLE 2. CANNABINOIDS AND ESTROGEN RECEPTORS—COMPETITIVE BINDING ASSAYS WITH THC, CANNABIDIOL OR MARIHUANA EXTRACT.

Competitor	Receptor[a]	% displacement[b]
DES		
2.8×10^{-7} M	Rhesus monkey	100
1.4×10^{-8} M	Rhesus monkey	96 ± 2
2.8×10^{-8} M	Rhesus monkey	80 ± 6
1.4×10^{-9} M	Rhesus monkey	48 ± 3
2.8×10^{-9} M	Rhesus monkey	13 ± 1
THC		
2.8×10^{-6} M	Rhesus monkey	None detected
Cannabidiol		
2.8×10^{-6} M	Rhesus monkey	None detected
Marihuana extract		
2.8×10^{-6} M	Rhesus monkey	None detected

a. Competitive binding assay using uterine cytosol from ovariectomized Rhesus monkeys. Protein concentrations of the cytosols were 5 mg/ml. The assays are described in the text.

b. The percent displacement of ^{3}H-estradiol (2×10^{-9} M) was recorded as the mean of the results obtained with tissue from three different monkeys.

their presence since the concentration of these receptors in the hypothalamus would be expected to be relatively low.

The observation that THC does not compete with estradiol for estrogen receptors *in vitro* is a most significant one. The fact that THC does not compete for estrogen receptors in the primate uterus and pituitary implies that it will not compete with estrogen for similar receptors in the hypothalamus if they are indeed present. This is supported by studies in the rat which have shown that the cytoplasmic estrogen receptors in estrogen target tissues such as endometrium, myometrium, vagina, anterior pituitary, anterior hypothalamus, and posterior hypothalamus all appear to possess similar properties(11). Similarly, we have shown that estrogen receptors from calf endometrium, myometrium and pituitary have similar K_D's and ligand binding specificities. Thus it can be concluded that, although there are differences in receptors from different species, within a particular species the hormonal receptors have similar properties. It is believed that estrogen controls gonadotropin secretion after interacting with estrogen receptors in the hypothalamus and/or pituitary. Since THC does not interact with the intracellular estrogen receptor, its inhibitory effect on gonadotropin secretion cannot be interpreted as an intracellular estrogenic effect, therefore, an alternative mechanism must be sought. Pietras and Szego have shown that estrogen receptors are also present in the cell membranes of target cells(19) and our studies do not preclude a mechanism by which THC interacts with membrane receptors and alters the secretion of gonadotropin releasing factor or gonadotropins.

b. *In vivo studies of cannabinoids and estrogen receptors*

To determine whether THC was converted to an estrogenic substance *in vivo*, which could then interact with estrogen receptors and translocate these receptors to the nucleus of estrogen responsive target cells, the following study was performed. Ovariectomized Rhesus monkeys were administered estradiol (100 μg/day), THC (2.5 mg/kg/day) or vehicle daily for 14 days. Before treatment was begun (day 0), half of each uterus was removed and the uterine tissue was frozen in liquid nitrogen. At the end of the 14-day treatment period, the remaining uterine tissue was removed. Pap smears were done on each monkey, before and after the 14-day treatment period. Cytosol was obtained from the uterine tissue and Scatchard analyses were performed. The concentration of high affinity cytoplasmic estrogen binding sites was determined in each uterine specimen using the samples from day 0 as controls. As expected when day 14 was compared to day 0, the group of animals administered estradiol showed a decrease in the concentration of cytoplasmic estrogen receptors, whereas, the group of animals administered THC or vehicle showed no significant differences. It must be concluded therefore that THC is not converted *in vivo* to an estrogenic substance, since it does not result in the translocation of cytoplasmic estrogen receptors to the nucleus of the target cells.

Confirmation of this lack of estrogenic activity was shown by the examination of the uterine histology and vaginal cytology in these monkeys. The results of this study are shown in Plates 1 through 12. Plates 1 and 2 show the vaginal cytology of the estrogen pre-treatment and post-treatment group. The effect of estrogen on vaginal epithelium is maturation. In the absence of estrogen in an ovariectomized monkey, intermediate and blue stained basal cells (Papanicolaou stain) are seen (Plate 1). After 14 days of estrogen administration, mature cornified superficial cells that stain pink are seen (Plate 2). The

Plates 1–4. Estrogen Administration (100 μg × 14 days)

PRE-TREATMENT POST-TREATMENT

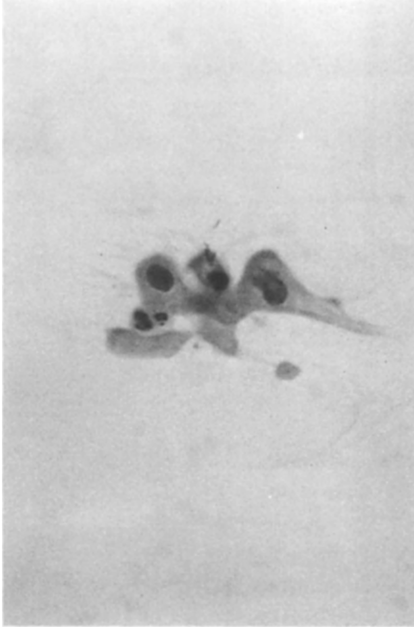


Plate 1

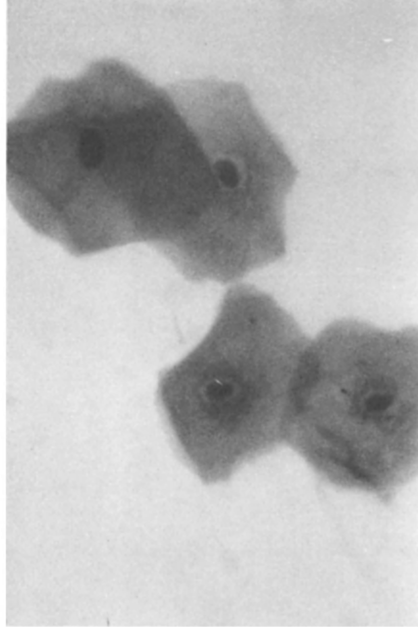


Plate 2

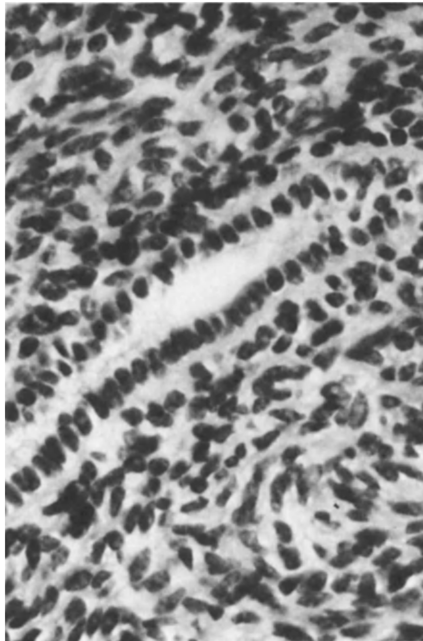


Plate 3

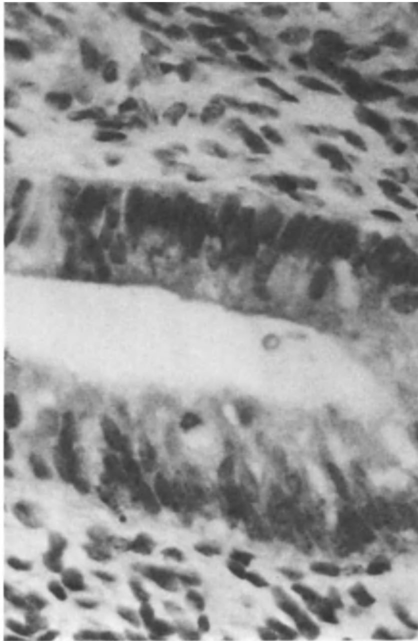


Plate 4

Plates 5–8. Vehicle Administration (2% Tween in saline × 14 days)

PRE-TREATMENT

POST-TREATMENT

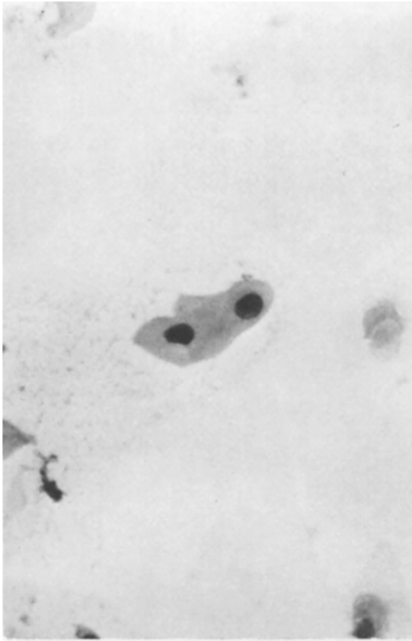


Plate 5

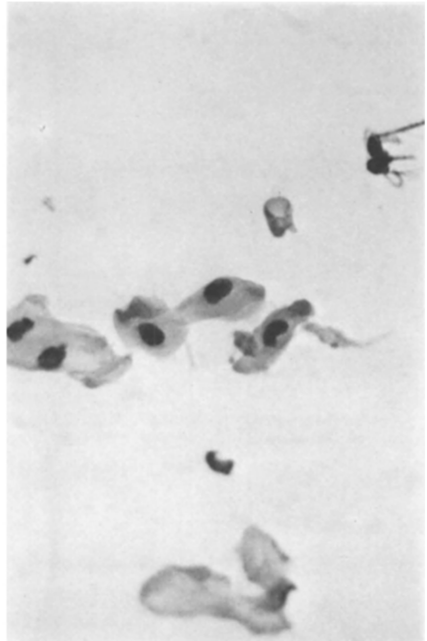


Plate 6

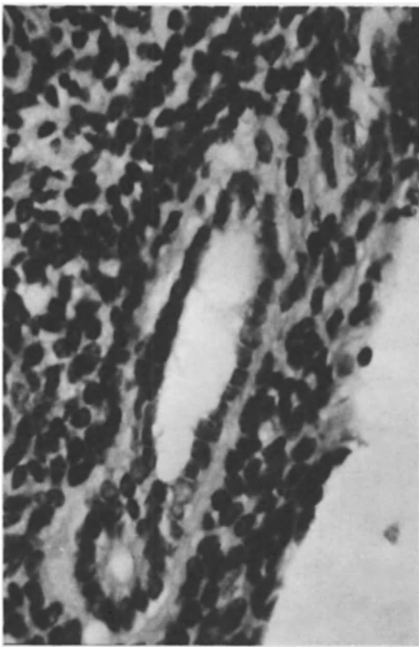


Plate 7

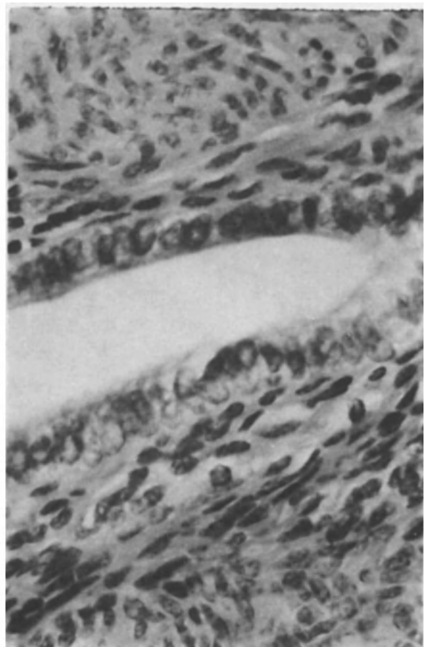


Plate 8

Plates 9–12. THC Administration (2.5 mg/kg × 14 days)

PRE-TREATMENT POST-TREATMENT

Plate 9

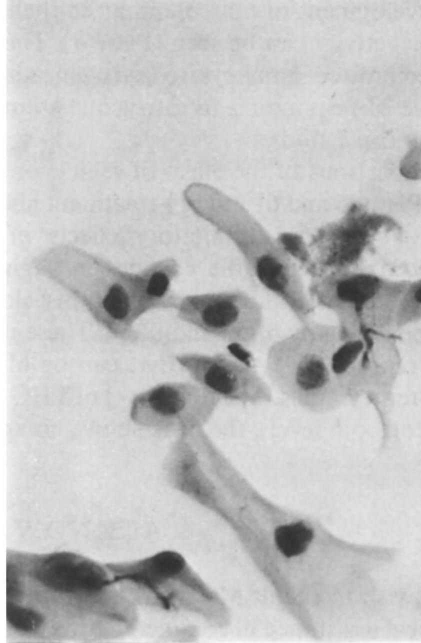


Plate 10

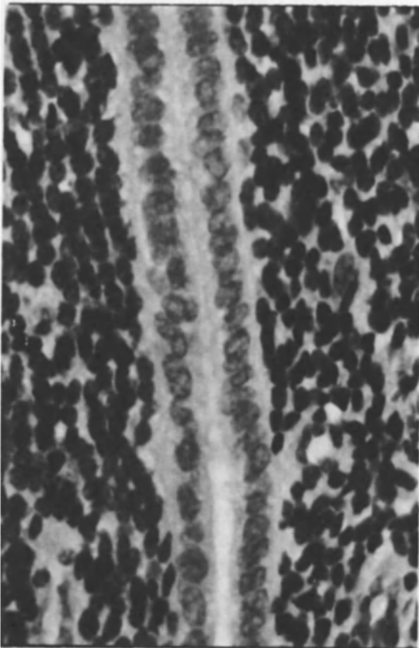


Plate 11

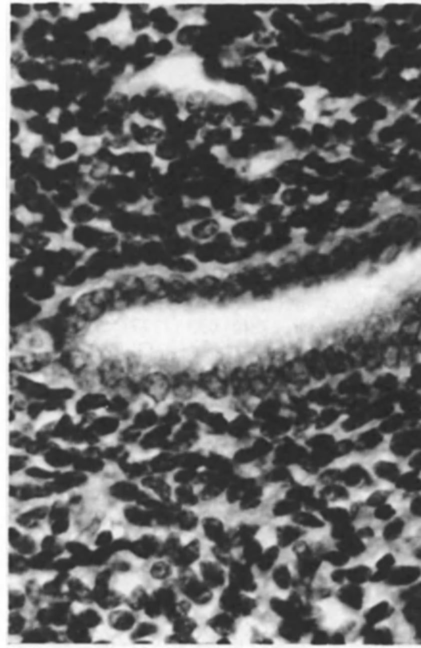


Plate 12

effect of estrogen on the endometrium is proliferation of the glandular epithelium. In the absence of estrogen, the cells of the glandular epithelium are short, the nuclei are stratified and no mitotic activity is seen (Plate 3). After 14 days of estrogen administration, the development of tall columnar epithelium is seen. The nuclei are pseudostratified, and mitotic activity can be seen (Plate 4). These first four plates show the typical response of ovariectomized monkeys to estrogen administration. They indicate that the tissues are capable of responding to estrogenic stimulation, and that adequate treatment time was used in these studies.

Comparisons of the effect of vehicle on pre-treatment and post-treatment vaginal cytology (Plates 5 and 6) and pre-treatment and post-treatment endometrial histology (Plates 7 and 8) show no stimulatory effects of vehicle administration. THC administration produced no detectable changes in vaginal cytology (Plate 9, pre-treatment; Plate 10, post-treatment) and endometrial histology (Plate 11, pre-treatment; Plate 12, post-treatment) in any of the monkeys. These results indicate that THC has no direct effects on these reproductive tissues that can be observed by histological examination, at least in short-term studies. At a dose level of THC that is sufficient to cause a marked depression in gonadotropin levels, the drug shows no apparent estrogenic effects on target organ tissues.

ACKNOWLEDGEMENTS

We wish to thank Ms. Mary Jo Ruppert and Mr. Charles E. Moore for their excellent technical assistance in completing this work. We also wish to thank Dr. John H. Littlefield, Division of Educational Resources, University of Texas Health Science Center, San Antonio, Texas, for his guidance and assistance in the statistical analysis of the data. This study was supported by a NIDA Grant (RO1DA02063) and USUHS.

REFERENCES

1. Asch, R. H., Fernandez, E. O., Smith, C. G. and Pauerstein, C. J., Δ-9-tetrahydrocannabinol inhibits the ovulation reflex in rabbits. *Fertility and Sterility*; in press.
2. Ayalon, D., Nir, I., Cordova, T., Bauminger, S., Puder, M., Naor, Z. and Lindner, H. R., Acute effect of Δ^1-tetrahydrocannabinol on the hypothalamo-pituitary-ovarian axis in the rat. *Neuroendocrinology* **23**,31 (1977).
3. Besch, N. F., Smith, C. G., Besch, P. K. and Kaufman, R. H., The effect of marihuana (delta-9-tetrahydrocannabinol) on the secretion of luteinizing hormone in the ovariectomized Rhesus monkey. *Am. J. Obstet. Gynecol.* **128**(6),635 (1977).
4. Boorman, G. A., Niswender, G. D. and Gay, V. L., Radioimmunoassay for follicle stimulating hormone in the Rhesus monkey using anti-human FSH serum and rat FSH-I^{131}. *Endocrinology* **92**,618 (1973).
5. Clark, S. G. and Smith, R. G., Characterization and partial purification of the estrogen receptor from human uterus. *Clin. Chem.* **22**(7),1182 (1976).
6. Dixit, V. P., Sharma, V. N. and Lohiya, N. K., The effect of chronically administered cannabis extract on the testicular function in mice. *Eur. J. Pharmacol.* **26**(1),111 (1974).
7. Dixit, V. P., Gupta, C. L. and Agrawal, M., Testicular degeneration and necrosis induced by chronic administration of cannabis extract in dogs. *Endokrinologie* **69**(3),299 (1977).
8. Duncan, D. B., Multiple range and multiple *f*-tests. *Biometrics* **11**(1),1 (1955).
9. Harmon, J. and Aliapolios, M. A., Letter: Gynecomastia in marihuana users. *New Eng. J. Med.* **287**,936 (1972).
10. Hembree, W. C., Zeidenberg, P. and Nahas, G. G., Marihuana's effect on human gonadal function. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas, *et al.*, eds.) Springer-Verlag, New York, pp. 521–532, 1976.

11. KING, R. J. B. and MAINWARING, W. I. P., *Steroid-Cell Interactions* University Park Press, Baltimore, 1974.
12. KOLODNY, R. C., MASTERS, W. H., KOLODNER, A. B. and TORO, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *New Eng. J. Med.* **290**,872 (1974).
13. MARKS, B. H., Δ^1-THC and luteinizing hormone secretion, "Progress in Brain Research," Vol. 39, Drug Effects on Neuroendocrine Regulation (E. Zimmerman, *et al.*, ed.) Elservier, Amsterdam, 1973.
14. MIYACHI, Y., VAITUKAITIS, J. L., NEISCHLAG, E. and LIPSETT, M., Enzymatic radioiodination of gonadotropins. *J. Clin. Endocrinol. Metab.* **34**,23 (1972).
15. NIR, I., AYALON, D., TSAFRIRI, A., CORDOVA, T. and LINDER, H. R., Suppression of the cyclic surge of luteinizing hormone secretion and of ovulation in the rat by Δ^1-tetrahydrocannabinol. *Nature* **243**,470 (1973).
16. NISWENDER, G. D., MONROE, S. E., PECKNAM, W. D., MIDGLEY, A. R., KNOBIL, E. and REICHERT, L. E., Radioimmunoassay for Rhesus monkey luteinizing hormone (LH) with anti-ovine LH serum and ovine LH-^{131}I. *Endocrinology* **88**,1327 (1971).
17. OKEY, A. B. and BONDY, G. P., Is Δ-9-tetrahydrocannabinol estrogenic? *Science* **195**,904 (1977).
18. OKEY, A. B. and BONDY, G. P., Δ^9-tetrahydrocannabinol and 17β-estradiol bind to different macromolecules in estrogen target tissues. *Science* **200**,312 (1978).
19. PIETRAS, R. J. and SZEGO, C. M., Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. *Nature* **265**,69 (1977).
20. ROSENTHAL, H. E., A graphic method for the determination and presentation of binding parameters in a complex system. *Anal. Biochem.* **20**,525 (1967).
21. SCATCHARD, G., The attractions of proteins for small molecules and ions. *Ann. N.Y. Acad. Sci.* **51**,660 (1949).
22. SHOEMAKER, R. H. and HARMON, J. W., Suggested mechanism for the demasculinizing effect of marihuana. *Fed. Proc.* **36**,345 (1977).
23. SMITH, C. G., MOORE, C. E., BESCH, N. F. and BESCH, P. K., The effect of marihuana (delta-9-tetrahydrocannabinol) on the secretion of sex hormones in the mature male Rhesus monkey. *Clin. Chem.* **22**(7),1120 (1976).
24. SMITH, H. E., SMITH, R. G., TOFT, D. O., NEERGAARD, J. R., BURROWS, E. P. and O'MALLEY, B. W., Binding of steroids to progesterone receptor proteins in chick oviduct and human uterus. *J. Biol. Chem.* **249**,5924 (1974).
25. SMITH, R. G., CLARK, S. G. and ZALTA, E., Two estrogen receptors in reproductive tissues. *J. Steroid Biochem.* (in press).
26. SMITH, R. G., SMITH, C. G., BESCH, N. F. and BECH, P. K., Is the inhibition of gonadotropin release by Δ-9-tetrahydrocannabinol (THC) receptor mediated? *The Physiologist* **20**(4),512 (1977).
27. SMITH, R. G., IRAMAIN, C. A., BUTHRAM, V. C. and O'MALLEY, B. W., Purification of human uterine progesterone receptor. *Nature* **253**,271 (1975).
28. SOLOMON, J., COCCHIA, M. A. and DIMARTINO, R., Effect of delta-9-tetrahydrocannabinol an uterine and vaginal cytology of ovariectomized rats. *Science* **195**,875 (1977).

THE EFFECT OF A CRUDE MARIHUANA EXTRACT ON EMBRYONIC AND FOETAL DEVELOPMENT OF THE RABBIT

D. D. COZENS, R. CLARK and A. K. PALMER

Department of Reproductive Toxicology, Huntingdon Research Centre, Huntingdon, England

N. HARDY and G. G. NAHAS

Laboratoire de Toxicologie Cellulaire INSERM U26, Paris, France

D. J. HARVEY

Department of Pharmacology, University of Oxford, England

INTRODUCTION

CRUDE marihuana extract and/or the principal psychoactive component of marihuana, Δ^9-tetrahydrocannabinol (THC) have been examined for foetotoxic effects in the rabbit, by several research groups: more recent papers have included that of Fournier, Rosenberg, Hardy and Nahas who reported studies using the fauve de Bourgogne rabbit[1] and that of Wright, Smith, Keplinger, Calandra and Braude who reported observations in the New Zealand White rabbit[2].

As discussed by the authors of the above papers, the significance of the apparently conflicting results obtained in earlier work is difficult to assess due to the lack of analytical data characterising the constituents of the crude extracts examined.

METHOD

The effect of a crude marihuana extract (CME) upon the foetal development of the New Zealand White rabbit was studied using procedures employed by the Huntingdon Research Centre over the last sixteen years in safety evaluation studies of more than 350 compounds. (Fig. 1.) These studies included an examination of the skeleton of the fetus, using a slightly modified version of the technique described by Dawson[4]. The CME provided by the National Institute on Drug Abuse (Lot No C1931N) was contained in 1.5 gm lots of resin in dark glass vials. Original analysis performed by Research Triangle Institute gave the following composition: delta-9-THC 19%, cannabidiol (CBD) 3%, cannabinol (CBN) 1%. To each lot, 10 ml sesame oil was added and emulsion produced by placing the vials into an ultrasonic bath for 3 hours. Re-analysis (Fig. 2) performed by DJH at the time of the experiment, 2 years after the original analysis, indicated that this solution contained 3% THC, 2.5% CBD, 15% CBN and 2.5% cannabichromene.

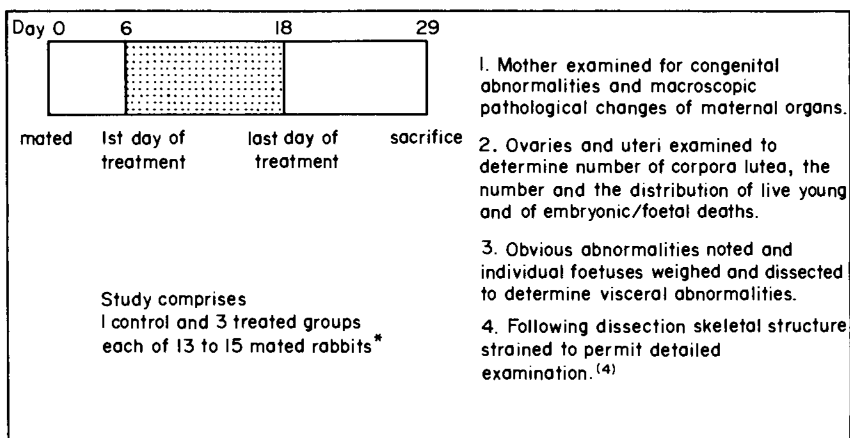


FIG. 1. Rabbit teratology study (Segment 2 Safety Evaluation Study).

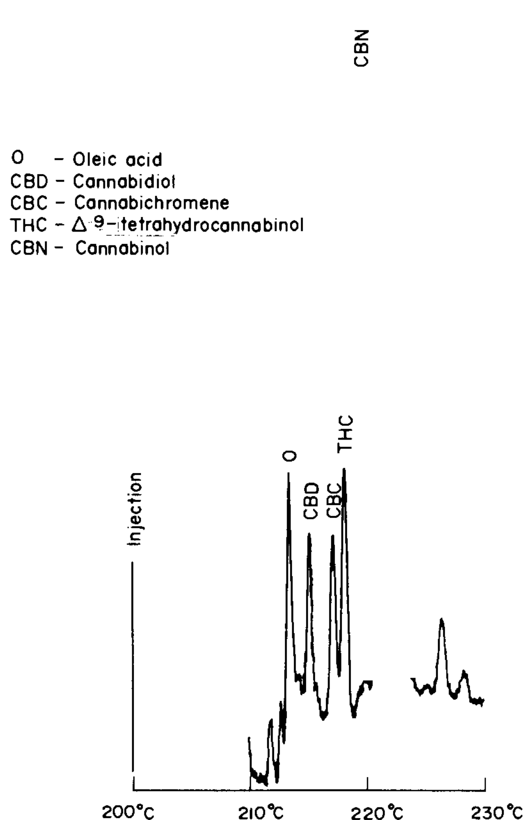


FIG. 2. Total ion chromatogram of material administered.

Solutions were prepared weekly and stored at 4°C in dark glass bottles. Dosages of either 1.0 ml/kg bodyweight or 0.2 ml/kg bodyweight were administered daily from day 6 through to day 18 of gestation where the day of mating was termed day 0. Control animals were given 1.0 ml sesame oil/kg bodyweight/day.

RESULTS AND DISCUSSION

1. PARENTS

Parent females showed no obvious clinical signs of treatment apart from the following effects upon bodyweight. Six of the fourteen females given 1.0 ml CME/kg/day showed actual weight loss during treatment providing a group mean weight loss during days 10 to 19 (Fig. 3). At 0.2 ml CME/kg/day group mean bodyweight gain was retarded. Bodyweight gain post treatment was essentially comparable for all groups. These observations are in accord with those of Wright *et al.*, who in an essentially similar study but using a CME containing 16% THC found bodyweight loss with dosages equivalent to, or greater than 5 mg THC/kg/day and reduced bodyweight gain with dosages equivalent to 1.5 mg THC/kg/day. These authors also noted that the administration of their CME produced a more severe effect upon bodyweight gain than that produced by comparable dosages of THC alone. The weight loss reported in our study with 1 ml CME/kg/day equivalent to only 3 mg THC/kg, but also equivalent to 15 mg CBN and to 2.5 mg CBC and 2.5 CBD/kg further emphasises the need to consider the heterogeneous nature of marihuana and to bear in mind that THC may not be the only toxic component[3].

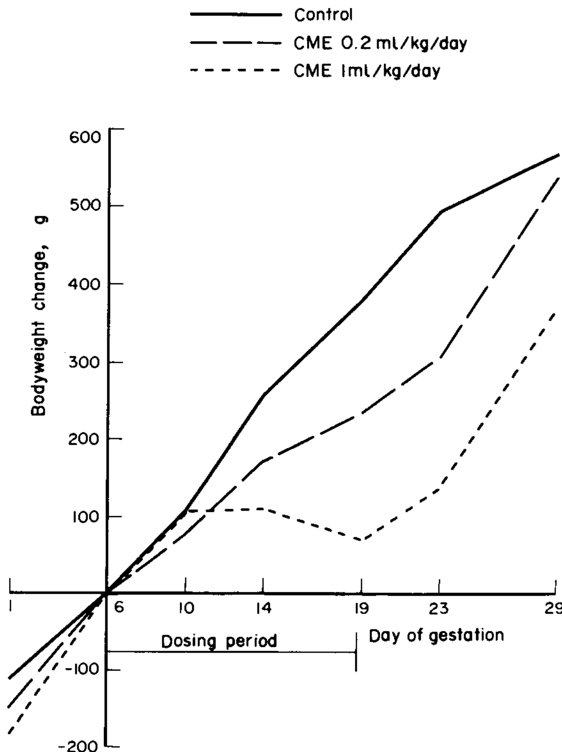


FIG. 3. Group mean bodyweight change of dams with viable young.

2. OFFSPRING

Before discussing effects upon offspring it should be mentioned that it is the practice in the Department of Reproductive Toxicology at HRC to consider the litter and not the individual pup as the experimental unit. (See Weil, 1970[5], Staples and Haseman, 1974[6], Palmer, 1974[7] for further discussion of this topic.)

This being so, for all values expressed as a percentage, values are first calculated within the litter and the group value derived as a mean of the individual litter percentages. The statistical analyses are then performed using the litter as the basic sample unit and non-parametric methods such as those described by Kruskal and Wallis (1952)[8] or Wilcoxon, (1945)[9].

Group mean values for litter size, including pre- and post-implantation losses and for litter and mean foetal weight are shown in Table 1. As can be seen, there was an adverse effect upon mean foetal weight for the group given 1 ml of our CME/kg/day (equivalent to 3 mg THC/kg/day, 15 mg CBN/kg/day, 2.5 mg CBC/kg/day and 2.5 mg CBD/kg/day). Wright *et al.* recorded reduced foetal weight with their CME equivalent to 15 mg THC/kg but not with dosages equivalent to 5 mg THC/kg or less. The dissimilarity of the composition of the extracts may be a reason for the slightly differing results in the two studies.

Examination of the foetuses on the 29th day of pregnancy revealed two obvious abnormalities (Table 2). One of the seven foetuses in a control litter was found to have an abnormal rib cage associated with a displaced fat pad. The skin overlying the thorax also showed discrete areas of thickening associated with lobular areas of adipose tissue in the dermis and/or subcutis. Examination of the skeletal structures showed the 3rd, 5th and 7th left ribs and costal cartilage to be thickened with ossification irregularities of the 3rd and 10th thoracic vertebrae. There were also lesser ossification irregularities of the 6th cervical, 1st sacral, and 5th caudal vertebrae.

The second obvious abnormality occurred in a litter produced by a dam given 0.2 ml CME/kg/day. One of the twelve foetuses was found to have lumbo-sacro-caudal spina bifida. Other anomalies of this animal included asymmetric sternebrae, connected 4th and 5th sternebrae, extra centre of sternebral ossification between sternebrae 5 and 6 fused to the 6th sternebra. There was also a small protrusion in the occipital region with some slight irregularity of the interparietal.

In respect of less obvious structural changes, detailed examination revealed, for the group given 1 ml CME/kg/day an increase in the number of foetuses showing a reduced degree of ossification of sternebrae, phalangeal bones and astragali, a pattern indicative of slight immaturity and reflecting the above mentioned reduced mean foetal weight.

There was also an apparent increase in all treated groups of the number of foetuses with more than the "normal" 12 pairs of ribs. However, rib configuration in the rabbit (and in other species) is subject to natural variation, see Sawin *et al.*[10], Cozens 1965[11], Palmer 1968[12], 1977[13]. Within the last year, we have observed a mean value of 43% of control pups having more than 12 pairs of ribs, but in individual experiments the control group mean has been as low as 29% and as high as 69%.

Other structures, subject to natural variation in the rabbit population include the presence or absence of sutural bones (item 5, Table 2) and the morphology of the gall bladder (item 10, Table 2). Interestingly, one of the mothers given 1 ml of our CME/kg/day was seen at autopsy to have a bilobed gall bladder as did one of her nine foetuses.

TABLE 1. GROUP MEAN LITTER DATA

Group treatment	1 Control (sesame oil)	2 CME	3 CME
ml/kg/day	1.0	0.2	1.0

Group	Number of animals	Viable young ♂	Viable young ♀	Viable young Total	Resorptions Early	Resorptions Late	Resorptions Total	Implantations	Corpora lutea	Pre-implantation loss %	Foetal loss %	Litter wt. (g)	Mean foetal wt. (g)
1	12[a]	3.9	4.3	8.2	1.0	0.3	1.3	9.4	10.8	11.8	9.6	334.3	42.0
2	16	4.6	4.0	8.6	0.6	0.7	1.3	9.9	11.3	12.0	12.0	334.8	39.7
3	14	4.6	4.1	8.6	0.6	0.7	1.4	10.0	11.1	9.4	12.7	$229.5^{(+)}$	34.7^{++b}
Jonckheere's test												$p < 0.05$	$p < 0.01$
Kruskal-Wallis test "H" statistic												NS	$p < 0.01$

Intergroup difference from control values statistically significant — Kruskal Wallis: $p < 0.05^{+}$, $p < 0.01^{++}$; NS $p > 0.05$

Parenthetic statistical significance indicates intergroup comparison not supported by a significant H statistic.

a. Excludes 1 animal which aborted its young.

b. Lowest control value recorded during the previous year 38.5 g

TABLE 2. MORPHOLOGICAL ANOMALIES: GROUP MEAN % INCIDENCE IN LITTER

Group	1	2	3
treatment	Control (sesame oil)	CME	
ml/kg/day	1.0	0.2	1.0

Item	Group 1	Group 2	Group 3
1. Pups with obvious abnormalities (see text)	1.2	0.5	0
2. Pups with anomalies reflecting immaturity	11.6	13.7	22.1
3. Pups with more than 12 pairs of thoracic ribs	34.9	53.4	59.8
4. Pups with less than 12 pairs of thoracic ribs	0.8	2.3	0
5. Pups with sutural bone(s)	4.5	4.8	1.7
6. Pups with skeletal anomalies of cranium, other than above	0	0.5	8.7[a]
7. Pups with anomalies of the thoracic skeleton	9.6	15.2	15.8
7.1 Slight scoliosis	2.8	1.1	0
7.2 Other thoracic vertebral anomalies	4.7	2.2	2.6
7.3 Fused ribs	3.5	0.6	3.5
7.4 Branched rib(s)	0	0.5	0.9
7.5 Thickened rib(s)	1.2	0	0
7.6 Fused sternebrae	2.1	4.1	3.4
7.7 Additional centres of sternebral ossification	1.2	3.8	3.7
7.8 Bipartite, asymmetric or bifurcated sternebrae	2.0	7.0	7.4
8. Pups with anomalies of sacro-caudal skeleton	0.8	2.4	0.9
9. Pups with corneal or lenticular opacities	1.0	0.8	7.9[a]
10. Pups with anomalies of the gall bladder	3.0	1.9	1.7
11. Pups with renal anomalies	0.8	0	0

Pups may be included in more than one category but only occur once where categories are combined, eg. item 7.
[a] 100% effect in one litter (see text)
Differences from control values all NS, ie. $P > 0.05$ Kruskal Wallis or χ^2 test for linear trend in proportions.

An impression was gained during the tabulation of these skeletal observations that there were more anomalies of the thoracic skeleton in the treated animals than in the control animals, perhaps fostered by the observation of Wright *et al.*[2] who noted "skeletal abnormalities involving primarily rib and sternum changes but that were similar in incidence to those observed in cumulative controls".

However, although in our study the mean percentage incidence of foetuses with anomalies of the thoracic skeleton (item 7, Table 2) was higher in the two treated groups, the differences from the control values did not attain statistical significance. Nevertheless, as discussed by Palmer in 1977[13] it is often useful to examine the loci at which minor defects occur for evidence of more serious defects. In this study, whilst there were isolated foetuses with more serious defects of the thoracic skeleton, there appeared to be no relationship to treatment. These included:

a. One foetus of three in a control litter with slight scoliosis which affected the 7th cervical and 1st thoracic and also the right 6th and 7th thoracic vertebrae and ribs.
b. In the group given 0.2 ml CME/kg/day one litter of eleven contained 5 foetuses with minor anomalies of the 1st and 2nd ribs and vertebrae, a relatively uncommon site,

although see above. Similar defects were seen in another litter of 12 in this group, involving one foetus which also showed slight scoliosis. Slight scoliosis involving a 9th thoracic vertebra and rib was also seen in one foetus of ten in another litter.

c. In the group given 1.0 ml CME/kg/day one litter contained one foetus of nine with partial fusion of the 1st and 2nd costal cartilage elements.

Other defects of the axial skeleton additional to those above and to those obvious at sacrifice and described earlier included disorganised caudal vertebrae seen in one of eleven control foetuses, similar findings were made in two foetuses of eleven given 0.2 ml CME/kg/day and also in one of eight foetuses in a second litter of this group (item 8, Table 2).

One litter in each group contained one foetus showing corneal or lenticular opacities (item 9, Table 2). These ocular anomalies may be seen prior to fixation, but are difficult to detect subsequently. In addition, all seven foetuses comprising the litter of a dam given 1 ml CME/kg/day showed both these ocular anomalies and increased size of the fontanelle with apparent slight protrusion of the frontal bones. It was interesting to note that this mother had shown actual bodyweight loss during pregnancy.

SUMMARY

1 ml/kg/day of this CME containing 15 mg CBN, 3 mg THC, 2.5 mg CBC and 2.5 mg CBD produces an adverse effect upon bodyweight gain of the parent female New Zealand White rabbit, with associated reduced bodyweight and signs of skeletal immaturity in the foetus. There was no other conclusive evidence that this treatment had increased the incidence or influenced the type of abnormalities observed. With 0.2 ml of this CME/kg/day the effects upon bodyweight gain of the pregnant female and upon foetal bodyweight were reduced.

APPENDIX

It is to be hoped that a more complete appreciation of the results from teratology studies of marihuana in the rabbit such as the one reported here, will emerge when they are accompanied by studies of the maternal distribution, and of the placental transfer of the substances administered. To this end, a feasibility study was carried out administering our CME to New Zealand White rabbits from days 15 to 18 of pregnancy inclusive. Four animals were given 0.5 ml CME/kg/day and plasma samples obtained 30 minutes after the last dose from two animals and after 60 minutes from the other two. The remaining two rabbits were given 1 ml CME/kg/day and samples obtained after 30 minutes from one animal and after 60 minutes from the other.

The analytical data obtained are shown in Table 3.

In addition, one whole foetus from each litter was subjected to the assay procedure. No cannabinoids were detected (detection limit was 5 mg/g of tissue).

Any consideration of these analytical data, in particular the failure to detect cannabinoids in the foetus must include an awareness of the small number of animals involved and of the reduced period of treatment, both necessitated by the small amount of extract

TABLE 3. PLASMA CANNABINOID CONCENTRATIONS

Rabbit number	Dosage ml/kg/day days 15–18	Sampling time (hours) following administration on day 18	ng/ml		
			CBN	THC	CBD
51	0.5	0.5	0	DL	0
52	0.5	1.0	13.0	4.0	DL
53	0.5	0.5	2.5	3.0	0
54[a]	0.5	1.0	4.5	70.0	4.5
55	1.0	0.5	3.0	4.5	0
56	1.0	1.0	4.3	4.5	0

a. Not pregnant
DL = just detected, detection limit 0.5 ng/ml

remaining after completion of the main teratology study. It should also be recalled that THC has been recovered from foetal mice and rats following administration to the parent female[14, 15, 16]. Finally, the necessity of reanalyzing the material used, before any experimental study is clearly apparent. The instability of THC to light, temperature and oxygen is well established.

ACKNOWLEDGEMENT

The authors wish to thank those staff members at Huntingdon and Oxford who were involved in the conduct of this study. This work was made possible by a grant from INSERM, Paris (ATPA 58).

REFERENCES

1. FOURNIER, E., ROSENBERG, E., HARDY, N. and NAHAS, G., Teratologic Effects of Cannabis Extracts in Rabbits: A Preliminary Study pp. 457–468 in "Marihuana: Chemistry, Biochemistry and Cellular Effects." ed. Nahas, G. G. Springer-Verlag, New York, 1976.
2. WRIGHT, P. L., SMITH, S. H., KEPLINGER, M. L., CALANDRA, J. C. and BRAUDE, M. C., Reproductive and Teratologic Studies with Δ^9-Tetrahydrocannabinol and Crude Marihuana Extract. *Toxicology and Applied Pharmacology*, **38**,223–235 (1976).
3. BRAUDE, M. C., Genetics and Reproduction, introduction in "Pharmacology of Marihuana" Vol 2, ed. Braude, M. C. and Szara, S. Raven Press, New York, 1976.
4. DAWSON, A. B., Note on the staining of the skeleton of cleared skeletal specimens with alizarin red S. *Stain Techn.*, **1**,123–4 (1926).
5. WEIL, C. S., Selection of the valid number of sampling units and a consideration of their combination in toxicological studies involving reproduction, teratogenesis or carcinogenesis. *Fd. Cosm. Toxic.* **8**,177–182 (1970).
6. STAPLES, R. E. and HASEMAN, J. K., Selection of appropriate experimental units in teratology. *Teratology* **9**,259-260 (1974).
7. PALMER, A. K., Statistical Analysis and Choice of Sample Units. *Teratology*, **10**,301–302 (1974).
8. KRUSKAL, W. H. and WALLIS, W. A., Use of ranks in one criterion variance analysis. *Journal of American Statistical Association*, **47**,583–621 (1952).
9. WILCOXON, F., Individual comparisons by ranking methods. *Biometrics.* **1**,80–83 (1945).
10. SAWIN, P. B., Numerous papers from 1932, specifically: SAWIN, P. and CRARY, D. (1964) Genetics of Skeletal Deformities in the Domestic Rabbit. *Clin. Orthopaedics*, **33**,71–90 (1964).
11. COZENS, D. D., Abnormalities of the External Form and of the Skeleton in the New Zealand White Rabbit. *Fd. Cosm. Toxic.* **3**,695–700 (1965).
12. PALMER, A. K., Spontaneous malformations of the New Zealand White Rabbit: The Background to Safety Evaluation Tests. *Lab. Anim.* **2**,195–206 (1968).

13. PALMER, A. K., Incidence of Sporadic Malformations, Anomalies and Variations in Random Bred Laboratory Animals. In "Methods in Prenatal Toxicology", ed. Neubert, D. *et al.* Georg Thieme Publishers, Stuttgart, pp. 52–71, 1977.

14. HARBISON, R. D., Maternal Distribution and Placental Transfer of ^{14}C-Δ^9-Tetrahydrocannabinol in Pregnant Mice. *Toxicology & Applied Pharmacology*, **19**,413–414 (1971).

15. KENNEDY, J. S. and WADDELL, W. J., Whole-body autoradiography of the pregnant mouse after administration of ^{14}C-Δ^9-THC. *Toxicol. Appl. Pharmacol.* **22**,252–258 (1972).

16. PACE, H. B., DAVIS, W. M. and BORGEN, L. A., Teratogenesis and marijuana. *Ann. N.Y. Acad. Sci.* **191**,123–131 (1971).

EFFECTS OF CANNABIS ON FETAL DEVELOPMENT OF RODENTS

HARRIS ROSENKRANTZ

Department of Biochemical Pharmacology, E G & G Mason Research Institute, Worcester, Massachusetts 01608, U.S.A.

Summary. The majority of teratologic studies on marihuana and Δ^9-THC in rodents have been negative but serious observations of cleft palate in mice and eventrations in rabbits remain unexplained. There has been agreement on the increased incidence of *in utero* deaths. To clarify the potential effects of cannabinoids on fetal development, studies were performed in rodents using the inhalation and oral routes. The Δ^9-THC doses in marihuana smoke were 0.8–3.8 mg/kg which correlated with plasma Δ^9-THC levels of 73–297 ng/ml and carboxyhemoglobin levels between 21 and 60%. Oral Δ^9-THC doses were 5–600 mg/kg for mice and 12–50 mg/kg for rats. An automatic inhalator provided a 50-ml puff from each of 3 NIDA cigarettes in a 2-sec puff, retention of smoke for a 30-sec exposure interval followed by a 30-sec fresh air purge each min. Exposure to smoke was performed during days 6 to 15 of gestation. Oral studies included a similar treatment protocol but in addition the number of treatments and days of treatment were varied to establish the time of greatest effect on fetal development. The embryocidal effect of marihuana and Δ^9-THC was demonstrated by 2 routes and in 2 rodent species. In fact, whole litter resorption was encountered and 2–5 treatments around days 7 to 9 of gestation were sufficient to induce embryotoxicity. At least in mice the fetocidal effect appeared to be related to vaginal bleeding, possibly a consequence of interrupted development of fetoplacental circulation and deranged hormone balance. No drug-related teratogenic effects were found.

INTRODUCTION

OVER the past ten years cannabis and a few cannabinoid constituents have been investigated for potential effects on the reproductive process and fetal development in animals. Despite assessment of the complete spectrum of reproductive function from coital performance in parents to postnatal behavior of progeny, controversial conclusions remain[54]. The disparity in teratogenic findings, including chromosomal aberrations, not always could be attributed to variation in species, dose or route of administration. No argument exists as to the need to resolve any discrepancies in reproductive findings. It is essential that some estimate of physiologic risk of marihuana use be obtained as a guideline for human females in the gravid state.

In the evaluation of cannabis for potential influence on fetal development, it is necessary to consider three possibilities of pharmacologic action of cannabinoids: (1) individual cannabinoid effects; (2) changes related to pure cannabinoid interaction; and (3) alterations initiated by interaction of cannabinoid and non-cannabinoid components present during pyrolysis of marihuana. Whereas the teratogenic assessment of pure cannabinoids,

individually or in combination, may be achieved in a straightforward fashion, the evaluation of the contribution of pyrolytic products, like carbon monoxide, in marihuana smoke is subject to greater complexity. It cannot be over-emphasized that reproductive risk in man must be related to use of cannabis in the usual manner of smoking marihuana.

In the instance of pure cannabinoids, the oral or intraperitoneal (i.p.) routes of administration have been favored, but both routes are unnatural and require large quantities of drug[23]. It would have been more reasonable to utilize the intravenous (i.v.) route since acute LD 50 values of Δ^9-tetrahydrocannabinol (Δ^9-THC) are similar for i.v. and inhalation routes[79]. A second advantage is that much smaller doses of pure cannabinoids are needed as compared with other routes and suitable i.v. drug formulations are available[80]. In addition, the onset and duration of behavioral effects are similar by i.v. and inhalation routes. However, it should be mentioned that in great part Δ^9-THC oral effects have adequately compared with those by inhalation[73, 76].

The use of marihuana smoke in contrast to pure cannabinoids affords the advantage of similarity to use of cannabis in man. However, simulation of human marihuana smoking can be compromised by variation in marihuana potency based on Δ^9-THC content and by variability in the ratios of cannabinoid constituents depending on the source of cannabis. For example, the Mexican variety contains much more Δ^9-THC than cannabidiol (CBD) or cannabichromene (CBCH), while in Turkish marihuana, the latter two predominate[56, 75, 78].

Other factors beside cannabinoid content exert influences that must be considered in order to mimic human marihuana smoking. Among the chemical factors to be considered are transformations of cannabinoid structure during pyrolysis, cannabinoid destruction during the burning process, generation of carbon monoxide (CO) and the chemical profile of non-cannabinoid pyrolytic products. The influence of the latter can be evaluated by comparison with a suitable placebo marihuana (which at the moment is not available) and the others are amenable to direct measurement[74].

Biological factors that must be standardized in order to reliably reproduce conditions for the inhalation of marihuana smoke include puff volume, puff duration, puff frequency, duration of exposure and appearance and clearance of carboxyhemoglobin (COHb). In animal paradigms, cannabinoid doses relevant to man should be documented by determination of plasma cannabinoid levels. In general, the biological factors may be controlled through the use of automatic smoking machines. The measurement of cannabinoid and CO in the smoke chamber in conjunction with analyses of COHb and plasma cannabinoid in the animals can establish greater credibility of marihuana inhalation studies[73, 77].

In relationship to studies of cannabis effects on fetal development, additional factors of animal species, strain and source provide problems of standardization. Particularly in the mouse, the incidences of reported cleft palate evoked by Δ^9-THC varied from 0 to 70 percent[23]. The mouse appears to have a greater susceptibility to cleft palate than other species[5, 6]. It is difficult to estimate genetic drift of a mouse strain from breeder to breeder. It would appear that knowledge of circulating cannabinoid would aid in resolving differences in incidences of cleft palate where disagreement exists (Table 1).

At the moment, in the absence of plasma Δ^9-THC analyses, only the disparities and not the reasons for the discrepancies can be reviewed. When cannabis resin or extract was tested, runting was observed in mouse treated i.p.[67] and in hamster treated subcutaneously (s.c.)[29]. However, the results in hamster could not be confirmed[66]. In the rat treated i.p., syndactyl and encephalocoele were seen[68] but oral treatment with a similar

TABLE 1. FETAL DEVELOPMENT AFTER ADMINISTRATION OF CANNABINOIDS DURING ORGANOGENESIS

Cannabinol preparation	Dose mg/kg	Species	Route	Literature references	
				Teratogenicity (type)	No teratogenicity
Cannabis, resin or extract	16	Mouse	IP	Persaud & Ellington, 1967 (stunting)	
	4	Rat	IP	Persaud & Ellington, 1968 (syndactyl, encephalocele)	
	0.5–15	Rat	PO		Miras, 1965; Haley *et al.* 1973; Keplinger *et al.*, 1973; Wright *et al.*, 1976
	23–300	Hamster	SC	Geber &Schramm, 1969 (runting)	Pace *et al.*, 1971
	15–500	Rabbit	SC, PO	Fournier *et al.*, 1976 (eventration, hairlip)	Geber & Schramm, 1969; Wright *et al.*, 1976
THC	50–300	Mouse	IP	Mantilla-Plata *et al.*, 1975, 1976; Harbison *et al.*, 1977 (cleft palate)	Philips *et al.*, 1971; Fleischman *et al.*, 1975
	3–300	Mouse	IV, SC		Joneja, 1976; Maker *et al.*, 1974
	400	Mouse	PO	Joneja, 1976 (exencephaly, cleft palate)	
	1–250	Rat	PO, SC		Borgen *et al.*, 1971, 1973; Pace *et al.*, 1971; Uyeno, 1973; Banerjee *et al.*, 1975; Vardaris *et al.*, 1976; Wright *et al.*, 1976
	25–500	Hamster	PO		Joneja, 1977
	1–15	Rabbit	PO		Haley *et al.*, 1973; Wright *et al.*, 1976
	2.4	Monkey	PO		Sassenrath & Chapman, 1975
	10 ppm	Zebra fish	IV, PO	Thomas, 1975 (curved spine, bulb tail)	
	1–2	Chick	IP		Jakubovic *et al.*, 1976
Marihuana (1.1% Δ^9-THC)	1–3	Rat	Lung		Fried 1976
(2.6% Δ^9-THC)	1–4	Rat	Lung		Rosenkrantz *et al.*, 1978
	1–4	Mouse	Lung		Rosenkrantz *et al.*, 1978

dose was negative[34, 45, 59]. Low doses of oral crude marihuana extract or Δ^9-THC given to albino rat were also not teratogenic[99]. Eventrations and hairlip were reported for rabbits given cannabis extract s.c. or orally[24] but earlier and recent studies in this species failed to implicate cannabis as a teratogen[29, 99].

Equally confusing but serious reports of teratogenicity induced by pure Δ^9-THC have been published. High doses of Δ^9-THC (50–400 mg/kg) administered to mice i.p.[52, 53] or orally[42] have evoked a 30–70 percent incidence of cleft palate. However, recently the group that consistently found a high incidence of cleft palate in mice have reported other findings on the Harlan Swiss mouse[36]. Δ^9-THC doses of 200 mg/kg only on days 12 and 13 or 50 mg/kg on days 10 and 11 initiated a 9–10 percent incidence of cleft palate. No cleft palate occurred when 50 mg/kg was given on days 8 and 9 and 12 and 13 or when

200 mg/kg was administered on day 8 and 9 and 10 and 11. The absence of cleft palate induction by Δ^9-THC in mice was also noted by others using various routes of administration[23, 51, 69]. Joneja[42], who did observe cleft palate formation after oral Δ^9-THC, could not substantiate this in mice treated i.v. or s.c.

Administration of Δ^9-THC (25–500 mg/kg) orally to hamsters was also essentially devoid of teratogenic activity[43]. Δ^9-THC given to rats orally or s.c.[4, 8, 9, 66, 92, 94], to rabbits orally[34], to monkeys orally[82] and to chickens i.p.[41] was not teratogenic. Zebrafish placed in a medium containing 10 ppm of Δ^9-THC produced offspring with curved spines and bulbous tails[91].

Despite the disparity in the findings of cannabis-evoked teratogenic effects, a common observation has been an increased incidence of *in utero deaths*[22, 23, 26, 42, 53, 54, 77, 93]. Although cannabinoid-induced embryotoxicity is of paramount importance as a risk factor in the use of marihuana, the observations of cleft palate formation in mice and eventrations in rabbits cannot simply be dismissed.

Because of the controversial findings of cannabinoid-induced abnormalities in rodent progeny, the present studies were performed. Both the route of inhalation and oral treatment were utilized to permit comparison of crucial findings in two rodent species by two routes of administration. No teratogenic effects were observed in rodents after exposure to marihuana smoke[26, 27, 77] but embryotoxicity was prevalent in mice[77].

METHODS AND MATERIALS

The inhalation investigation was conducted to establish whether marihuana smoke itself containing doses of Δ^9-THC relevant to man could elicit embryotoxicity. Other studies with larger oral doses of pure Δ^9-THC were performed to delineate the time of greatest fetal sensitivity to cannabinoid-induced fetal resorption. In addition, experiments were designed to clarify the finding of cannabinoid-evoked whole litter resorption which compromised confirmation of pregnancy of rodent dams[22].

The inhalation and oral studies were performed at different times but, where possible, the same personnel were used and environmental factors were kept similar.

ANIMAL HOUSING, CARE AND MATING

All animals were procured from the same source (Charles River Breeding Laboratories, Wilmington, Ma). Two strains of mice, CD-1 and Swiss-Webster, and Fischer 344 rats were studied. Female mice weighing 30–40 gm were housed 5/cage and female rats weighing 135–150 gm were housed 3/cage. The dimensions of the wire-mesh suspension cages were 24 × 18 × 17 cm. Individual sexually mature males of either species were kept in similarly constructed but larger breeding cages. Mice in advanced pregnancy were transferred to plastic cages to prevent loss of pups if early parturition occurred.

Commercial rodent chow pellets and water were freely available. Each animal room had a 12-hr light/dark circadian cycle to minimize the effects of hormonal surges. Ambient room temperature was 23 ± 2°C and there were 6–8 air changes per hour.

Mating of rodents for the inhalation studies were performed in-house. One shipment of each species was used. The estrus cycle was determined in rats by vaginal smear and 2

females at proestrus were transferred to the breeding cage of 1 fertile male. Positive mating was confirmed by the presence of sperm in the vaginal tract and the day of this occurrence was considered day 0 of gestation. As pregnant rats were obtained, they were arbitrarily randomized into control and treated groups till there were approximately 30 pregnant rats/group. The estrus cycles of mice were too variable to depend on mating during proestrus; 2 females were permitted to remain with 1 male for 1–3 days until sperm was found in the vagina. Randomization was like that for the rat.

In the oral studies rodents were obtained 3-days pregnant from the breeder, whose larger breeding colony could provide more accurate conception rates and adequate numbers of rodents as needed. As above, day 0 of gestation was the day the presence of a vaginal plug and/or sperm was found the morning after mating.

CANNABINOID MATERIALS AND DOSES

Marihuana cigarettes contained approximately 2.8 percent Δ^9-THC and less than 0.2 percent of each of the major cannabinoids. Cannabinoid-extracted marihuana served as placebo cigarettes. Oral preparations were sesame oil solutions of 96 percent pure synthetic (−)-*trans* Δ^9-THC; drug formulations of 3.75–15 mg/ml were used for rats and 15–60 mg/ml for mice. All cannabinoids were supplied by the National Institute on Drug Abuse: marihuana cigarettes were lot RTI 2055–53; placebo cigarettes were lot RTI 2055-34; and pure Δ^9-THC was lot QCD-84924. Cannabinoid materials were stored at 5°C but before use, cigarettes were placed at 60 percent humidity and 23°C for 24–48 hr.

The doses selected for inhalation studies were estimated on the basis of rodent tidal volumes, respiration rates, duration and number of exposure periods (puffs) and concentration of Δ^9-THC in the smoke as previously described[73]. These calculated values were corrected for pyrolytic losses and for losses due to entrapment in nasal passages. For example in the rat: 3.3 μg/ml (Δ^9-THC concentration in smoke) × 0.8 ml (tidal volume) × 60 (respiration rate) × 12 puffs = 1.9 mg/200 g rat or 9.5 mg/kg; 50% loss in rat nasal turbinates and 20 percent loss in lungs yield a dose of approximately 3.8 mg/kg. In the mouse: 3.3 μg/ml × 0.16 ml × 60 × 12 puffs = 0.38 mg/40 gm mouse or 9.5 mg/kg; losses similar to those in rat yield a dose of approximately 3.8 mg/kg.

The rodent inhalation doses were relevant to marihuana use in man. Assuming a 50 kg body weight and correcting for Δ^9-THC losses during smoking, it has been estimated that Δ^9-THC doses of marihuana with 2.6 percent Δ^9-THC would be approximately between 0.3–1.8 mg/kg in man depending on consumption of 1–6 marihuana cigarettes per day[73]. On the basis of body surface area, these human doses would be similar to 2–20 mg/kg in rats and mice. Since oral doses in man needed to elicit similar effects to those by inhalation are known to be 3–6 times larger, relevant oral doses in rodents would be approximately 6–70 mg/kg in rat and 12–200 mg/kg in mice corrected for body surface areas[73]. The acute inhalation LD 50 in these rodent species was approximately 40–50 mg/kg and the acute oral LD 50 was approximately 1000–2000 mg/kg, the mouse being less sensitive than rat to Δ^9-THC given by the oral route[73]. Furthermore, female rodents were more sensitive to oral Δ^9-THC than males.

Dosimetry in the present inhalation studies was directly monitored (suborbital blood specimens) by determination of COHb by the Dubowski spectrophotometric method[17]

and plasma Δ^9-THC levels were measured by a gas chromatographic procedure through the courtesy of Battelle Columbus Laboratories.

TREATMENT PROTOCOLS

Of considerable importance in the inhalation studies was to simulate smoking conditions in rodents to closely mimic those in man. In addition, it was anticipated to obtain plasma Δ^9-THC levels and relate these to reported circulating levels in man. Cannabis consumers inspire a puff volume of 50–200 ml during a 2–10-sec period and retain the smoke in their lungs for 15–60 sec[73]. In the rodent teratology studies, an automatic smoking machine was used which delivered a 150-ml puff volume (50 ml from each of 3 cigarettes) over a 2-sec period and the smoke was retained in a 400-ml constant volume smoke chamber for 30 sec. The smoke was displaced with a fresh air purge for 30 sec. The complete cycle was repeated each min and was equivalent to 1 puff/min.

Multiple rodents were exposed simultaneously by attachment of a circular array of 4–9 cone-shaped lucite holders to each side of the smoke chamber. The Swiss-Webster mouse strain and the Fischer 344 rat strain were used in inhalation studies. The former was selected because of the report of others of a high incidence of Δ^9-THC-evoked cleft palate[53]. The rat strain was chosen because of a considerable archive of marihuana data on the Fischer rat[76]. Groups of successfully mated rodents were exposed during days 6 to 15 of gestation to 4 puffs (0.8 mg/kg), 8 puffs (2.6 mg/kg) or 12 puffs (3.8 mg/kg). Two control groups were used, one being exposed to 8 puffs of placebo smoke and the second group was placed in the inhalator in the absence of smoke (sham treated). A reduced number of placebo puffs, compared with the marihuana high dose, were used because cannabinoid-extracted marihuana has been found to generate approximately 30 percent more carbon monoxide and induce mortality.

The oral treatment protocols were varied in accordance with exploration of the mechanism of embryotoxicity: (1) effect of number of Δ^9-THC treatments on fetal viability in rats and mice; (2) determination of gestational days of greatest susceptibility to embryotoxicity in rats; and (3) confirmation of complete fetal resorption in rats whose pregnancies were unequivocally established by laparotomy. The rats were of the Fischer 344 strain and the mice were the CD-1 strain. The gavage volume of drug formulation administered to rats was 0.5 ml/150 gm body weight and that for mice was 0.2 ml/20 gm body weight.

In the first oral study, weekly shipments of 3-day pregnant rats were randomized to achieve approximately 50 rats/group, who received Δ^9-THC doses of 12.5, 25 or 50 mg/kg. One control group received the sesame oil vehicle and a second control group were sham treated. Weekly shipments of 3-day pregnant mice were also randomly assigned to 2 treatment protocols: (1) approximately 75 mice/group were orally administered Δ^9-THC doses of 5, 15 or 50 mg/kg or were treated with vehicle; and (2) approximately 90 mice/group received oral Δ^9-THC doses of 150, 300 or 600 mg/kg and control groups were given vehicle or were sham treated. Rats and mice were treated either for 2, 5, 8 or 10 days commencing on day 6 of gestation, the longest duration of treatment terminating on day 15 of gestation. Rodents on the shorter treatment schedules were sacrificed the day after the last treatment.

In the second oral study approximately 200 rat dams were distributed among 10 groups. One-half the groups received an oral Δ^9-THC dose of 50 mg/kg and the others were given

vehicle, matched treated and control groups being treated on days 5 to 7, 6 to 8, 7 to 9, 8 to 10 or 9 to 11 of gestation and all were sacrificed on day 14.

In the third oral study, pregnancy was confirmed by laparotomy, an exact count of embryos was recorded and approximately 20 dams/group were orally administered Δ^9-THC at 50 mg/kg or vehicle or were sham treated on days 6–9 of gestation. Approximately one-half the rats in each group were sacrificed on day 12 and the other half on day 16 of gestation.

TERATOLOGY PROCEDURES

Except for the early sacrifices in the variable treatment schedules of oral studies 1 and 2 and on laparotomized dams in study 3, all other animals underwent caesarian delivery on days 18 to 20. Euthanasia was by carbon dioxide inhalation and standard observations were made for the classical teratology experiments and abbreviated ones for the studies on the mechanism of embryotoxicity. Briefly, the abdominal wall of each dam was incised and reflected to expose the uterine horns; fetal swellings and metrial glands were counted under a magnifier illuminator; the uterine horns were opened from apex to cervix and the numbers of live, dead and resorbed fetuses were determined; each fetus was individually weighed and placed upon a sponge relative to its position in the uterine horns; and, each fetus was sexed and examined for external defects.

Approximately equal numbers of male and female pups were randomly transferred into labeled vials containing 95 percent ethanol for bone clearing or Bouin's solution for fetal slicing. As a general procedure, the first and second fetuses were placed in alcohol and the third in Bouin's solution. When gross skeletal defects were evident, the fetus went into alcohol; fetuses with external indication of soft tissue injury were immersed in Bouin's solution. Approximately one-third of the fetuses were preserved for evaluation of soft tissue changes and two-thirds were prepared for the evaluation of skeletal anomalies.

Examination of fetuses for soft tissue anomalies was performed according to the Wilson technique[98]. Bouin's solution (8 days) was replaced by 80 percent ethanol (5–6 days) which in turn was replaced by distilled water prior to cutting the fetus. Legs and tail were clipped from their trunk junctures and the head severed at the level above the ears by slicing through the mouth. Five to 6 slices were made between front of eyes to ears. Transverse slices (0.5–2 mm) were made of the trunk from the regions of the shoulders to a caudal area beyond the kidneys. Included were the heart, liver, kidney and genitourinary regions. A dissecting microscope was used for closer viewing of slices and the sections were stained with 0.1 percent aqueous cresyl violet mixed 1 : 20 with 2 percent acetic acid. Differential coloration yielded a purple stain for bone, red for cartilage, dark blue for glands, blue-green for other tissue (background color was yellow). The coloration lasted approximately 4 hr in water and stained sections were returned to 80 percent ethanol.

Bone clearing was accomplished by fixing fetuses in 95 percent ethanol for 48–96 hr after which they were transferred to 1 percent potassium hydroxide until the skeleton was clearly visible (24–72 hr). A subsequent transfer into alkaline alizarin R (1 : 100; 1 percent aqueous alizarian R in 1 percent potassium hydroxide) for 2 days provided a violet-red staining of the skeletal tissue. The stained fetuses were immersed in Mall's solution (20 percent glycerol in 1 percent potassium hydroxide) for 1–2 days to remove excess stain and

were placed into a 35 percent glycerol solution for inspection. Storage of specimens was in 50 percent glycerin.

Statistical analysis of the significance of change of the usual teratologic parameters were based on the Student t test. Chi Square analysis was performed using the numbers of embryos before and after treatment with experimental N unit[5]. Whole litter resorption was subjected to Fisher's Exact Probability using dams as the experimental unit. Percent whole litter resorption and fetal mortality were based on embryo counts made at laparotomy.

RESULTS

It is of some importance to mention behavioral changes observed in the rodent species at the cannabis doses used since prolonged gross intoxication can compromise the teratological findings. Approximately 30 percent of the Swiss-Webster mice exposed to marihuana smoke exhibited a dose-related CNS-inhibition manifested by ataxia, dyspnea and inactivity at the high dose in the first 2 hr from which the animals rapidly recovered by the third hr. This temporal pattern of events occurred for 4–5 days postexposure but tolerance to CNS-inhibition developed thereafter. The majority of placebo-exposed mice displayed similar, but more intense, behavioral changes which included instances of tremors, convulsions and lethality. The observation of cyanosis in many placebo mice suggested their behavioral changes were related to CO intoxication. There were minor incidences of CNS-stimulation at all doses of marihuana before and after development of tolerance to CNS-inhibition. Sham treated mice behaved normally.

Approximately 20 percent of rats at the mid-dose and 50 percent at the high dose of marihuana responded only with a depression of activity and all animals behaved normally by the end of the day. About 10 percent of the low-dosed rats exhibited hyperactivity. As in the instance of mice, many placebo rats were depressed and some were cyanotic. In general, tolerance developed to the CNS-inhibition in marihuana-exposed groups between days 3–5 and the usual second phase of CNS-stimulation was a minor occurrence. Sham-treated rats behaved normally.

In the oral studies with pure Δ^9-THC, mice predominantly exhibited a dose-related decrease in voluntary activity to which tolerance developed in 3–4 days. Hypersensitivity was noted in approximately 25 percent of high-dosed mice during days 5 to 7 of treatment. Control mice behaved normally. Less than 20 percent of the high-dosed rats vocalized and were sedated or depressed after the first treatment. Thereafter, approximately 75 percent of high-dosed rats were depressed during 2 subsequent treatments but all groups of Δ^9-THC-treated rats behaved normally during continued treatment. One-half of the mid-dosed animals and approximately 30 percent of the low-dosed rats were depressed during the first 3 oral treatments while an equal number displayed hyperactivity. Some vocalization occurred at the lower doses and approximately 35 percent of the rats in these groups behaved aggressively after 5 treatments. Δ^9-THC groups were normal during continued treatment while control rats behaved normally throughout treatment with sesame oil vehicle.

A pilot study in rats using oral Δ^9-THC doses of 75–300 mg/kg revealed considerable cannabis intoxication and mortality. Therefore, none of these doses were considered realistic or relevant in the rat teratology investigations and were abandoned.

REPRODUCTIVE FINDINGS

Because most of the teratology data have or will soon appear in other publications, such data will not be presented in detail here[22, 77]. Instead, simplified tables refering to the significance of changes observed in dams and fetuses in all studies have been provided herein. More stress has been placed on the embryotoxicity observations and appropriate data have been supplied on this aspect of fetal development.

1. *Dam changes*

In the inhalation studies on both rodent species, there were no significant adverse effects on conception rate, dam growth rate, total number of implants and number of implants per dam (Table 2). On the other hand, the number of dams with early fetal resorptions was significantly increased in a dose-related fashion among marihuana-exposed Swiss-Webster mice. In contrast, marihuana inhalation did not evoke a similar significant change of this parameter in rats. There was no significant difference between treated and control groups of either species in regard to the numbers of dams with late fetal resorptions or dead fetuses.

In the oral studies on CD-1 mice, who were procured 3-days pregnant, a dose-related interruption of pregnancy occurred (Table 2). At the larger doses (150–600 mg/kg), dam growth rate was significantly inhibited but loss in dam weight was related to resorption of fetuses and not dam intoxication. Total number of implants and number of implants per dam were unchanged at all Δ^9-THC doses. The number of dams with early fetal resorptions, but not with late resorptions or dead fetuses, was significantly increased at doses greater than 25 mg/kg in a non-dose related manner.

Orally treated rat dams exhibited pregnancy interruption but the finding was of borderline significance (Table 2). This was also true for dam growth rate. As in the instance of mice, a non-dose-related increase in the number of dams with early fetal resorptions occurred at higher doses in rat dams.

2. *Fetal changes*

In the inhalation studies on rodents, total litter weight, mean fetal weight and sex ratio were unaltered for both species (Table 2). In contrast, there was a dose-related elevation in early resorption of mouse fetuses but not rat fetuses. The increase in total fetal mortality was attributable to early fetal resorption alone since there were no significant changes in late fetal resorption or fetal deaths.

Fetal changes among mice treated orally with pure Δ^9-THC substantiated the findings obtained after marihuana inhalation (Table 2). In addition to the increase in early fetal resorption, and thus fetal mortality, the larger doses of Δ^9-THC (150–600 mg/kg) induced a significant decrease in total litter weight and mean fetal weight. These changes originated in the excessive loss of fetuses due to early resorptions. Similarly, rats treated orally with Δ^9-THC had a significant increase in early resorptions concomitant with decrements in total litter weight and mean fetal weight.

A summary of the rodent fetal abnormalities encountered is outlined in Table 3. There

TABLE 2. TERATOLOGY PARAMETERS EVALUATED ON CD-1 MICE, SWISS-WEBSTER MICE AND FISCHER RATS ORALLY TREATED OR EXPOSED TO INHALATION OF Δ^9-TETRAHYDROCANNABINOL DURING GESTATION DAYS 6 TO 15 AND SACRIFICED NEAR TERM[a].

Parameters evaluated	CD-1 mice		Swiss-Webster mice	Fischer 344 rats	
	Oral Δ^9-THC 5–50 (mg/kg)	Oral Δ^9-THC 150–600 (mg/kg)	Inhalation Δ^9-THC 0.8–3.8 (mg/kg)	Oral Δ^9-THC 12.5–50 (mg/kg)	Inhalation 0.8–3.8 (mg/kg)
Dams (N = 30–50):					
Effect on pregnancy[b]	$P < 0.01$	$P < 0.01$	NS	$P < 0.1$	NS
Premature delivery	NS	NS	NS	NS	NS
Growth rate	NS	$P < 0.01$	NS	$P < 0.1$	NS
Number implants/dam	NS	NS	NS	NS	NS
Number with					
(a) Early resorption	$P < 0.05$[c]	$P < 0.01$	$P < 0.01$	$P < 0.05$[c]	NS
(b) Late resorption	NS	NS	NS	NS	NS
(c) Dead fetuses	NS	NS	NS	NS	NS
Fetuses (N = 350–550):					
Litter weight	NS	$P < 0.01$	NS	$P < 0.01$	NS
Mean fetal weight	NS	$P < 0.05$	NS	$P < 0.05$	NS
Sex ratio	NS	NS	NS	NS	NS
Early resorptions	$P < 0.05$[c]	$P < 0.05$	$P < 0.01$	$P < 0.05$	NS
Late resorptions	NS	NS	NS	NS	NS
Dead non-resorbed	NS	NS	NS	NS	NS
Total fetal mortality	$P < 0.05$	$P < 0.01$	$P < 0.01$	$P < 0.01$	NS

a. NS = non-significant compared with sham and/or sesame oil controls in oral studies or with sham and placebo marihuana (free of cannabinoids) controls in inhalation studies.
b. This parameter relates to pregnancy interruption in oral studies and to inhibition of conception rate in inhalation studies.
c. Only at higher doses.

were no significant differences in the numbers of incidences or types of external defects, soft tissue anomalies or skeletal abnormalities for either species exposed to marihuana smoke or treated orally with pure Δ^9-THC. Among the orally treated CD-1 mice, the major external findings in both treated and control fetal groups were skin hemorrhages and backwardly curved hindlimbs. Soft tissue anomalies in all groups included hydronephrosis, hydrocephalus, a rare observation of cleft palate, hemothorax, hemoperitoneum and occasional hepatic focal pale areas. Skeletal aberrations encompassed missing 5th sternebrae, a hole in the 6th sternebrae and an extra right or left rib. Orally treated rats had fetuses virtually free of teratological signs.

Swiss-Webster mice exposed to marihuana smoke did not have fetuses with external or skeletal defects but there were a few incidences of soft tissue anomalies of hydronephrosis and hydrocephalus in both treated and control groups. The fetuses of rats exposed to marihuana smoke had similar insignificant changes to those observed for mice.

TABLE 3. SUMMARY OF FETAL ANOMALIES IN CD-1 MICE, SWISS-WEBSTER MICE AND FISCHER 344 RATS ORALLY TREATED OR EXPOSED TO INHALATION OF Δ^9-TETRAHYDROCANNABINOL DURING GESTATION DAYS 6 TO 15 AND SACRIFICED NEAR TERM

Species	Route of administration	Δ^9-THC dose (mg/kg)	Number of fetuses	Percent anomalies (all types)					
				External	Types	Soft tissue	Types	Skeletal	Types
CD-1 mice	Oral	Sham	755	2.2	2[a]	10.5	5[b]	7.5	3[c]
		Vehicle	1265	2.1	2[a]	13.2	7[b]	7.9	3[c]
		5-50	1310	2.5	2[a]	18.7	7[b]	16.2	3[c]
		150–600	795	2.3	2[a]	13.3	5[b]	14.5	3[c]
Swiss-Webster mice	Inhalation	Sham	263	0		1.1	2[d]	0	
		Placebo	202	0		1.0	2[d]	0	
		0.8–3.8	853	0		0.6	2[d]	0	
Fischer rats	Oral	Sham	677	0					
		Vehicle	563	0					
		12.5–50	1472	0					
	Inhalation	Sham	287	0		1.0	2[d]	0	
		Placebo	230	0		0.4	2[d]	0	
		0.8–3.8	799	0		0.4	2[d]	0	

a. Major findings were skin hemorrhages and backwardly curved hindlimbs.
b. Major anomalies were hydronephrosis, hemorrhages, hydrocephalus, cleft palate, hemothorax, hemoperitoneum and hepatic focal pale areas.
c. Major abnormalities were missing 5th sternebrae, hole in 6th sternebrae and extra right or left ribs.
d. Major anomalies were hydronephrosis and hydrocephalus; 1 low-dosed female rat had cleft palate.

MECHANISM OF EMBRYOTOXICITY

The prevalent observation of embryotoxicity in both inhalation and oral teratology studies stimulated closer examination of this adverse effect of cannabis. Based on a sufficient number of studies, it was apparent that the pregnant state of some animals was always in doubt. There was a lack of sufficient residual embryonic tissue for identification. This was a crucial circumstance since it compromised the significance of cannabis-related pregnancy interruption or inhibition of conception rate. In order to understand the reason for the doubtful pregnancies and to relate them to the process of embryotoxicity, all reproductive data were pooled from similar studies and were organized on the basis of known pregnancy rates for each rodent species (Table 4 and 5). The theoretical pregnancy rates were 55–70 percent for mice and 80–95 percent for rats. These pregnancy rates were equally applied to treated and control groups.

In the oral studies on mice, there were approximately 1–8 percent pregnancies in doubt in control groups. On the contrary, treated groups had a nearly dose-related 42–90 percent doubtful pregnancies at doses above 10 mg/kg. These doubtful pregnancies resulted in pregnancy rates not being in agreement with the theoretical one for dams treated with Δ^9-THC doses of 150–600 mg/kg. The percentage of doubtful pregnancies correlated with the percentage of dams having fetal resorptions. Moreover, there was a close correlation between the incidence of doubtful pregnancies and the percentage of dams with whole litter resorptions. There were no doubtful pregnancies in the inhalation study with mice (Table 4). There also were no incidences of whole litter resorption but there was a significant increase in the percentage of dams with fetal resorptions.

TABLE 4. PREGNANCY RATES AND NUMBER OF MOUSE DAMS EXHIBITING FETAL RESORPTION AFTER TREATMENT WITH Δ^9-TETRAHYDROCANNABINOL DURING GESTATION DAYS 6 TO 15 AND SACRIFICED NEAR TERM

Mouse strain	Route of adminis-tration	Δ^9-THC dose (mg/kg)		Number of expected pregnancies: Theoret-ical (N)	Found (N)	In doubt (N)	Pregnancy rate[a] (%)	Percent with early resorptions: One or more resorption	All resorbed
CD-1 mice	Oral	Sham	(3)[b]	71	65	6	67	47	4
		Veh.	(4)	114	107	7	60	40	9
		5	(1)	40	40	0	71	50	0
		10	(1)	40	40	0	68	65[c]	0
		50	(1)	40	38	2	56	66[c]	5
		150	(4)	111	64	47	26[d]	82[d]	65[d]
		300	(3)	67	20	47	20[d]	96[d]	76[d]
		600	(3)	68	7	61	3[d]	100[d]	95[d]
Swiss-Webster mice	Inhala-tion	Sham	(1)	23	23	0	62	52	0
		Placebo	(1)	19	19	0	63	63	0
		0.8	(1)	29	29	0	62	66	0
		2.6	(1)	27	27	0	70	70	0
		3.8	(1)	30	30	0	70	73[c]	0

a. Based on actual numbers of mice mated and unequivocally established pregnant by presence of at least one fetus.
b. Values in parenthesis indicate number of studies performed.
c. $P < 0.05$.
d. $P < 0.01$.

TABLE 5. PREGNANCY RATES AND NUMBER OF RAT DAMS EXHIBITING FETAL RESORPTION AFTER TREATMENT WITH Δ^9-TETRAHYDROCANNABINOL DURING GESTATION DAYS 6 TO 15 AND SACRIFICED NEAR TERM[a]

Rat strain	Route of adminis-tration	Δ^9-THC dose (mg/kg)	Number of expected pregnancies: Theoret-ical (N)	Found (N)	In doubt (N)	Pregnancy rate[b] (%)	Percent with early resorptions: One or more resorptions	All resorbed
Fischer 344	Oral	Sham	70	70	0	94	21	0
		Vehicle	65	65	0	94	32	0
		12.5	63	55	8	79	45	12[d]
		25.0	64	55	9	77	50[c]	18[d]
		50.0	61	51	10	69[c]	74[d]	25[d]
Fischer 344	Inhala-tion	Sham	31	31	0	91	13	0
		Placebo	30	30	0	85	30	0
		0.8	30	30	0	86	27	0
		2.6	30	30	0	77	23	0
		3.8	33	33	0	92	21	0

a. Oral results are the mean of three studies and there was one inhalation study.
b. Based on actual numbers of rats mated and unequivocially established pregnant by presence of at least one fetus.
c. $P < 0.05$.
d. $P < 0.01$.

A similar assessment of reproductive data was performed for the rat studies (Table 5). There were no doubtful pregnancies in oral vehicle control groups. On the other hand, approximately 13–16 percent of doubtful pregnancies was possible among orally treated rat dams. However, only the high-dosed group had a pregnancy rate significantly different

from that of control groups. Of more certainty was a dose-related increase in the percentage of dams with fetal resorptions and whole litter resorptions.

The doses of Δ^9-THC used in the rat marihuana inhalation studies were insufficient to initiate significant changes in fetal resorption. There were no pregnancies in doubt (Table 5).

1. Temporal pattern of embryotoxicity

The effect of the number of treatments on fetal viability was established by administration of 2–10 oral treatments during the critical phase of organogenesis (Table 6). Since the embryotoxicity response was not dose related, the data on Δ^9-THC groups were pooled for simplicity. After 2 treatments on days 6 and 7 of gestation in mice, there were no dams with resorptions in vehicle or sham-treated controls. In contrast, 74 percent of treated dams had fetal resorptions. Treatment for 5 days in mice elicited fetal resorptions in 50–64 percent of controls and 100 percent in Δ^9-THC groups. A total of 8 oral treatments resulted in 60–67 percent control dams having fetal resorptions. Ten consecutive treatments during gestation days 6 to 15 evoked fetal resorptions in approximately 42 percent control dams and 100 percent in Δ^9-THC groups.

A similar temporal pattern of dams with fetal resorptions was observed in rats receiving more than 2 treatments with Δ^9-THC (Table 6). After 2 treatments, no control or treated

TABLE 6. TEMPORAL PATTERNS OF EMBRYOTOXICITY IN CD-1 MICE AND FISCHER RATS GIVEN ORAL Δ^9-TETRAHYDROCANNABINOL

Treatment on gestation days	Sacrifice on gestation day	Number of dams with resorptions/total dams					
		CD-1 mice[a]			Fischer 344 rats[a]		
		Sham	Vehicle	> 100 mg/kg	Sham	Vehicle	> 12 mg/kg
6–7	8	0/12	0/13	20/27	0/8	0/8	0/23
6–10	11	7/14	7/11	15/15	0/10	0/10	13/28
6–13	14	8/12	9/15	16/18	2/10	2/9	10/22
6–15	17–19	7/16	6/15	17/17	5/21	7/19	27/44

a. Results on Δ^9-THC groups, 150, 300 and 600 mg/kg, for mice and 12.5, 25 and 50 mg/kg for rats, were pooled for a simpler presentation.

dams had fetal resorptions. Continued treatment for 5 days instituted no resorptions in control rats but 46 percent of Δ^9-THC dams had resoptions. When 8 consecutive treatments were administered, approximately 16 percent control dams had resorptions while 45 percent of treated dams were involved. Ten treatments produced fetal resorptions in 24–37 percent control dams and 61 percent in Δ^9-THC dams.

When whole litter resorptions were related to the number of treatments, there was a significant dose-related increase in whole litter resorption among mice (53–100 percent) after 2–5 dosages of oral Δ^9-THC[22]. Vehicle and sham-treated control mice had approximately a 6 percent incidence of whole litter resorptions. In the instance of rats, the response was not strictly dose related and the incidences of whole litter resorption was 18–32

percent after 5 treatments. Both control groups had a 0–2 percent incidence of whole litter resorption.

In a study in which rats were orally treated with 50 mg/kg on days 5 to 7, 6 to 8, 7 to 9, 8 to 10 or 9 to 11 and sacrificed on day 14 of gestation, optimal susceptibility to embryotoxicity occurred between days 7 to 9 of gestation(22). At this time interval, 36 percent of Δ^9-THC-treated dams had whole litter resorptions and none of the vehicle controls did.

In the investigation on rats in which pregnancy was unequivocally confirmed by laparotomy, 50 mg/kg given orally on days 6 to 9 of gestation induced whole litter resorption in 27 percent of dams sacrificed on day 12 and in 42 percent of dams sacrificed on day 16. Neither vehicle control or sham-treated dams exhibited whole litter resorption at either sacrifice interval(22).

2. *Embryotoxicity and vaginal bleeding*

Vaginal bleeding at mid-gestation has been considered a sign of pregnancy in rodents(95) but in the present investigation, vaginal bleeding correlated with decreases in uterine weights and fetal resorption in mice (Table 7). An oral Δ^9-THC dose of 150 mg/kg induced approximately a 21 percent incidence of fetal resorptions in conjunction with a 94 percent reduction in dam uterine weights and a 9 percent incidence in dam vaginal bleeding. At 300 mg/kg 68 percent fetal resorptions occurred concomitantly with a 93 percent decline in uterine weights and a 33 percent incidence of vaginal bleeding in dams. The highest Δ^9-THC dose of 600 mg/kg had corresponding values of 100 percent fetal resorptions, a 97 percent reduction in uterine weights and 36 percent of dams exhibited vaginal bleeding. There was approximately 8 percent fetal resorptions, normal uterine weights and only 1/185 dams displayed vaginal bleeding in combined control groups. Of the 56 treated mice exhibiting vaginal bleeding, 18 had 1 or 2 resorptions, 8 had multiple resorptions and 30 had whole litter resorptions.

TABLE 7. INTERRELATIONSHIP OF MOUSE EMBRYOTOXICITY, DAM UTERINE WEIGHTS AND DAM VAGINAL BLEEDING EVOKED BY Δ^9-TETRAHYDROCANNABINOL ADMINISTERED DURING ORGANOGENESIS

Mouse strain	Route of administration	Δ^9-THC dose (mg/kg)	Early fetal resorptions		Uterine weights mean ± S.D. (g)	Dam vaginal bleeding	
			Resorption/ total	Fetuses (%)		Bled/ total	Dams (%)
CD-1 mice[a]	Oral	Sham	77/755	10.2	5.8 ± 6.3	0/71	0
		Vehicle	83/1343	6.2	7.6 ± 7.8	1/114	1
		150	120/565	21.2[b]	0.4 ± 0.6[b]	10/111	9[b]
		300	123/181	68.0[b]	0.5 ± 0.8[b]	22/67	33[b]
		600	87/87	100.0[b]	0.2 ± 0.1[b]	24/68	36[b]
Swiss-Webster mice	Inhalation	Sham	17/284	6.0		0/31	0
						0/45	0
		Placebo	18/229	7.9		0/33	0
		0.8	46/331	13.9[b]		0/33	0
		2.6	37/352	10.5[b]		0/33	0
		3.8	46/332	13.9[b]		0/39	0

a. Mean results of three studies.
b. Results significant at $P < 0.05–0.01$.

Among the Swiss-Webster mice exposed to marihuana smoke during days 6 to 15 of gestation, none were observed to have vaginal bleeding. Similarly none of the control dams, placebo-exposed or sham treated dams had signs of vaginal bleeding.

DOSIMETRY

It was not feasible to estimate plasma Δ^9-THC levels during treatment. However, for the sake of monitoring equivalence of smoke exposure of treated and control dams, COHb concentrations were determined (Table 8). It should be pointed out that the COHb analy-

TABLE 8. CORRELATION OF ESTIMATED Δ^9-THC DOSE, PLASMA Δ^9-THC LEVELS AND CARBOXYHEMOGLOBIN CONCENTRATIONS[a]

Dosimetry		Levels of plasma Δ^9-THC and carboxyhemoglobin						
		Pregnant mouse		Pregnant rat		Non-pregnant rat[b]		Human male
Estimated Δ^9-THC (mg/kg)	Puffs (3 cigs.)	THC (ng/ml)	COHb (%)	THC (ng/ml)	COHb (%)	THC (ng/ml)	COHb (%)	THC (ng/ml)
Placebo	6	0	33 ± 15	0	54 ± 4			
0.8	4	73 ± 15	29 ± 11	81 ± 60	21 ± 12			
2.6	8	123 ± 21	43 ± 13	183 ± 54	45 ± 9			
3.8	12	200 ± 34	60 ± 16	297 ± 75	54 ± 10			
Placebo	12					0	49 ± 9	
0.4	4					76 ± 27	15 ± 4	40–180[c]
1.1	8					179 ± 81	25 ± 10	
2.2	16					319 ± 135	42 ± 9	200–500[d]

a. Marihuana (2–2.8% Δ^9-THC) inhalation between days 6 and 15 of gestation for pregnant animals and 20 consecutive days for non-pregnant rats. Males used for mating were not exposed to marihuana.

b. Male and female rats not used in reproductive studies; literature values after single inhalation or i.v. radiolabeled dose (5 mg/kg) were 68–270 ng/ml for mouse and rat (Gill *et al.*, 1974; Leighty, 1973; Willinsky *et al.*, 1974).

c. Acute study using one radiotracer labeled marihuana cigarette (Skinner, 1972; Galanter *et al.*, 1972; Agurell *et al.*, 1973; Lemberger, 1973; Rosenfeld *et al.*, 1974).

d. Chronic marihuana users consuming unknown number of cigarettes (Gross *et al.*, 1974; Teale *et al.*, 1974).

tical procedure used tended to have an approximately 10 percent error on the high side. Furthermore, animals exposed to smoke develop some tolerance to smoke intoxication during continued treatment and symptoms of smoke toxicity diminished with time. In effect, COHb levels are tolerated that initially are debilitating. For example, a single continuous exposure of rodents, particularly mice, to marihuana or tobacco smoke for a period of time necessary to induce COHb levels of 35–45 percent evoked lethality. With prolonged treatment, these and higher levels of COHb did not induce mortality. It has been demonstrated that CO is cleared from the blood within 2 hr after each exposure.

The COHb concentrations attained in the present study were dose related and COHb levels of treated and placebo groups were similar (Table 8). In rats, mice, hamsters and guinea pigs exposed to single or multiple doses of tobacco smoke, non-lethal mean COHb values between 25 and 60 percent have been reported[7, 61, 71].

Upon completion of the marihuana inhalation studies, it was found that plasma Δ^9-THC levels were dose related (Table 8). Moreover, the range of plasma Δ^9-THC concentrations in the rodents approximated those reported for man[2, 28, 33, 50, 72, 86, 90].

For the sake of comparison, plasma Δ^9-THC findings from a chronic inhalation study in non-pregnant rats are included in Table 8[22]. Others have reported a range of 68–270 ng/ml for mice and rats after a single exposure to marihuana smoke or a Δ^9-THC i.v. dose of 5 mg/kg[30, 49, 97].

POSTNATAL DEVELOPMENT

In a separate pilot experiment in which dams were treated with an oral Δ^9-THC dose of 1, 5 or 10 mg/kg or sesame oil vehicle during 21 days of gestation, they were permitted to deliver their offspring. The pups remained with their natural mothers until they achieved a body weight of 30–40 gm. At this time one-half the offspring of each treated and control group was given a single oral dose of Δ^9-THC of 10 mg/kg. The other one-half of pups received sesame oil. Nearly all pups from Δ^9-THC-treated mothers exhibited a dose-related hypersensitivity and approximately 10–25 percent of them responded with involuntary vertical jumping ("popcorn" response). The half receiving vehicle behaved normally. None of the offspring from vehicle control dams responded to sesame oil in an abnormal fashion. Administration of Δ^9-THC evoked CNS-inhibition.

DISCUSSION

The unequivocal embryocidal effect of marihuana and its major psychoactive agent, Δ^9-THC, has been demonstrated in rodents. The ability of cannabis products to elicit whole litter resorptions in which no vestigal embryonic tissue could be discerned was also established. Although the embryocidal effect was not always dose-related, it occurred at Δ^9-THC doses that were relevant to heavy, chronic marihuana use in man.

It was determined that two oral treatments of Δ^9-THC in mice or five in rats commencing on day 6 of gestation were adequate to induce embryotoxicity. The period of greatest susceptibility to fetal resorption was between days 7 to 9 of gestation which was in agreement with the findings of other investigators[42, 53].

The mechanism of embryotoxicity was not identified but there was a correlation between mid-gestation vaginal bleeding and excessive fetal resorption. This observation suggested that there may have been a direct effect of cannabis on the endometrium and associated development of the fetoplacental circulatory tree. On the other hand, a similar result could have been accomplished indirectly by a deficiency of hormones necessary to maintain normal pregnancy. In regard to a potential direct effect by cannabis, it is known that Δ^9-THC traverses the placental barrier of mice[25, 35, 44, 81], rats[94], hamsters[39] and dogs[55], and deposits in yolk sac, fetal tissues and amniotic fluid.

In the instance of a potential indirect effect through alteration of hormone secretion, both tracer disposition studies and hormone measurements have associated marihuana components with endocrine glands. Δ^9-THC doses penetrate the blood-brain barrier[1, 83] and reach the critical areas of the pituitary gland[48, 55, 58, 81] and hypothalamus[18, 37, 84] of a variety of species including monkey. An anti-gonadotrophic effect of Δ^9-THC was not mediated through the pituitary[87] but there is ample evidence that growth hormone levels[15, 46], LH levels[88, 89], FSH levels[19] and prolactin

levels[11,13,47] are suppressed in rats. Adrenal steroidogenesis is inhibited by cannabinoids[96] as well as steroid metabolism[57].

A number of effects have been elicited by cannabis products directly on the rodent uterus. Ovulation has been inhibited[63], uterine weights depressed[16,65] and water and glycogen content were deranged[12]. The earlier confusion revolving around the uterotropic effect of Δ^9-THC has been partially resolved by the demonstration of different binding sites for estrogens and cannabinoids[64]. Although not investigated directly on the uterus, Δ^9-THC has been shown to influence nucleic acid and protein synthesis and the activity of lysosomes[10,60].

One additional point must be addressed and that is the contribution of CO on fetal development in inhalation studies with marihuana. In the present studies there was a significant difference between the number of fetal resorptions in treated groups compared with placebo groups. However, it can be reasoned that CO may have contributed to the extent of embryotoxicity because of diminution in the available oxygen to fetuses. Other investigators have reported toxic changes in fetal rats and pups at COHb concentrations less than 20 percent. Persistent neurotoxic effects[20], cardiac hypertrophy[70] and reduced birth rates[21] have been seen. Slight numbers of fetal mortality were observed in rats exposed to carbon monoxide[32] but virtually none after exposure to tobacco[71]. Neonatal mortality was significantly increased (35 percent versus 1 percent in controls) among rabbits at COHb levels of 18 percent[3]. Fetal brain injury occurred, but no embryotoxocity, in monkeys exposed to carbon monoxide intoxication[31]. There is a paucity of data on the mouse in part due to their greater sensitivity to carbon monoxide[38]. Minimal effects on pregnant rats and their fetuses were observed after exposure to tobacco smoke[62].

Despite the discrepancies in cannabis-evoked teratological signs, the conclusion that cannabinoids are embryotoxic cannot be avoided. Furthermore the deposition of cannabinoids in fetal tissue for periods long enough to affect postnatal development must be reckoned with since fetal cannabinoid levels may be augmented during lactation[14,25,40]. The residual sensitivity of offspring to Δ^9-THC has been demonstrated in the present studies on rats and EEG changes in fetal guinea pigs have extended into the postnatal period[85].

ACKNOWLEDGEMENT

The author recognizes the extensive contributions of both professional and technical staff members to these studies: Dr. Robert W. Fleischman, John R. Baker, Jean Fredette, R. Jeffrey Grant, Phyllis Hughes, Rachel Haskell, Jack Metterville and Forrest Tibbetts.

Supported in part by NIDA Contracts HSM 42-71-79 and 271-76-3320.

REFERENCES

1. Agnew, W. F., Rumbaugh, C. L. and Cheng, J. T., The uptake of Δ^9-tetrahydrocannabinol in choroid plexus and brain cortex *in vitro* and *in vivo*. *Brain Res.* **109**,335–366 (1976).
2. Agurell, S., Gustafsson, B. and Holstedt, B., Quantitation of Δ^1-tetrahydrocannabinol in plasma from cannabis smokers. *J. Pharm. Pharmacol.* **25**,554–558 (1973).
3. Astrup, P., Olsen, H. M., Trolle, D. and Kjeldsen, K., Effect of moderate carbon-monoxide exposure on fetal development. *The Lancet*, pp. 1220–1222 (1972).

4. BANERJEE, B. M., GALBREATH, C. and SOFIA, R. D., Teratologic evaluation of synthetic Δ^9-tetrahydrocannabinol in rats. *Teratology* **11**,99–102 (1975).
5. BECKER, B. A., In "Toxicology," Louis J. Casarett and John Doull, eds., Macmillan, New York, pp. 313–332, 1975.
6. BIDDLE, F. G., 6-Aminonicotinamide-induced cleft palate in the mouse: the nature of the difference between the A/J and C57BL/6J strains in frequency of response and its genetic basis. *Teratology* **16**,301–312 (1977).
7. BINNS, R., BEVEN, J. L., WILTON, L. V. and LUGTON, W. G. D., Inhalation toxicity studies on cigarette smoke. II. Tobacco smoke inhalation dosimetry studies on small laboratory animals. *Toxicology* **6**,197–206 (1976).
8. BORGEN, L. A., DAVIS, W. M. and PACE, H. B., Effects of synthetic Δ^9-tetrahydrocannabinol on pregnancy and offspring in the rat. *Toxicol. Appl. Pharmacol.* **20**,480–486 (1971).
9. BORGEN, L. A., DAVIS, W. M. and PACE, H. B., Effects of prenatal Δ^9-tetrahydrocannabinol on the development of rat offspring. *Pharmacol. Biochem. Behav.* **1**,203–206 (1973).
10. BRAUDE, M. C. and SZARA, S., "Pharmacology of Marihuana." Raven Press, New York, Vol. 2, 461–492, 1976.
11. BROMLEY, B. and ZIMMERMAN, E., Divergent release of prolactin and corticosterone following Δ^9-tetrahydrocannabinol injection in male rats. *Fed. Proc.* **35**,220 (1976).
12. CHAKRAVARTY, I. and GHOSH, J. J., Effect of cannabis extract on uterine glycogen metabolism in prepubertal rats under normal and estradiol-treated conditions. *Biochem. Pharmacol.* **26**,859–863 (1977).
13. CHAKRAVARTY, I., SHETH, A. R. and GHOSH, J. J., Effect of acute Δ^9-tetrahydrocannabinol treatment on serum luteinizing hormone and prolactin levels in adult female rats. *Fertil. Steril.* **26**,947–948 (1975).
14. CHAO, F.-C., GREEN, D. E., FORREST, I. S., KAPLAN, J. N., WINSHIP-BALL, A. and BRAUDE, M. C., The passage of ^{14}C-Δ^9-tetrahydrocannabinol into the milk of lactating squirrel monkeys. *Res. Comm. Chem. Pathol. Pharmacol.* **15**,303–317 (1976).
15. COLLU, R., LETARTE, J., LEBOEUF, G. and DUCHARME, J. R., Endocrine effects of chronic administration of psychoactive drugs to prepubertal male rats. I. Δ^9-Tetrahydrocannabinol. *Life Sci.* **16**,533–542 (1975).
16. DIXIT, V. P., ARYA, M. and LOHIYA, N. K., The effect of chronically administered cannabis extract on the female genital tract of mice and rats. *Endokrinologie* **66**,365–368 (1975).
17. DUBOWSKI, K. M., Measurement of hemoglobin derivatives. In Sunderman, F. W. and Sunderman, F. W., Jr., eds., "Haemoglobin: its precursors and metabolites," J. B. Lippincott, Philadelphia, pp. 49–60, 1964.
18. ERDMANN, G., JUST, W. W., THEL, S., WERNER, G. and WIECHMANN, M., Comparative autoradiographic and metabolic study of Δ^8 and Δ^9-tetrahydrocannabinol in the brain of the marmoset callithrix jacchus. *Psychopharmacol.* **47**,53–58 (1976).
19. ESBER, H. J., ROSENKRANTZ, H., KUO, E. H. and BRAUDE, M. C., Serum hormone levels in non-pregnant rats after chronic oral treatment with Δ^9-tetrahydrocannabinol. *Fed. Proc.* **34**,783 (1975).
20. FECHTER, L. D., Persistent neurotoxic effects of mild prenatal carbon monoxide exposure. *17th Ann. Meet. Soc. Toxicol.*, p. 105 (1978).
21. FECHTER, L. D. and ANNAU, Z., Toxicity of mild prenatal carbon monoxide exposure. *Science* **197**,680–682 (1977).
22. FLEISCHMAN, R. W., HAYDEN, D. W., NAQVI, R. H., ROSENKRANTZ, H. and BRAUDE, M. C., The embryotoxic effects of cannabinoids in rats and mice. *J. Environ. Pathol. Toxicol.* (being reviewed).
23. FLEISCHMAN, R. W., HAYDEN, D. W., ROSENKRANTZ, H. and BRAUDE, M. C., Prenatal effects of Δ^9-tetrahydrocannabinol in mice including a review of the literature. *Teratology* **12**,47–50 (1975).
24. FOURNIER, E., ROSENBERG, M., HARDY, N. and NAHAS, G. G., Teratologic effects of cannabis extracts in rabbits. In "Marihuana: Chemistry, Biochemistry and Cellular Effects," G. G. Nahas, ed., Springer-Verlag, New York, pp. 457–468, 1976.
25. FREUDENTHAL, R. I., MARTIN, J. and WALL, M. E., Distribution of Δ^9-tetrahydrocannabinol in the mouse. *Brit. J. Pharmacol.* **44**,244–249 (1972).
26. FRIED, P. A., Short and long-term effects of prenatal cannabis inhalation upon rat offspring. *Psychopharmacol.* **50**,285–291 (1976).
27. FRIED, P. A. and NIEMAN, G. W., Inhalation of cannabis smoke in rats. *Pharmacol. Biochem. Behav.* **1**,371–378 (1973).
28. GALANTER, M., WYATT, R. J., LEMBERGER, L., WEINGARTNER, H., VAUGHAN, T. B. and ROTH, W. T., Effects on humans of delta-9-tetrahydrocannabinol administered by smoking. *Science* **176**,934–936 (1972).
29. GEBER, W. F. and SCHRAMM, L. C., Effect of marihuana extract on fetal hamsters and rabbits. *Toxicol. Appl. Pharmacol.* **14**,276–282 (1969).
30. GILL, E. W. and LAWRENCE, D. K., Blood and brain levels of Δ^1-tetrahydrocannabinol in mice. The effect of 7-hydroxy-Δ^1-tetrahydrocannabinol. *Biochem. Pharmacol.* **23**,1140–1143 (1974).
31. GINSBERG, M. D. and MYERS, R. E., Fetal brain injury after maternal carbon monoxide intoxication. *Neurology* **26**,15–23 (1976).
32. GRABOWSKI, C. T., Postnatal effects on rats of prenatal exposure to carbon monoxide. *Teratology* **13**,23a (1976).
33. GROSS, S. J., SOARES, J. R., WONG, S. L. R. and SCHUSTER, R. E., Marijuana metabolites measured by a radioimmune technique. *Nature* **252**,581–582 (1974).

34. HALEY, S. L., WRIGHT, P. L., PLANK, J. B., KEPLINGER, M. L., BRAUDE, M. C. and CALENDRA, J. C., The effect of natural and synthetic Δ^9-tetrahydrocannabinol on fetal development. *Toxicol. Appl. Pharmacol.* **25**,450 (1973).
35. HARBISON, R. D. and MANTILLA-PLATA, B., Prenatal toxicity, maternal distribution and placental transfer of tetrahydrocannabinol. *J. Pharmacol. Exp. Ther.* **180**,446–453 (1972).
36. HARBISON, R. D., MANTILLA-PLATA, B. and LUBIN, D. J., Alternation of Δ^9-tetrahydrocannabinol-induced teratogenicity of stimulation and inhibition of its metabolism. *J. Pharmacol. Exp. Ther.* **202**,455–465 (1977).
37. HATTORI, T. and MCGEER, P. L., Electron microscopic autoradiography and Δ^8-(^{3}H)-tetrahydrocannabinol localization in brain tissue. *Toxicol. Appl. Pharmacol.* **39**,307–311 (1977).
38. HILADO, C. J. and CUMMING, H. J., Effect of carbon monoxide on Swiss albino mice. *J. Combust. Toxicol.* **4**,216–230 (1977).
39. IDANPAAN-HEIKKILA, J. E., FRITCHIE, G. E., ENGLERT, L. F., HO, B. T. and MCISAAC, W. M., Placental transfer of tritiated-1-Δ^9-tetrahydrocannabinol. *New Eng. J. Med.* **281**,330–332 (1969).
40. JAKUBOVIC, A., HATTORI, T. and MCGEER, P. L., Radioactivity in suckled rats after giving carbon-14-labeled tetrahydrocannabinol to the mother. *Eur. J. Pharmacol.* **22**,221–223 (1973).
41. JAKUBOVIC, A., MCGEER, P. L. and FITZSIMMONS, R. C., Effects of Δ^9-tetrahydrocannabinol and ethanol on body weight protein and nucleic acid synthesis in chick embryos. *J. Toxicol. Environ. Health* **1**,441–447 (1976).
42. JONEJA, M. G., A study of teratologic effects of intravenous, subcutaneous and intragastric administration of Δ^9-tetrahydrocannabinol in mice. *Toxicol. Appl. Pharmacol.* **36**,151–162 (1976).
43. JONEJA, M. G., Effects of Δ^9-tetrahydrocannabinol on hamster fetuses. *J. Toxicol. Environ. Health* **2**,1031–1040 (1977).
44. KENNEDY, J. S. and WADDELL, W. J., Whole-body autoradiography of the pregnant mouse after administration of ^{14}C-Δ^9-THC. *Toxicol. Appl. Pharmacol.* **22**,252–258 (1972).
45. KEPLINGER, M. L., WRIGHT, P. L., HALEY, S. L., PLANK, J. B., BRAUDE, M. C. and CALANDRA, J. C., The effect of natural and synthetic delta-9-tetrahydrocannabinol on reproductive and lactation performance in albino rats. *Toxicol. Appl. Pharmacol.* **25**,449 (1973).
46. KOKKA, N. and GARCIA, J. F., Effects of Δ^9-THC (tetrahydrocannabinol) on growth hormone and ACTH secretion in rats. *Life Sci.* **15**,329–338 (1974).
47. KRAMER, J. and BEN-DAVID, M., Suppression of prolactin secretion by acute administration of Δ^9-THC in rats. *Proc. Soc. Exp. Biol. Med.* **17**,482–484 (1974).
48. LAYMAN, J. M. and MILTON, A. S., Distribution of tritium labelled Δ^1-tetrahydrocannabinol in the rat brain following intraperitoneal administration. *Brit. J. Pharmacol.* **42**,308–310 (1971).
49. LEIGHTY, E. G., Metabolism and distribution of cannabinoids in rats after different methods of administration. *Biochem. Pharmacol.* **22**,1613–1621 (1973).
50. LEMBERGER, L., Tetrahydrocannabinol metabolism in man. *Drug Metab. Dispos.* **1**,461–468 (1973).
51. MAKER, B. S., KHAN, M. A. and LEHRER, G. M., The effect of self regulated Δ^9-tetrahydrocannabinol consumption of pregnant mice and offspring. *Fed. Proc.* **33**,540 (1974).
52. MANTILLA-PLATA, B., CLEWE, G. L. and HARBISON, R. D., Δ^9-Tetrahydrocannabinol-induced changes in prenatal growth and development of mice. *Toxicol. Appl. Pharmacol.* **33**,333–340 (1975).
53. MANTILLA-PLATA and HARBISON, R. D., Influence of alteration of tetrahydrocannabinol metabolism on tetrahydrocannabinol-induced teratogenesis. In "Pharmacology of Marihuana," Vol. 2, M. C. Braude and S. Szara, eds., Raven Press, New York, 733–746, 1976.
54. Marihuana and Health, Sixth Annual Report to Congress, Department of Health, Education and Welfare, Publ. No. (ADM) 77-443 (1977).
55. MARTIN, B. R., DEWEY, W. L., HARRIS, L. S. and BECHNER, J. S., ^{3}H-Δ^9-tetrahydrocannabinol distribution in pregnant dogs and their fetuses. *Res. Comm. Chem. Pathol. Pharmacol.* **17**,457–470 (1976).
56. MARTIN, B. R., HARVEY, D. J. and PATON, W. D. M., Biotransformation of cannabidiol in mice: identification of new acid metabolites. *Drug. Metab. Disp.* **5**,259–267 (1977).
57. MASKARINEC, M. P., SHIPLEY, G., NOVOTNY, M., BROWN, D. J. and FORNEY, R. B., Different effects of synthetic delta-9-tetrahydrocannabinol and cannabis extract on steroid metabolism in male rats. *Experientia* **34**,88–89 (1978).
58. MCISAAC, W. M., FRITCHIE, G. E., IDANPAAN-HEIKKILA, J. E., HO, B. T. and ENGLERT, L. F., Distribution of marihuana in monkey brain and concomitant behavioral effects. *Nature* **230**,593–594 (1971).
59. MIRAS, C. J., Some aspects of cannabis action. In "Hashish: its Chemistry and Pharmacology." G. E. W. Wolstenholme and J. Knight eds., Little, Brown, Boston, pp. 37–52, 1965.
60. NAHAS, G. G., PATON, W. D. M. and IDANPAAN-HEIKKILA, J. E., "Marihuana, Chemistry, Biochemistry and Cellular Effects." Springer-Verlag, New York, pp. 3–556, 1976.
61. NETTESHEIM, P., GUERIN, M. R., KENDRICK, J., RUBIN, I., STOKELY, J., CREASIA, D., MADDOX, W. and CATON, J. E., Control and maximization of tobacco smoke dose in chronic animal studies. In Proceedings of the Tobacco Smoke Inhalation Workshop on Experimental Methods in Smoking and Health Research. DHEW Publ. No. (NIH) 75-906. G. B. Gori, ed., pp. 17–26 (1975).

62. NIKITOVITCH-WINER, M. B. and FERRY, J. D., Influence of smoking on reproductive process in the rat. *Proc. Un. Kent. Tobacco & Health Res. Inst. Rep.* **4**,337-359 (1973).
63. NIR, I., AYALON, D., TSAFRIRI, A., CORDOVA, T. and LINDNER, H. R., Δ^1-Tetrahydrocannabinol: suppression of the cyclic surge of LH secretion and of ovulation in the rat. *Nature* **243**,470 (1973).
64. OKEY, A. B. and BONDY, G. P., Δ^9-Tetrahydrocannabinol and 17β-estradiol bind to different macromolecules in estrogen target tissue. *Science* **200**,312-314 (1978).
65. OKEY, A. B. and TRUANT, G. S., Cannabis-demasculinizes rats but is not estrogenic. *Life Sci.* **17**,1113-1118 (1975).
66. PACE, H. B., DAVIS, W. M. and BORGEN, L. A., Teratogenesis and marihuana. *Ann. N.Y. Acad. Sci.* **191**,123-131 (1971).
67. PERSAUD, T. V. N. and ELLINGTON, A. C., Cannabis in early pregnancy. *Lancet* **2**,1306 (1967).
68. PERSAUD, T. V. N. and ELLINGTON, A. C., Teratogenic activity of cannabis resin. *Lancet* **2**,406-407 (1968).
69. PHILLIPS, R. N., TURK, R. F. and FORNEY, R. B., Acute toxicity of Δ^9-tetrahydrocannabinol in rats and mice. *Proc. Soc. Exp. Biol. Med.* **136**,260-263 (1971).
70. PRIGGE, E. and HOCHRAINER, D., Effects of carbon monoxide inhalation on erythropoiesis and cardiac hypertrophy in fetal rats. *Toxicol. Appl. Pharmacol.* **42**,225-228 (1977).
71. RECKZEH, G., DONTENWILL, W. and LEUSCHNER, F., Testing of cigarette smoke inhalation for teratogenicity in rats. *Toxicology* **4**,289-295 (1975).
72. ROSENFELD, J. J., BOWINS, B., ROBERTS, J., PERKINS, J. and MACPHERSON, A. S., Mass fragmentographic assay for Δ^9-tetrahydrocannabinol in plasma. *Anal. Chem.* **46**,2232-2234 (1974).
73. ROSENKRANTZ, H., The immune response and marihuana. In "Marihuana: Chemistry, Biochemistry and Cellular Effects." G. G. Nahas ed., Springer-Verlag, New York, pp. 441-456, 1976.
74. ROSENKRANTZ, H. and BRAUDE, M. C., Acute, subacute and 23-day chronic marihuana inhalation toxicities in the rat. *Toxicol. Appl. Pharmacol.* **28**,428-441 (1974).
75. ROSENKRANTZ, H. and BRAUDE, M. C., Rat inhalation toxicity of Turkish marihuana. *Pharmacologist* **17**,181 (1975).
76. ROSENKRANTZ, H. and BRAUDE, M. C., Comparative chronic toxicities of Δ^9-tetrahydrocannabinol administered by inhalation or orally in rat. In "The Pharmacology of Marihuana." M. C. Braude and S. Szara eds., Raven Press, New York, Vol. II pp. 571-584, 1976.
77. ROSENKRANTZ, H., FLEISCHMAN, R. W. and BAKER, J. R., Embryotoxicity of marihuana by inhalation. *Fed. Proc.* **37**,737 (1978).
78. ROSENKRANTZ, H., HAYDEN, D. W. and BRAUDE, M. C., Inhalation toxicity of Turkish marihuana, cannabichromene and cannabidiol. *Fed. Proc.* **35**,643 (1976).
79. ROSENKRANTZ, H., HEYMAN, I. A. and BRAUDE, M. C., Inhalation, parenteral and oral LD 50 values of Δ^9-tetrahydrocannabinol in Fischer rats. *Toxicol. Appl. Pharmacol.* **28**,18-27 (1974).
80. ROSENKRANTZ, H., THOMPSON, G. R. and BRAUDE, M. C., Oral and parenteral formulations of marihuana constituents. *J. Pharm. Sci.* **61**,1106-1112 (1972).
81. RYRFELDT, A., RAMSAY, C. H., NILSSON, I. M., WIDMAN, M. and AGURELL, S., Whole-body autoradiography of Δ^1-tetrahydrocannabinol in the rat. *Psychopharmacol.* **27**,141-156 (1973).
82. SASSENRATH, E. N. and CHAPMAN, L. F., Tetrahydrocannabinol-induced manifestations of the "marihuana syndrome" in group-living macaques. *Fed. Proc.* **34**,1666-1670 (1975).
83. SCHOU, J., PROCKOP, L. D., DAHLSTROM, G. and ROHDE, C., Penetration of delta-9-tetrahydrocannabinol and 11-hydroxy-delta-9-tetrahydrocannabinol through the blood-brain barrier. *Acta Pharmacol. Toxicol.* **41**,33-38 (1977).
84. SHANNON, M. E. and FRIED, P. A., The macro- and microdistribution and polymorphic electroencephalographic effects of Δ^9-tetrahydrocannabinol in the rat. *Psychopharmacol.* **27**,141-156 (1972).
85. SINGER, P. R., SEIBETTA, J. J. and ROSEN, M. G., Simulated marihuana smoke in the maternal and fetal guinea pig. *Am. J. Obs. Gyn.* **117**,331-340 (1973).
86. SKINNER, R. F., Determination of submicrogram amounts of Δ^9-THC (tetrahydrocannabinol) and its metabolites. *Proc. West. Pharmacol. Soc.* **15**,136-138 (1972).
87. SMITH, C. G., BESCH, N. F., SMITH, R. G. and BESCH, P. K., Mechanism for the anti-gonadotrophic action of tetrahydrocannabinol (THC). *Fed. Proc.* **37**,724 (1978).
88. SMITH, C. G. and MOORE, C. E., Effect of delta-9-tetrahydrocannabinol (THC) on the secretion of male sex hormone in the rhesus monkey. *The Pharmacologist* **18**,248 (1976).
89. SYMONS, A. M., TEALE, J. D. and MARKS, V., Effect of Δ^9-tetrahydrocannabinol on the hypothalamic-pituitary-gonadal system in the maturing male. *J. Endocrin.* **68**,43P-44P (1976).
90. TEALE, J. D., FORMAN, E. J., KING, L. J. and MARKS, V., Radioimmunoassay of cannabinoids in blood and urine. *Lancet* **2**,533-555 (1974).
91. THOMAS, R. J., Toxicologic and teratologic effects of Δ^9-tetrahydrocannabinol in the zebrafish embryo. *Toxicol. Appl. Pharmacol.* **32**,184-190 (1975).
92. UYENO, E. T., Δ^9-Tetrahydrocannabinol administration during pregnancy of the rat. *Proc. West. Pharmacol. Soc.* **16**,64-67 (1973).
93. UYENO, E. T., Δ^9-Tetrahydrocannabinol administered to pregnant rats. *Pharmacologist* **17**,181 (1975).

94. VARDARIS, R. M., WEISZ, D. J., FAZEL, A. and RAWITCH, A. B., Chronic administration of delta-9-tetrahydrocannabinol to pregnant rats: studies of pup behavior and placental transfer. *Pharmacol. Biochem. Behav.* **4**,249–254 (1976).
95. VENABLE, J. W., Intrauterine bleeding in the pregnant albino rat. The "placental sign". *Anat. Rec.* **74**,273–293 (1939).
96. WARNER, W. and HARRIS, L. S., Inhibition of corticosteroidogenesis by delta-9-tetrahydrocannabinol. *Endocrinology* **101**,1815–1820 (1978).
97. WILLINSKY, M. D., KALANT, H., MERESZ, O., ENDRENYI, L. and WOO, N., Distribution and metabolism *in vivo* of carbon 14-labeled tetrahydrocannabinol in the rat. *Eur. J. Pharmacol.* **27**,106–119 (1974).
98. WILSON, J. C. and WARKARY, J., Methods of administering agents and detecting malformations in experimental animals. In "Teratology, Principles and Techniques," eds., University of Chicago Press, 1964.
99. WRIGHT, P. L., SMITH, S. H., KEPLINGER, M. L., CALANDRA, J. C. and BRAUDE, M. C., Reproductive and teratologic studies with Δ^9-tetrahydrocannabinol and crude marihuana extract. *Toxicol. Appl. Pharmacol.* **38**,223–235 (1976).

REPRODUCTION IN RHESUS MONKEYS CHRONICALLY EXPOSED TO DELTA-9-TETRAHYDROCANNABINOL

E. N. SASSENRATH, L. F. CHAPMAN, and G. P. GOO

Department of Behavioral Biology, University of California Medical School, and California Primate Research Center, Davis, California

Summary. Data is reported from 46 matings between 11 undrugged adult rhesus monkey breeders and 8 comparable breeders which had received daily oral doses of Δ^9-THC at 2.4 mg per kg for a 5-year period. Matings of drugged breeders showed no decrease in conceptions, but a 42 percent reproductive loss for matings of THC-treated females compared to an 8 to 11 percent loss for matings of undrugged females. The observed losses were not related to parity of mother or duration of drug intake and were non-specific relative to stage of pregnancy; i.e., they occurred as resorptions, abortions, fetal deaths, stillbirths and neonatal deaths. Non-viable term offspring appeared grossly normal, but exhibited a variety of non-specific abnormalities on histophathological evaluation.

Viable offspring of drugged mothers also appeared grossly normal. However, male infants had significantly lower birth rates. In general, offspring of THC-treated mothers tended to show subtle behavioral differences from control offspring in both responsivity to environmental stimuli and adaptability in peer social environments.

The non-specific and variable nature of these effects in a controlled primate test system suggests that such effects would be difficult to document in relation to marihuana use in the human population.

INTRODUCTION

DURING the last decade a number of studies have focused on the question of the possibility that marihuana use may present a significant risk for the offspring of exposed parents. Only a few, inconclusive, observations have been reported on humans[4, 15]. Almost all of these studies have utilized rodents, in part because of their obvious practical advantages in terms of low cost, large number of animals per litter, and relatively brief gestation period. In general, these studies have utilized an experimental model in which marihuana extracts or synthetic THC has been administered to pregnant females for a relatively brief time (assumed to be a critical period). The results of such studies have recently been summarized in a number of articles, including Fleishman *et al.*[7], Braude[3], Fournier *et al.*[8], and Harbison *et al.*[14], among others. In brief, some studies reported abnormalities and others did not. Differences in species and strains, routes of administration, dosage, stage of gestation and duration of exposure during pregnancy, as well as environmental conditions, details of experimental design and other factors have been suggested as possible explana-

tions of the divergence in the implications of specific studies for evaluation of potential hazards to human reproduction.

Several early studies[9, 10, 18, 19] reported embryotoxicity, fetal toxicity, and specific teratological malformations in rats, guinea pigs, hamsters and rabbits associated with exposure to natural cannabis extracts during pregnancy. In general, the amounts of the agents reported to result in frank teratology were well beyond the range used by humans. A number of later studies with synthetic THC failed to produce specific congenital malformations[12, 17], even with relatively large amounts of the agent. However, some (but not all) investigators have continued to report an increase in embryotoxicity and fetal toxicity[5, 7, 8, 13, 14, 20, 26]; the amounts used have often been large, but the observation of dose-dependent increases in fetal and embryonic deaths emphasizes the importance of assessing the possibility that lower levels of exposure might result in significant human risk.

While the method of administering suspected teratogenic agents only during presumed critical periods is an important and valid component of the assessment of reproductive hazards associated with a given pharmacologically active agent, in some instances it may not result in complete assessment. For example, the usual pattern of marihuana exposure is not duplicated by heavy dosage limited to brief periods during pregnancy: a much more common pattern is chronic exposure beginning months and years before pregnancy. In addition to the possibility of direct damage to male or female gametes, the capacity of the mother to develop adequate fetal life-support following conception could be adversely affected by changes in any of the variety of systems that contribute (endocrine, hematological, vascular, etc.). The perinatal period is also pertinent; exposure to THC interferes with maternal milk production[17].

Laboratory primates offer obvious advantages as animal models for assessment of human reproductive hazards. The principal disadvantages of using laboratory primates for this purpose lie in the slow rate of reproduction and high costs associated with controlled studies on sufficient numbers of animals. To our knowledge, only a single study with laboratory primates has been reported. Grilly, Ferraro and Braude[11] examined the reproductive histories of 8 chimpanzees which had been exposed to various amounts of cannabis or THC from 5 to over 150 times during a period which ended 1.5 months to 1.5 years before conception. One of the mothers experienced a miscarriage at 3 months. The remaining infants appeared normal at birth.

In 1973 we began a series of observations on possible social behavior changes associated with chronic long-term exposure to THC[23, 24]. These behavioral studies generated an opportunity to observe certain aspects of reproductive capacity under the condition of long-term chronic exposure in laboratory primates. The program has also permitted exploration of possible behavioral and developmental abnormalities in the live-birth offspring of THC-treated parents.

There is evidence from rodent studies to indicate that exposure of the pregnant rat to THC (10 mg/kg) on days 10–12 of gestation results in behavioral and developmental changes in the offspring. Cross fostering did not reduce these adverse effects, indicating a direct action on the developing fetus.

Although studies with non-human species inevitably must be evaluated with caution in terms of their relevance to humans, the rhesus monkey is a particularly attractive animal model for exploration of potential human reproductive risks from exposure to THC. For example, in addition to the obvious similarities common to other simian species, it is

known that the metabolism of THC in the rhesus follows pathways more closely related to those of man than those utilized by certain other simians[27].

We report here a summary of current data from ongoing studies. At this time, several major conclusions are apparently well established, with adequate numbers of animals for statistical evaluation. For other questions, the number of animals studied is still too small to permit firm conclusions, and other components (notably observations of the offspring of THC-treated parents) remain incomplete; nevertheless, in view of the paucity of information regarding reproductive function in primates treated with cannabis, we have included brief descriptions of these incomplete studies as a preliminary report.

METHODS

SUBJECTS

Breeders were 19 sexually mature colony-born rhesus monkeys, progressing from an age range of 3 to 4 years to 8 to 9 years during the 5-year study.

All females were mated in 3 to 5 sequential years during the study; all were primiparous at first conception. The males also were inexperienced breeders on first matings. In all matings, breeding behavior appeared normal. After timed matings, sperm plugs were observed and/or sperm recovered by vaginal swabbing of the female.

DRUG ADMINISTRATION

Δ^9-THC was given orally daily on preferred food to 5 of 12 female breeders and to 3 of the 7 male breeders, starting 3 to 18 months prior to first mating. During 4 of the 5 breeding seasons THC was given at 2.4 mg per kg per day. To clarify the reproductive effect of dosage, the level was raised to 4.8 mg per kg for both males and females prior to the third breeding season (October, 1975) and maintained at that level for females until weaning or loss of infant, after which the 2.4 mg per kg dose level was reinstituted.

BREEDING

During the first 3 breeding seasons, all conceptions occurred in group settings of 4 to 6 monkeys per cage. Breeding groups were left intact through births and weaning until second season conceptions were confirmed. Thereafter, all group-bred females were removed to individual cages after conception to assure standardization of pre-natal and post-natal mother-infant environments.

During the fourth and fifth breeding seasons, additional timed-matings were instituted for indoor-caged breeders. Females were caged in the same room as the male, and moved to his cage for 2 to 4 hours daily for 5 to 7 days mid-menses. This interval was calculated to cover oestrus for each female on the basis of daily records of vaginal bleeding to define menses.

Pregnancies were initially confirmed by cessation of regular menses together with the appearance of "placental sign" vaginal discharge. After 30 days, pregnancies were eval-

uated periodically by bimanual palpation (for size) and by detection of fetal heart-beat (for viability).

There were a total of 46 matings for 5 breeding seasons: i.e., 19 each for THC-treated and control females; and 27 each for THC-treated and control males.

RESULTS

REPRODUCTIVE POTENTIAL OF THC-TREATED BREEDERS

No differences in sexual function were observed between breeders under long-term daily treatment with THC at 2.4 mg per kg per day, compared to the undrugged controls with respect to cyclic endocrine changes or observable responses to sexual stimuli. The regularity and duration of menses were similar for drugged and undrugged females, as were the behavioral sexual receptivity and the regularity of conceptions in a group breeding environment or in timed matings of breeder pairs. Cyclic changes in plasma levels of progesterone and estrogen in the females were also within the normal range.

Similarly, outdoor-caged THC-treated males were comparable to undrugged controls, both in mean elevation of plasma testosterone levels during the breeding season and in the annual rhythm of this hormone. For both males and females, if THC-related effects on these measures were present during long-term chronic drug exposure, they were overshadowed by inter-individual variability in these measures.

REPRODUCTIVE DEFICIT FOR THC-TREATED BREEDERS

As shown in Table 1, based on 46 matings among 8 THC-treated and 11 non-drugged

TABLE 1. COMPARISON OF REPRODUCTIVE LOSS FOR 46 PREGNANCIES FROM MATINGS BETWEEN THC-TREATED AND UNDRUGGED (CONTROL) MALES AND FEMALES.

Matings		Births			Group difference	
Mother × father	N	Lost	Viable	Percent loss	Comparison matings	χ^2
THC × cont	14	6	8	42%	Cont-Cont	$P < .05$
THC × all	19	8	11	42%	Cont-All	$P < .005$
Cont × THC	14	2	12	14%	Cont-Cont	N.S.
All × THC	19	4	15	21%	All-Cont	N.S.
Cont × Cont	13	1	12	8%		
Cont × All	27	3	24	11%		
All × Cont	27	7	20	26%		

THC = THC-treated parent
Cont = undrugged parent
All = drugged + undrugged parent
P = Significance from contingency tables for chi-square test[6].

control breeders, the percent of pregnancies which did *not* result in viable offspring surviving to 6 months of age was higher for THC-treated mothers mated with undrugged fathers (42% loss) than for undrugged females mated with undrugged males (8%). This deficit did not change when matings with drugged fathers was included for both THC-treated and undrugged females. The chi square test for significance of group differences confirms that reproductive deficit differs from control levels only when considering matings with THC-treated mothers and regardless of the drug status of the father. In this study, matings between drugged females and drugged males have been too few to permit evaluation of possible interaction effects.

The distribution of reproductive loss occurred throughout the course of pregnancy and parturition as shown in Table 2. Losses occurred at a variety of stages in development: including *in utero*, as abortions and fetal deaths; at term, as stillbirths and neonatal deaths; and as post-natal infant deaths. Although the number of animals studied is small, the data indicate a non-specific distribution of conceptus losses during gestation and shortly after birth for pregnancies of THC-treated females.

TABLE 2. DISTRIBUTION OF REPRODUCTIVE LOSS FOR DIFFERENT STAGES OF PREGNANCY RELATIVE TO DRUG STATUS OF PARENT.

Parents			Offspring mortality					
Drug status			*In utero*			Perinatal		Post-natal
Mother	Father	Number matings	RE	AB	FD	SB	ND	ID
THC	× THC or Cont	19	2(1)	1^{T}	2(2)		1^{T}	2(1)
Cont	× THC	14			1		1	
Cont	× Cont	13					1	

RE = Resorption SB = Stillbirth
AB = Abortion ND = Neonatal death
FD = Fetal death ID = Infant death
T = Mating with THC-treated father
( ) = Number of females contributing to deficit

If reproductive loss is viewed relative to parity of mother, as in Table 3, the distribution again appears random. There is no evidence of greater loss during first pregnancies than during subsequent pregnancies. Neither is there evidence of increasing or decreasing risk with successive pregnancies.

To evaluate effects of environmental differences during successive birthing seasons, the success of individual matings is shown in Table 4 as they occurred for each female in each breeding season. Again, losses appear to be randomly distributed. It is of interest, however, that, in the year of higher THC dosage (1976) pregnancy losses occurred earlier in pregnancy as abortions and resorptions rather than later fetal, neonatal, or infant deaths.

The higher dose of THC does not appear to increase the contribution of drugged males (noted by T superscript) to reproductive loss. Further, no effect related to drugged fathers appeared during the last two seasons when THC-treated males were bred only to undrugged control females.

TABLE 3. REPRODUCTIVE DEFICIT FOR SUCCESSIVE PREGNANCIES OF INDIVIDUAL FEMALE BREEDERS RELATIVE TO PARITY OF MOTHER.

Reproductive deficit and parity of mother						
Females		Number of pregnancies				
Drug	I.D.	1	2	3	4	5
THC	6586	+^{T}	(F)	+		
	6604	(A)^{T}	+	(F)		
	6627	+	(I)	(I)	+	+
	6668	(N)^{T}	+^{T}	+^{T}	+	
	6840	+	(R)	+	(R)	
		2/5 40%	3/5 60%	2/5 40%	1/3 33%	0/1
None	6666	(A)	+	+	(F)^{T}	+^{T}
	6737	+	+	+	+^{T}	+^{T}
	6756	+^{T}	(N)^{T}	+^{T}	+	+
	6989	+	+^{T}	+^{T}		
	7024	+	+^{T}	+^{T}		
	7054	+	+	+^{T}	+^{T}	
	6962	+^{T}	+			
		1/7 14%	1/6 16%	0/6 0%	1/4 25%	0/3 0%

A = abortus N = neonatal death
F = fetal death R = resorption
I = infant death
T = mating with THC-treated male

One major difference between THC-treated and undrugged control female breeders was apparent in assessment of body weight changes during the course of the study. The mean body weight gain of THC-treated females during all pregnancies which came to full term was significantly less than that of undrugged control females: i.e., the percent body weight gain for 12 term pregnancies in THC-treated females was 10 ± 5 compared to 20 ± 9 for 21 term pregnancies in undrugged controls ($t = 3.11$; $P = 0.005$). In contrast, the average non-pregnant body weight gain per year was comparable between THC-treated and control females when individuals were equated for age and weight data were taken only during months when females were neither pregnant or lactating. The annual whole body weight gains for all females on this study also fell in the range of the norms established for colony-born rhesus monkeys by other workers[(2)].

PATHOLOGICAL OBSERVATIONS

Histopathological evaluation of all offspring on this study has not been completed: many offspring are still under observation for behavioral evaluation. However, observations on a limited number of offspring are completed and of interest here.

Of 7 early fetal and neonatal deaths prior to 1978, 3 were partially autolysed and one was a mummified fetus from an *in utero* death. Lesions observed in 2 offspring of THC-treated mothers and 1 offspring of a control mother mated with a THC-treated father are

TABLE 4. REPRODUCTIVE DEFICIT IN SUCCESSIVE BREEDING SEASONS FOR INDIVIDUAL FEMALE BREEDERS. THC DOSAGE WAS DOUBLED (TO 4.8 mg PER kg) DURING PREGNANCY AND LACTATION IN THE 1976 BIRTHING SEASON ONLY.

Reproductive deficit in successive breeding seasons						
Females		Breeding season (year of birth)				
Drug	I.D.	1974	1975	1976	1977	1978
THC	6586			$+^{T}$	(F)	+
	6604			$(A)^{T}$	+	(F)
	6627	+	(I)	(I)	+	+
	6668	$(N)^{T}$	$+^{T}$	$+^{T}$		+
	6840		+	(R)	+	(R)
		1/2 50%	1/3 33%	3/5 60%	1/4 25%	2/5 40%
Control	6666	(N)	+	+	$(F)^{T}$	$+^{T}$
	6737	+	+	+	$+^{T}$	$+^{T}$
	6756	$+^{T}$	$(N)^{T}$	$+^{T}$	+	+
	6989			+	$+^{T}$	$+^{T}$
	7024			+	$+^{T}$	$+^{T}$
	7054	+	+		$+^{T}$	$+^{T}$
	6962				$+^{T}$	+
		1/4 25%	1/4 25%	0/5 0%	1/7 14%	0/7 0%

A = abortus N = neonatal death
F = fetal death R = resorption
I = infant death
T = mating with THC-treated male

shown in Table 5. These included hydrocephalus, inguinal hernia, ectopic pancreas, and umbilical and myocardial degeneration. Two infants showing hydrocephalus were offspring of a THC and a control mother mated with the same drug-treated male in different birth years.

Since monkey births occur primarily at night and females normally eat the placental tissue after birth, fresh placentas were not usually available for examination. However, it has been possible to obtain one fresh placenta by caesarian section within 24 hours after death of a late third trimester fetus (140 days) of a THC-treated mother. This had gross morphological and vascular abnormalities of both lobes and was massively infarcted. Placentas from caesarian section deliveries of viable term infants of 2 undrugged control mothers (with vaginal prolapses) showed no comparable abnormality. This pathology has not been observed in placentas obtained from caesarian-section delivery of term pregnancies in the rhesus colony.

INFANTS OF THC-TREATED PARENTS

Surviving infants of THC-treated parents appeared grossly normal. However, birth weights of male offspring of THC-treated mothers were significantly smaller than offspring

TABLE 5. PATHOLOGICAL CHANGES NOTED IN THREE OF THE EARLY NEONATAL AND INFANT DEATHS IN THIS STUDY.

Offspring	Mother	Father	Pathological changes
No.			
7932	THC	THC	Stillbirth with hydrocephalus. umbilical artery degeneration; myocardial degeneration; renal cortical necrosis.
16088	THC	Cont	Infant, $2\frac{1}{2}$ mo. with inguinal hernia (resulting in intestinal obstruction); ectopic pancreas within the intestinal wall and pancreatic atrophy.
8239	Cont	THC	Neonatal death with hydrocephalus and inguinal hernia.

of control mothers. They were also significantly smaller than a larger reference sample of colony male offspring with comparable pre- and post-natal environments, as shown in Table 6. All birth weights of male offspring of THC-treated females fell below the mean and median of the reference sample. Birth weights of male offspring of THC-treated fathers (with control mothers) or non-drugged parents (experimental controls), however, were distributed throughout the range of birth weights of the reference sample, as were the weights of female offspring of THC-treated mothers.

Surviving offspring from this study have also been subjected to a series of behavioral characterizations during their first year of life. The behavior of the first season's offspring of THC-treated mothers was suggestive of hyperactivity and over-responsiveness to envir-

TABLE 6. COMPARISON OF MEAN BIRTH WEIGHTS OF INFANTS BORN TO THC-TREATED AND UNDRUGGED PARENTS.

Experimental group		N	Birth weight (g.)		Comparison group	Significance	
Offspring	Parents		(mean)	(S.D.)		*t*	P
Males	THC-mothers	7	430.6	52.1	Control mothers	2.59	0.025
					Experimental controls	2.83	0.01
					Colony controls	3.07	0.005
	THC-fathers	3	483.3	123.3	Experimental controls	0.61	N.S.
					Colony controls	0.80	N.S.
	Control mothers	7	527.8	84.4	Colony controls	0.27	N.S.
	Experimental controls*	2	540.0	7.1	Colony controls	0.38	N.S.
	Colony controls	54	519.6	74.1			
Females	THC-mothers	6	471.7	48.8	Control mothers	0.77	N.S.
					Experimental controls	0.36	N.S.
					Colony controls	0.56	N.S.
	THC-fathers	6	510.8	72.1	Experimental controls	0.62	N.S.
	Control mothers	15	496.3	72.0	Colony controls	0.50	N.S.
	Experimental controls	8	485.0	79.6	Colony controls	0.06	N.S.
	Colony controls	48	486.7	63.3			

* Experimental controls: Offspring of undrugged parents.

onmental stimuli, together with a lack of appropriate cautious or avoidance behaviors in novel environments. In order to study this further, the gestation, parturition, and preweaning environments have been carefully standardized for subsequent mother-infant pairs to minimize the contribution of early environmental differences to subsequent observed behavioral differences in offspring.

To date, we have characterized 9 infants from 1 week to 1 year of age and are studying an additional 8 newborns. The data suggest that offspring of THC-treated mothers show (1) enhanced responsivity—both autonomic and behavioral—to visual and auditory stimuli, (2) less fear and avoidance behavior in a novel environment, and (3) over-stimulation and assertiveness in response to peer socialization. Sex differences in these infant responses are apparent as well as differences in maternal behavior between THC-treated and undrugged mothers; data on a larger number of mother-infant pairs is essential to assess adequately these drug-related effects on offspring behavior.

DISCUSSION

This study has examined the reproductive outcome of 5 breeding seasons in rhesus monkeys exposed to THC at a level equivalent to moderately heavy marihuana usage in the U.S. The number of animals treated was necessarily small in comparison to rodent studies, and the data are insufficient to answer a number of important questions. However, in terms of statistical significance, several findings can be expressed with confidence.

First, it was clearly established that daily maternal oral intake of THC in the range of 2.4 to 4.8 mg/kg resulted in significant reproductive casualties. The rate of reproductive loss in the treated animals was approximately 40%; reproductive loss in specific control animals and in a large breeding program at the same center approximated 10%. There was some evidence that the higher dosage (4.8 mg/kg) resulted in a greater and earlier loss. Reproductive losses occurred from the first trimester to early infant death. The pattern of reproductive deficit indicated embryonic and fetal toxicity and was not characterized by specific consistent congenital anomalies. Survivors appeared morphologically normal. Exposure of the father (but not the mother) to chronic THC in the same dose range did not result in significant reproductive loss beyond control levels.

Secondly, a sufficient number of animals were studied to permit the conclusion, with a high level of statistical confidence, that the birth weight of male infants born to THC-treated mothers is less than comparable control weights. The birth weights of all male infants born to THC-treated mothers were all less than the mean or median birth weights of control animals. The mean birth weight of female offspring of THC-treated mothers was not significantly less than mean control birth rate. This finding is in keeping with the greater vulnerability of male conceptions to adverse prenatal, perinatal and neonatal influences in macaque monkeys and in man[22]. The probable pathological basis for the observed increase in reproductive loss remains a matter of speculation.

A non-specific, variable drug action on gametes of long-term THC-treated parents might be relevant; however, the evidence from these studies is not supportive of such a hypothesis. There was no evidence of a deficit related to drug exposure of the sperm of THC-treated fathers. Also, evidence for a contribution of direct damage to the ova is lacking. Our studies revealed no evidence for a cumulative effect over sequential pregnancies in individual drugged females.

The absence of frank teratology and the non-specific nature of pregnancy interruption and morphological changes suggest that a proximal cause of drug-related decrements may be impairment of the maternal support for the fetus, possibly due to alterations of the complex mechanisms involved in establishing an optimally functioning placental circulation and other aspects of the overall fetal support system. The observations of placental infarction and umbilical degeneration in pathological evaluation of a few late pregnancy deaths support this possibility, as does the lower body weight gain during pregnancy of THC-treated mothers.

In broad terms, the present study indicates that in the rhesus monkey, chronic exposure to moderately large amounts of THC results in embryotoxicity and fetotoxicity. There was no evidence of specific teratology in the sense of a specific reproducible pattern of congenital malformation. Surviving infants were grossly normal in appearance. This overall pattern of findings is closely similar to that recently observed by Rosenkrantz *et al.*[21] in rodents. They found that exposure to relatively small (0.9–3.5 mg/kg) amounts of THC inhaled in smoke on days 5 and 6 of gestation resulted in significant embryotoxicity in mice, but not in specific teratological patterns.

Although behavioral characterization of viable offspring from this study is still in progress, current data suggest that the offspring of THC-treated mothers show altered autonomic and behavioral responsiveness to visual, auditory, and social environmental stimuli.

The limitations and qualifications which must be applied in extrapolating findings based on animal models in evaluating potential human health hazards are well known. However, the results of the present study with laboratory primates, and those of a number of investigators who have observed increased embryotoxicity and fetotoxicity with moderate levels of THC exposure, raise the possibility that exposure of the human female to marihuana in amounts in relatively common use may be associated with an increased risk of reproductive loss. There is also evidence suggesting that surviving infants may also be at increased risk for subsequent behavioral and developmental abnormalities, although there is no evidence of increased risk of physical abnormality in these offspring.

If, indeed, marihuana use in the moderately heavy range is associated in humans with increased reproductive hazard, a question arises as to why the increased risk has not been readily recognized in clinical practice, since marihuana use during childbearing age is widespread. Clues to the answers may lie in the observations with animal models that (1) viable offspring do not appear physically unusual, (2) the embryotoxic and fetotoxic actions are variable in this dosage range; one pregnancy may terminate early, but the next may result in a live birth, and finally, (3) animals that die *in utero* do not exhibit a consistent teratological pattern.

These factors combine in such a manner that the type of increased reproductive risk suggested by the studies with animal models would be unusually difficult to recognize in the human population. A number of associated problems concerned with identifying the amount of marihuana exposure as well as problems of multiple drug exposure add to the difficulties of designing and conducting studies to assess potential reproductive risks in humans.

The delayed definition of the fetal alcohol syndrome may also be instructive. Despite a long history of recognition that heavy alcohol consumption might be related to increased reproductive risks, a specific syndrome was not defined until a decade ago; it is now recognized that this syndrome is not rare.

Although there are many difficult problems in conducting human clinical studies on this question, these considerations underscore the need for additional information regarding the possibility that increases in reproductive hazards may be associated with human exposure to marihuana.

ACKNOWLEDGEMENT

We wish to acknowledge the continuing cooperation and support of the administration and staff of the California Primate Research Center, especially members of Pathology Services of the Primate Medicine Unit, which performed all autopsy and histopathology evaluations of subjects in this study.

Δ^9-THC has been supplied by the National Institute on Drug Abuse.

This investigation was supported by USPHS grant DA-00135. Additional partial support has been provided by USPHS grants RR-00169 to the California Primate Research Center and RR-05684 to the School of Medicine, University of California, Davis.

REFERENCES

1. Borgen, L. A., Davis, W. M. and Pace, H. B., Effects of prenatal delta-nine-tetrahydrocannabinol on the development of rat offspring. *Pharmacol. Biochem. Behav.* **1**,203 (1973).
2. Bourne, G. H., Collected anatomical and physiological data from the rhesus monkey. In "The Rhesus Monkey." Vol. 1. "Anatomy and Physiology." Ed. G. H. Bourne. Academic Press, New York, 1975.
3. Braude, M. C., Genetics and Reproduction, Introduction. In "Pharmacology of Marihuana." Vol. 2. Ed. M. C. Braude and Szara. Raven Press, New York, 1976.
4. Carakushansky, G., Neu, R. L. and Gardner, L. I., Lysergide and cannabis as possible teratogens in man. *Lancet* **1**,150 (1969).
5. Clarren, S. K. and Smith, D. W., The fetal alcohol syndrome. *New Eng. Jour. Med.*, **298**,1063 (1978).
6. Finney, D. J., Latscha, R., Bennett, B. M. and Hsu, P., "Tables for testing significance in a 2×2 contingency table." University Press, Cambridge, 1963.
7. Fleischman, R. W., Hayden, D. W., Rosenkrantz, H. and Braude, M. C., Teratologic evaluation of Δ^9-tetrahydrocannabinol in mice, including a review of the literature. *Teratology*, **12**,47 (1975).
8. Fournier, E., Rosenberg, E., Hardy, N. and Nahas, G., Teratologic effects of cannabis extracts in rabbits: a preliminary study. In "Marihuana: Chemistry, Biochemistry, and Cellular Effects." Ed. G. G. Nahas. Springer-Verlag, New York, 1976.
9. Geber, W. F. and Schramm, L. C., Effects of marihuana extract on fetal hamsters and rabbits. *Toxic. Appl. Pharmacol.* **14**,276 (1969).
10. Geber, W. F. and Schramm, L. C., Teratogenicity of marihuana extract as influenced by plant origin and seasonal variation. *Arch. Int. Pharmacol. Ther.*, **177**,224 (1969).
11. Grilly, D. M., Ferraro, D. P. and Braude, M. C., Observations on the reproductive activity of chimanzees following long-term exposure to marihuana. *Pharmacology* **11**,304 (1974).
12. Haley, S. L., Wright, P. L., Plank, J. B., Keplinger, M. L., Braude, M. C. and Calandra, J. C., The effect of natural and synthetic delta-9 tetrahydrocannabinol on fetal development. *Toxicol. Appl. Pharmacol.* **25**,450 (1972).
13. Harbison, R. D. and Mantilla-Plata, B., Prenatal toxicity, maternal distribution and placental transfer of tetrahydrocannabinol. *Jour. Pharmacol. Exptl. Ther.* **180**,446 (1972).
14. Harbison, R. D., Mantilla-Plata, B. and Lubin, D. J., Alteration of Δ^9-tetrahydrocannabinol-induced teratogenicity by stimulation and inhibition of its metabolism. *Jour. Pharm. Exptl. Ther.* **202**,455 (1977).
15. Hecht, F., Beals, R. K., Lees, M. H., Jolly, H. and Roberts, P., Lysergic acid diethylamide and cannabis as possible teratogens in man. *Lancet* **2**,1087 (1968).
16. Joneja, M. G., Effects of Δ^9-tetrahydrocannabinol on hamster fetuses. *Jour. Tox. Envir. Health*, **2**,1031 (1977).
17. Pace, H. B., Davis, W. M. and Borgen, L. A., Teratogenesis and marijuana. *Ann. N.Y. Acad. Sci.* **191**,123 (1971).
18. Persaud, T. V. N. and Ellington, A. C., Cannabis in early pregnancy. *Lancet* **2**,1306 (1967).

19. PERSAUD, T. V. N. and ELLINGTON, A. C., Teratogenic activity of canabis resin. *Lancet*, **2**,406 (1968).
20. PHILLIPS, R. N., TURK, R. F. and FORNEY, R. B., Acute toxicity of delta-nine tetrahydrocannabinol in rats and mice. *Proc. Sco. Exp. Biol. Med.* **136**,260 (1971).
21. ROSENKRANTZ, H., FLEISHMAN, R. W. and BAKER, J. R., Embryotoxicity of marihuana by inhalation. *Fed. Proc.* **37**(3),737 (Abstract) (1978).
22. SACKETT, G., HOLM, R. and LANDESMAN-DWYER, S., Vulnerability for abnormal development: pregnancy outcomes and sex differences in macaque monkeys. In "Aberrant Development in Infancy: Human and Animal Studies." Ed. Norman R. Ellis. John Wiley and Sons, New York, 1975.
23. SASSENRATH, E. N. and CHAPMAN, L. F., Tetrahydrocannabinol-induced manifestations of the "marihuana syndrome" in group-living macaques. *Fed. Proc.* **34**,1666 (1975).
24. SASSENRATH, E. N. and CHAPMAN, L. F., Primate social behavior as a method of analysis of drug action: studies with THC in monkeys. *Fed. Proc.* **35**,2238 (1976).
25. SASSENRATH, E. N., GOO, G. P. and CHAPMAN, L. F., Behavioral effects of acute and long-term chronic exposure to Δ^9-THC in group-living rhesus monkeys. (See This Symposium pp. 693–712).
26. UYENO, E. T., Delta-nine tetrahydrocannabinol administration during pregnancy of the rat. *Proc. West. Pharmacol. Soc.* **16**,64 (1973).
27. WURSCH, M. S., OTIS, L., GREEN, D. E. and FORREST, I. S., ^{3}H-Δ^9-tetrahydrocannabinol metabolism in rhesus and squirrel monkeys. *Proc. West. Pharmacol. Soc.* **15**,68 (1972).

CANNABIS AND REPRODUCTION: A SUMMARY

H. TUCHMANN-DUPLESSIS

EXPERIMENTAL investigations as well as clinical observations described in the nine papers presented at this session demonstrated the harmful effect of cannabis on the testis, the ovary and the hypothalamic-pituitary axis. These investigations also describe the developmental effects of cannabis in rodents and primates.

TESTIS

In rodents, Delta-9-THC and cannabidiol significantly decrease testosterone and impair spermatogenesis. Such effects may be related to a direct effect of cannabinoids on the gonads through a decrease in RNA synthesis, and also to an inhibition by THC of the gonadotropin function of the pituitary.

OVARY

Ovarian function is also inhibited. In the female rhesus monkey, THC produces a dose-related depression of ovarian function, with a decrease in gonadotropic hormones, LH and FSH. During the luteal phase, THC administration impairs progesterone production and produces anovulatory cycles. In rodents, THC or cannabis extract induces ovarian and uterine atrophy.

PRENATAL DEVELOPMENT

Different cannabinoids are embryotoxic and produce foetal resorptions in rats, mice and rabbits, but they are not grossly teratogenic. When THC is administered before mating to female rhesus monkeys, the incidence of abortion and neonatal mortality is 3 to 4 times higher than in control animals. The offspring from THC-treated mothers present abnormal behaviour to sensory stimuli.

CLINICAL OBSERVATION

In man, one observes a depression of reproductive functions with intermittent decrease of testosterone and presence of morphologic abnormalities of spermatogenesis.

Although considered by some as a soft, if not innocuous, drug, cannabis is undoubtedly harmful to man.

The papers and lively discussions of this session resulted in a clear picture of a problem which had given rise to contradictory statements.

RÉSUMÉ

LORS du Symposium de Reims sur la Marihuana, neuf communications furent consacrées à l'analyse des retentissements des principes actifs du cannabis sur la reproduction.

Les investigations experimentales, de même que les observations cliniques démontrent l'action nocive du cannabis sur le testicule, sur l'ovaire et sur l'axe hypophyso-hypothalamique. Elles mettent également en lumière les effets sur le développement embryonnaire des Rongeurs et des Primates.

TESTICULE

Chez le Rat, le Delta-9-tetrahydrocannabinol et le cannabidiol, déterminent une forte baisse de la production de testosterone et altèrent la spermatogénèse. Il s'agit non seulement d'une action directe sur la gonade attribuée à une synthèse réduite de RNA mais également d'une inhibition de la fonction gonadotrope de l'antéhypophyse.

OVAIRE

La fonction ovarienne est également inhibée. Chez la Guenon (singe Rhésus), on note une dépression de l'activité ovarienne. Le taux des hormones gonadotropes LH et FSH est diminué.

Pendant la phase lutéale, l'administration de cannabis perturbe la production de progesterone et détermine des cycles anovulatoires. Chez le Rongeur, le THC et les extraits de cannabis induisent une atrophie utérine et ovarienne.

DEVELOPPEMENT PRENATAL

Les différentes fractions de cannabis ont un effet abortif et embryoléthal chez le Rat, la Souris et le Lapin, mais elles ne s'avèrent pas tératogènes.

Lorsque le cannabis est donné chez la Guenon avant l'accouplement, le taux des avortements et de la mortalité périnatale est 3 à 4 fois plus élevé que chez les témoins. De plus, la descendance des mères traitées, présente une certaine déficience physique sensorielle et comportementale.

OBSERVATIONS CLINIQUES

Chez l'Homme, on observe également une dépression des fonctions reproductrices avec baisse de la testosterone et présence d'anomalies morphologiques de la spermatogénèse.

Considéré par certains comme "une drogue légère" sinon anodine, le cannabis est inconstestablement nocif pour l'homme.

Les exposés et les discussions très animées de la Réunion de Reims organisée par le Professeur Nahas ont permis de faire une synthèse claire d'un problème qui avait donné lieu à des affirmations contradictoires.

ULTRASTRUCTURAL AND BIOCHEMICAL CHANGES IN CNS INDUCED BY MARIHUANA

P. L. McGEER and A. JAKUBOVIC

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, B.C., V6T 1W5, Canada

Summary. The effect of Δ^9-tetrahydrocannabinol (THC) on some metabolic processes and cell structures in rat brain were studied *in vitro* and *in vivo*. The intracellular distribution and binding of labeled THC in brain tissue was also determined. The following results were observed:

a. The incorporation of radioactive precursors into protein and nucleic acid of rat brain cortical slices was significantly inhibited by THC. The inhibition was age related, with the most marked effect being in the infant (3-day-old) rat.

b. Acute administration of THC and other cannabinoids brought about morphological changes, viz. a reduction in the number of nuclear membrane-attached ribosomes (NMAR) in infant rat brain. The reduction of NMAR was dose and time related, and reversible. The pharmacologically active cannabinoids had a highly significant effect, but the pharmacologically inactive had only a slight effect. These ultrastructural changes are consistent with the biochemical effects of THC on protein and RNA synthesis.

c. Following the administration of radioactive THC to lactating rats, THC and its metabolites were identified in the organs of suckling infants. The amount of cannabinoids transferred from the lactating mother to suckling infant produced a highly significant decrease of NMAR in brain cells, which was still detectable 24 h after the injection. The prolonged supply of THC and/or active metabolites from the milk probably accounted for this lengthy action.

d. Intracellular distribution studies with labeled THC show highly preferential binding to particulate fractions in the brain cells, mainly the crude mitochondrial fraction. This localization is supported by electron microscope studies which show preferential binding of labeled THC to nerve endings.

The subcellular distribution and binding of the cannabinoids and their effects on synthesis processes may play important roles in their physiological and pharmacological effects.

INTRODUCTION

CANNABINOIDS are widely utilized by man for their euphoriant effects. A significant segment of the general population considers this to be a safe practice on the assumption that marihuana and its derivatives are relatively mild and harmless agents. Furthermore, it is argued by many that there should be no proscription against their use since only traditional fears of habit-forming drugs, and exaggerated claims of potential physiological damage stand in the way of their acceptance as recreational agents.

Much of the reassurance which is offered with respect to the safety of cannabinoids is related to the transient and reversible nature of their obvious pharmacological effect. The

pleasurable sensations, which are accompanied by only mild physiological changes such as increased heart rate, come on within minutes of inhalation and last for only a few hours[21, 22, 23]. This experience can be repeated many times with little apparent adaption to the effects. Although impaired motor and cognitive functioning accompany higher doses, they are also quickly reversible, and there is almost no danger of death from profound overdose.

The advocates of marihuana overlook persistent reports of other, more subtle changes which accompany human cannabis use and which have prompted investigations extending beyond the short term pharmacological effects. The amotivational syndrome, for example, is hard to explain either on the basis of a voluntary decision with regard to lifestyle, or the acute, reversible pharmacological effects. So is the reported memory deficit[10, 22]. The reports of depressed sexual functioning[20] and even gynecomastia[6] in heavy male users must be disassociated from the euphoriant effect.

The euphoriant effect has not yet been explained, and nothing is known so far about what CNS receptors might be involved. But it seems clear even at this stage of our knowledge that such receptors form only part of the cannabis story and that other processes must be explored to explain the more subtle effects. Furthermore, the transient, pleasurable sensations of marihuana are associated with only certain cannabis derivatives, particularly Δ^9-tetrahydrocannabinol (Δ^9-THC). Others, derivatives such as cannabidiol and cannabigerol, are psychogenically inactive[2, 3, 24]. But they have in common with the euphoriant derivatives, an inhibitory effect on cellular anabolic processes which may lie behind many of the described subtle and long term effects of cannabis use. These inhibitory effects, which seem to be a general property of cannabis derivatives, include inhibition of the fundamental processes of protein and nucleic acid synthesis[7, 8, 11, 16, 17, 18, 25] will have widespread physiological effects that first manifest themselves in those processes most sensitive to protein and nucleic acid synthesis. Since sexual functioning depends on new protein synthesis and memory is also thought to do so, it might be anticipated that these could be processes particularly affected by cannabis derivatives.

In this paper we summarize data we have obtained on the effects of Δ^9-THC and other cannabis derivatives on brain tissue. Part of the data relate to the distribution of labeled THC in brain. This distribution follows the pattern to be expected if receptor sites of the kind that interact with neurotransmitters were involved and which might well be involved in the obvious euphoriant effects. The remainder of the data relate to the effect of cannabis derivatives on protein and nucleic acid synthesis, including some structural changes that have been observed in rats. These latter effects may be involved in some of the non-euphoriant CNS actions of cannabis. In a companion paper in this monograph we discuss some of these same effects on rapidly anabolizing testicular tissue, which may explain some of the sexual effects.

ACTION OF THC IN THE BRAIN

a. *Influence of THC on metabolism of rat cerebral cortex slices*

These experiments were undertaken to study the possibility of biochemical alterations caused by THC *in vitro* in brain cortex slices of rats of different ages, as distinct from pharmacological actions on receptor sites.

Brain slices, no more than 0.4 mm thick, and usually weighing 60–80 mg wet weight were prepared with a Stadie-Riggs tissue slicer. Infant rat brain cortex slices were prepared by hand cutting. Incubation was carried on for 60 minutes in a Warburg manometric apparatus, in Krebs-Ringer phosphate buffer with 5 mM glucose, pH 7.4, under O_2, in a final volume of 3 ml. The tissue fractionation was carried out as previously described[13]. The rate of metabolism and incorporation of the radioactive substrate was linear for at least 2 hrs.

Since most of the metabolic and biosynthetic processes in the brain differ quantitatively with the age of mammals[4, 5, 12], rats of various ages were used. The results in Table 1 show that neither the respiration nor the radioactivity in the soluble fraction was affected by the presence of THC for any of the age groups. However, the incorporation of radioactivity into protein was significantly reduced. The reduction varied with age, the effect being greater in the younger rats. Under control conditions the slices from infant rat brain showed less O_2 uptake and radioactivity in the soluble fraction but a 10-fold higher incorporation into protein as compared with slices from adult rat brain.

TABLE 1. EFFECT OF THC ON THE RESPIRATION AND METABOLISM OF L-[U-^{14}C] LEUCINE IN ADULT AND INFANT RAT BRAIN CORTEX SLICES.

Incubation conditions	O_2 uptake (μmol/100 mg wet weight)	ng-atoms ^{14}C/100 mg initial wet wt/h		
		$^{14}CO_2$ evolved	Soluble fraction	Proteins
8–10 g rats (3-day-old)				
Control	4.1 ± 0.2	7.16 ± 0.41	55.7 ± 2.3	67.3 ± 4.8
THC	4.3 ± 0.4	6.49 ± 1.34	56.2 ± 2.3	46.2 ± 2.4^a
175–200 g rats				
Control	9.06 ± 0.4	7.22 ± 0.62	115 ± 6	6.98 ± 0.42
THC	9.58 ± 0.1	7.16 ± 0.82	110 ± 4	4.05 ± 0.21^b
275–325 g rats				
Control	10.4 ± 0.41	9.2 ± 1.0	107 ± 3	6.23 ± 0.58
THC	10.76 ± 0.15	8.7 ± 0.9	108 ± 3	5.23 ± 0.31^c

a. $p < 0.01$
b. $p < 0.02$
c. $p < 0.05$
Incubation was carried out in an oxygen atmosphere in Krebs-Ringer phosphate medium containing 5 mM glucose. THC, 0.1 mM in 10 μl alcohol or 10 μl alcohol only was added to the medium at the same time as the tissue. L-[U-^{14}C] leucine, 33 μM (10 mCi/mmol, 2.2×10^6 dpm per incubation) was tipped from the side tube into the main vessel after 7 min equilibration at 37°C to commence the experiment. Radioactivity is expressed as nanogram-atoms of ^{14}C incorporated per 100 g initial wet weight of tissue per time of incubation (mean $\pm$ S.D., $N = 4$).

In similar experiments THC also brought about marked decreases in the ^{14}C-uridine incorporated into RNA in infant, young and adult rat brain cortex slices (Table 2) with the least inhibition occurring in the oldest rats aged about 100–120 days. As with protein synthesis, the incorporation of ^{14}C-uridine into RNA for the 3-day-old rats was 3–4 times higher than that for the other groups. In contrast with the results with ^{14}C-leucine, however, THC also caused a decrease in the radioactivity in the soluble fraction in incubations with slices from infant or young rats[13].

TABLE 2. EFFECT OF THC ON THE METABOLISM OF [2-^{14}C] URIDINE IN ADULT AND INFANT RAT BRAIN CORTEX SLICES

Incubation conditions	Incubation time (min)	O_2 uptake (μmol/100 g wet weight)	pmol/100 mg initial wet wt./time of incubation	
			Soluble fraction	Nucleic acid
8–10 g rats (3-day-old)				
Control	60	4.8 ± 0.2	1565 ± 130	291 ± 2.9
THC	60	4.5 ± 0.1	1176 ± 44[a]	206 ± 3.6[a]
175–200 g rats				
Control	60	10.8 ± 0.1	1233 ± 45	89.0 ± 4.3
THC	60	10.5 ± 0.3	962 ± 18[a]	52.3 ± 4.3[a]
275–325 g rats				
Control	60	10.1 ± 0.2	1022 ± 47	77.3 ± 2.5
THC	60	9.9 ± 0.4	992 ± 35	66.5 ± 5.1[b]

a. $p < 0.01$
b. $p < 0.02$
Incubation condition was that described in Table 1 except that [2-^{14}C] uridine, 5.4 μM (62 μCi/μmol 2.56 × 10^6 dpm per incubation) was used as substrate and times of incubation were as indicated; *p* values in each case are for the THC test compared with the matched control conditions.

In order to study the effect of THC on brain metabolism *in vivo*, infant rats (5 rats in each group) received simultaneously a single s.c. dose of THC (20 mg/kg) and a 1 μl intracerebral injection containing [2-^{14}C] uridine (0.5 μC) (Fig. 1) or L-[U-^{14}C] leucine (0.5 μC) (Fig. 2). With [2-^{14}C] uridine (Fig. 1) as the tracer, the results show that after 3–4 h there is a significant decrease of radioactivity in both fractions and at 30 min and after 20 h there is no difference in the radioactivity of the fractions. With L-[U-^{14}C] leucine (Fig. 2) as the tracer, there is a decrease in incorporation into brain proteins 3 h after the injection in the THC-treated rats.

b. Influence on the number of nuclear membrane-attached ribosomes (NMAR) in infant rat brain

In subsequent experiments the possibility of structural changes related to the biochemical effect of cannabinoids on the brain was studied. All the acute experiments were done with 3-day-old rats. The cannabinoids were dissolved in 10 μl of 95% alcohol and injected subcutaneously. Control rats were injected with 10 μl of alcohol. At different times after the administration of the agents, the brains were fixed by intraventricular injections of ice-cold phosphate buffer containing 4% glutaraldehyde and slices of the occipital cortex were examined for NMAR[7, 8].

Control slices had about 1/3 of the total length of the nuclear membrane loaded with ribosomes. However, 30 min, 1 h and 3 h after the administration of 10 mg/kg of Δ^9-THC there was a 42%, 50% and 63% depletion of the NMAR, respectively. After 6 h, no depletion could be observed. Significant depletions were also seen with 5 mg/kg Δ^9-THC or 10 mg/kg of Δ^8-THC, cannabigerol or 11-OH-Δ^9-THC, with the latter being particularly

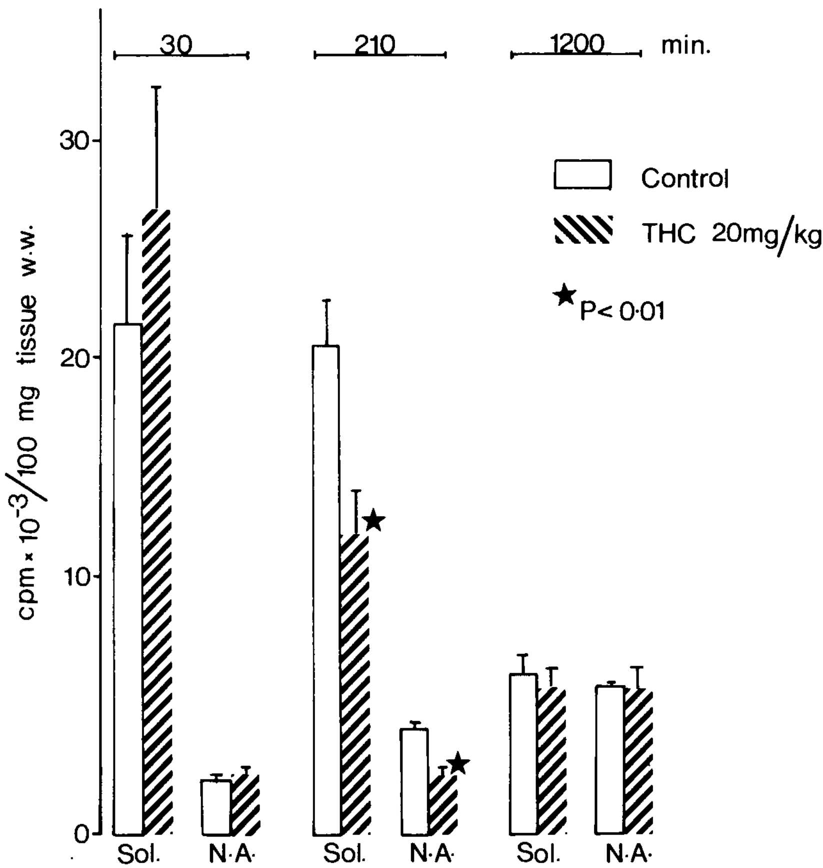


FIG. 1. Effect of THC on the metabolism of [2-^{14}C] uridine in infant rat brain *in vivo*. Infant rats (3 days old) received simultaneously a single s.c. dose of THC and intracerebral injection of 0.5 μCi [2-^{14}C] uridine, and were sacrificed at the time as indicated. Values are means $\pm$ S.D.; $n = 5$.

effective (Table 3). The losses of NMAR seen after 10 mg/kg of cannabidiol or cannabinol were not significant and alcohol alone had almost no effect (Table 3).

A significant reduction of NMAR was observed in the neurons of suckling 3-day-old rats after THC was injected into the mother rats[18]. A marked depletion of NMAR occurred as early as 4 h after the administration of THC to the lactating mother and was still significant even 24 h after injection of the drug to the mother (Table 4). This effect was more prolonged than that observed with an acute injection of THC directly into infant rats (Table 3). The higher dose of THC used and the repeated supply of THC and/or its active metabolites from the milk probably accounted for the prolonged action.

c. Tissue and intracellular distribution of THC:

Infant rats were sacrificed at 4, 8, 24 and 72 h after the injection of 53 mg/kg of 2,4-^{14}C-Δ^9-THC (2.5 $\times$ 10^7 dpm per rat) into the lactating mother. The total radioactivity in individual tissues was extracted with 95% alcohol and alcohol: ether (3 : 1), and radioactivity assayed in the combined supernatants[14].

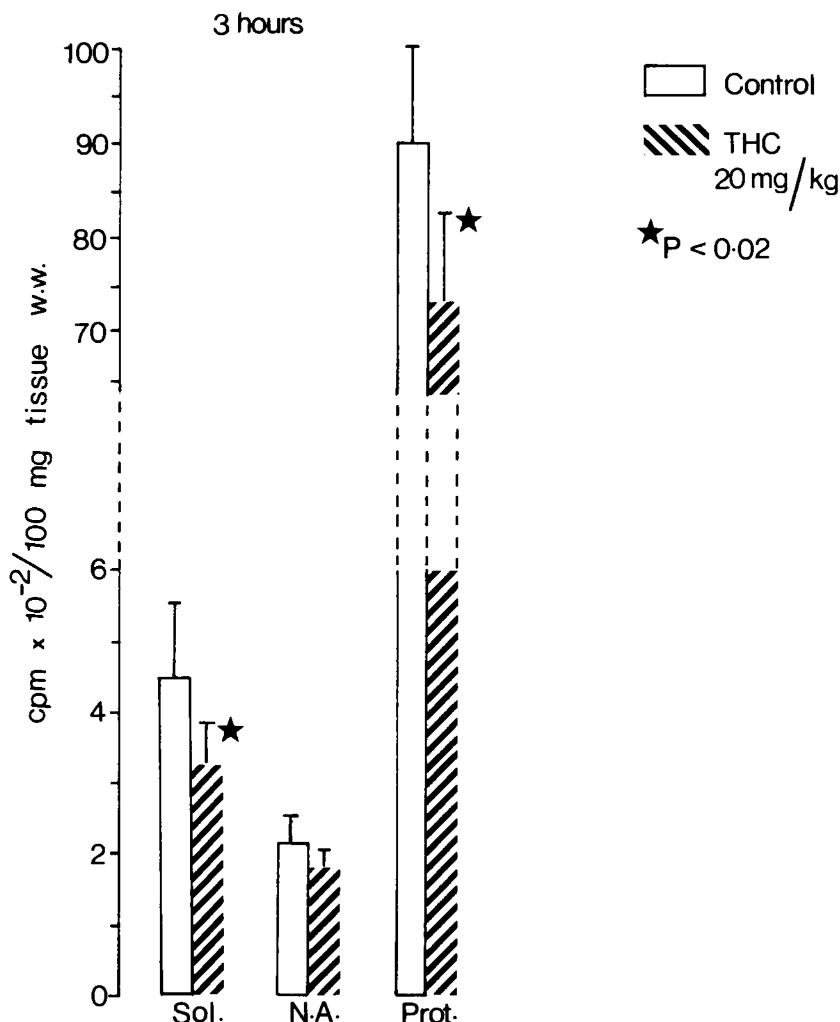


FIG. 2. Effect of THC on the metabolism of L-[U-^{14}C] leucine in infant rat brain *in vivo*. Experimental conditions were as described in Fig. 1 except that 0.5 μCi of L-[U-^{14}C] leucine was the substrate. Values are means $\pm$ S.D.; $n = 5$.

As early as 4 h after the administration of the labeled THC to the mother, radioactivity was present in all studied organs of the suckling rats (Table 5). After 72 h the highest radioactivity was in the stomach indicating the presence of a constant supply of the label via the mother's milk. Substantial amounts of the label were present in the liver, brain, spleen, heart and lungs.

For the study of intracellular distribution in rat organs ^{3}H-Δ^8-THC, 400 μg/kg (10^7 dpm; 465 mC/mmol) in 20 μl alcohol, was injected into the vena cava of 15-day-old (17 g) rats. The animals were sacrificed 20 min later and the brains, livers and kidneys removed, divided into left and right parts, homogenized in cold 0.32 M sucrose containing 1 mM $MgCl_2$ and 10 mM K-phosphate buffer, pH 7.4, and subcellular fractions obtained by differential centrifugation[15]. As shown in Table 6, the drug was almost exclusively

TABLE 3. EFFECT OF CANNABINOIDS ON NUCLEAR MEMBRANE-ATTACHED RIBOSOMES IN INFANT RAT BRAIN.

Substrate and dose (mg/kg)	Time after injection (h)	Percentage nuclear membrane-attached ribosomes	Percentage inhibition
Control	3	38 ± 6	0
Δ^9-THC, 10	0.5	19.5 ± 3	42
Δ^9-THC, 10	1	19 ± 3[a]	50
Δ^9-THC, 10	3	14 ± 2[a]	63
Δ^9-THC, 10	6	34 ± 2	11
Δ^9-THC, 5	1	35 ± 1	6
Δ^9-THC, 5	3	24 ± 2[a]	37
Δ^9-THC, 1	1	40 ± 3	+3
Δ^8-THC, 10	3	16 ± 2[a]	58
Cannabigerol, 10	3	23 ± 4[a]	40
Cannabidiol, 10	3	29 ± 3	24
Cannabinol, 10	3	30 ± 10	21
11-OH-Δ^9-THC, 10	1	16 ± 4[a]	58
11-OH-Δ^9-THC, 10	3	8 ± 3[a]	79
11-OH-Δ^9-THC, 10	6	26 ± 9	32
10 μl alcohol	0.5	33.5 ± 5	12

a. $p < 0.01$

TABLE 4. NUCLEAR MEMBRANE ATTACHED RIBOSOMES (NMAR) IN THE OCCIPITAL CORTEX OF SUCKLED RATS.

Time after THC administration	No. of rats	Percentage NMAR	Percentage inhibition
0 (control)	4	40 ± 1.1	—
4	4	21 ± 2.7[a]	48
24	4	25 ± 0.5[a]	38

Female rats on the third day postpartum were injected s.c. with 53 mg/kg Δ^9-THC and suckling rats were sacrificed as indicated.

The mean lengths of the nuclear membrane surface of the neurons measured were 0 h, 33.4 μm; 4 h, 34.1 μm; 24 h, 34.4 μm.

a. $p < 0.005$, in each group compared with the 0 h time control.

associated in the brain with the mitochondrial fraction, which also contains synaptosomes. In the kidney, the nuclear fraction, which also contains tissue debris, had the highest radioactivity, while in the liver, the distribution was practically the same in all three fractions (Table 6).

When each precipitate was washed twice with 2 ml and then twice more with 1 ml of 95% alcohol, the majority of the radioactivity was removed from all fractions and only "firmly bound" radioactivity remained with the cellular components (Table 7). The retention was highest in the microsomal fraction of the brain and in the mitochondrial fractions of the liver and kidney (Table 7). These results show differences in intracellular distribution

TABLE 5. DISTRIBUTION OF RADIOACTIVITY IN THE TISSUES OF SUCKLING RATS.

Tissue	μg original ^{14}C-THC/g wet infant tissue[a] Time (h)			
	4	8	24	72
Liver	9.8 (1)	0.43 (1)	0.21 (5)	0.37 (4)
Brain	0.45 (1)	0.34 (1)	0.16 (5)	0.08 (4)
Stomach	0.24 (2)	2.06 (3)	1.47 (5)	2.94 (3)
Spleen	0.38 (1)	1.37 (1)	0.62 (2)	0.08 (1)
Heart	0.03 (1)	1.52 (1)	0.70 (2)	0.19 (1)
Lung	0.32 (1)	1.83 (1)	0.77 (2)	0.06 (1)

a. Values represent averages with the number of determinations in brackets. In some cases tissues from more than one infant were combined.

Female rats on the third day postpartum were injected s.c. with 53 mg/kg of 2,4-^{14}C-Δ^9-THC and suckling rats were sacrificed as indicated.

TABLE 6. RADIOACTIVITY IN SUBCELLULAR FRACTIONS OF RAT BRAIN, LIVER AND KIDNEY AFTER I.V. ADMINISTRATION OF ^3H-Δ^8-THC.

Organ		dpm/g wet wt. $\times 10^{-3}$	Percentage injected radio-activity	Nuclei	Mito-chondria	Micro-somes	Soluble
Brain	1	186 ± 1.9	1.7	30	48	10	12
	2	199 ± 3.8	1.9	32	49	10	9
	3	287 ± 1.2	2.7	35	47	11	8
Liver	1	968 ± 6.4	9	33	37	30[a]	
	2	1100 ± 9.6	10	32	34	34	
	3	1340 ± 58	12	35	33	32	
Kidney	1	331 ± 35	3	51	17	33[a]	
	2	410 ± 4	3.8	50	17	34	
	3	450 ± 20	4.2	53	17	31	

Each value represents the mean ± S.E. from the left and right part of the organ of a rat.
a. Whole post-mitochondrial fraction.

as well as binding capacity for the drug in the brain and non-neural tissues (Tables 6 and 7), which may be of importance in determining the physiological and pharmacological effects.

Electron microscopic studies were begun in an attempt to define more exactly the intracellular localization of THC in brain. Adult rats were injected intraventricularly, and infant rats (14-day-old) were injected intravenously with ^3H-Δ^8-THC (16 μCi in 20 μl of 95% alcohol; 450 mCi/mmol). They were sacrificed either 30 min or 2 h later by perfusion and the occipital cortex was fixed as previously described[9].

Most of the radioactivity was lost during fixation-dehydration but the terminal boutons

TABLE 7. RADIOACTIVITY IN THE SUBCELLULAR FRACTIONS AFTER EXTRACTION WITH ALCOHOL.

Organ		Percentage Radioactivity left in fraction		
		Nuclei	Mitochondria	Microsomes
Brain	1	2.6 ± 0.3	3.2 ± 0.0	9.1 ± 0.6
	2	3.0 ± 0.2	3.0 ± 0.0	9.1 ± 0.3
	3	2.4 ± 0.4	2.7 ± 0.2	8.1 ± 0.6
Liver	1	2.6 ± 0.6	5.1 ± 0.3	—
	2	3.1 ± 0.1	6.3 ± 0.1	—
	3	2.1 ± 0.4	4.9 ± 0.4	—
Kidney	1	4.0 ± 0.0	9.1 ± 0.9	—
	2	4.1 ± 0.6	9.6 ± 1.0	—
	3	3.1 ± 0.3	10.7 ± 0.4	—

Each value represents the mean ± S.E. from the left and right part of the organ of a rat.

in the adult central gray contained about 30% of the residual radioactivity at both 30 min and 2 h after the administration of the ^{3}H-THC (Table 8). Terminal boutons were the only structures which showed a relative grain density significantly greater than 1.0 (Fig. 3).

In young rats (Table 9), the labeling of cell somata was somewhat greater than in the adult rat cells (Table 8). About 60% of those grains which labeled cell soma were situated around the Golgi apparatus and Nissl bodies[9].

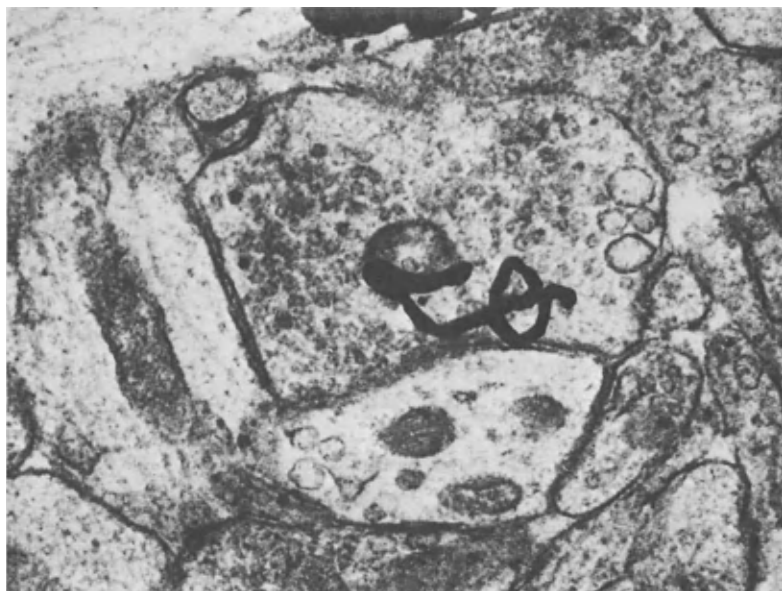


FIG. 3. Labeled bouton in central gray of adult rat contained moderately packed small round vesicles (35–45 nm in diameter) making an axodendritic synaptic contact. × 48,850.

TABLE 8. THE GRAIN DISTRIBUTION IN CENTRAL GRAY OF ADULT RATS 30 MIN AND 2 h AFTER AN INTRAVENTRICULAR INJECTION OF ^{3}H-Δ^{8}-TETRAHYDROCANNABINOL.

	Percentage grain		Percentage area		Relative grain density (percentage grain/ percentage area)	
	30 min[a]	2 h[c]	30 min[b]	2 h[d]	30 min	2 h
Terminal bouton	29	33	11	12	2.6	2.8
Dendrite	32	28	28	27	1.1	1.0
Axon	25	26	30	28	0.8	0.9
Soma	7	11	20	20	0.4	0.6
Others	7	2	11	13	0.6	0.2

a. Total grains counted: 207
b. Total area measured: 2931 μm^2
c. Total grains counted: 257
d. Total area measured: 3780 μm^2

TABLE 9. GRAIN DISTRIBUTION IN OCCIPITAL CORTICES OF 14-DAY-OLD RATS 30 MIN AFTER INTRAVENOUS INJECTION OF ^{3}H-Δ^{8}-TETRAHYDROCANNABINOL.

	Percentage grain[a]	Percentage area[b]	Relative grain density (percentage grain/ percentage area)
Terminal bouton	12	7	1.7
Dendrite	36	31	1.2
Axon	22	25	0.9
Soma	25	26	1.0
Others	5	11	0.5

a. Total grains counted: 148
b. Total area measured: 2011 μm^2.

DISCUSSION

The studies described here demonstrate that THC and other cannabinoids bring about significant changes in the biosynthesis of vital macromolecules in rat brain cells. However, the interference of cannabinoids with biosynthetic pathways is not unique to brain cells. Similar inhibition has been reported in a variety of cellular systems[11, 16, 17, 23, 25] with various labeled precursors of protein, DNA, RNA and lipid synthesis.

It has been shown that incorporation is several times higher in brain slices of infant rats than in those of adult animals[1, 2, 3]. Our present control results are consistent with these observations. The degree of *in vitro* inhibitions obtained with cannabinols of incorporation of labeled precursor into the protein (Table 1) and RNA (Table 2) of brain cortex slices is also age related, with the greatest effects being seen in infant rats.

The depressed incorporation of ^{14}C-leucine into protein is probably the result of THC inhibition of anabolic pathways rather than altered transport mechanisms. The decreased

radioactivity in RNA may be related to a reduced synthesis of uridine nucleotides, caused by THC[13].

The results from the *in vivo* experiments (Figs. 1 and 2), are in agreement with the *in vitro*, suggesting that similar interactions of THC and/or biosynthetic processes occur.

The effect of THC on protein and RNA synthesis is consistent with the ultrastructural evidence indicating depletion of ribosomes at the nuclear membrane. The effect of acute administration of pharmacologically active cannabinoids[3, 24] (e.g. Δ^9-THC, Δ^8-THC and 11-OH-Δ^9-THC) on the reduction of NMAR is dose-related, rapid and reversible. With the pharmacologically inactive[3, 24] CBN, CBD and CBG, only the last reduced the NMAR in the rat occipital cortex (Table 3).

Our results with suckling rats (Tables 4 and 5) show that: (1) THC and/or its metabolites can be transferred via the milk from the lactating mother to the infant; (2) the amount of the drug which is transferred is capable of bringing about structural and biochemical changes in the suckling rat brain cells.

The studies on the intracellular localization of labeled THC (Tables 6 and 7) show preferential association of the drug with particulate fractions of the brain, mainly (48%) in the crude mitochondrial fraction, which also contains synaptosomes. Similar results on the distribution of THC in the brains of rats, dogs and mice, were recently reported[1].

The preferential localization of the label, after intraventricular injections of ^{3}H-Δ^8-THC into adult rats, in the terminal boutons (Tables 8 and 9) corresponds with the high radioactivity found in the crude mitochondrial fraction of the brain cells (Table 6). This suggests the possibility of particularly tight binding of THC to nerve endings (Fig. 3).

It seems unlikely, however, that this preferential binding to nerve endings plays a major role in the longer term more subtle effects of THC usage which are more probably related to the inhibitory effects of THC and its metabolites on general cell metabolism. These inhibitory effects are marked and carry ominous implications for the physiological effects of chronic THC usage.

ACKNOWLEDGEMENT

This work was supported by a grant from the Non-Medical Use of Drugs Directorate, Health and Welfare Canada.

REFERENCES

1. DEWEY, W. L., MARTIN, B. R., BECKNER, J. S. and HARRIS, L. S., A comparison of the subcellular distribution of cannabinoids in the brains of tolerant and non-tolerant dogs, rats and mice after injecting radio labeled Δ^9-tetrahydrocannabinol. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.) Springer-Verlag, New York, p. 349, 1976.
2. DOMINO, E. F., Neuropsychopharmacologic studies of marijuana: some synthetic and natural THC derivatives in animals and man. *Ann. N.Y. Acad. Sci.* **191**,166 (1971).
3. EDERY, H., GRUNFELD, Y., BEN-ZVI, Z. and MECHOULAM, R., Structural requirements for cannabinoid activity. *Ann. N.Y. Acad. Sci.* **191**,40 (1971).
4. GLAZER, R. I. and WEBER, G., Incorporation of [6-^{3}H] glucose into lipid, protein, RNA and DNA of slices of differentiating rat cerebral cortex. *J. Neurochem.* **18**,1569 (1971).
5. GUROF, G. and BRODSKY, M., Enzyme and nuclei acid metabolism in the brains of young and adult rats. *J. Neurochem.* **18**,2077 (1971).
6. HARMON, J. and ALIAPOULIOS, M. A., Gynecomastia in marihuana users. *W. Engl. J. Med.* **287**,936 (1972).

7. HATTORI, T., JAKUBOVIC, A. and McGEER, P. L., Reduction in number of nuclear membrane-attached ribosomes in infant rat brain following acute Δ^9-tetrahydrocannabinol administration. *Exp. Neurol.* **36**,207 (1972).
8. HATTORI, T., JAKUBOVIC, A. and McGEER, P. L., The effect of cannabinoids on the number of nuclear membrane-attached ribosomes in infant rat brain. *Neuropharmacology* **12**,995 (1973).
9. HATTORI, T. and McGEER, P. L., Electron microscopic autoradiography on Δ^8-(^{3}H)-tetrahydrocannabinol localization in brain tissue. *Toxicol. Appl. Pharmacol.* **39**,307 (1977).
10. HOLLISTER, L. E., Marijuana in man: three years later. *Science* **172**,21 (1071).
11. HUOT, J., Cellular and biochemical alterations induced *in vitro* by Δ^1-tetrahydrocannabinol: Effects on cell proliferation, nucleic acids, plasma cell membrane ATPase, and adenylate cyclase. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.) Springer-Verlag, New York, p. 313, 1976.
12. ITOH, T. and QUASTEL, J. H., Ribonucleic acid biosynthesis in adult and infant rat brain *in vitro*. *Science* **164**,79 (1969).
13. JAKUBOVIC, A. and McGEER, P. L., Inhibition of rat brain protein and nucleic acid synthesis by cannabinoids *in vitro*. *Can. J. Biochem.* **50**,654 (1972).
14. JAKUBOVIC, A., HATTORI, T. and McGEER, P. L., Radioactivity in suckled rats after giving ^{14}C-tetrahydrocannabinol to the mother. *Eu. J. Pharmacol.* **22**,221 (1973).
15. JAKUBOVIC, A. and McGEER, P. L., Intracellular contribution of ^{3}H-Δ^8-tetrahydrocannabinol in rat organs after i.v. administration. *Res. Commun. Chem. Pathol. Pharmacol.* **9**,197 (1974).
16. JAKUBOVIC, A. and McGEER, P. L., *In vitro* inhibition of protein and nucleic acid synthesis in rat testicular tissue by cannabis. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.) Springer-Verlag, New York, p. 223, 1976.
17. JAKUBOVIC, A. and McGEER, P. L., Biochemical changes in rat testicular cells *in vitro* produced by cannabinoids and alcohol. Metabolism and incorporation of labeled glucose, amino acids, and nucleic acid precursors. *Toxicol. Appl. Pharmacol.* **41**,473 (1977).
18. JONES, R. T., Tetrahydrocannabinol and the marijuana-induced social "high", or the effects on the mind of marijuana. *Ann. N.Y. Acad. Sci.* **191**,155, (1971).
19. KOLANSKY, H. and MOORE, W. T., Effect of marihuana on adolescents and young adults. *J. Am. Med. Soc.* **216**,486 (1971).
20. KOLODNY, R. C., MASTERS, W. H., KOLODNER, R. M. and TORO, G., Depression of plasma testosterone levels after chronic intensive marihuana use. *N. Engl. J. Med.* **290**,872 (1974).
21. LEMBERGER, L., AXELROD, J. and KOPIN, I. J., Metabolism and disposition of tetrahydrocannabinols in naive subjects and chronic marihuana users. *Ann. N.Y. Acad. Sci.* **191**,142 (1971).
22. MELGES, F. T., TINKLENBERG, J. R., HOLLISTER, L. E. and GILLESPIE, H. K., Marijuana and temporal disintegration. *Science* **168**,1118 (1970).
23. NAHAS, G. G., DESOIZE, B., HSU, J. and MORISHIMA, A. A., Inhibitory effects of Δ^9-tetrahydrocannabinol on nucleic acid synthesis and protein in cultured lymphocytes. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, eds.). Springer-Verlag, New York, p. 299, 1976.
24. PEREZ-REYES. M., TIMMONS, M. C., LIPTON, M. A., CHRISTENSEN, H. D., DAVIS, K. H. and WALL, M. E., A comparison of the pharmacological activity of Δ^1-tetrahydrocannabinol and its monohydroxylated metabolites in man. *Experientia* **29**,1009 (1973).
25. ZIMMERMAN, A. M., and ZIMMERMAN, S. B., The influence of marihuana on eukaryote cell growth and development. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, *et al.*, ed.). Springer-Verlag, New York, p. 195, 1976.

BRAIN BIOCHEMICAL ALTERATIONS IN NEONATES OF DAMS TREATED ORALLY WITH Δ^9-TETRAHYDROCANNABINOL DURING GESTATION AND LACTATION*

YUGAL K. LUTHRA

Department of Biochemical Pharmacology, Mason Research Institute, Worcester, Massachusetts 01609

Summary. Brain protein and nucleic acid levels were measured in neonate rats of dams treated orally with Δ^9-THC doses of 0, 1, 5 or 10 mg/kg during gestation or gestation and lactation. These doses were calculated to represent human use, were non-teratogenic and did not provoke adverse effects on the dams, as determined by reproductive data and endocrine organ and body weights. Treatment during gestation with 5 mg/kg induced 11% decline in protein, whereas 10 mg/kg dose regimen caused approximately 13% decrease in protein, RNA and DNA in less than one day old offsprings. In 7-day-old pups of dams administered 10 mg/kg of Δ^9-THC during gestation, cerebral protein fell by 10% and RNA level decreased by 14%. When the drug was given during gestation and lactation, the analysis of brains from 7-day-old offsprings revealed 10–12% fall in protein and RNA concentration in the 5 mg/kg group. At 10 mg/kg dose, a 15% decrease occurred in protein and nucleic acid levels. This evidence for the effect of Δ^9-THC on the neonate brain macromolecules could be determinant factors in producing behavioral aberrations in the developing organism.

INTRODUCTION

NEURAL systems possess a complex structural and metabolic arrangement; the maintenance of a balanced dynamic equilibrium and co-ordination within the individual metabolic reactions is a basic requirement for neural function.

Drug action on the mature or developing brain may alter this chemical environment and consequently modify cerebral function. The change in the biochemical architecture may last for a short time, persist longer or indeed may remain irreversible.

During the course of investigation of chronic toxicity with high doses of cannabinoids administered orally to adult rats (Thompson *et al.*, 1973) and at doses calculated to be relevant to human consumption (Fleischmann *et al.*, 1975; Rosenkrantz and Braude, 1976) whole or anatomically different areas of the brain were analysed (Luthra and Rosenkrantz, 1974; Luthra *et al.*, 1975a; Luthra *et al.*, 1976). In general, a pattern of dose dependent and sex-specific behavioral manifestations and of development of tolerance to these signs were

* A Preliminary communication was presented at the meeting of French Society of Toxicology (Paris, January 1978).

observed during treatment. Alterations in neurochemical measurements of protein, RNA and enzymes at specific time intervals coincided with the behavioral aberrations. Moreover, while normal behavior was exhibited by those rats which were left untreated for 20–30 days, neurochemical alterations were only partially reversed. Other neurochemically adverse effects induced by marihuana or Δ^9-tetrahydrocannabinol (Δ^9-THC), the psychoactive constituent, were changes in glycolytic metabolites and related enzymes (Luthra *et al.*, 1973; *et al.*, 1975b).

The relationship between and possible implication of cannabinoid effects on the developing brain have been reported by various authors. Hattori *et al.* (1972) reported ultrastructural changes and decrease in the number of nuclear membrane-attached ribosomes following acute Δ^9-THC treatment. Whole-body autoradiographic studies of pregnant mice by Kennedy and Waddell (1972), demonstrated that ^{14}C-Δ^9-THC concentration in neural tissue was highest of all the other fetal tissue. More recently, Hattori and McGeer (1977) reported greater localization of ^3H-Δ^8-THC in neural subcellular structures of young rats.

Vardaris and co-workers (1976) have provided direct evidence of ^3H-Δ^9-THC transfer across the placenta. The chemical was administered orally at a dose level of 2 mg/kg to rats during gestation. Average drug level in the pup was 20 ng/gm of tissue. Earlier Jakubovic *et al.* (1974), reported the detection of ^{14}C-Δ^9-THC and metabolites in the milk of lactating ewes given a single i.v. injection of 0.2 or 1 mg/kg dose. Radioactivity was also present in urine and feces of suckling lambs.

In one study (Jakubovic *et al.*, 1976) the investigators reported some evidence for brain biochemical changes induced by Δ^9-THC in the chick embryo. Drug treatment was on specific days and did not include the full period of incubation. In addition, this model may not be analogous to mammalian systems.

The results of the study presented here provide the first direct evidence for neurochemical alterations in new born and suckling pups from rats treated with Δ^9-THC during gestation and lactation.

METHOD AND MATERIALS

ANIMAL AND DESIGNATION OF PREGNANCY

Fisher rats were purchased from Charles River Breeding Laboratories, Wilmington, Massachusetts. Females (135 $\pm$ 10 g) were mated overnight (two females/males), and the presence of sperm in vaginal smears, taken the next morning, was used to designate day zero of pregnancy.

EXPERIMENTAL DESIGN

Pregnant rats were arranged into three groups of 40 animals each. Each group contained 10 females per dose level. Δ^9-Tetrahydrocannabinol (Δ^9-THC, 96% pure), provided by National Institute on Drug Abuse, Rockville, Maryland, was administered orally by intubation at 1, 5 and 10 mg/kg. Controls in each group received sesame oil, the vehicle, in dose

volume of 1 ml/100 g body weight. Drug and vehicle were administered daily according to the following protocol:

a. Group I was treated during gestation (21–22 days); neonates and mothers were sacrificed within 24 hours of birth and parturition respectively.
b. Group 2 was treated during gestation (21–22 days) only; pups and dams were sacrificed on day 7. This group represented sucklings from females left untreated during lactation.
c. Group 3 was treated during gestation (21–22 days) and in addition for 6 days. Offspring and dams were sacrificed on day 7. This group consisted of suckling pups from dams treated during lactation.

Neonates were sacrificed by snipping the cephalic region directly into liquid nitrogen. All specimens were stored at −120°C till analyzed. Dams were sacrificed by decapitation.

Parameters monitored for dams were: final/initial body weight ratio and pituitary, adrenal and ovarian weights. Data on offspring consisted of numbers of implants and pups; number of viable pups and incidence of resorption. Pups were also examined for external deformities. Neurochemical measurements determined were protein and nucleic acids according to the methods previously described (Shibko *et al.*, 167; Luthra *et al.*, 1975a; Luthra *et al.*, 1976). Sixteen individual brain samples per dose level were analysed from each of the three groups.

Statistical significance ($P < .05$) of data was evaluated by analysis of variance and Newman-Keuls test for multiple comparisons (Zivin and Bartko, 1977).

RESULTS

Body and organ weights and offspring data are described briefly in terms of mean range values. Neurochemical results are presented in Tables 1, 2, and 3. These data will be described separately for each group.

The mean body weight ratios and organ weights within each group did not reveal significant differences. For dams sacrificed soon after gestation (Group 1), the range of body weight ratio was 1.17 ± 0.04 to 1.23 ± 0.05; pituitary weights ranged between 9.1 ± 1.7 to 9.4 ± 1.8 mg; adrenal weights fell within 55.1 ± 5.1 to 60.9 ± 5.5 mg; and ovaries weighed between 96.4 ± 10.9 to 111.7 ± 12.6 mg. The range of body weight ratios of dams in Groups 2 and 3 were 1.29 ± 0.10 to 1.34 ± 0.04; pituitaries weighed between 7.2 ± 2.3 to 9.4 ± 1.9 mg; adrenals were between 53.4 ± 2.7 to 57.8 ± 6.4 mg and ovaries weighed between 81.2 ± 10.6 to 86.7 ± 3 mg.

Analysis of offspring data revealed 8 ± 2 to 10 ± 2 numbers of implants; 8 ± 3 to 10 ± 2 of pups and 7 ± 3 to 10 ± 1 number of viable pups per dam in Groups 1, 2 and 3. Within-group differences were not significant for these parameters. Examination of the pups did not reveal any apparent external malformation.

NEUROCHEMICAL ANALYSIS

Group 1 (*Table 1*). In neonates sacrificed within 24 hours of birth, protein declined by 11% ($P < 0.005$) and 15% ($P < 0.005$) at 5 and 10 mg/kg dose levels, respectively. RNA fell by 16% ($P < 0.025$) and DNA by 15% ($P < 0.05$) at 10 mg/kg dose.

TABLE 1. BRAIN PROTEIN AND NUCLEIC ACIDS OF NEONATE RAT PUPS FROM FISHER DAMS TREATED ORALLY WITH Δ^9-THC.[a]

Dose[b] (mg/kg)	N	Protein	RNA	DNA
		(mg/g of wet wt.)		
Veh. cont.	16	68.2 ± 4.3	1.88 ± 0.37	2.23 ± 0.44
1	16	68.8 ± 5.0	1.86 ± 0.39	2.21 ± 0.45
5	16	60.9[c] ± 6.7	1.60 ± 0.34	1.91 ± 0.46
10	16	58.3[c] ± 3.6	1.58[d] ± 0.32	1.89[e] ± 0.38

a. Values are mean ± S.D. Sacrifice Day: < 24 hours.
b. Dams were treated daily during gestation only.
c. $P < 0.005$;
d. $P < 0.025$;
e. $P < 0.05$
Statistical significance was evaluated by Analysis of Variance and Newman-Keuls Test for multiple comparison. (Treated *vs.* control groups)

TABLE 2. BRAIN PROTEIN AND NUCLEIC ACIDS OF NEONATE PUPS FROM FISHER DAMS TREATED ORALLY WITH Δ-^{9}-THC.[a]

Dose[b] (mg/kg)	N	Protein	RNA	DNA
		(mg/g of wet wt.)		
Veh. cont.	16	75.0 ± 5.0	1.74 ± 0.34	1.85 ± 0.36
1	16	74.6 ± 7.1	1.75 ± 0.34	1.83 ± 0.39
5	16	73.4 ± 5.0	1.70 ± 0.33	1.79 ± 0.38
10	16	67.8[c] ± 5.9	1.50[d] ± 0.31	1.79 ± 0.34

a. Values are mean ± S.D. Sacrifice Day: 7
b. Dams were treated during gestation only.
c. $P < 0.005$;
d. $P < 0.05$
Statistical significance was evaluated by Analysis of Variance and Newman-Keuls Test for multiple comparison. (Treated *vs.* control group).

Group 2 (*Table 2*). Suckling offspring from dams not treated during lactation and sacrificed on day 7 revealed 10% ($P < 0.005$) fall in protein and 14% ($P < 0.05$) decline in RNA levels at the high dose.

Group 3 (*Table 3*). Suckling neonates from dams treated during lactation and sacrificed on day 7 had a dose-related decline of 12–15% ($P < 0.005$) for proteins at 5 and 10 mg/kg

TABLE 3. BRAIN PROTEIN AND NUCLEIC ACIDS OF NEONATE PUPS FROM FISHER DAMS TREATED ORALLY WITH Δ^9-THC.[a]

Dose[b] (mg/kg)	N	Protein	RNA	DNA
		(mg/g of wet wt.)		
Veh. cont.	16	76.8 ± 4.2	1.77 ± 0.25	1.82 ± 0.37
1	16	74.8 ± 6.5	1.82 ± 0.27	1.81 ± 0.38
5	16	67.3[c] ± 3.6	1.59[d] ± 0.23	1.60 ± 0.33
10	16	65.3[c] ± 3.9	1.50[e] ± 0.19	1.54[d] ± 0.31

a. Values are mean ± S.D. Sacrifice Day: 7.
b. Dams were treated daily during gestation and given 6 additional daily treatments during lactation.
c. $P < 0.005$;
d. $P < 0.05$;
e. $P < 0.01$
Statistical significance was evaluated by Analysis of Variance and Newman-Keuls Test for multiple comparison. (Treated *vs*. control group).

doses. Decrease of 10% ($P < 0.05$) at 5 mg/kg and of 15% ($P < 0.01$) at 10 mg/kg was evident for RNA. A 15% ($P < 0.05$) fall in DNA occurred at the high dose of 10 mg/kg.

DISCUSSION

The results of this study provide direct evidence of biochemical changes induced in immature brain by Δ^9-THC administered orally to pregnant Fisher rats. Significantly, the doses administered were nonteratogenic and calculated to represent extrapolation to human use (Rosenkrantz and Braude, 1976). Moreover, these doses did not provoke significant drug-related changes on the endocrine organ weights or body weights of dams. Offspring parameters, other than neurochemical, also did not show significant effects attributable to the drug. Brain biochemical changes can, however, be ascribed to the access by Δ^9-THC to the neural tissue through transplacental transport and exposure via milk (Jakubovic *et al*., 1974; Vardaris *et al*., 1976).

The macromolecules assayed soon after birth represent the period when cell proliferation and division predominate. At this time neurons nearly reach their adult value. The sacrifice interval for the sucklings falls in the phase (0–10 days) when growth of cells, axons and dendrites is evident. Glial cell proliferation and myelination is minimal during this period. This segment of developmental cycle represents a very active stage in nucleic acid and protein metabolism (McIlwain and Bachelard, 1971; Richter, 1970).

In view of this, alterations in the rates of synthesis and degradation and, in structural modification of the protein could lead to neurological functional deficit. Damage to a type of neuronal population may also affect that particular cerebral function.

Learning deficits have been demonstrated in 21 and 65 day old pups from rats treated with cannabis or Δ^9-THC during gestation (Gianutsos and Abbatiello, 1972; Vardaris *et al.*, 1976). The interpretation of these findings may be important in the light of the role of protein and nucleic acids in the processes of learning and memory (Glassman, 1969; Roberts and Flexner, 1969; Barondes, 1970). Other functions such as membrane maintenance, synaptic transmission and energy metabolism seem to be related to these macromolecules (Richter, 1970).

Other important points emerge from this study. Protein and nucleic acids declined by 15% at the high dose in the neonates sacrificed soon after birth, as well as in pups from mothers treated during lactation. Thus, prolonged treatment caused no further decrease in neurochemical parameters. Secondly, cessation of treatment during lactating period considerably reduced the adverse cannabinoid-induced brain biochemical alterations. These two observations suggest that the developing brain is capable of recovery and can initiate adaptive and/or protective mechanisms. The induction of such processes was also postulated for the adult brain (Luthra *et al.*, 1974; Luthra *et al.*, 1975a; Luthra *et al.*, 1976). This observation conforms with the earlier report that protein degradation in adult, as well as in developing brain contributes to metabolic regulation and adaptation (McIlwain and Bachelard, 1971). It remains speculative whether the recovery and/or initation of protective mechanisms shield the developing organism from cerebral dysfunction. Evidence suggests that the developing brain is the most susceptible of all organs and the effects persist the longest (Sparber, 1972).

Thus, it has been shown that Δ^9-THC administered to pregnant rats by the oral route is capable of altering the biochemistry of the brain in developing offspring. The extent, mode and duration of these changes could be the decisive factors in determining the type, degree and magnitude of behavioral aberrations.

ACKNOWLEDGEMENT

The author is grateful for the help and encouragement received from Dr. Harris Rosenkrantz and Dr. Monique C. Braude; for the competent technical assistance of Miss Phyllis Hughes and Mrs. Rachel Haskell and the typing of the manuscript by Ms. Margaret Roop and Ms. Elizabeth Parsons.

REFERENCES

1. BARONDES, S. H., Cerebral protein synthesis inhibitors block long-term memory. *Int. Rev. Neurobiol.* **12**,177–187 (1970).
2. FLEISCHMAN, R. W., HAYDEN, D. W., BRAUDE, M. C. and ROSENKRANTZ, H., Chronic marihauna inhalation toxicity in rats. *Toxicol. Appl. Pharmacol.* **34**,467–478 (1975).
3. GIANUTSOS, A. and ABBATIELLO, E. R., The effect of prenatal cannabis sativa on maze learning ability in the rat. *Psychopharmacol.* **27**,117–122 (1972).
4. GLASSMAN, E., The biochemistry of learning: An evaluation of the role of RNA and protein. *Ann. Rev. Biochem.* **38**,605–620 (1969).
5. HATTORI, T., JAKUBOVIC, A. and MCGEER, P. L., Reduction in number of nuclear membrane attached ribosomes in infant rat brain following acute Δ^9-tetrahydrocannabinol administration. *Exptl. Neurol.* **36**,207–211 (1972).
6. HATTORI, T. and MCGEER, P. L., Electron microscopic autoradiography on Δ^8-^{3}H tetrahydrocannabinol localization in brain tissue. *Toxicol. Appl. Pharmacol.* **39**,307–311 (1977).

7. JAKUBOVIC, A., TAIT, R. M. and MCGEER, P. L., Excretion of THC and its metabolites in ewes' milk. *Toxicol. Appl. Pharmacol.* **28**,38–43 (1974).
8. JAKUBOVIC, A., MCGEER, P. L. and FITZSIMMONS, R. C., Effects of Δ^9-tetrahydrocannabinol and ethanol on body weight, protein and nucleic acid synthesis in chick embryo. *J. Toxicol. Env. Hlth.* **1**,441–447 (1976).
9. KENNEDY, J. S. and WADDELL, W. J., Whole body autoradiography of the pregnant mouse after administration of ^{14}C-Δ^9-THC. *Toxicol. Appl. Pharmacol.* **22**,252–258 (1972).
10. LUTHRA, Y. K. and ROSENKRANTZ, H., Cannabinoids: Neurochemical aspects after chronic oral administration to rats. *Toxicol. Appl. Pharmacol.* **27**,158–168 (1974).
11. LUTHRA, Y. K., ROSENKRANTZ, H., HEYMAN, I. A. and BRAUDE, M. C., Levels of rat brain protein, RNA and glycolytic metabolites after chronic oral administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) for 14–28 days. *166th ACS National Meeting, Abst. No. 183* (1973).
12. LUTHRA, Y. K., ROSENKRANTZ, H., HEYMAN, I. A. and BRAUDE, M. C., Differential neurochemistry and temporal pattern in rats treated orally with Δ^9-tetrahydrocannabinol for periods up to six months. *Toxicol. Appl. Pharmacol.* **32**,418–431 (1975).
13. LUTHRA, Y. K., ROSENKRANTZ, H. and BRAUDE, M. C., Activities of rat cerebral glycolytic enzymes after chronic marihuana inhalation. *Fed. Proc., Fed. Amer. Soc. Exp. Biol.* **34**,664 (1975).
14. LUTHRA, Y. K., ROSENKRANTZ, H. and BRAUDE, M. C., Cerebral and corebellar neurochemical changes and behavioral manifestations in rats chronically exposed to marihuana smoke. *Toxicol. Appl. Pharmacol.* **35**,445–465 (1976).
15. MCILWAIN, H. and BACHELARD, H. S., Chemical and enzymatic make-up of the brain during development. In "Biochemistry and the central nervous system," pp. 406–444. Churchill Livingstone, London, (1971).
16. ROBERTS, R. B. and FLEXNER, L. B., The biochemical basis of long-term memory. *Q. Rev. Biophys.* **2**,135 (1969).
17. RICHTER, D., Protein metabolism and functional activity. In "Protein Metabolism of the Nervous System" (Ed. A. Lajtha). pp. 241–257. Plenum Press, New York, 1970.
18. ROSENKRANTZ, H. and BRAUDE, M. C., Comparative chronic toxicities of Δ^9-tetrahydrocannabinol administered by inhalation or orally to rats. In "The Pharmacology of Marihuana" (M. C. Braude and S. Szara, Eds.). pp. 571–584. Raven Press, New York, 1976.
19. SHIBKO, S., KOIVISTOINEN, P., TRATNYEK, C. A., NEWHALL, A. R. and FREIDMAN, L., A method for sequential quantitative separation and determination of protein, RNA, DNA, lipid and glycogen from single rat liver homogenate or from subcellular fraction. *Anal. Biochem.* **19**,514–528 (1967).
20. SPARBER, S. B., Effects of drugs on the biochemical and behavioral responses of developing organisms. *Fed. Proceed.* **31**,74–80 (1972).
21. THOMPSON, G. R., MASON, M. M., ROSENKRANTZ, H. and BRAUDE, M. C., Chronic oral toxicity of cannabinoids in rats. *Toxicol. Appl. Pharmacol.* **25**,373–390 (1973).
22. VARDARIS, R. M., WEISZ, D. J., FAZEL, A. and RAWITCH, A. B., Chronic administration of Delta-9-tetrahydrocannabinol to pregnant rats: Studies of pup behavior and placental transfer. *Pharmacol. Biochem. Behav.* **4**,249–254 (1976).
23. ZIVIN, J. A. and BARTKO, J. J., Statistics for disinterested scientists, *Life Sci.* **18**,15–26 (1977).

CANNABIS AND THE BRAIN WITH SPECIAL REFERENCE TO THE LIMBIC SYSTEM

LOREN L. MILLER

Veterans Administration Hospital, Lexington, Kentucky

Summary. An hypothesis is presented which suggests that the cognitive, subjective-emotional and perceptual changes produced by psychoactive cannabinoids may be mediated in part by actions of these agents on limbic system structures and limbic cortical connections. The major cognitive alterations which are found following cannabinoid intoxication are impaired memory functioning, lapses in attention, altered speech production and general problems with complex information processing. Perceptual anomalies consist of alterations in time perception with moderate doses, and visual distortions and hallucinations with high doses of cannabinoids. Subjective-emotional changes reported following cannabinoid intoxication include feelings of euphoria, drowsiness, a sensation of well being with low to moderate doses and feelings of derealization and depersonalization with higher doses. The role of the limbic system structures such as septum, hippocampus, amygdala and hypothalamus in the mediation of these cannabinoid effects along with feedback loops running between limbic structures, thalamus and frontal and temporal cortices was reviewed and analyzed.

ALTHOUGH a plethora of research studies have attempted to describe the subjective, behavioral and pharmacological effects of cannabinoid derivatives in man, there exists a paucity of data concerning a comprehensive neural model which might explain brain mechanisms underlying the effects of these agents. This state of affairs is due in part to (1) the complex pharmacopoeia offered by cannabinoids and (2) the fact that a myriad of scientific disciplines while all contributing to a basic understanding of the cannabinoids, have not attempted any synthesis of viewpoints. We seem to be faced with another case of the blind men and the elephant with the biases of each discipline determining which part of the elephant will be seen.

An adequate understanding of how brain-behavior relationships are altered by cannabinoids must entail an understanding of interacting brain centers and the complex neural and neurochemical processes mediating their functions. It is important to build bridges between disciplines seeking to establish causal relationships between the behavior of the total organism and the physiological and biochemical changes taking place in the organism coincidentally with the behavior. The purpose of this paper is to present some integrating themes in an effort to "bridge the gap" between disciplines.

CANNABIS-CLINICAL OBSERVATIONS

Clinical observations concerning acute intoxication with cannabinoids vary from describing these agents as mild intoxicants to classifying them as hallucinogens. However, it is known that the continuum of effects produced by cannabis is due for the most part to the dosage of Δ^9-tetrahydrocannabinol (Δ^9-THC) contained in the preparation, along with setting, route of administration, history of use and current psychological state of the user.

On a subjective level, low to moderate doses of THC produce euphoria; the intoxicated individual describes himself as feeling contented, relaxed and exhilarated. These feelings are wave-like, occurring in rushes, and are often facilitated by social interactions. Usually, when smoking, these feelings last 30 minutes to one hour. Later, they may give way to feelings of listlessness and indolence. With higher doses of THC, abrupt fluctuations in mood may occur along with incongruity of affect. Dysphoric reactions which include feelings of depression, anxiety, apprehension and paranoia may also be present. Finally, sedation and drowsiness may later replace states of excitement[136].

On a sensory-perceptual level, an awareness of sensory stimuli becomes more acute with a preoccupation with stimuli for which some amount of habituation has accrued. Feelings of warmth, light-headedness, floating and drifting are common. These experiences wax and wane. With higher doses, derealization and depersonalization occur as well as visual distortions and hallucinations[136, 157].

The most consistently reported perceptual change with low to moderate doses of THC is an alteration of time sense. According to early descriptions of the French psychiatrist Moreau, time drags slowly in the intoxicated state—the whole concept of time becomes more elusive with the past and present becoming confused. Translated into movements in space, many objects look as if they are being viewed through the large end of a telescope. Sensations follow each other so closely in time that true appreciation of time is not possible.

On a cognitive level, thought processes are described as being speeded up. Ames[10] spoke of fragmented thought processes and interruptions in the stream of thought so that what was said a few seconds earlier was forgotten. According to Bromberg[17], marijuana intoxication produces

> "an astounding feeling of lightness to the limbs and body. Elation continues: he laughs uncontrollably and explosively for brief periods of time without, at times, the slightest provocation: if there is a reason, it quickly fades, the point of the joke is lost immediately. Speech is rapid, flighty . . . ideas flow quickly . . . . These flighty ideas are not deep enough to form an engram that can be recollected—hence the confusion that appears on trying to remember what was thought"

Continuous thought is difficult because of the intrusion of irrelevant associations. Thoughts wander and conversation is often difficult to follow. Integration of facts is impaired as well as the focusing of attention and concentration.

While the above summary of the effects of cannabis on subjective feeling states, sensation-perception and cognition is brief, it does illustrate the variety of changes which occur and suggests that cannabis probably affects central nervous system functioning at a number of levels.

EXPERIMENTAL CONFIRMATION OF CLINICAL OBSERVATIONS

The clinical observations discussed previously indicate that cannabinoids alter a variety of mental phenomena including mood and affect, sensory and perceptual changes, especially the time sense and various aspects of cognition including memory, attention and psychomotor performance.

As with many drug studies attempting to measure subjective changes resulting from administration of pharmacological agents, scaling techniques are usually employed. Measuring the subjective effects of cannabinoids has varied from employing direct subjective reports or simple scales in which a subject is required to indicate how "high" he is to the use of more sophisticated scales such as the Profile of Mood States (POMS), Subjective Drug Effects Questionnaire (SDEQ), Addiction Research Inventory (ARCI) and Cornell Medical Index. Each of these scales differ slightly in their emphasis with considerable overlap. Hollister and Overall[73] based on a factor analysis of questionnaire data have suggested that the subjective effects of marijuana are multidimensional. They found that the expected subjective effects of marijuana could be described in terms of five factors. Factor 1 represented a dimension of functional interference which was associated with the sedative or hypnotic effects of the drug. Subjective effects comprising this factor were fatigue, drowsiness, and confusion. Factor 2 represented euphoria which consisted of a sensation of well-being and adequacy. Factor 3 reflected psychological distress which consisted of a generalized feeling of distress, despondency and anxiety. Factor 4 represented perceptual alteration consisting of unpleasant mental changes. Hollister and Overall[73] emphasized that the factors they reported represented primary dimensions of individual differences in the subjective experiences of marijuana users. The results of this investigation as well as those of other investigators support the subjective reports previously offered by Tart[157].

Subjective symptoms as assessed by the SDEQ are similar to those classified by Hollister and Overall[73]. Jones[83] has also noted that responses to this questionnaire can be substantially influenced by the setting in which the marijuana is taken, expectations and past experience with the drug. All of these factors determine how the experience of intoxication will be labeled and reported. Subjective ratings as measured by the POMS appear to be more modestly changed in comparison to the SDEQ and less sensitive to the actions of marijuana[84]. The ARCI, a questionnaire similar to the MMPI, has been used to study the activity of cannabinoids in man[81]. It was demonstrated that THC produced marijuana-like symptoms on a marijuana scale and with increasing doses, subjective state as measured by a "psychotomimetic" scale was similar to that seen with LSD. Feelings of derealization, depersonalization, visual distortions and hallucinations were all reported with higher doses of Δ^9-THC. On a sensory level, perceptual changes produced by cannabinoids have proved difficult to validate empirically in man[128]. Various objective measures of visual and auditory perception such as tests of visual acuity, depth perception, critical flicker fusion as well as Seashore Rhythm have been shown to be little affected by marijuana[136]. Experimentally the most consistently induced perceptual change occurs for the perception of time. This phenomenon has been aptly demonstrated by Melges and associates[112, 113] employing the Goal Directed Serial Alternation Task (GDSA), a task which requires subjects to serially coordinate and keep track of information in immediate

memory. Adequate performance on this task depends on the integration of current impressions with both preceding and subsequent experiences. Following intoxication with oral THC, Melges *et al.* found that subjects often described feelings of derealization and depersonalization, the magnitude of which correlated with impaired performance on the GDSA. It was reasoned that the time line extending from past to present appears discontinuous to the intoxicated subject. As a result time passes more slowly. Thus, marijuana may speed up an "internal biological clock"[159]. It is possible that under marijuana there is an increase in the level of sensory information that reaches the central nervous system in a given period of "objective" time.

According to Paton and Pertwee[136], three techniques have been used to study time perception following intoxication with cannabinoids. These methods differ in terms of whether "felt time" or "clock time" is the dependent variable. The former method involves estimating the duration of a time interval while the latter consists of generating or producing a given interval. These two methods differ in terms of whether the judgment is made before or after the passage of a time interval. In the method of reproduction, the experimenter demonstrates a given time interval and the subject is required to reproduce it. Employing the method of estimation, marijuana intoxication results in an overestimation of the passage of time[27, 86, 167]. For example, if 30 seconds has elapsed, a subject might estimate that the interval was 40 seconds in length. When a time interval is produced following intoxication it usually is underestimated. Therefore, if a subject is asked to demonstrate when 30 seconds has elapsed, he might produce 20 seconds[86, 159, 170]. With either time estimation or time production methods, the rate of reported time to actual elapsed time increases under marihuana. On time reproduction tasks, marijuana apparently has little effect[40]. Reproduction tasks require a subject to manually reproduce an interval of time which is demonstrated by the experimenter. No subjective estimate of time is involved. Thus, accuracy of time perception may not be affected by cannabinoids whereas estimates of elapsed time are.

Perhaps one of the more consistently reported effects of marijuana is on various aspects of the memory process[115]. Conceptually, the human memory system can be described as having two major components, a short term and a long term storage component[88, 153]. This model has achieved empirical validation from mainly two areas, cognitive psychology and neuroanatomy. The short term memory store represents an individual's working memory. It is responsible for holding the trace of an external stimulus and at the same time matching the memory trace of the stimulus with a previous representation of the stimulus from the long term component. The short term and long term components differ mainly in both information capacity and duration. Only a small number of items can be maintained in the short term store at any given time. Maintenance can be increased by various control processes including rehearsal, imagery or use of mnemonics. When information resides in short term storage for a reasonable period of time, it is transferred to long term memory.

A number of investigators employing this model and analyzing the effects of cannabinoids on the free recall of verbal material have found that the amount of information both learned and recalled during the period of intoxication is reduced. Recall is not affected if subjects acquire information prior to drug treatment even though recall occurs in the drug state. This suggests that once information is encoded, retrieval of that material will occur during the period of intoxication[34, 39]. Thus, a major effect of marijuana may be to produce faulty consolidation. This effect appears to be more pronounced during the recall of prose[118, 124] a task which may require intact sequential abilities. Recognition memory

is largely unaffected by marijuana[39, 119, 126]. Two studies have suggested that cues do not eliminate the recall deficits produced by marijuana[117, 118]. Yet, cues do eliminate state dependent retrieval deficits produced by the drug[46]. The former finding suggests that less information is available under marijuana and not simply inaccessible while the latter finding suggests that recall deficits following intoxication are cue dependent. However, another study has indicated that in some instances storage of information may be slower following intoxication but the same amount is eventually encoded. But, information appears to be in a state of flux if consistency of recall is employed as a criterion of adequate remembering. That is, marijuana produces lapses in recall[116].

An interesting aspect of the memory data is the finding that marijuana increases the incidence of inclusive errors. In recall experiments, this usually involves the introduction of intralist or extralist intrusions[22, 126, 142], the introduction or unrelated extraneous material during the recall of prose[124] or an increase in false alarms rather than misses in recognition memory tasks[1, 173]. According to Dornbush (1974), following intoxication with marijuana, a subject may lose his ability to discriminate between old and new material thereby lowering his criterion of acceptability. Thus, it is possible that at least some of the memory loss found with cannabis may be due to enhanced imagery or thought flow which potentiates interference due to the intrusion or irrevelant associations. Miller and Cornett[116] have suggested that memory retention following intoxication is difficult because marijuana may alter the manner in which encoding takes place. That is, the number and type of operations performed on stimulus input are affected by the drug. Thus, marijuana may affect the encoding processes by interfering with attention or any number of as yet unspecified coding operations.

The lapses in recall as well as intrusions are often reflected in conversational speech following intoxication. An analysis of speech samples indicates that marijuana produces more free verbalizations and associations, vivid imagery and a decreased awareness of a listener[166]. The distortions in speech do not appear to occur on a syntaxical level. However, the intoxicated subject displays lapses in memory for what was said as well as a tendency to be tangential. Other studies have shown that smoking increased latency of verbal response, produced fewer syllables per phrase and a prolongation of syllables suggesting a deficit in information processing and a decreased ability to focus verbal communication[137, 173].

A number of studies have suggested that numerous aspects of drug-induced performance deficits can be accounted for by understanding how marijuana affects the process of attention. DeLong and Levy[37] have accounted for the effects of cannabis on attention in terms of three processes, degree of selectivity, resistance to distraction and shifting. Degree of selectivity refers to the ability to articulate or make sharp distinctions concerning environmental stimuli. Thus, cannabis may cause the intoxicated subject to become more field dependent. However, drug effects on tests of field dependence such as the Embedded Figures Test and Rod and Frame test have produced mixed findings. On the other hand, Miller *et al.*[121] found that subjects were more affected on a free recall task depending on their degree of field dependence with field dependent subjects displaying poorer recall.

Resistance to distraction refers to a continuum ranging from great concentration to extreme inability to maintain a focus of attention. The Lilly group reported that under conditions of delayed auditory feedback a number of simple cognitive problems were affected adversely by the drug at rather low doses[92, 107, 108]. These results suggest that the intoxicated subject may be more easily distracted by external stimuli. This variable

appears to be very salient when one considers that marijuana has little effect on tasks which are automatized or require minimal attention[138].

A third attention component is shifting. This refers to the ability to shift the current focus of attention and establish a new one. It is measured on a continuum ranging from voluntary control to perseveration or the inability to stop focusing. Trail making performance is thought to be a reasonable measure of this ability and two studies have found some evidence that marijuana at low doses will increase time taken to complete this task as well as the number of errors made[43, 93]. Pursuit rotor performance may be affected if subjects perseverate and indeed a number of studies have reported impaired performance on this task following marijuana intoxication[107, 108]. An interesting study by Casswell and Marks[25] employed a divided attention task which appears to measure both vigilance and the ability to shift attention. Subjects were surrounded by lights and told to press a button every time they detected a break in the flashes. At the same time a peripheral light also flashed to which a subject was required to press a second button. Results indicated that significantly more central and peripheral light flashes were missed under marijuana in comparison to placebo.

Feeney[50] has suggested that a basic effect of marijuana is to increase the variability of an organism's reaction to its environment. This variability in behavior is associated with a variability in neural responsiveness. A review of a number of experiments employing cannabinoids indicated that measures of variability are significantly affected by these agents rather than measures of central tendency. He further suggested that atypical neural responses are induced by cannabinoids which result in novel perceptions and experiences. Relating this to the popularity of marijuana he suggests that increased variability of neural responses may retard the habituation of classical reinforcers such as food and sex and that once common events will produce novel effects which are reinforcing. Habituation in animals is slowed, following administration of cannabinoids. For example, it was shown that in rats both THC and anticholinergics attenuated the effects of previous exposure to an experimental chamber[18]. That is, the habituation of normal exploratory tendencies was reduced under both drugs. Rats habituated to running wheels become dishabituated following treatment with THC[44]. These studies offer some support for the contention of Feeney[50] that information processing by the CNS is more variable than normal under the influence of cannabinoids and consequently behavioral responding is more variable.

It should be noted that a variety of cognitive and emotional processes have been hypothesized to be affected by the cannabinoids. Whatever function appears to be most influenced by these agents is usually defined by or dependent on the particular task employed and the function it is thought to measure. Thus, marijuana may affect attention or recall depending on the question asked. This suggests that cannabinoids produce a basic change in the overall functioning of the brain. Brain mechanisms subserving many of the functions discussed are not totally understood. However, there is neuroanatomical and neurophysiological evidence which suggests that a number of subcortical structures collectively defined as the limbic system subserve many of the functions described. These areas include such structures as the septum, hippocampus, amygdala, mammillary bodies, anterior thalamic nuclei and hypothalamus. A graphic representation of the limbic system is represented in Fig. 1.

While there is no satisfactory general concept of how the limbic system is organized, it is known that the former structures are interconnected anatomically in a complex fashion. The close interrelationships between various structures is thought to result in closed

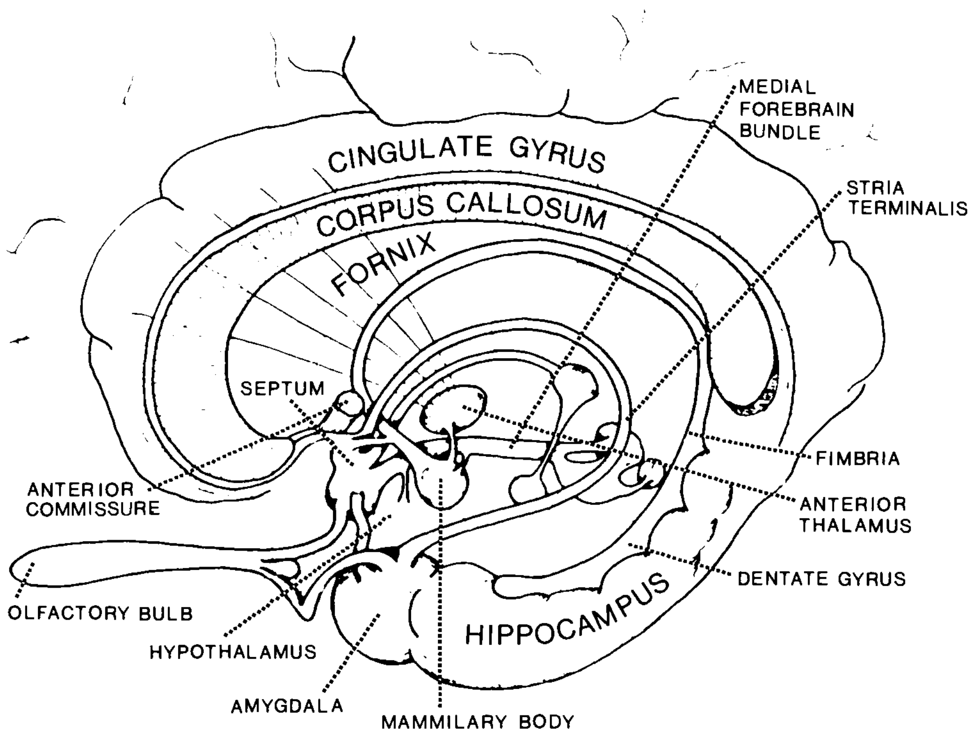


FIG. 1. The limbic system

circuits. That is, impulses generated in a particular nucleus may induce inactivity that feeds back upon the nucleus. For example, Papez[135] described a circuit containing fibers projecting from the hippocampus via the fornix to the mammillary bodies of the hypothalamus which send fibers via the mammillothalamic tract to the anterior thalamic nuclei. These nuclei send fibers to the cingulate cortex from which fibers project to the entorhinal cortex. The circuit is completed by fibers arising in the entorhinal cortex projecting back to the hippocampus via the temporoammonic tract. Papez felt that this anatomical pathway played a critical role in the control of emotional reactivity. While conclusive evidence regarding the function of this circuit in emotion does not exist, the existence of such circuits is one reason the limbic system has been considered a functional whole[96]. Although details of limbic anatomy cannot be extensively described here, certain anatomical features are important: (1) the hypothalamus receives numerous inputs from the hippocampus via the fornix, medial forebrain bundle, stria terminalis and mammillary peduncle; (2) there are many reciprocal connections between limbic structures and the midbrain reticular formation; (3) there is rich indirect sensory input which suggests a modulating influence of limbic system rather than a direct controlling function; and (4) there are a number of complex interconnections between limbic structures and thalamic nuclei. These relationships suggest that the limbic system is involved in central autonomic and motivational control and in the processes of arousal and attention[96].

The septum is a small structure located at the base of the anterior horn of the lateral ventricle rostral to the anterior commissure. In man, this structure is thought to mediate feelings of pleasure and levels of awareness. EEG changes consisting of high amplitude

spindling in the septal area are correlated with reported feelings of pleasure and contentment in humans[65, 66]. Fried[56] has suggested on the basis of infrahuman studies that this structure may function as a response suppression system which is responsible for reducing the habit strength of responses developed during motivational situations thereby allowing new responses to become established. Thus, septal damage may cause animals to overrespond to motivating stimuli and to be less able to inhibit responses that have previously been reinforced. This response suppressing function is thought not to operate at the level of independent muscle tonicity but at a higher level of complex, organized goal directed behavior. Fried also suggests that many of the effects of septal dysfunction are probably traceable to its multiple anatomical connections with the hypothalamus and hippocampus.

The amygdala consists of a collection of nuclei found in the anterior portions of the temporal lobes. In humans, stimulation of this structure is thought to produce visceral sensations and confused speech while ablation results in docility and calmness. Lesions of this structure in nonhuman primates results in a reduction in their social interactions[59] and an increased threshold for the elicitation of aggressive behaviors in rodents[60]. The structure also seems to be intimately involved in food intake. Thus, amygdala damage may produce an insensitivity to certain environmental changes, greater tameness and placidity, and less interest in social cues presented by peers. The initiation of new behaviors is reduced and the giving up of established patterns is facilitated.

Perhaps the most studied limbic structure when one considers both human and infrahuman investigations is the hippocampus. Lesions of this structure have been reported to produce a variety of cognitive changes in both man and animals. These include striking deficits in the acquisition of new material which have been hypothesized to be a function of faulty transfer of information from short term to long term memory storage[35, 139]. Meissner[111] has suggested that memory impairments found in temporal lobe and Korsakoff patients may reflect an inability to habituate or to ignore trivial stimuli while Warrington[164] suggests that hippocampal damage results in an inability to inhibit or dissipate irrevelant stored information which may result in interference with new coding. Thus, the hippocampus may be instrumental in suppressing the effect of irrelevant memories which may intrude during the consolidation of new memories. Whether or not the intrusion of irrelevant material is the sole determiner of the memory defect found with damage to hippocampus is still an open question. In infrahumans the basic defect has been described by Douglas[41] as impaired gating. Gating is defined as the process of excluding "stimulus patterns from attention through a process of efferent control of sensory reception". In animals, gating acts mainly to inhibit the perception of stimuli associated with non-reinforcement. This would be important to such processes as error reduction, habituation and extinction. Thus, the organism with hippocampal damage may be less apt in inhibiting its responses to initially prepotent environmental stimuli and hence display less flexible and therefore less adaptive behavior.

Alterations in perception and changes in mood have been consistently reported following epileptic discharges arising in the limbic cortex of the insula and hippocampal formation[106]. MacLean[103] has indicated that all sensory systems feed into the hippocampal formation. He reported that lesions in the lateral geniculate body produce degeneration in the hippocampal gyrus. It is also known that the parahippocampal cortex transmits impulses to the hippocampus which has rich connections with the hypothalamus as well as other brainstem structures. Mahl *et al.*[105] have shown that stimulation of the temporal lobe areas produces hallucinatory experiences which relate to the patients mental

content before the stimulation. It was concluded that the stimulation produced a state of "consciousness which makes it more probable that primary process modes of functioning will prevail."

Feelings of depersonalization or what Hughlings Jackson has termed "mental diplopia" as well as derealization are found with discharges near the basal limbic cortex. Somesthetic sensations, apparent changes in auditory and visual sensation as well as distorted time perception occur with epileptic discharge or electrical stimulation in limbic areas[104]. Horowitz and Adams[77] have suggested that disruption in temporal lobe functioning may impair the capacity to form thought in words because of the capacity to organize cognitive bits of information in sequence. This would lead to a tendency towards derailment of lexical thinking and result in a state in which primitive thinking in imagery would be more likely to occur. This may be especially true of Korsakoff patients who display a disorganization in thinking along a temporal dimension. These patients display particular difficulty in comparing present information with preceding and subsequent experiences. The sequential disturbances of memory in Korsakoff patients have been described as "agnosia of succession"[171].

The hypothalamus is an important structure not only because of its regulation of homeostatic brain mechanisms but because it is a place of convergence for other limbic structures. It is also considered to be important because of its many pathways which are thought to be involved in the mediation of rewarding and pleasurable effects. These effects were noted earlier by Olds and Milner[131] who found that animals will perform various behavioral acts to receive electrical stimulation in lateral hypothalamic and medial forebrain areas. Other portions of the hypothalamus such as the ventromedial nucleus and posterior hypothalamus may serve as aversive centers because animals will perform various behaviors to escape from stimulation of these areas. Various neurotransmitter systems have been thought to mediate these effects with the pleasure systems being noradrenergic and the aversive systems being cholinergic in nature.

Relatively undifferentiated patterns of behavior are probably organized by the hypothalamus and reticular formation. Elaboration and integration of these patterns occur through extensive limbic connections with hypothalamus which in turn mediate neocortical control. The hypothalamus may be concerned with reorganizing and recoding instructions received from hippocampus and amygdala into appropriate sympathetic and parasympathetic concomitants of behavior[154].

It is thought that the main overall function of the limbic system is to relate information from drive centers of the organism with information about the external environment and information about the internal environment. In a general sense, the limbic system is an area in which the current drive state of an organism gives emotional tone to what the senses perceive. In conjunction with the hypothalamus and reticular formation, these regions may predispose an organism to specific behaviors based on an interplay between the current environmental situation and the current needs of the organism. The control of attention and of the level of arousal or alertness is important in these functions[155].

CANNABINOIDS AND THE LIMBIC SYSTEM

Numerous electroencephalographic studies have been performed with the cannabinoids in an effort to determine sites of action. Klonoff and Low[93] in a review of these studies

have indicated that only minor measurable changes occur with the EEG in humans. There appears to be a generalized reduction in cortical EEG voltage following an acute dosage of cannabinoids. There is also an increase in alpha activity, a slight decrease in the peak frequency of alpha rhythm and a decrease in beta activity[54, 75, 146, 162]. There have been a few reports of desynchronized EEG[86, 101, 170]. Klonoff and Low[93] suggest that this is a dose related phenomenon with higher doses of THC producing this latter effect. An interesting report by Koukkou and Lehmann[94] suggested that the effects of THC on EEG were related to the existing functional state of the organism and indicated that since the subjective effects fluctuate over time, it was difficult to arrive at any general consensus concerning electrophysiological changes produced by this drug. In their study the EEG reflected less alpha and more beta and theta frequencies following a 200 mcg/kg dose of THC during periods of hallucinations and body image disturbances. A group exhibiting a high frequency of these disturbances displayed faster alpha and slower theta and beta waves than a group of subjects reporting a low frequency of these disturbances. These authors suggest that their data lend support to models of hallucinations which posit that during hallucinatory periods there is a shift of attention from external to internal input[168] or a loosening of control of organization of cortical function possibly due to an alteration of reticular system activation. Alteration of these functions could result in errors of integrative evaluation of input data. While high doses of cannabinoids do produce significant cortical EEG changes, less potent preparations of the drug produce few profound effects, although cognitive and subjective changes are present.

In animals, Δ^9-THC as well as its chemical analogs produce high voltage slow wave cortical EEG activity indicative of sedation[38]. EEG arousal consisting of high voltage fast activity has been reported in the rat[143] and squirrel monkey[4]. Gross behavior is characterized by excitation with a desynchronized EEG[38, 76]. High voltage spindle activity superimposed on low voltage activity has been reported suggesting increased excitability of neurons[129]. Therefore, an organism may appear sedated but display central nervous system arousal. The effects of THC on rabbit EEG are dependent on environmental variables[30]. Rabbits well adapted to an experimental chamber displayed high voltage slow wave activity in response to THC while nonadapted rabbits showed predominately slow voltage fast activity. Behavioral and postural components of arousal accompanied high voltage slow cortical activity. Thus, an arousing novel situation may overcome the sedative actions of THC. With repeated administration of cannabinoids, tolerance develops to EEG effects which reflect sedation but not to changes associated with central nervous system activation[54].

A number of studies have assessed the effects of cannabinoids on sensory evoked responses and contingent negative variation. The latter measure consists of a slowly changing brain potential which may reflect the activity of brain mechanisms subserving processes of attention, motivation and preparatory set[158]. The amplitude and latency of visual, auditory and somatosensory evoked potentials have been shown to be altered by cannabinoids[93, 97, 148]. In a simple reaction time study CNV magnitude has been reported to increase following a low dose of THC but is unchanged by a high dose[16, 159]. This suggests that brain mechanisms subserving attention are enhanced rather than diminished. These results would appear discordant with reported behavioral effects of cannabinoids. However, Roth *et al.*[148] has suggested that the CNV may be enhanced by mild intoxicants during the performance of repetitive tasks. However, at higher levels of intoxication, this increase in alertness may be offset in the declining ability to focus one's

attention. These authors noted that evoked potentials in the 280–380 degree range were reduced under THC and ethanol which may reflect these agents' effects on response selection-organization stages of information processing.

Since only minor changes in human EEG occur following intoxication, it may be that subcortical, medial or basal brain structures are implicated in the actions of cannabinoids on human behavior(93). The most interesting work of Heath and his associates(67, 68) on the effects of cannabis on human and infrahuman electrical changes from deep brain structures supports this conjecture. Electrophysiological changes occurred mainly in the septal area of a human subject intoxicated with marijuana with high voltage slow wave activity predominating and being most pronounced during mood elevation and rushes. This was replaced by rhythmical lower amplitude activity when the rushes subsided. In nonhuman primates following THC administration slow wave activity interspersed with sharp wave activity in the septum, reticular formation and lateral thalamic areas was reported. Bursts of high amplitude spindles were also noted in septum, temporal cortex, deep cerebellar nuclei, orbital cortex and hippocampus. Diffuse electrical activity in deep structures such as amygdala, hypothalamus and hippocampus was reported to occur in cats(70) and rats(26). Since stimulation of the septum has been shown to heighten awareness and induce pleasurable feelings, it was suggested that marijuana may act mainly on this limbic area to produce its effects. Furthermore, its connection with sensory relay nuclei of the cerebellum and posteroventral lateral thalamus could account for reported distortions of body image and somatic sensations following intoxication. Its connections with the hippocampus may also be significant.

The hippocampus has also been implicated in the mediation of the effects of cannabis(45, 122). Drug effects on this structure could have profound implications for behavior since the hippocampus is integral to the functions of the limbic forebrain-limbic midbrain system. The limbic forebrain limbic midbrain system serves as a reciprocating network interlinking limbic forebrain areas such as the hippocampus, amygdala and septum with the mesencephalic reticulum and gray matter of the brain stem. This reciprocating circuitry makes passage through the hypothalamus. Thus, the limbic system is a receiving and integrating center for information from all the sensory modalities and may be conceptualized as an anterior extension of the reticular formation. Subsequently, this information is "refluxed" back to the hypothalamus and reticular fields. This system would seem to modulate multimodal inputs and regulate filtering in information channels at several integrative levels in the brain stem(127). According to Adey(5) "the hippocampus appears to influence, and to be influenced by activity in sensory systems and to be responsible for the establishment in extra-hippocampal structures of the psychological set that is requisite for the storage of information therein."

Cannabinoids have been reported to disrupt hippocampal theta rhythm(57, 98, 152, 172). Hippocampal theta is associated with the performance of voluntary movements(160) and with a general state of arousal(100). Theta activity may be a correlate of a brain state that is associated with consolidation of information into long term memory(48). Other agents such as barbiturates, neuroleptics, anticholinergics and hallucinogens which are known to impair learning and retention also disrupt theta rhythm.

Izquierdo *et al.*(79) have reported that cannabidiol, cannabinol and Δ^8- and Δ^9-THC decreased rat dorsal hippocampal seizure discharges induced by electrical stimulation. These workers found that cannabidiol effectively blocked K^+ efflux from the afferently stimulated hippocampus suggesting that an interference with hippocampal K^+ release

may reflect one mechanism of action of the cannabinoids. Izquierdo and Nasello[78] found that cannabidiol depressed the hippocampal facilitation of evoked responses in the rat as well as their post-tetanic potentiation—Cannabidiol blocked the normal increase in hippocampal RNA levels pursuant to afferent stimulation and impaired the acquisition of a conditioned avoidance response. THC has also been found to enhance evoked responses recorded from CA1 fields of the dorsal hippocampus[161] and polysensory neocortex of squirrel monkeys[15]. The action of any drug on the hippocampus may have profound implications for the workings of related structures since the hippocampus may modulate activity in a number of brain areas. For example, the fornix system which is the main efferent pathway of the hippocampus distributes fibers to the septum, hypothalamus and midbrain. The circuit of Papez connects the hippocampus with the mammillary bodies, anterior thalamus and cingulate gyrus. Association fibers from the cingulate reach a large portion of the cerebral cortex. Thus, a drug acting on the hippocampus might produce a variety of psychotropic effects. If the hippocampus has inhibitory actions on the hypothalamus, the release of the hypothalamus from inhibition could stimulate the reticular activating system. This could account for disinhibitory effects sometimes seen with THC[45] and the occasionally reported EEG activation.

With regard to the hypothalamus, there is some evidence that cannabinoids can depress its activity. THC has been found to reduce self stimulation by rats of lateral hypothalamic areas and eliminate the normal increase in water consumption which usually follows stimulation of the lateral hypothalamus. Kilbey *et al.*[89] reported that THC may act on cholinergic drinking centers within the hypothalamus. Rats administered THC parenterally in conjunction with carbachol injections to the anterior hypothalamus blocked the increase in water consumption normally produced by carbachol. In a second experiment, THC and atropine were administered to the lateral hypothalamus. Both drugs blocked deprivation induced drinking. Blood flow to the hypothalamus as well as the hippocampus, cerebellum and basal ganglia was reported to be reduced in rats made cataleptic by THC. No changes in cortical blood flow were reported. These changes were thought to reflect functional and metabolic activity[62].

The structural distribution of Δ^9-THC in nonhuman primates along with its change in distribution over time correlates highly with many of the behavioral changes produced by the drug. McIsaac *et al.*[110] found elevated concentrations of tritiated Δ^9-THC in the frontal cortex, lateral and medial geniculate, superior and inferior colliculi and limbic areas including amygdala and hippocampus. These authors concluded that "the extremely high concentration of Δ^9-THC in the frontal cortex together with the hippocampal accumulation makes it tempting to suggest that the interactions between these two areas plays an important part in associating stimuli into a temporal context."

A number of studies have suggested that THC has antiepileptic effects[31, 87]. These effects appear paradoxical when one considers that THC also produces epileptiform activity in hippocampus[151] as well as other brain structures. It has been shown that THC excacerbated seizure activity in epileptic dogs along with the production of myoclonic jerks[51]. The threshold for producing hippocampal-evoked after-discharges in cats employing a weak stimulus was elevated but a marked increase in variance of discharge was observed with strong stimuli with an increase in frequency of long duration after-discharge occurring[52]. An increased variability of neural responses may lead to an increase in variability of experience. In humans, this may be reflected in variability of higher-order information processing and reduced habituation to novel stimuli. Therefore, one reason

for cannabis use may be that it makes the commonplace novel and the trivial unique[50].

Although cannabinoids do appear to have direct cortical actions, any effects they exert on subcortical structures especially limbic system would have profound implications for behavior. Any or all of the cognitive and emotional consequences of cannabis intoxication previously described including mood and sensory changes, impaired attention, reduced memory and an increase in inclusive errors, subjective changes in perception of time, impaired speech and conversational ability, difficulty with the complex, impaired habituation and increased behavioral variability, could all be accounted for by actions of these agents directly on limbic structures and/or limbic cortical connections.

Delgado[36] conceives the limbic system as a set of organizing centers which modulate functions of other cerebral structures. Limbic structures may be seen as planning stations which activate other brain structures, organizing their functions in space and time according to determined sequences, processing at the same time sensory information which interplays with the organization of the response. McKay[102] has called the limbic system a meta-organizational system which evaluates and appraises the activities of the cortex. Bornstein[14] has suggested that the limbic system acts as a dampening device for the neopallium, regulating endogenous and exogenous stimuli before they can become fully effective. These explanations emphasize the interplay of external and internal environments with the limbic system being involved in subjective evaluation. According to Sommerhoff[155] behavior in a given situation is dependent on the subjective evaluation system being able to trigger reactions which outlast the duration of the stimuli that aroused them. That is, stimuli must endure beyond specific situations for a behavior to occur. A reverberation of images in representational brain areas (i.e. neocortex) must occur causing lasting changes in the central state of the organism. Mechanisms based on positive feedback loops such as the Papez circuit may mediate this reverberation. Any drug may affect a particular feedback loop depending on both individual and environmental factors. These feedback loops are likely to run from cortical areas to limbic areas in which a representation of an event is appraised with reference to the drive state of the organism and then back to cortex where the representations would be fixed. Thus, if cognitive processes such as memory consolidation are dependent on the degree of attention given to an event, the internal replay of that event would be "jammed" if any interference with essential links in these loops occurred.

Heath[67] has shown that cannabis produces significant electrical changes in septal activity and that these changes are correlated with alterations in emotional and bodily perceptual changes. The action of cannabis on septum may produce not only emotional changes but cognitive changes as well. The septum may function as the most rostral portion of the reticular activating system. It has rich interconnections with the hippocampus and may be concerned with arousal/inhibitory relationships between the reticular formation and hippocampus. It may also mediate certain aspects of emotionality by inhibiting the pituitary adrenal stress mechanism via the hippocampus. Thus, direct action of cannabis on septum may produce changes in other aspects of brain functioning which could have significant ramifications for cognitive-emotional changes.

One way in which cannabinoids may impair various cognitive functions is to attenuate the hippocampal theta rhythm which is evoked via reticular activation by a pathway running through the hypothalamus, septum and fornix. The medial septal nucleus is thought to be a pacemaker for the theta responses of the hippocampus[141]. If cannabinoids attenuate the hippocampal theta rhythm by actions on septum or hippocampus, the

normal function of the hippocampus which may be to inhibit responses to insignificant stimuli, would be impaired. This might result in a failure of habituation to novel stimuli and hence an overaroused, hypersensitive organism.

Adey and Dunlop[8] have suggested that theta may signal the active involvement of hippocampus in the processing, storage and retrieval of information or more specifically, the disposition of memory traces in extrahippocampal systems. Adey *et al.*[7] have tested this implication by examining the effect of psychotomimetic drugs on visual discrimination and noted that these drugs suppressed theta and at the same time interfered with performance. The presence of theta seems to be important to performance of a delayed response[9] and cannabinoids have been shown to interfere with the delayed matching in nonhuman primates[53, 150, 174]. Although disruption of theta activity may be an important mechanism by which cannabis produces its effect on memory, the presence of theta is inversely related to the amount of neocortex present in an organism. Thus, theta activity in humans may not be a significant determinant of memory consolidation.

A second and perhaps more specific mechanism by which marijuana may affect cognitive, emotional and perceptual functions is related to the manner in which limbic system is interfaced between cerebral cortex and thalamic nuclei. Anterior thalamic nuclei have numerous reciprocal connections with limbic structures. Thus, limbic structures and thalamus provide much functional feedback. The cingulate cortex which receives connections from thalamic nuclei projects via association fibers to various cortical areas. These cortical receiving areas in turn project to thalamic nuclei. This interface which has been proposed by Powell and Hines[144] may have important implications for the understanding of how cannabinoids affect internal and external factors involved in information processing. Anteriorly the limbic system is composed of the dorsal hippocampus and septum which are interconnected via the fornix. The anterior section is in close association with the anterior parts of the cingulate gyrus and prefrontal cortex which is thought to be involved in abstract thought and temporal sequencing. A second interface consists of the ventral hippocampus and amygdala which are interconnected via the subiculum and temporoammonic tracts which are in close association with the temporal lobes. These circuits may be particularly crucial to learning and memory as well as to sensory integration. Information concerning the environment is probably passed from primary cortical sensory areas and then topographically modulated with the limbic interface to the thalamus and is then recycled through various feedback circuits including the neocortex. Limbic connections with thalamus and cortex carry impulses which modulate emotional input into the cerebral system. Both interfaces are presented in Fig. 2.

Based on the distribution of THC in the brain[110] as well as the numerous behavioral effects produced by cannabinoids, the feedback loops within the anterior limbic interface could be affected by these agents. Although few tests of abstract reasoning have been employed to test the effects of cannabis, there is some suggestion that problem solving ability is retarded by the drug. These deficits are seen with anagram problems[2], simple arithmetic[25, 107, 165], performance on the Category test of the Halstead-Reitan battery[93] and on the GDSA, the performance of which probably involves arithmetic ability and short term memory abilities[112, 113]. Tasks which require sequential integration of responses including prose learning[118, 124], GDSA[112, 113] as well as alternation responses in animals[125] are influenced by cannabinoids. Both problem solving behavior as well as the ability to sequentially integrate information may be mediated by these anterior limbic circuits. Thus, the characterization of the acute intoxication syndrome, especially looseness

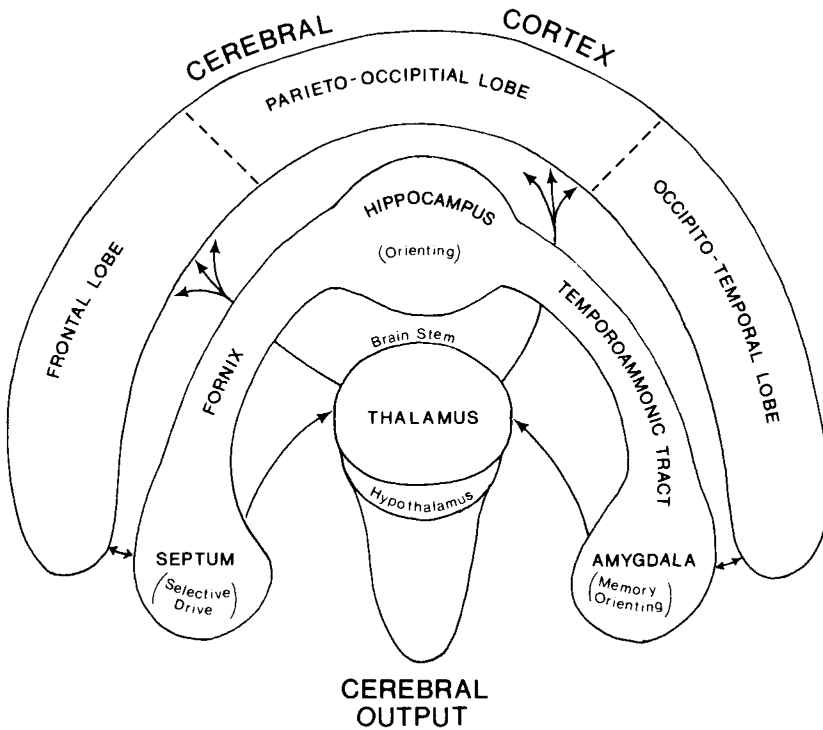


FIG. 2. Limbic system interface between the cerebral hemispheres and thalamus. Redrawn from Powell and Hines[144].

of associations, poorly integrated speech and excess joviality as a mild form of "frontal lobishness" may be accurate neurally as well as behaviorally[146].

The cognitive, perceptual and emotional effects of cannabinoids may also be mediated through the posterior section of limbic interface with temporal cortex. Meissner[111] has suggested that dysfunction within "temporo-ammonic" circuits will result in intact sensory functions and the sparing of simple cognitive skills and retention of automatized behaviors. These functions are spared in the intoxicated individual. However, complex skills are likely to be impaired and this is the usual finding with cannabis. The deficit produced by cannabinoids may arise at an intermediate level of awareness and training. If, as Meissner[111] suggests, TA circuits influence organizational and integrative capacity so that stimulus input is recognized as familiar and is associated with a specific response pattern, the characteristic effects of cannabinoids on the human memory process can be understood by appealing to this schema.

As cannabis preparations become more potent, the temporal cortex may be affected. This cortex may be important as a detailed memory store in which a continuous record of our experiences can be compared with present information. Feelings of derealization and depersonalization found with high doses of THC may be related to possible drug effects in this cortical area. The temporal lobes may also be involved in the ability to make temporal judgments. It has long been believed that the brain's clock may lie below the temporal artery, since the artery owes its name to the fact that ancients used its pulsations to

measure time[155]. Distortions of perception induced by cannabis could arise from dysfunction within limbic structures themselves or might result from effects of limbic perturbations on primary sensory areas via thalamic nuclei.

In discussing perceptual and emotional pathology, Kluver[95] suggests that limbic structures mediate "intimate" rather than "defining" senses, that is senses which do not define things spatially but interpret events as desirable or agreeable. Intimate senses fluctuate and oscillate but are devoid of constancies. Shifts, fluctuations and inconstancies are related to the levels of reality within which one operates. Shifts in affective phenomena or levels of reality are termed poikilofunctions and were thought by Kluver to be mediated by temporal lobe and limbic areas. Drugs such as marijuana may manifest their effects in increasing the variability and intensification of emotional and behavioral fluctuations by inducing a gamut of emotions which fluctuate and alternate. Cannabinoids may interfere with the regulatory role of the limbic system in these functions so that fluctuations and oscillations no longer remain within normal limits. These observations parallel those of Feeney[50].

The deficit in language produced by cannabis could be described as a mild form of semantic aphasia. Brown[20] has indicated that this disorder produces conversational speech which can be very tangential with derailments into unexpected avenues of discourse. Comprehension is also impaired. Patients with this disorder display affective and behavioral changes showing a euphoric attitude and sometimes manic states. Interestingly, this disorder has been associated with left temporal lobe pathology and bilateral limbic cortical lesions and as an aftermath of carbon monoxide poisoning. It is unlikely that cannabis produces these effects by direct cortical actions since the ability to speak is not impaired, articulation is normal, and there is no mutism or agrammatism.

In a number of the cognitive deficits produced by cannabinoids, the hippocampus may play a crucial role since this structure has been termed the hub of the limbic system. A drug acting on this structure could produce both memory and attention impairments. It was previously suggested that the hippocampus forms a significant link in which reticular arousal evoked by novel stimuli habituates and gives way to focused attention if the stimuli are deemed significant. The intact hippocampus may be instrumental in suppressing reactions to novel stimuli. An effect of marijuana on this structure might lessen its tonic inhibitory influence. Cannabinoids may cause the intoxicated subject to be less able to give up a focus of attention once established and at the same time reduce the ability to refocus once a distracting stimulus is introduced. In tests of memory, intoxicated individuals consistently demonstrate the intrusion of irrelevant material which may contribute to long term memory problems. In a free recall task, attention processes may be labile so that information in short term memory is "dumped" before transfer to long term memory occurs. Thus, the internal replay or reverberation of an event in some of the cortical-limbic loops previously described would be interfered with.

Jarvik[80] has suggested that perceptual alterations such as hallucinations which are produced by a variety of agents may be due to a deficit in information processing by the brain rather than being due to impaired sensation. Drugs such as cannabis may actually enhance sensation and perception. Hallucinations may consist of long term memories which are inappropriately retrieved. That is, retrieval is no longer conducted via a systematic strategy. This retrieval problem is termed vicarious retrieval or uninhibited retrieval. Thus, information may enter the nervous system during the period of intoxication, but may be poorly integrated with material from long term memory.

A demonstration of the kinds of effects marijuana can have on the memory process may

be appropriate at this point. The data to be described are strikingly similar to those found with patients experiencing amnesia due to herpes simplex encephalitis[140] or Korsakoff syndrome[58]. Herpes complex is thought to affect the limbic system via disruption of cholinergic limbic pathways[42, 140, 147], while Korsakoff syndrome may be due to lesions in the hippocampus, mamilliary bodies and/or dorsalmedial nucleus of the thalamus[111]. The task employed was a free recall task in which words in a list are presented individually until recall of the word occurs once. Following initial recall, words are not presented again so that eventually recall trials occur without any further presentation of items. All words are recalled on each trial. Buschke[21] has argued that storage and retrieval of items in memory cannot be evaluated when all items are presented before every recall attempt because immediate recall of items does not demonstrate that an item resides in long term memory. The basic dependent variables in this paradigm are long term storage which consists of the number of items encoded on a given trial while retrieval consists of the number of items recalled on each trial that are considered to be in long term storage. Sixteen male volunteers were run in a crossover design with each receiving both smoked marijuana (10.5 mg Δ^9-THC) or placebo in successive sessions separated by one week. In each session, a different 30-item word list was employed with words being presented at a 3-second rate. Recall testing occurred for 12 trials. The results of this study are presented in the following three figures and only the most salient features will be emphasized. In Fig. 3, it can be seen that the number of items eventually encoded under marijuana and placebo were essentially the same although it took more recall trials for the same number of items to be encoded under drug. However, retrieval of items from long term storage was significantly impaired under drug and this was due to the fact that more memory lapses or recall failures took place following intoxication. That is, intoxicated subjects displayed an inconsistency in recall. For example, under marijuana an encoded word might be retrieved on a given trial following which a 3 to 4 trial lapse in recall would occur before the word would be recalled again. In Fig. 4, these effects are highlighted further. Items in random storage consist of those items which are encoded but not yet consistently recalled. Items from random storage are added to the group of consistently recalled words (additional list learning) at a much slower rate following intoxication. In Fig. 5, it can be seen that intrusion error rates are significantly elevated in the drug condition in comparison to placebo. Intrusions consisted of the number of different extralist words which were emitted. What intoxicated subjects tended to do was commit an intrusion error, encode the word and repeat it on the majority of recall trials or if they committed one error, they might drop that word and substitute another. Thus, extraneous words from long term memory are introduced and may interfere with recall although total recall of words is not highly correlated with number of intrusion errors[126].

These data suggest that the basic problem in recall of the intoxicated individual may be in his capacity to integrate material in some meaningful fashion for recall to occur. Buschke[21] suggests that when items of information are consistently retrieved in memory they are integrated with the retrieval of other items in the list. Thus, items in memory are encoded within a given context. Information about a target item and its relationship to other words in the semantic system provides a basis of organization. That is, the learner imposes structure on information to be recalled by employing integrating strategies based on information already in long term memory. Marijuana may affect an individual's capacity to make use of information in his semantic memory to employ efficient recall strategies. Thus, the depth to which information is processed following cannabis intoxica-

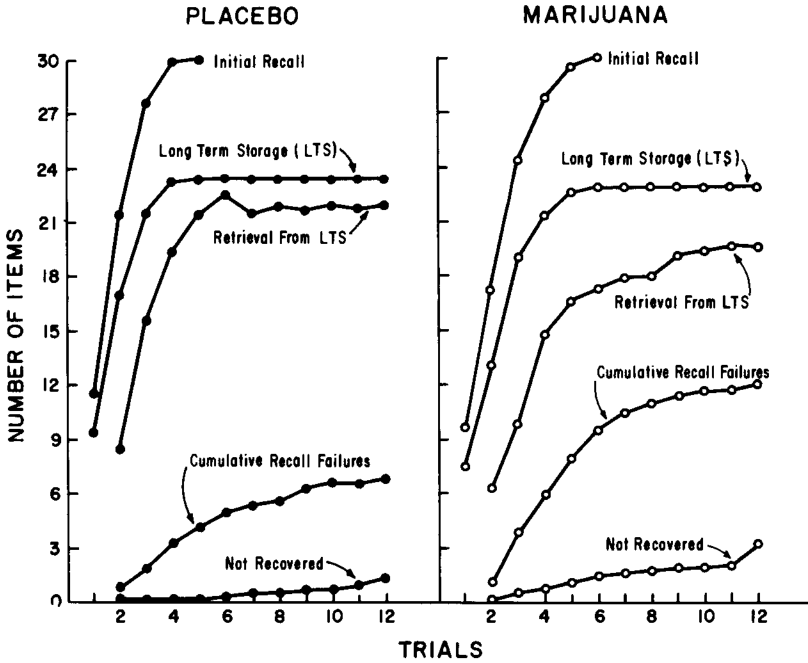


FIG. 3. Analysis of free recall by restricted reminding for placebo and marijuana conditions. Initial recall, LTS, retrieval from LTS, cumulative recall failures, and number of items not recovered from LTS.

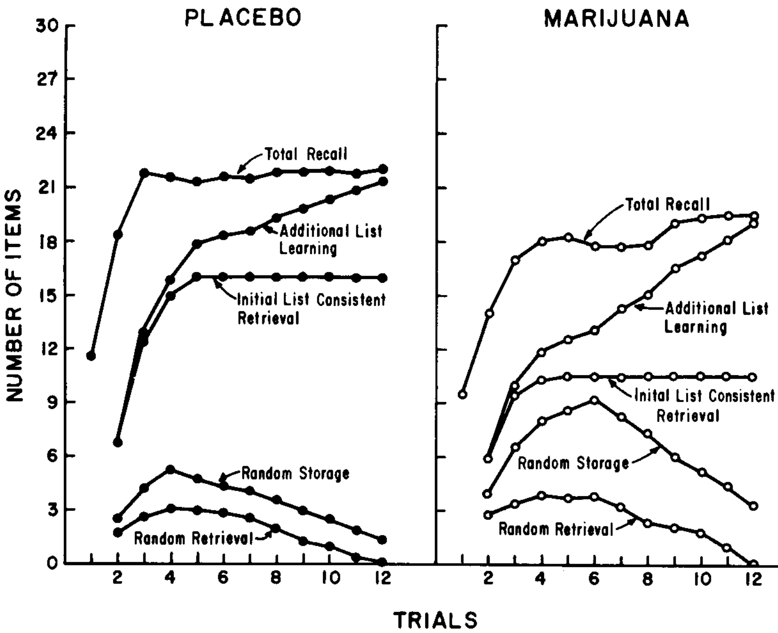


FIG. 4. Analysis of free recall by restricted reminding for placebo and marijuana conditions. Total recall, additional list learning, initial list consistent retrieval, random storage, and random retrieval.

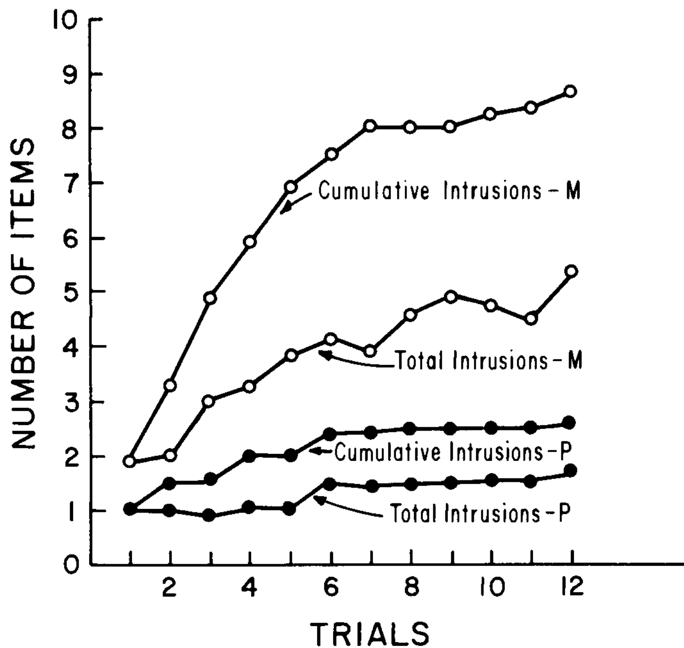


FIG. 5. Total number of intrusion errors and cumulative intrusion errors for placebo and marijuana conditions.

tion may be an important mechanism by which recall is impaired. Intrusion errors may be seen as being secondary to organizing and integrating deficiencies since information from long term memory which is the basis for semantic organization intrudes on the recall process.

BIOCHEMICAL MECHANISMS

The literature which is beginning to evolve concerning the biochemical mechanisms by which cannabinoids mediate behavior is too vast to cover adequately in this paper. Brain neurotransmitters especially the biogenic amines have been implicated as mediators.

Virtually all neurotransmitters have been reported to be in some way affected by cannabinoids but it has been suggested that many findings are contradictory and inconclusive[(63)]. However, if cannabis does influence limbic-cortical relationships, the various amine systems would be likely to be involved.

Many of the physiological reactions as well as numerous behavioral effects of cannabis are similar to those found with anticholinergic drugs such as atropine or scopolamine. These reactions include dry mouth, decreased salivation, and tachycardia[(82)]. Cardiovascular studies have suggested a possible atropine-like effect of cannabis employing moderate dose levels in humans. One study suggested that marijuana induces tachycardia by inhibiting vagal tone[(28)], while another reported that cannabis suppressed sinus arrhythmia presumably by the same mechanism[(145)]. Unlike anticholinergics, however, cannabis has distinct hypotensive actions[(74)].

On a behavioral level, the similarity of cannabinoids to anticholinergic muscarinic drugs such as atropine or scopolomine has been reviewed elsewhere[(45)]. Like cannabis, anticho-

linergics are found to impair various aspects of the memory process, produce state-dependent learning and impair performance on tasks which may require some inhibitory control. Some studies have also noted that the behavioral effects of cannabinoids can be blocked by anticholinesterase type drugs[18]. Two human studies reported a failure to block the effects of cannabinoids by physostigmine suggesting that cannabinoids may not exert significant actions on post-synaptic receptors[49, 55]. However, it has been suggested that cannabinoids may act at a different step by blocking release of acetylcholine or by affecting uptake mechanisms[12, 45]. Complicating the picture further, it has been reported that THC has anticholinesterase-like activity[19].

Miller and Drew[122] have suggested that cannabinoids may act on cholinergic inhibitory centers within the limbic system especially the hippocampus. Hippocampal gating mechanisms proposed by Douglas[41], which are thought to be cholinergic in nature may be particularly sensitive to the effects of cannabinoids. Nonspecific gating is responsible for the exclusion of extraneous stimuli during concentration of attention protecting memory traces from interference during consolidation while specific gating acts to inhibit the perception of stimuli associated with nonreinforcement. Carlton[24] has tried to explain the action of anticholinergic drugs on behavior by hypothesizing that these agents interfere with an inhibitory process common to a variety of situations and the hippocampus is thought to contain a cholinergic inhibitory system[163].

Other brain amines implicated as mediators of cannabinoids are the catecholamines and serotonin. In man, the neuronal system comprising norepinephrine has been associated with changes in affective state, especially depression. Dopamine deficiency is related to Parkinsonism, while an excess results in abnormal motor and mood states. Some of the more effective neuroleptics block the action of dopamine which results in an alleviation of psychotic episodes. Serotonin has been implicated in sleep mechanisms and depression[29].

There are a number of reasons why the catecholamines and serotonin have been thought to be involved in the mechanism of action of cannabinoids. First, the early human studies on the physiological and behavioral reactions to THC noted that high doses (150–200 mcg/kg orally) produced subjective effects similar to those found for LSD-25. Since it has been shown that serotonin mediates some of the effects of LSD[71], it seemed reasonable to assess the actions of cannabinoids on these amines. Secondly, early comparisons of dimethylheptylpyran (DMHP) with reserpine and amphetamine, agents which act via catecholamine systems, lent credence to the idea that cannabinoids might exert their effects through the same mechanisms[33]. DMHP, like reserpine, induces a prolonged decrease in blood pressure in cats along with a reduction in heart rate and respiration[32].

In man, the most prominent physiological reactions following intoxication which may be related to changes in amine activity are tachycardia and hypotension while behavioral reactions include changes in affective states and general cognitive deficits. In animals, the major physiological reactions noted include bradycardia, hypotension and hypothermia, while behavioral reactions consist of stimulation and depression of activity, catelepsy, circling and stereotyped behaviors, squealing, reaction time to aversive stimulation, aggression and stress reactions. All of the above physiological and behavioral reactions have been hypothesized to be mediated by, or at least correlated with, changes in catecholamines and serotonin. One problem with infrahuman studies in this area is that abnormally high dose levels of cannabinoids have been employed in order to produce pharmacological changes. A question arises as to what minimum effective dose of cannabis must be employed to produce a given change in amine levels and behavior. Intravenous doses of

THC which produce significant alterations in numerous rat behaviors such as squealing, catalepsy and hot plate and tail flick reaction time, do not alter the serotonin content of forebrain and brainstem or brain levels of 5HIAA. This suggests a dissociation between behavior and changes in amine concentration[61]. Two human studies have reported no changes in endogenous secretion of catecholamines and serotonin except for a transient rise in norepinephrine secretion which was attributed to anticipatory stress of the experiment or to the rapid onset of unfamiliar symptoms[72, 114]. Both pulse rate as well as a number of behavioral and emotional changes produced by cannabinoids have been reported to be attenuated by propranolol suggesting that the effects of cannabis may be mediated by a beta adrenergic mechanism[156]. A number of studies have been conducted to determine the interaction of THC and amine activity with aggression and stress. In animals, the effects of cannabinoids on irritable, predatory and spontaneous aggression have been extensively studied[3]. Whether cannabinoids increase or decrease aggression depends on the paradigm employed, species, sex, acute or chronic treatment, environmental factors, as well as other variables. This area is exceedingly complex and becomes more so in attempting to identify brain mechanisms mediating changes in aggression. Acute doses of THC usually reduce aggressiveness except under conditions of stress (i.e. deprivation, cold environment) where aggression is increased. Prolonged food deprivation as well as chronic treatment can increase aggression. Cannabis may potentiate irritable aggression and suppress predatory and inter-male aggression. Whole brain serotonin levels are reported to be increased following a THC-induced decrease in predatory aggression[91] whereas decreased brain levels are found in midbrain, hypothalamus and cerebral hemispheres in stressed rats in which aggressiveness is elicited by THC[134]. It has also been reported that PCPA, a serotonin inhibitor and DOPA pretreatment potentiate aggressiveness in starved rats but not in rats fed *ad lib* following THC treatment. Increased aggression was not found following treatment with PCPA or DOPA alone. It was concluded that PCPA and DOPA were "preparatory" agents for aggressiveness while THC acted as a "releasing" agent[133]. Eichelman and Thoa[47] suggest that irritable aggression may be related to alterations in norepinephrine metabolism, spontaneous aggression to dopamine metabolism and predatory aggression to serotonin depletion. The effect of cannabinoids on catecholamines and serotonin in relation to aggressive behavior are at this point not well established. A survey of the literature suggests that cannabinoids in most instances decrease aggression (discounting the introduction of stress) and may therefore be similar to anticholinergic drugs which are effective in reducing different forms of animal aggression[47].

The interaction of environmental variables and concomitant biochemical alterations following cannabinoid treatment has been studied infrequently. One study has indicated that rats stressed by immobilization and treated chronically with THC displayed an increase in dopamine hydroxylase activity. Dopamine hydroxylase arises from sympathetic nerve terminals and catalyzes the formation of norepinephrine from dopamine. Interestingly, THC had synpatholytic effects in nonstressed rats[130]. THC also increases circling behavior in rats subjected to mild auditory or tactile stimulation. This appears to be associated with a change in the ratio of dopamine levels between striati without affecting the mean striatal levels. Finally, the cataleptic and hypothermic actions of THC may be related to blockade of dopamine receptors[99].

Some promising research concerning the effects of cannabinoids on 2-phenylethylamine (PEA) has been performed by Sabelli and associates[149]. PEA is a brain amine thought to

facilitate arousal and excitement and is structurally related to amphetamine. It is possible that increases in brain PEA levels or activation of central PEA receptors mediate the stimulant and depressive actions of a wide variety of drugs. In a series of experiments, THC increased brain levels of PEA which could be responsible for the euphorant effects of the drug. The sedative actions of THC were reversed by MAO inhibitors producing subsequent jumping and vocalization and pronounced aggression. It was suggested that the stimulation and postural arrest produced by THC is a result of facilitation of endogenous DOPA, a catecholamine precursor. Alpha-methyltyrosine facilitated THC-induced jumping and vocalization suggesting that its stimulant effects are not mediated by the catecholamines.

It is known that the catecholamines and serotonin are distributed in numerous brain areas. Norepinephrine and serotonin are distributed widely in the brain stem, reticular formation and limbic system while dopamine is distributed mainly in basal ganglia. Although the present review of the effects of cannabinoids on amine systems is cursory, it is nevertheless evident that these agents may influence all of the amine systems. As is the case with other pharmacological agents, cannabis may alter the interaction between the various amine systems. For example, the antidepressant, imipramine, may potentiate the action of brain catecholamines at central adrenergic receptors and at the same time exhibit anticholinergic activity by blocking cholinergic effects. Thus, the mood-altering effects of the drug may be related to its adrenergic effects while changes in cognitive functions may be related to cholinergic mediation. Understanding how cannabinoids might produce an imbalance in neurohumoral systems and how this relates to behavioral changes may be more important than specifying absolute changes in brain amine levels. It should also be noted that cannabinoids may not only affect different neurotransmitters, but may act on any of the numerous steps in neural firing such as synthesis, storage, transport, release or uptake. The actions of these agents may not be limited to neurotransmitters but may also be mediated by peptides, nucleic acids as well as metabolic products.

The major neurotransmitters are distributed throughout the limbic system. Cannabis probably affects a number of behaviors mediated by limbic structures alone or in conjunction with cortex. While cannabis may influence a specific neurotransmitter such as acetylcholine to produce a given change in behavior, it is likely that the balance among several neurotransmitter systems is altered by cannabinoids. The behavioral effects of these agents are beginning to be well defined; the neural and biochemical mechanisms by which these behavioral changes are affected provides questions for future research.

REFERENCES

1. ABEL, E. L., Marijuana and memory: acquisition or retrieval? *Science*, **173**,1038–1040 (1971).
2. ABEL, E. L., Effects of marijuana on the solution of anagrams, memory and appetite. *Nature*, **231**,260–261 (1971).
3. ABEL, E. L., Cannabis and aggression in animals. *Behav. Biology*, **14**,1–20 (1975).
4. ADAMS, P. M. and BARRATT, E. S., Role of biogenic amines in the effects of marijuana on EEG patterns in cats. *Electro. Clin. Neurophysiol.*, **39**,621–625 (1975).
5. ADEY, W. R., Hippocampal states and functional relations with cortico-subcortical systems attention and learning. *Prog. Brain Research*, **27**,228–245 (1967).
6. ADEY, W. R., Neurophysiological action of LSD in "Neurophysiological and behavioral aspects of psychotropic drugs", Karczmar, A. G. and Koella, W. P., Eds., pp. 5–26, C. C. Thomas, Springfield, 1969.
7. ADEY, W. R., BELL, F. R. and DENNIS, B. J., Effects of LSD-25, psilocybin and psilocin on temporal lobe EEG patterns and learned behavior in the cat. *Neurology*, **12**,591–602 (1962).

8. ADEY, W. R. and DUNLOP, C. W., The action of certain cyclohexamines on hippocampal system during approach performance in the cat. *J. Pharmacol. Exper. Therap.*, **130**,418-426 (1960).
9. ADEY, W. R., DUNLOP, C. W. and HENDRIX, C. E., Hippocampal slow waves, distribution and phase relationships in the course of approach learning. *Arch. Neurology*, **3**,74-90 (1960).
10. AMES, F. A., A clinical and metabolic study of acute intoxication with cannabis sativa and its role in the model psychoses. *J. Ment. Science*, **104**,972-999 (1958).
11. ARNOLD, M. B., Brain function in emotion: a phenomenological analysis in "Physiological correlates of emotion", Black, Perry Eds., pp. 261-285, Academic Press, New York, 1969.
12. ASKEW, W. E., KIMBALL, A. P. and HO, B. T., Effect of tetrahydrocannabinols on brain acetylcholine. *Brain Research*, **69**,375-378 (1974).
13. AVIS, H. F., The neuropharmacology of aggression: a critical review. *Psychol. Bull.*, **81**,47-63 (1974).
14. BORNSTEIN, W., Zur physiologie und pathologie des rhinencephalon. *Scheweiz. Arch. Neurol. Psychiatry*, **67**,264-274 (1951).
15. BOYD, E. S., BOYD, E. H. and BROWN, L. E., The effects of some drugs on evoked responses in polysensary cortex. *J. Pharmacol. Exp. Therap.*, **189**,748-758 (1974).
16. BRADEN, W., STILLMAN, R. C. and WYATT, R. J., Effects of marijuana on contingent negative variation and reaction time. *Arch. Gen. Psychiatry*, **31**,537-541 (1974).
17. BROMBERG, W., Marijuana intoxication. *Amer. J. Psychiatry*, **91**,303-330 (1934).
18. BROWN, H., Some anticholinergic-like behavioral effects of trans $(-)$-Δ^8-tetrahydrocannabinol. *Psychopharmacologia*, **21**,294-301 (1971).
19. BROWN, H., Possible anticholinesterase-like effects of trans $(-)$-Δ^8 and Δ^9-tetrahydrocannabinols as observed in the general motor activity of mice. *Psychopharmacologia*, **27**,111-116 (1972).
20. BROWN, J. W., The neural organization of language: aphasia and lateralization. *Brain and Language*, **3**,482-494 (1976).
21. BUSCHKE, H., Retrieval in verbal learning. *Trans. N.Y. Acad. Science*, **236**,721-729 (1974).
22. CAPPELL, H. D. and PLINER, P. L., Volitional control of marijuana intoxication: a study of the ability to "come down" on command. *J. Abn. Psychology*, **82**,428-434 (1973).
23. CARLINI, E. A., LINDSEY, C. J., MUSTY, R. E. and MONTI, J. M., Marijuana aggressiveness in REM-sleep-deprived rats: neurochemical and neurophysiological correlates in "The pharmacology of marijuana", Braude, M. C. and Szara, S. Eds., pp. 515-530, Raven Press, New York, 1976.
24. CARLTON, P. L., Brain acetylcholine and inhibition in "Reinforcement and behavior", Tapp, J. T. Ed., pp. 286-327, Academic Press, New York, 1969.
25. CASSWELL, S. and MARKS, D.: Cannabis induced impairment of performance of a divided attention task. *Nature*, **241**,60-61 (1973).
26. CHRISTIANSEN, H. D., FRUEDENTHAL, R. I., GIDLEY, J. T., ROSENFELD, R., BOEGLI, G., TESTINO, L., BRINE, D. R., PITT, C. G. and WALL, M. E., Activity of Δ^8- and Δ^9-tetrahydrocannabinol and related compounds in the mouse. *Science*, **172**,165-167 (1971).
27. CLARK, L. D., HUGHES, R. and NAKASHIMA, E. N., Behavioral effects of marijuana: experimental studies. *Arch. Gen. Psychiatry*, **23**,193-198 (1970).
28. CLARK, S. C., GREENE, C., KARR, G. W., MACCONNELL, K. L. and MILSTEIN, S. L., Cardiovascular effects of marijuana in man. *Can. J. Physiol. Pharmacology*, **52**,706-719 (1974).
29. CLARK, W. G. and DEL GIUDICE, J., "Principles of Psychopharmacology," Academic Press, New York, 1970.
30. CONSROE, P. F., JONES, B. C. and CHIN, L., Δ^9-tetrahydrocannabinol, EEG, and behavior: the importance of adaptation to the testing milieu. *Pharmacol. Biochem. Behav.*, **3**,173-177 (1975).
31. CONSROE, P., JONES, B., LAIRD, H. II and REINKING, J., Anticonvulsant-convulsant effects of delta-9-tetrahydrocannabinol in "The Therapeutic Potential of Marihuana", S. Cohen and R. C. Stillman Eds., pp. 363-382, Plenum Press, New York, 1976.
32. DAGIRMANJIAN, R. and BOYD, E. S., Some pharmacological effects of two tetrahydrocannabinols. *J. Pharmacol. Exp. Therap.*, **135**,25-33 (1962).
33. DAGIRMANJIAN, R. and HODGE, H. C., Are biogenic amines involved in the mechanism of action of the tetrahydrocannabinols. *Agents and Actions*, **1**,46-48 (1969).
34. DARLEY, C. F. and TINKLENBERG, J. R., Marihuana and memory in "Marijuana: effects on human behavior", L. L. Miller, Ed., pp. 73-102, Academic Press, New York, 1974.
35. DEJONG, R. N., The hippocampus and its role in memory. *J. Neurol. Sciences*, **19**,73-83 (1973).
36. DELGADO, J. M. R., "Physical Control of the Mind," Harper and Row, New York, 1969.
37. DELONG, F. L. and LEVY, B. I., A model of attention describing the cognitive effects of marijuana in "Marijuana: effects on human behavior", L. L. Miller, Ed., pp. 103-120, Academic Press, New York, 1974.
38. DOMINO, E. F., Neuropsychopharmacologic studies of marihuana, some synthetic and natural THC derivatives in animals and man. *Ann. N.Y. Acad. Science*, **191**,166-191 (1971).
39. DORNBUSH, R. L.: Marijuana and memory: effects of smoking on storage. *Trans. N.Y. Acad. Science*, **234**,94-100 (1974).

40. DORNBUSH, R. L., FINK, F. and FRIEDMAN, A. J., Marihuana, memory and perception. *Am. J. Psychiatry*, **128**,194–197 (1971).
41. DOUGLAS, R. J., The hippocampus and behavior. *Psychol. Bull.*, **67**,416–442 (1967).
42. DRACHMAN, D. A. and ADAMS, R. D., Herpes simplex and acute inclusion-body encephalitis. *Arch. Neurology*, **7**,61–79 (1962).
43. DREW, W. G., KIPLINGER, G. F., MILLER, L. L. and MARX, M., Effects of propranolol on marihuana-induced cognitive dysfunctioning. *Clin. Pharmacol. Therap.*, **13**,526–533 (1972).
44. DREW, W. G. and MILLER, L. L., Differential effects of Δ^9-THC on locomotor behavior in activity-wheel habituated and nonhabituated rats. *Pharmacology*, **9**,41–51 (1973).
45. DREW, W. G. and MILLER, L. L., Neural mechanisms and behavior—a theoretical review. *Pharmacology*, **11**,12–32 (1974).
46. EICH, J. R., WEINGARTNER, H., STILLMAN, R. C. and GILLIN, J. C., State-dependent accessibility of retrieval cues in the retention of a categorized list. *J. Verb. Learn. Verb. Behav.*, 14,408–417 (1975).
47. EICHELMAN, B. S. and THOA, N. B., The aggressive monoamines. *Biol. Psychiatry*, **6**,143–164 (1973).
48. ELAZAR, A. and ADEY, W. R., Spectral analysis of low frequency components in the electrical activity of the hippocampus during learning. *Electro. Clin. Neurophysiol.*, **23**,225–240 (1967).
49. EL-YOUSEF, M. K., JANOWSKY, D. S., DAVIS, J. M. and ROSENBLATT, J. E., Induction of severe depression by physostigmine in marijuana intoxicated individuals. *Brit. J. Addictions*, **68**,321–325 (1973).
50. FEENEY, D. M., The marijuana window: a theory of cannabis use. *Behav. Biology*, **18**,445–471 (1976).
51. FEENEY, D. M., SPIKER, M. and WEISS, G. K., Marijuana and epilepsy: activation of symptoms by delta-9-THC in "The therapeutic potential of marihuana", S. Cohen and R. C. Stillman, Eds., pp. 343–362, Plenum Press, New York, 1976.
52. FEENEY, D. M., WAGNER, H. R., MCNAMARA, M. C. and WEISS, G., Effects of tetrahydrocannabinol on hippocampal evoked after discharges in rats. *Exp. Neurology*, **41**,357–365 (1973).
53. FERRARO, D. P. and GRILLY, D. M., Lack of tolerance to Δ^9-tetrahydrocannabinol in chimpanzees. *Science*, **179**,490–492 (1973).
54. FINK, M., Affects of acute and chronic inhalation of hashish, marijuana, and Δ^9-tetrahydrocannabinol on brain electrical activity in man: evidence for tissue tolerance. *Ann. N.Y. Acad. Science*, **282**,387–398 (1976).
55. FREEMAN, F. R., ROSENBLATT, J. E. and EL-YOUSEF, M. K., Interaction of physostigmine and delta-9-tetrahydrocannabinol in man. *Clin. Pharmacol. Therap.*, **17**,121–126 (1975).
56. FRIED, P. A., Septum and behavior: a review. *Psychol. Bull.*, **78**,292–310 (1972).
57. FRIED, P. A. and MCINTYRE, D. C., Electrical and behavioral attenuation of the anti-convulsant properties of delta-9-THC following chronic administration. *Psychopharmacologia*, **31**,215–227 (1973).
58. FULD, P. A., Storage, retention and retrieval in Korsakoff's syndrome. *Neuropsychologia*, **14**,225–236 (1976).
59. FULLER, J. L., ROSVOLD, H. E. and PRIBRAM, K. H., The effect on affective and cognitive behavior in the dog of lesions of the pyriform-amygdala-hippocampal complex. *J. Comp. Physiol. Psychology*, **50**,89–96 (1957).
60. GALEF, B. G., Aggression and timidity: responses to novelty in feral Norway rats. *J. Comp. Physiol. Psychology*, **70**,370–381 (1970).
61. GALLAGER, D. W., SANDERS-BUSH, E. and SULSER, F., Dissociation between behavioral effects and changes in metabolism of cerebral serotonin following Δ^9-tetrahydrocannabinol. *Psychopharmacologia*, 337–345 (1972).
62. GOLDMAN, H. R., DAGIRMANJIAN, R., DREW, W. G., and MURPHY, S., Δ-9-tetrahydrocannabinol alters flow of blood to subcortical areas of the conscious rat brain. *Life Sciences*, **17**,477–482 (1975).
63. HARRIS, L. S., Cannabis: a review of progress in "Psychopharmacology: A Generation of Progress", M. A. Lipton, A. DiMascio, K. F. Killam, Eds., pp. 1565–1574, Raven Press, New York, 1978.
64. HAERTZEN, C. A., Development of scales based on patterns of drug effects using the Addiction Research Center Inventory (ARCI). *Psychol. Rep.*, **18**,163–194 (1966).
65. HEATH, R. B., Definition of the septal region in "Studies in schizophrenia", R. G. Heath, Ed., pp. 3–5, Harvard University Press, Cambridge, 1954.
66. HEATH, R. G., Pleasure response of human subjects to direct stimulation of the brain: physiologic and psychodynamic considerations in "The role of pleasure in behavior", pp. 219–243, Harper Row, New York, 1964.
67. HEATH, R. G., Marijuana: Effects on deep and surface electroencephalograms of man. *Arch. Gen. Psychiatry*, **26**,577–584 (1972).
68. HEATH, R. G., Effects on deep and surface electroencephalograms of rhesus monkeys. *Neuropharmacology*, **12**,1–14 (1973).
69. HERRICK, C. J., The functions of the olfactory parts of the cerebral cortex. *Proc. Nat. Acad. Science*, **19**,7–14 (1933).
70. HOCKMAN, C. H., PERRIN, R. G. and KALANT, H., Electroencephalographic and behavioral alterations produced by Δ^1-tetrahydrocannabinol. *Science*, **172**,968–970 (1971).
71. HOFFER, A. and OSMOND, H., "The Hallucinogens," Academic Press, New York, 1967.

72. HOLLISTER, L. E., MOORE, F., KANTER, S. and NOBLE, E., Δ^1-tetrahydrocannabinol, synhexyl and marihuana extract administered orally in man: catecholamine excretion, plasma cortisol levels and platelet serotonin content. *Psychopharmacologia*, **17**,354–360 (1970).
73. HOLLISTER, L. E. and OVERALL, J. E., Dimensions of marihuana experience. *Drug and Alcohol Dependence*, 155-161 (1975/76).
74. HOLLISTER, L. E., RICHARDS, R. K. and GILLESPIE, H. K., Comparison of tetrahydrocannabinol and synhexyl in man. *Clin. Pharmacol. Therap.*, **9**,783-791 (1968).
75. HOLLISTER, L. E., SHERWOOD, S. L. and CAVASINO, A., Marihuana and the human electroencephalogram. *Pharmacol. Res. Commun.*, **2**,305–308 (1970).
76. HOLTZMAN, D., LOVELL, R. A., JAFFE, J. H. and FREEDMAN, D. X., Δ^9-tetrahydrocannabinol: neurochemical and behavioral effects in the mouse. *Science*, **163**,1464–1467 (1969).
77. HOROWITZ, M. J. and ADAMS, J. E., Hallucinations on brain stimulation: evidence for revision of the Penfield hypothesis in "Origins and Mechanisms of Hallucinations", W. Keup, Ed., pp. 13–22, Plenum Press, New York, 1970.
78. IZQUIERDO, I. and NASELLO, A. G., Effects of cannabidiol and of diphenylhydantoin on the hippocampus and on learning. *Psychopharmacologia*, **31**,167–175 (1973).
79. IZQUIERDO, I., ORSINGHER, O. A. and BERARDI, A. C., Effect of cannabidiol and of other cannabis sativa compounds on hippocampal seizure discharges. *Psychopharmacologia*, **28**,95–102 (1973).
80. JARVIK, M., Drugs, hallucinations and memory in "Origin and mechanisms of hallucinations", pp. 277–301, Plenum Press, New York, 1970.
81. JASINSKI, D. R., HAERTZEN, C. A. and ISBELL, H., Review of the effects in man of marijuana and tetrahydrocannabinols on subjective state and physiologic functioning. *Ann. N.Y. Acad. Science*, **191**,196–206 (1971).
82. JOHNSON, S. and DOMINO, E. F., Some cardiovascular effects of marijuana smoking in normal volunteers. *Clin. Pharmacol. Therap.*, **12**,762–768 (1971).
83. JONES, R., Tetrahydrocannabinol and the marijuana induced social "high" or the effects of the mind on marijuana. *Ann. N.Y. Acad. Science*, **191**,155–165 (1971).
84. JONES, R. T. and BENOWITZ, N., The 30-day trip-clinical studies of cannabis tolerance and dependence in "Pharmacology of Marihuana", S. Szara and M. C. Braude, Eds., pp. 627–642, Raven Press, New York, 1976.
85. JONES, R. T., BENOWITZ, N. and BACHMAN, J., Clinical studies of cannabis tolerance and dependence. *Ann. N.Y. Acad. Science*, **282**,221–239 (1976).
86. JONES, R. T. and STONE, G. R., Psychological studies of marijuana and alcohol in man. *Psychopharmacologia*, **18**,108–117 (1970).
87. KARLER, R. and TURKANIS, S. K., The antiepileptic potential of the cannabinoids in "The Therapeutic Potential of the Cannabinoids", S. Cohen and R. C. Stillman, Eds., pp. 383–397, Plenum Press, New York, 1976.
88. KESNER, R., A neural system analysis of memory storage and retrieval. *Psychol. Bull.*, **80**,177–203 (1973).
89. KILBEY, M. M., FORBES, W. B. and OLIVETTI, C. C., Δ^9-Tetrahydrocannabinol: inhibition of deprivation and carbachol-induced water consumption in the rat after central and peripheral administration. *Behav. Biology*, **8**,679–685 (1971).
90. KILBEY, M. M., MCLENDON, D. M., FRITCHIE, G. E. and JOHNSON, K. M., Δ^9-tetrahydrocannabinol induced inhibition of attack behavior in the mouse. *Nature*, **283**,463–465 (1972).
91. KILBEY, M. M., MOORE, J. W. and HALL, M., Δ^9-tetrahydrocannabinol induced inhibition of predatory aggression in the rat. *Psychopharmacologia*, **31**,157–166 (1973).
92. KIPLINGER, G. F., MANNO, J. E., RODDA, B. E. and FORNEY, R. B., Dose-response analysis of the effects of tetrahydrocannabinol in man. *Clin. Pharmacol. Therap.*, **12**,650–657 (1971).
93. KLONOFF, H. and LOW, M. D., Psychological and neurophysiological effects of marijuana in man: an interaction model in "Marijuana: effects on human behavior", L. L. Miller, Ed., pp. 121–155, Academic Press, New York, 1974.
94. KOUKKOU, M. and LEHMANN, D., Human EEG spectra before and during cannabis hallucinations. *Biol. Psychiatry*, **11**,663–677 (1976).
95. KLUVER, H., Neurobiology of normal and abnormal perception in "Psychopathology of perception" P. H. Hoch and J. Zubin, Eds., pp. 1–40, Grune and Stratton, New York, 1963.
96. LEATON, R. N., The limbic system and its pharmacological aspects in "An introduction to psychopharmacology", R. H. Rech and K. E. Moore, Eds., pp. 137–174, Raven Press, New York, 1971.
97. LEWIS, E. G., DUSTMAN, R. E., PETERS, B. A., STRAIGHT, R. C. and BECK, E. C., The effects of varying doses of Δ^9-tetrahydrocannabinol on the human visual somatosensory evoked responses. *Electro. Clin. Neurophysiol.*, **35**,347–354 (1973).
98. LIPPARINI, F., SCOTTI DE CAROLIS, A., and LONGO, V. G., A neuropharmacological investigation of some trans-tetrahydrocannabinol derivatives. *Physiol. Behav.*, **4**,527–532 (1971).
99. LITTLETON, J. M. and MACLEAN, K. I., The effect of Δ^8-tetrahydrocannabinol (Δ^8-THC) on dopamine metabolism in the rat corpus striatum: the influence of environment. *Brit. J. Pharmacology*, **511**,117 (1974).

100. LONGO, V. G. and LOIZZO, A., Effects of drugs on the hippocampal theta rhythm in *Proc. Fifth Int. Cong. Pharmacology*, **4**,46–54 (1972).
101. LOW, M. D., KLONOFF, H. and MARCUS, A., The neurophysiological basis of the marihuana experience. *Canad. Med. Ass. J.*, **108**,157–164 (1973).
102. MACKAY, D. M., Cerebral organization and conscious control of action in "Brain and conscious experience", J. C. Eccles, Ed., Springer, New York, 1966.
103. MACLEAN, P. D., The limbic brain in relation to the psychoses in "Physiological correlates of emotion", Perry Black, Ed., pp. 129–146, Academic Press, New York, 1970.
104. MACLEAN, P. D., Sensory and perceptive factors in emotional functions of the triune brain in "Biological Foundations of Psychiatry" R. G. Grenell and S. Gabay, Eds., Vol. 1, pp. 177–198, Raven Press, New York, 1976.
105. MAHL, C. F., ROTHERBERG, A., DELGADO, J. M. R., and HAMLIN, H., Psychologic response in the human to human intracerebral electrical stimulation. *Psychosom. Medicine*, **26**,337–368 (1964).
106. MALAMUD, N., The epileptogenic focus in temporal lobe epilepsy from a pathological standpoint. *Arch. Neurology*, **14**,190–196 (1966).
107. MANNO, J., KIPLINGER, G. F., HAINE, S. E., BENNETT, I. F. and FORNEY, R. B., Comparative effects of smoking marihuana or placebo on human motor and mental performance. *Clin. Pharmacol. Therap*, **11**,808–815 (1970).
108. MANNO, J. E., MANNO, B. R., KIPLINGER, G. F., and FORNEY, R. B., Motor and mental performance with marijuana: Relationship to administered dose of Δ^9-tetrahydrocannabinol and its interaction with alcohol in "Marijuana: effects on human behavior", L. L. Miller, Ed., pp. 45–72, Academic Press, New York, 1974.
109. MARTZ, R., BROWN, D. J., FORNEY, R. B., BRIGHT, T. P., KIPLINGER, G. F., and RODDA, B. E., Propranolol antagonism of marihuana induced tachycardia. *Life Sciences*, 999–1005 (1972).
110. MCISAAC, W. M., FRITCHIE, G. E., IDANPAAN-HEIKKILA, J. E., HO, B. T. and ENGLERT, L. F., Distribution of marijuana in monkey brain and concomitant behavioral effects. *Nature*, **230**,593–594 (1971).
111. MEISSNER, W. W., Learning and memory in the Korsakoff syndrome. *Int. J. Neuropsychiatry*, **4**,6–20 (1968).
112. MELGES, F. T., TINKLENBERG, J. R., HOLLISTER, L. E. and GILLESPIE, H. K., Marihuana and temporal disintegration. *Science*, **168**,1118–1120 (1970).
113. MELGES, F. T., TINKLENBERG, J. R., HOLLISTER, L. E. and GILLESPIE, H. K., Marihuana and the temporal span of awareness. *Arch. Gen. Psychiatry*, **24**,564–567 (1971).
114. MESSIHA, F. S., and SOSKIN, R. A., Effect of cannabis sativa administered by smoking on biogenic amine excretions in man. *Res. Comm. Chem. Pathol. Pharmacology*, **6**,325–330 (1973).
115. MILLER, L. L., Marihuana and human cognition: a review of laboratory investigations in "The therapeutic potential of marihuana" S. Cohen and R. C. Stillman, Eds., pp. 271–292, Plenum Press, New York, 1976.
116. MILLER, L. L. and CORNETT, T. L., Marijuana: dose effects on pulse rate, subjective estimates of intoxication, free recall and recognition memory. *Pharmacol. Biochem. Behav.* (in press).
117. MILLER, L., CORNETT, T., BRIGHTWELL, D., MCFARLAND, D., DREW, W. G. and WIKLER, A., Marijuana and memory impairment: the effect of retrieval cues on free recall. *Pharm. Biochem. Behav.*, **5**,639–643 (1976).
118. MILLER, L., CORNETT, T., BRIGHTWELL, D., MCFARLAND, D. and DREW, W. G., Marijuana: effects on storage and retrieval of prose material. *Psychopharmacology*, **51**,311–316 (1977).
119. MILLER, L., CORNETT, T., DREW, G., MCFARLAND, D. and WIKLER, A., Marijuana: dose response effects on pulse rate, subjective estimates of potency, pleasantness and recognition memory. *Pharmacology*, **15**,265–275 (1977).
120. MILLER, L. L., CORNETT, T. and MCFARLAND, D., Marijuana: an analysis of storage and retrieval deficits in memory with the technique of restricted reminding. *Pharm. Biochem. Behav.*, **8**,327–332 (1978).
121. MILLER, L., CORNETT, T. and NALLAN, G., Marijuana: effect on nonverbal free recall as a function of field dependence. *Psychopharmacology* (in press).
122. MILLER, L. L. and DREW, W. G., Cannabis: neural mechanisms and behavior in "Marijuana: effects on human behavior", L. L. Miller, Ed., pp. 157–188, Academic Press, New York, 1974.
123. MILLER, L. L. and DREW, W. G., Cannabis: review of behavioral effects in animals. *Psychol. Bull.*, **81**,401–417 (1974).
124. MILLER, L., DREW, W. G. and KIPLINGER, G. F., Effects of marijuana on recall of narrative material and Stroop-colour word performance. *Nature*, **237**,172–173 (1972).
125. MILLER, L. L., DREW, W. G. and WIKLER, A., Comparison of Δ^9-THC, LSD-25 and scopolamine on non-spatial single alternation performance in the runway. *Psychopharmacologia*, **28**,1–11 (1973).
126. MILLER, L. L., MCFARLAND, D., CORNETT, T. L. and BRIGHTWELL, D., Marijuana and memory impairment: effect on free recall and recognition memory. *Pharmac. Biochem. Behav.*, **7**,99–103 (1977).
127. MORGANE, P. J. and STERN, W. C., Relationship of sleep to neuroanatomical circuits, biochemistry, and behavior. *Ann. N.Y. Acad. Science*, **193**,95–111 (1972).
128. MOSKOWITZ, H., SHARMA, S. and SCHAPERO, M., A comparison of the effects of marijuana and alcohol on visual functions in "Current Research in Marijuana", M. F. Lewis, Ed., pp. 129–150, Academic Press, New York, 1972.

129. NAHAS, G. G., Marihuana-Deceptive Weed, Raven Press, New York, 1973.
130. NG, L. K. Y., LAMPRECHT, F., WILLIAMS, R. B. and KOPIN, I. J., Δ^9-tetrahydrocannabinol and ethanol: differential effects on sympathetic activity in differing environmental settings. *Science*, **180**,1368–1369 (1973).
131. OLDS, J. and MILNER, P., Positive reinforcement produced by electrical stimulation of septal area and other regions of rat brain. *J. Comp. Physiol. Psychol.*, **47**,419–427 (1954).
132. OLDS, M. E. and OLDS, J., Anxiety-relieving drugs in hippocampus, reticular midbrain and pre-optic area in freely moving rat. *Int. J. Neuropsychiatry*, **8**,87–95 (1969).
133. PALERMO-NETO, J. and CARLINI, E. A., Aggressive behavior elicited in rats by cannabis sativa: effects of *p*-chlorophenylalanine and dopa. *Eur. J. Pharmacol.*, **17**,215–220 (1972).
134. PALERMO-NETO, J. and CARVALHO, F. V., The effects of chronic cannabis treatment on the aggressive behavior and brain 5-hydroxytryptamine levels of rats with different temperaments. *Psychopharmacologia*, **32**,383–392 (1973).
135. PAPEZ, J. W., A proposed mechanism of emotion. *Arch. Neurol. Psychiatry*, **38**,725–744 (1937).
136. PATON, W. D. M. and PERTWEE, R. G., The actions of cannabis in man in "Marijuana: chemistry, pharmacology, metabolism and clinical effects", R. Mechoulam, Ed., pp. 287–333, Academic Press, New York, 1973.
137. PAUL, M. I. and CARSON, I. M., Marijuana and communication. *Lancet*, 270–271 (1973).
138. PEEKE, S. C., JONES, R. T. and STONE, G. C., Effects of practice on marijuana-induced changes in reaction time. *Psychopharmacology*, **48**,159–163 (1976).
139. PENFIELD, W. and MILNER, B., The memory deficit produced by bilateral lesions in the hippocampal zone. *A.M.A. Arch. Neurol. Psychiatry*, **79**,475–497 (1957).
140. PETERS, B. H. and LEVIN, H. S., Memory enhancement after physostigamine treatment in the amnesic syndrome. *Arch. Neurology*, **34**,215–219 (1977).
141. PETSCHE, H., STUMPF, C. and GOGOLAK, G., The significance of the rabbit's septum as a relay station between midbrain and the hippocampus. I. The control of hippocampal arousal activity by the septum cells. *Electro. Clin. Neurophysiol.*, **14**,202–211 (1962).
142. PFEFFERBAUM, A., DARLEY, C. F., TINKLENBERG, J. R., ROTH, W. T. and KOPELL, B. S., Marijuana and memory intrusions. *J. Nerv. Ment. Disease*, **165**,381–386 (1977).
143. PIRCH, J. H., COHN, R. A., BARNES, P. R. and BARRATT, E. S., Effects of acute and chronic administration of marijuana extract on the rat electrocorticogram. *Neuropharmacology*, **11**,231–240 (1972).
144. POWELL, E. W. and HINES, G., The limbic system: an interface. *Behav. Biology*, **12**,149–164 (1974).
145. RENAULT, P. F., SCHUSTER, C. R., HEINRICH, R. and FREEMAN, D. X., Marihuana: standardized smoke administration and dose effect curves on heart rate in humans. *Science*, **174**,589–591 (1971).
146. RODIN, E. A., DOMINO, E. F. and PORZAK, J. P., The marijuana-induced "social high": neurological and electroencephaloic concomitants. *J.A.M.A.*, **213**,1300–1302 (1970).
147. ROSE, F. C. and SYMONDS, C. P., Persistent memory defect following encephalitis. *Brain*, **83**,195–211 (1960).
148. ROTH, W. T., TINKLENBERG, J. R. and KOPELL, B. S., Ethanol and marihuana effects on event-related potentials in a memory retrieval paradigm. *Electro. Clin. Neurophysiol.*, **42**,381–388 (1977).
149. SABELLI, H. C., PEDEMONTE, W. A., WRALLEY, C., MOSNAIM, A. D. and VAZQUEZ, A. J., Further evidence for a role of 2-phenylethylamine in the mode of action of Δ^9-tetrahydrocannabinol. *Life Sciences*, **14**,149–156 (1974).
150. SCHECKEL, C. L., BOFF, E., DAHLEN, P. and SMART, T., Behavioral effects in monkeys of racemates of two biologically active marihuana constituents. *Science*, **160**,1467–1469 (1968).
151. SEGAL, M., Central implantation of cannabinoids: induction of epileptiform discharges. *European J. Pharmacology*, **27**,40–45 (1974).
152. SEGAL, M. and KENNEY, A. F., Delta-1 and delta 1(6)-tetrahydrocannabinol: similarities and differences in central pharmacological effects. *Experientia*, **29**,816–819 (1971).
153. SHRIFFIN, R. M. and ATKINSON, R. C., Storage and retrieval processes in long term memory. *Psychol. Rev.*, **76**,179–193 (1969).
154. SMYTHIES, J. R., Brain Mechanisms and Behavior, Academic Press, New York, 1970.
155. SOMMERHOFF, G., Logic of the Living Brain, Wiley, New York, 1974.
156. SULKOWSKI, A., VACHON, L. and RICH, E. S., Propranolol effects on acute marihuana intoxication in man. *Psychopharmacology*, **52**,47–53 (1977).
157. TART, C. T., Marijuana intoxication: common experiences. *Nature*, **226**,701–704 (1970).
158. TEECE, J. J., Contingent negative variation (CNV) and psychological processes in man. *Psychol. Bull.*, **77**,73–108 (1972).
159. TINKLENBERG, J. R., KOPELL, B. S., MELGES, F. T. and HOLLISTER, L. E., Marihuana and alcohol: time production and memory functions. *Arch. Gen. Psychiatry*, **27**,812–815 (1972).
160. VANDERWOLF, C. H., Hippocampal electrical activity and voluntary movement in the rat. *Electro. Clin. Neurophysiol.*, **26**,407–418 (1969).
161. VARDARIS, R. M., WEISZ, D. J. and TEYLER, T. J., Δ^9-Tetrahydrocannabinol and the hippocampus: effects on CA1 field potentials in rats. *Brain Res. Bull.*, **2**,181–187 (1977).

162. VOLAVKA, J., DORNBUSH, R., FELDSTEIN, S., CLARE, G., ZAKS, A., FINK, M., and FREEDMAN, A. M., Marijuana, EEG and behavior. *Ann. N.Y. Acad. Science*, **191**,206–215 (1971).
163. WARBURTON, D. M., Effects of atropine on single alternation in hippocompectomized rats. *Physiol. Behav.*, **4**,641-644 (1969).
164. WARRINGTON, E. K., Neurological disorders of memory. *Brit. Med. Bull.*, **27**,243–245 (1971).
165. WASKOW, I. E., OLSSON, J. E., SOLZMAN, G. and KATZ, M. M., Psychological effects of delta-9-THC. *Arch. Gen. Psychiatry*, **22**,97–107 (1970).
166. WEIL, A. T. and ZINBERG, N. E., Acute effects of marihuana on speech. *Nature*, **222**,434–437 (1969).
167. WEIL, A., ZINBERG, N. and NELSON, J., Clinical and psychological effects of marijuana in man. *Science*, **162**,1234–1242 (1968).
168. WEST, L. J., A general theory of hallucinations and dreams in "Hallucinations", L. J. West, Ed., pp. 275–291, Grune and Stratton, New York, 1962.
169. WIKLER, A., Drug dependence in "Clinical Neurology", A. B. Baker and L. H. Baker, Eds., pp. 1–53, Harper and Row, Hagerstown, MD, 1971.
170. WILLIAMS, E. G., HIMMELSBACH, C. K., WIKLER, A., RUBLE, D. C. and LLOYD, B. J., Studies on marihuana and pyrahexyl compound. *Public Health Rep.*, **61**,1059–1083 (1946).
171. WILLIAMS, M. and ZANGWILL, O. L., Memory defects after head injury. *J. Neurol. Neursurg, Psychiatry*, **15**,54–58 (1952).
172. WILLINSKY, M. D., SCOTTI DE CAROLIS, A. and LONGO, V. G., EEG and behavioral effects of natural, synthetic and biosynthetic cannabinoids. *Psychopharmacologia*, **31**,365–374 (1973).
173. ZEIDENBERG, P., CLARK, W. C., JAFFE, J., ANDERSON, W. W., CHIN, S. and MALITZ, S., Effect of oral administration of delta-9-tetrahydrocannabinol on memory, speech and perception of thermal stimulation: results with four normal human volunteer subjects. Preliminary report. *Comp. Psychiatry*, **14**,549–556 (1973).
174. ZIMMERBERG, B., GLICK, S. D., JARVIK, M. E., Impairment of recent memory by marihuana and THC in rhesus monkeys. *Nature*, **233**,343–345 (1971).

CANNABIS AND BRAIN-STIMULATION REWARD

S. N. PRADHAN, ALOK K. BHATTACHARYYA, C. S. AULAKH
SIKTA PRADHAN, and PAUL T. BAILEY

Department of Pharmacology, College of Medicine, Howard University, Washington, D.C.

Summary. Effects of Δ^9-tetrahydrocannabinol (Δ^9-THC) were studied on self-stimulation (SS) behavior in rats with implanted electrodes at the posterior hypothalamus (PH) or area ventralis tegmentum (A10 area). Its effects were also studied with respect to spontaneous motor activity (SMA), rectal temperature, barbiturate sleeping time, development of tolerance and brain neurotransmitter levels.

Two patterns of Δ^9-THC effects were observed in these experiments. In one type, there was a gradual drug-induced depression that persisted for a prolonged period. Decrease in SS responding in non-aroused subjects, hypothermia, potentiation of barbiturate-induced sleep are examples. These effects may be due to a nonspecific generalized depression of the central nervous system. The other type, a triphasic effect is characterised by an initial depression followed by a stimulation and then again depression, and is observed in SS in aroused subjects and SMA. The effects of the stimulants such as amphetamine and cocaine are antagonised during the initial depressant phase and unaffected or even potentiated during the subsequent stimulant phase.

The neurochemical studies demonstrated an initial decrease in dopamine (DA) in the caudate nucleus and diencephalon-midbrain (DM) and increase of serotonin (5-HT) in DM and PM (pons-medulla) followed by increase in DA and decrease in 5-HT and then their reversal in respective brain areas. These effects can be well correlated to the triphasic behavioral effects.

Development of tolerance to the behavioral depressant effect in SS on repeated administration of Δ^9-THC in SS and lack of cross-tolerance to mescaline were also observed.

SELF-STIMULATION (SS) behavior in rats introduced by James Olds (see Olds, 1958, 1962) provides an excellent animal model for studying drug effects on the brain reward system. In this behavior an animal presses a lever in a Skinner box to be reinforced or rewarded by an electric stimulation of a discrete brain area through an implanted electrode. This behavior appears not only to provide a measure of centrally mediated positive reinforcement, but also an index of motivation. In an earlier study from this laboratory (Bailey and Pradhan, 1972), Δ^9-tetrahydrocannabinol (Δ^9-THC, 1–25 mg/kg, i.p.) the active principle of cannabis was shown to decrease SS responding of rats with electrodes implanted in their posterior hypothalamus (PH).

In earlier investigations, SS behavior was considered to be mediated through a central noradrenergic mechanism (Stein, 1964). However, subsequently, a central dopaminergic mechanism has been implicated to play a prominent role in this behavior (Roll, 1970; Pradhan, 1975, 1976; Hall *et al.*, 1977). Accordingly, the objectives of the present experiment were to further investigate the effects of Δ^9-THC on SS behavior in rats with electrodes implanted in one of the two electrode sites: the PH area that was used in our previous as well as in numerous other experiments and involves a number of neurotransmitter mechanisms including norepinephrine (NE), dopamine (DA), serotonin (5-HT) and acetylcholine (ACh), and area ventralis tegmentum (dorsal to the interpeduncular nucleus) described by Dahlström and Fuxe (1965) as A10 area that involves mainly DA mechanisms. Spontaneous motor activity (SMA), a nonreinforced behavior, was included in this study for comparison. Furthermore, attempts were made to correlate drug-induced behavioral changes with those in the levels of various neurotransmitters in discrete brain areas. Interaction of Δ^9-THC with some depressants and stimulants, and development of tolerance were also studied.

MATERIAL AND METHODS

Male albino rats (Wistar-derived Walter Reed strain) 200–250 g body weight were used throughout the experiment. The animals were given food and water *ad libitum*. For behavioral studies the rats were housed in individual cages.

METHODS

(*a*) *SS*. The procedures for electrode implantation in a brain area, behavioral training, drug administration, and data evaluation were essentially the same as described earlier (Pradhan and Bowling, 1971; Nimitkipaisan *et al.*, 1977), unless otherwise mentioned. Under pentobarbital (50 mg/kg, i.p.) anesthesia a set of bipolar stainless steel electrodes were stereotaxically implanted in five rats each in the PH or A10 area. The stereotaxic coordinates for the PH were 3.5 mm posterior to the bregma, 0.75 mm lateral to the midline, and 9 mm in depth from the top of the skull held flat according to the atlas of Hart (1969). The stereotaxic coordinates for the A10 area were 5.5 mm posterior to the bregma, 0.1 mm lateral to the midline, and 8.5 mm in depth from the top of the skull. The rats with electrodes at PH and A10 areas will be designated as PH and A10 rats respectively. Verification of the location of electrode tips was done histologically in representative rats upon completion of the experiments according to the atlas of König and Klippel (1963).

(*b*) *SMA*. SMA of individual rats was recorded by Selective Activity Meters (Model S, Columbus Instruments, Columbus, Ohio). A rat was kept in a plastic cage, placed over an Activity Meter and covered at the top with a wooden board with several holes. During 3-hour daily sessions, SMA was recorded every 10 min; the activity during the initial 10-min period was considered as exploratory activity.

(*c*) *Other parameters*. Rectal temperature (°F) was monitored by a Telethermometer (Model 44TD, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio) along with SMA in an individual rat kept in a plastic cage over an Activity Meter (same as described

under SMA). Recordings were made every 10 min before and after injection of Δ^9-THC or its vehicle during 3-hour daily sessions. Throughout the whole session, room temperature was kept constant at 83°F ± 1°F. Sleeping time was measured as interval between loss and appearance of righting reflex.

DRUGS

Δ^9-THC was given in 4% Tween in normal saline (as vehicle) in 1–20 mg/kg doses as scheduled in different experiments. For interaction studies, pentobarbital sodium (35 mg/kg), D-amphetamine sulfate (0.5 mg/kg), cocaine hydrochloride (5 mg/kg) and for cross-tolerance study, mescaline hydrochloride (18 or 25 mg/kg) dissolved in saline were used. All drugs were given i.p.

PROCEDURE

A week after recovery from surgery, the rats were trained to press the bar in a Skinner box to be reinforced with intracranial electrical stimulation. The box (22 cm × 27 cm × 47 cm) made of plastic was equipped with a bar at the center of a side wall 6 cm above the floor. The bar was connected with programming and recording equipment and each bar press delivered an electrical stimulus that was a sine wave consisting of train of pulses of 60 Hz and 0.4 sec duration. The current intensity (measured as rms) remained constant at slightly above the threshold level throughout the sessions. Bar pressing responses were usually recorded every 10 min during daily 3-hour sessions. The rats were subjected to a daily session for six days a week.

During some sessions of self-stimulation especially after drug treatment (either Δ^9-THC alone or in combination with a stimulant) rats were handled and placed on the bar every 10 min either to arouse them or to examine their behavior status. Handling was done also during the corresponding control period sessions.

When bar-pressing rate of self-stimulating rats became stable after 2 to 3 weeks of training, treatment with Δ^9-THC was started. For SMA, 5 to 7 days of adaptation to the experimental situation, during which the vehicle was injected daily, were needed for stabilization of activity before the drug injection. A dose of Δ^9-THC (5, 10, 15 or 20 mg/kg) was injected in increasing sequence at an interval of at least 3 days. The vehicle was given daily for 2 days before each drug day. A dose of a drug was injected only when the control data from two successive daily sessions reached the previous baseline level and were within 10% of their average. Injection was given immediately before the session, unless otherwise mentioned.

For the study of drug interactions, a dose of cocaine or D-amphetamine was injected either in conjunction with Δ^9-THC (10 mg/kg) before the session (0 min) or at 80 min post-THC. Prior to drug interactions, control recordings after injection of cocaine or D-amphetamine alone, at specified time (0 or 80 min after the vehicle) was recorded at least one week before each drug-interaction day.

For experiments on sleeping time, 3 groups of rats, 5 in each, were used. The rats were injected at 0 min with the vehicle in group I and Δ^9-THC (10 mg/kg) in groups II and III. Pentobarbital was injected 10 min later in groups I and II and 100 min later in group III.

For tolerance study, repeated doses were injected daily as described in the schedules.

NEUROCHEMICAL PROCEDURES

At specified intervals following injection of Δ^9-THC (10 mg/kg i.p.), naive rats were decapitated after being subjected to microwave radiation focused on the skull for 2 sec in a Litton Microwave Oven (Model No. 70/50; 3.5 kw; 2450 MHz; 1300 W output). The brain was quickly removed and different areas, such as the caudate nucleus (CN), the diencephalon-midbrain (DM) and the pons-medulla (PM) were dissected out at 4°. DM and PM were assayed for NE and 5-HT. DM and one CN were assayed for DA, and the other CN was used for ACh assay. The tissues were homogenized (100 mg/ml) in ice-cold 0.4 N perchloric acid, centrifuged in a Sorvall (RC2-B Model) at 4° for 20 min at 10,000 rpm. NE, DA and 5-HT were extracted simultaneously from the supernatant of the tissue homogenate according to the method of Cox and Perhach (1973). NE and DA were assayed by the method of Chang (1964) and Spano and Neff (1971). 5-HT was extracted and determined according to the procedure of Ansell and Beeson (1968) and Maickel *et al.* (1968). ACh was assayed according to the procedure of Shea and Aprison (1973) with slight modification (Pradhan *et al.*, 1978).

DATA ANALYSIS

In both behavioral and neurochemical experiments, the data of each parameter from several rats at a particular experimental condition were used to calculate the mean and the standard error of the mean ($\pm$ S.E.). Student's t test was performed to evaluate the statistical significance of the change from the control. Drug effects were calculated in terms of percent change from the controls in neurochemical and some behavioral experiments.

RESULTS

A. EFFECTS OF SINGLE ADMINISTRATIONS

(i) *Self-stimulation.* During the control sessions, self-stimulation responding varied from 3,400 to 5,160 per hour in PH rats and from 7,220 to 8,310 per hour in A10 rats. Following treatment with Δ^9-THC at 5 or 10 mg/kg doses the response rates decreased by 60% or more within 20 to 30 min and remained more or less at the same level for the rest of a 3-hour session. The decrease in responding was more rapid in A10 rats. The effects of 5 and 10 mg/kg doses did not appear to differ from each other (Fig. 1).

In other experiments, rats were handled and placed on the lever every 10 min, following administration of 5, 10, 15 or 20 mg/kg of Δ^9-THC. As in the previous experiments, respondings decreased progressively in both groups of rats reaching the minimum levels at 60 min, but later on gradually increased reaching their peaks between 120 to 160 min and then decreased again (Fig. 2 & 3). Handling during 80–160 min post-THC also induced behavioral outbursts, such as excitation, jumping and vocalization.

In order to compare the dose-response relation in both groups of rats, the effects of different doses were calculated during an early 60 min (20–80 min post-drug) period of decreased responding and later 60 min (100–160 min post-drug) period of temporarily enhanced responding (Fig. 4). It appears that a definite dose-response relation was lacking in either group. However, although the effects were similar at 5 and 10 mg/kg and also at

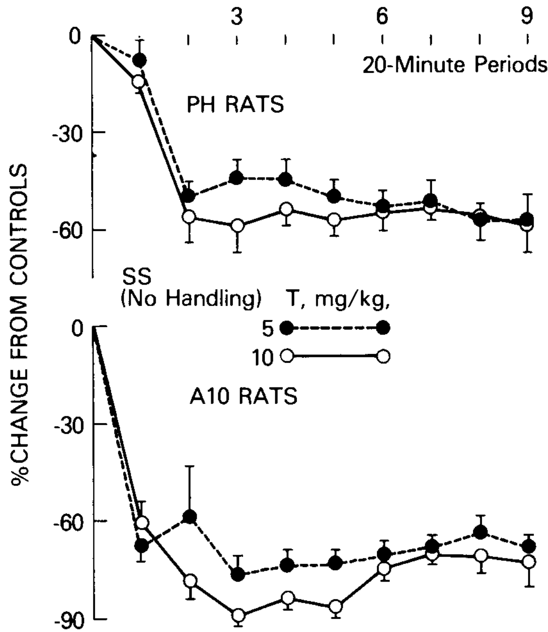


FIG. 1. Effects of Δ^9-THC (injected at 0 min) in nonaroused (no handling) PH and A10 rats on self-stimulation (SS) showing persistent depression.

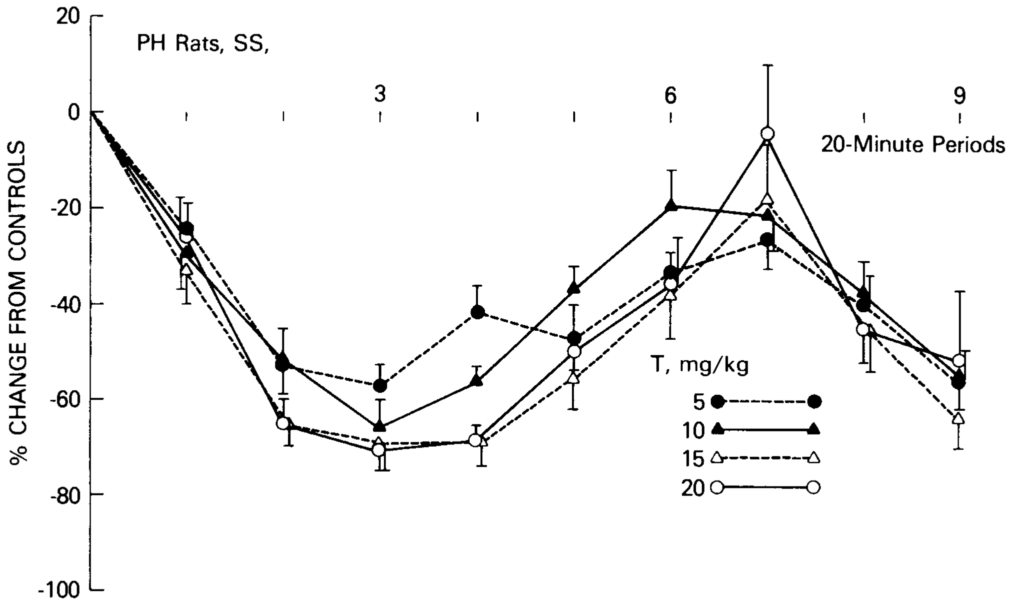


FIG. 2. Effects of Δ^9-THC (injected at 0 min) on SS in PH rats aroused (handled) every 10 min (as described in the text), showing a triphasic response.

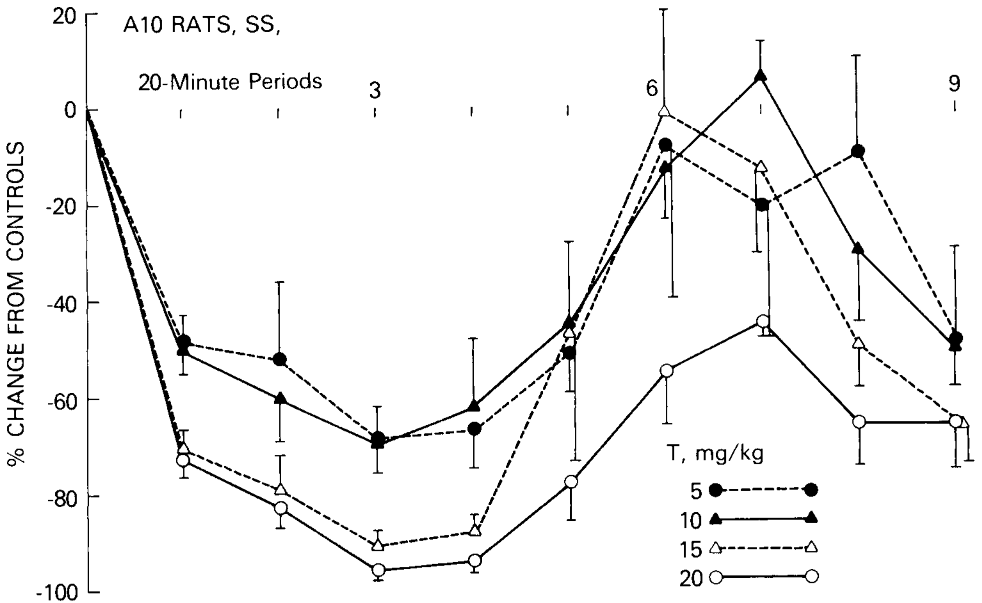


FIG. 3. Effects of Δ^9-THC in A10 rats. Details same as in Fig. 2.

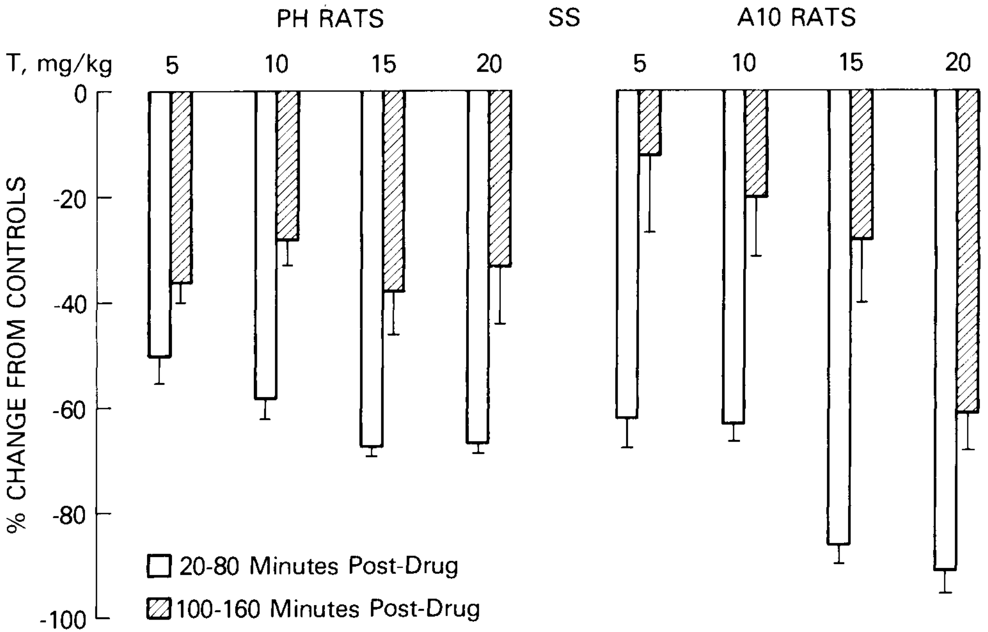


FIG. 4. Dose-response relationship in the depressant and stimulant phases of Δ^9-THC effect in PH and A10 rats.

15 and 20 mg/kg, the effects of the two lower doses appeared to differ significantly ($P < 0.05$ to $P < 0.01$) from those at the two higher doses.

(ii) *SMA*. In control sessions, SMA was high during the first 10–20 min, which can be considered as exploratory activity. After several days of adaptation of rats to the experimental situation, the SMA including the exploratory activity was decreased and stabilized. It varied from rat to rat and ranged between 1520 and 2060 per hour.

Following treatment with 5, 10 or 15 mg/kg of Δ^9-THC, the SMA usually decreased gradually reaching the minimum levels between 60 to 80 min post-drug following which it began to increase. SMA reached its peak between 120 to 140 min and then was decreased again (Fig. 5).

(iii) *Body temperature*. Rectal temperature of rats ($N = 4$) varied between 98.5 to 99.5°F. Following injection of 4% Tween in the controls there was a slight increase (0.5–1°F) of temperature during the first 20 min, after which it either returned to the normal level and/or fluctuated by 0.25–0.5°F. Following administration of 15 mg/kg of Δ^9-THC the temperature gradually decreased to its minimum (97–97.25°F) between 60 and 80 min and remained approximately at that level fluctuating by ± 0.25°F for the rest of the 180-min session. Motor activity recorded concomittantly in these animals showed the usual triphasic response as described earlier (Fig. 5).

(iv) *Neurotransmitter level*. The time-courses of effects of 10 mg/kg of Δ^9-THC and its vehicle (4% Tween) are shown in Fig. 6. While Tween itself failed to show any significant effect on the neurotransmitter levels in the discrete brain areas, Δ^9-THC produced some effects (Fig. 6). DA levels at CN and DM decreased significantly ($P < 0.01$) at 60 min, but increased ($P < 0.01$) at 90 and 120 (peak level) min and then decreased again. 5-HT levels in DM and PM increased at 60 min, but decreased significantly ($P < 0.05$ or less) at 90 and 120 min and then increased towards normal. NE at DM and PM, and ACh at CN decreased slightly at 60 min; ACh then gradually increased, whereas NE remained around the normal levels up to 150 min.

(v) *Drug interaction*. Because of the triphasic nature of the behavioral and neurochemical effects of Δ^9-THC some interaction studies were conducted with a depressant like pentobarbital and stimulants like amphetamine and cocaine during the depressant and stimulant phases of the drug action. Sleeping time for pentobarbital sodium (35 mg/kg) was enhanced when it was given either 10 or 100 min after Δ^9-THC (10 mg/kg), potentiation being more marked in the latter situation (Fig. 7).

The effects of amphetamine (0.5 mg/kg) or cocaine (5 mg/kg) on SS in PH and A10 rats were studied following their administration at 0 or 80 min after Δ^9-THC (10 mg/kg) injection (Figs. 8–11). Injected at 0 min (i.e. during the depressant phase of Δ^9-THC action) both the stimulants showed some degree of antagonism that was more marked in case of amphetamine (Figs. 8 & 9). On the other hand, injected at 80 min (i.e. during the apparent stimulant phase of Δ^9-THC action) the stimulants failed to produce any antagonism, rather showed some potentiation, the effects of the drugs or combinations being estimated in terms of the areas covered under the graphs (Figs. 10 & 11).

B. EFFECTS OF REPEATED ADMINISTRATIONS

Δ^9-THC injected once or twice daily at 1, 5, 10, 15 or 20 mg/kg doses showed development of tolerance with respect to its behavioral depressant effect. Figure 12 illustrates the

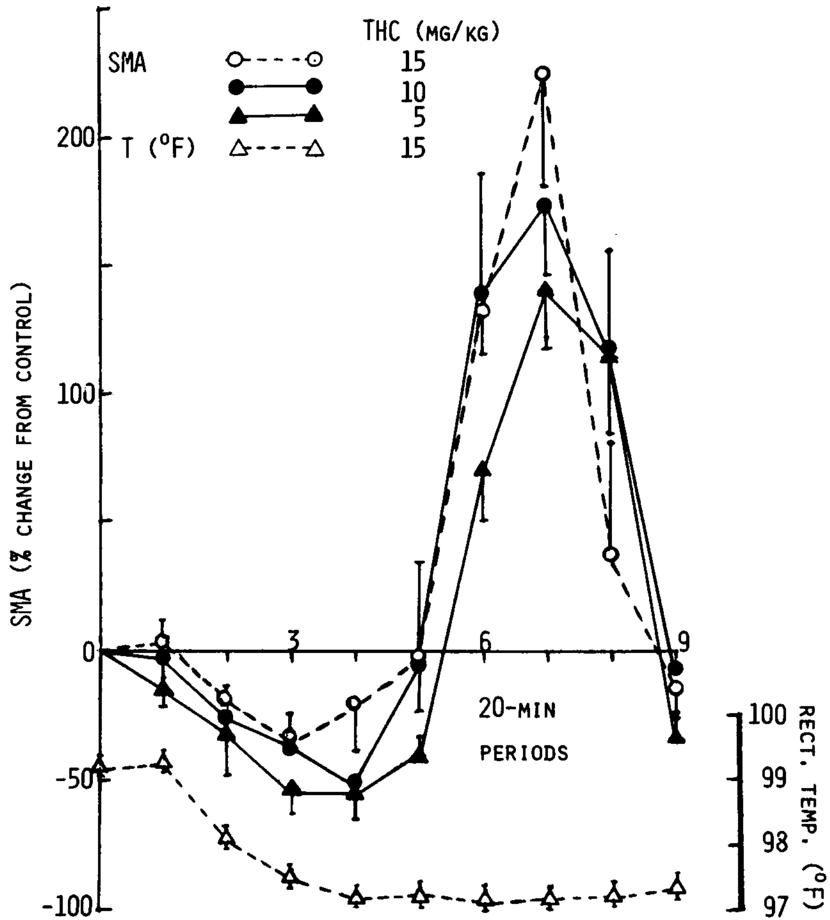


FIG. 5. Δ^9-THC-induced triphasic effect on SMA and persistent decrease of rectal temperature, showing a lack of correlation.

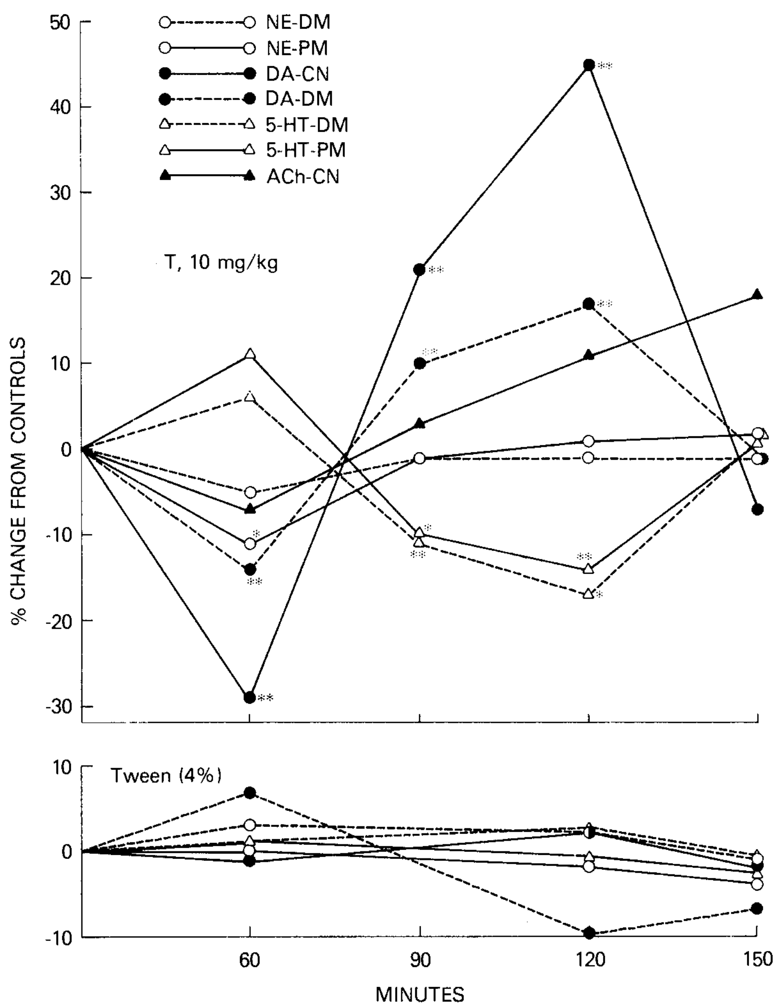


FIG. 6. Changes in the levels of various neurotransmitters (norepinephrine, NE; dopamine, DA; serotonin, 5-HT; acetylcholine, ACh) in discrete brain areas (CN, caudate nucleus; DM, diencephalon-midbrain; PM, pons-medulla) following injection of Tween or Δ^9-THC(T). Significant changes in the levels are indicated by *($P < 0.05$) or **($P < 0.01$).

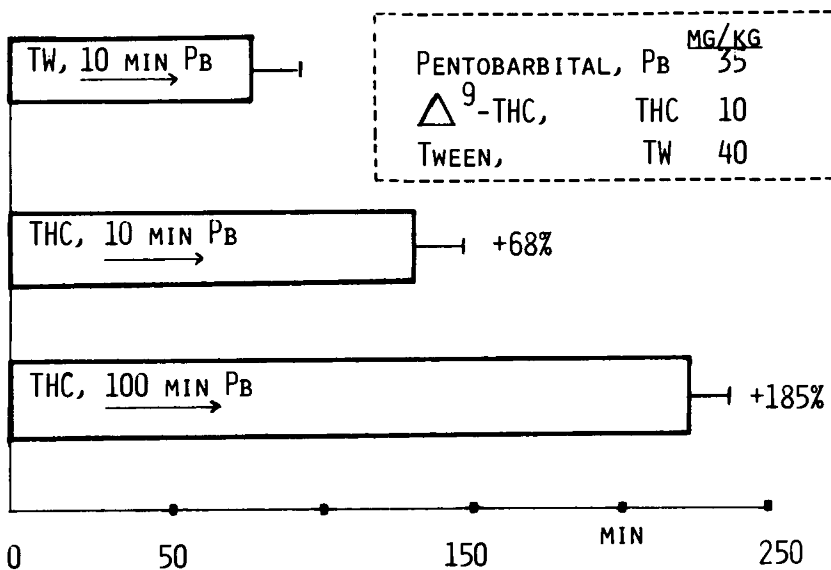


FIG. 7. Potentiation of pentobarbital-induced sleep by Δ^9-THC.

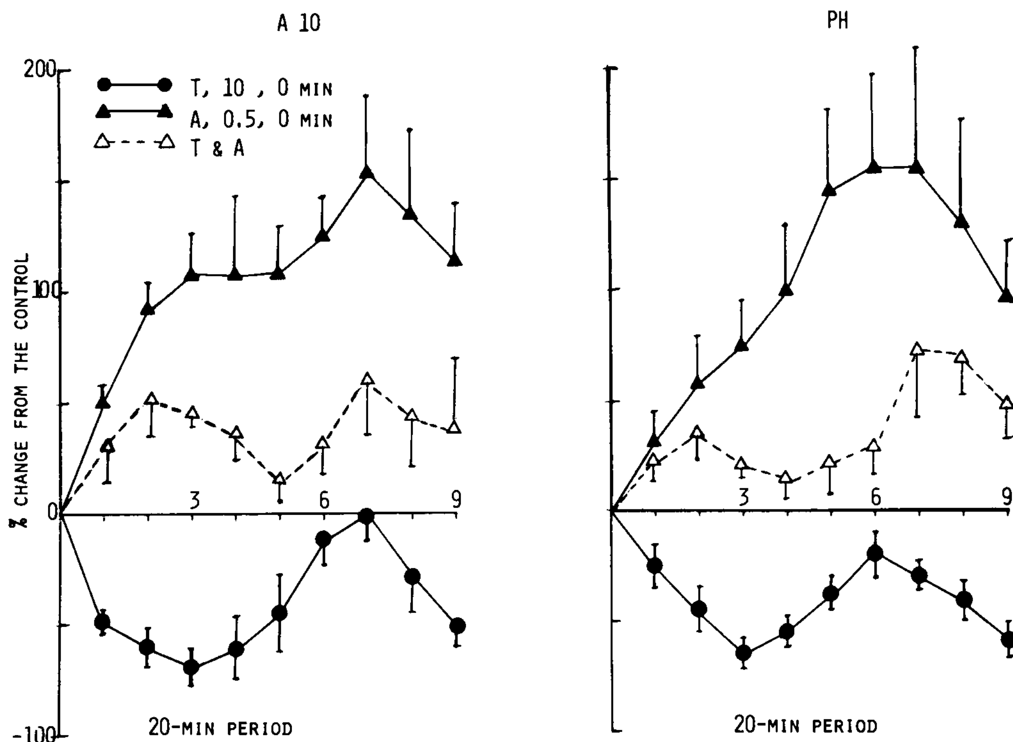


FIG. 8. Effects of Δ^9-THC (T, 10 mg/kg), D-amphetamine (A, 0.5 mg/kg) on their combination on SS respondings in PH and A10 rats. Both Δ^9-THC and amphetamine were injected simultaneously at 0 min, but separately. Data from both groups of rats show some degree of antagonism between the two drugs.

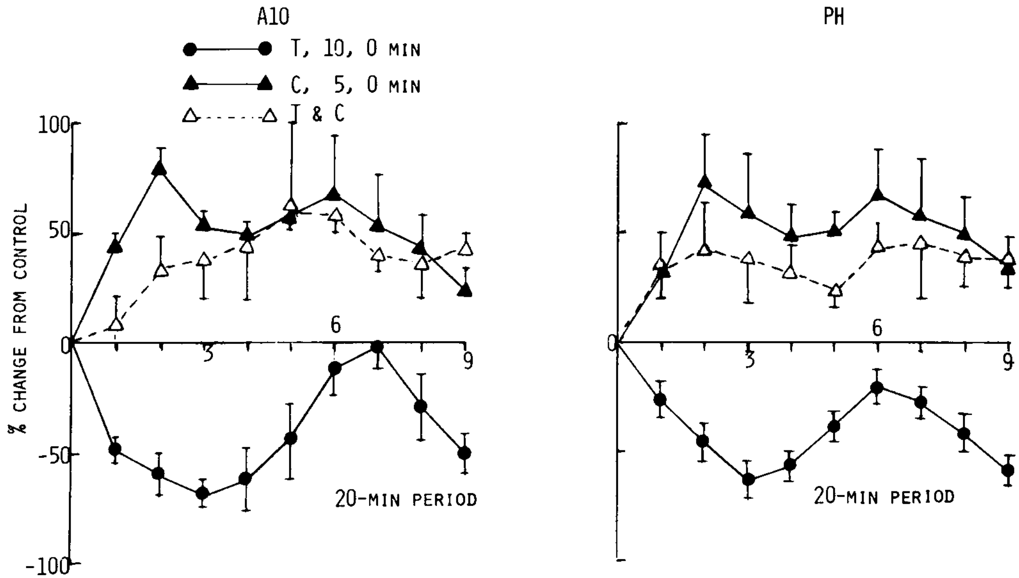


FIG. 9. Same as in Fig. 8 except that cocaine (5 mg/kg) was used in place of D-amphetamine.

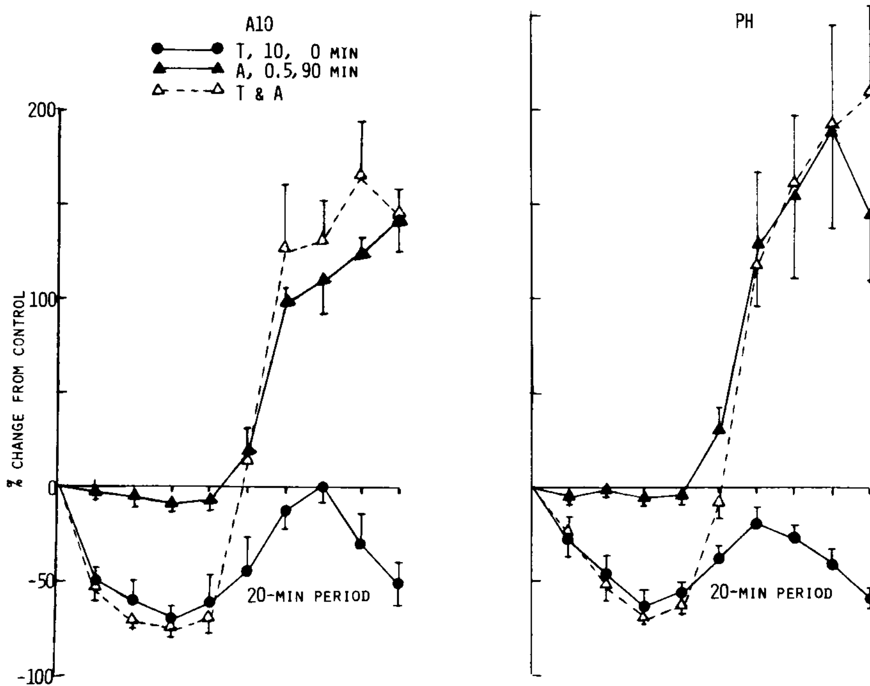


FIG. 10. Effects of Δ^9-THC (T, 10 mg/kg, injected at 0 min), D-amphetamine (A, 0.5 mg/kg, injected at 90 min) or their combination on SS responding in PH and A10 rats. Data from both groups failed to show any antagonism, rather indicated some potentiation of amphetamine effect by Δ^9-THC.

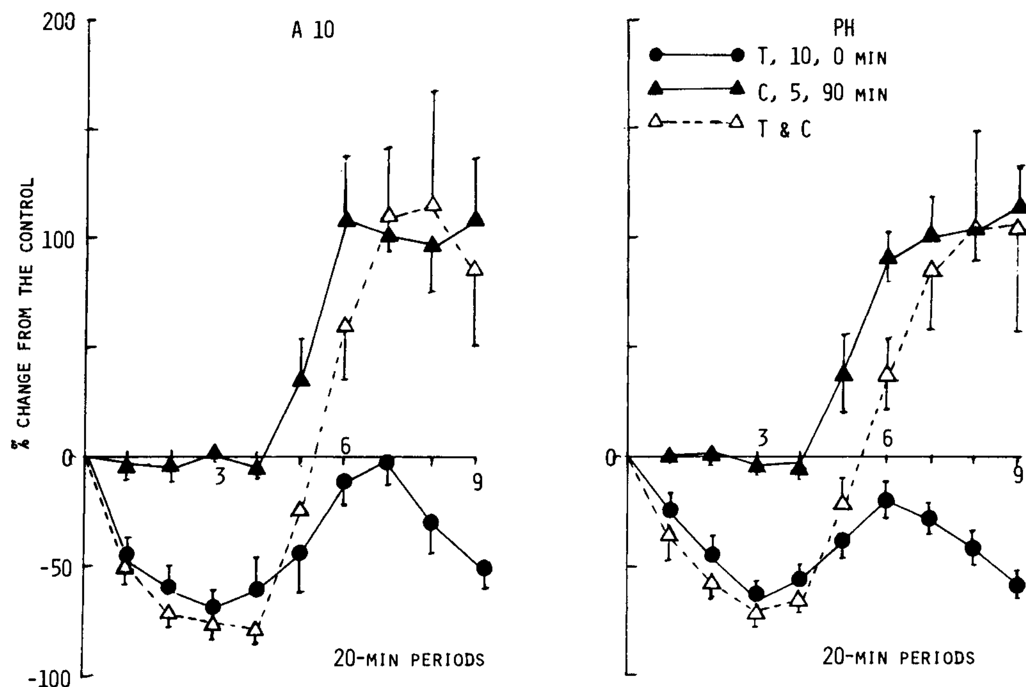


FIG. 11. Same as in Fig. 10 except that cocaine (5 mg/kg) was used in place of D-amphetamine.

SS responses of 4 PH rats following treatment with daily doses of 5, 10, 15, or 20 mg/kg of Δ^9-THC. The responses of these rats were decreased to a minimum in 1–3 days and then gradually returned very close to or above the baseline levels within 7 days. Figure 13 shows that following two daily injections (once before the experimental session and again after 8 hours) of Δ^9-THC at 10 mg/kg doses, the self-stimulation responding in both PH and A10 rats were decreased to their minimum by the 4th to 5th day, and increased gradually very close to or above the baseline levels between 8 and 10 days.

Cross-tolerance was also studied between Δ^9-THC and mescaline which also showed tolerance to its depressant effect on SS (Bailey and Pradhan, 1972). Figure 14 illustrates an experiment in two PH rats in which a test dose of Δ^9-THC (10 or 20 mg/kg) or mescaline (18 mg/kg) was injected at an interval of 5 days, the same dose of Δ^9-THC was injected showing development of tolerance between the 5th and 9th days. When these rats were challenged with the same test dose of mescaline, the usual decrease of responding was produced indicating thereby a lack of cross-tolerance to mescaline in Δ^9-THC-tolerant rats.

DISCUSSION

The present study, showing an initial depressant effect of Δ^9-THC on SS using both PH and A10 electrode sites, confirms our previous observation (Bailey and Pradhan, 1972) and extends to show a subsequent stimulant phase in its effects on rats aroused by handling. However, handling does not appear to modify its hypothermic effect or potentiation of

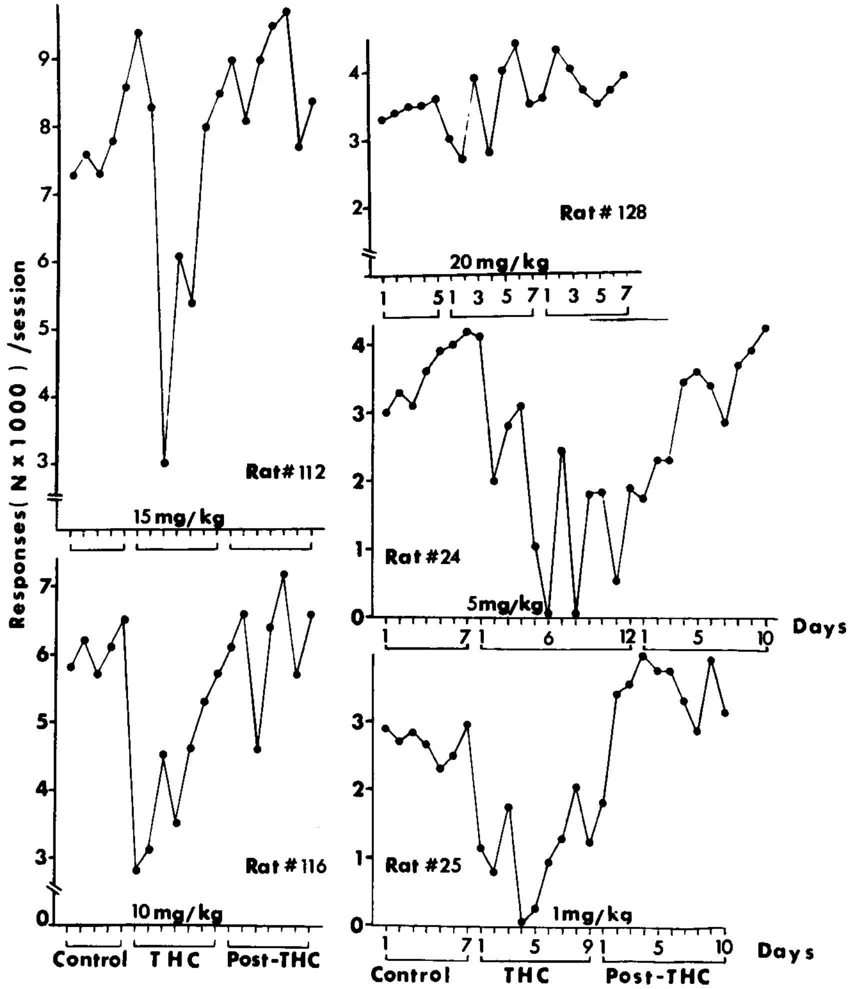


FIG. 12. Development of tolerance to depressant effect of Δ^9-THC following its single repeated daily doses (1–20 mg/kg) in PH rats.

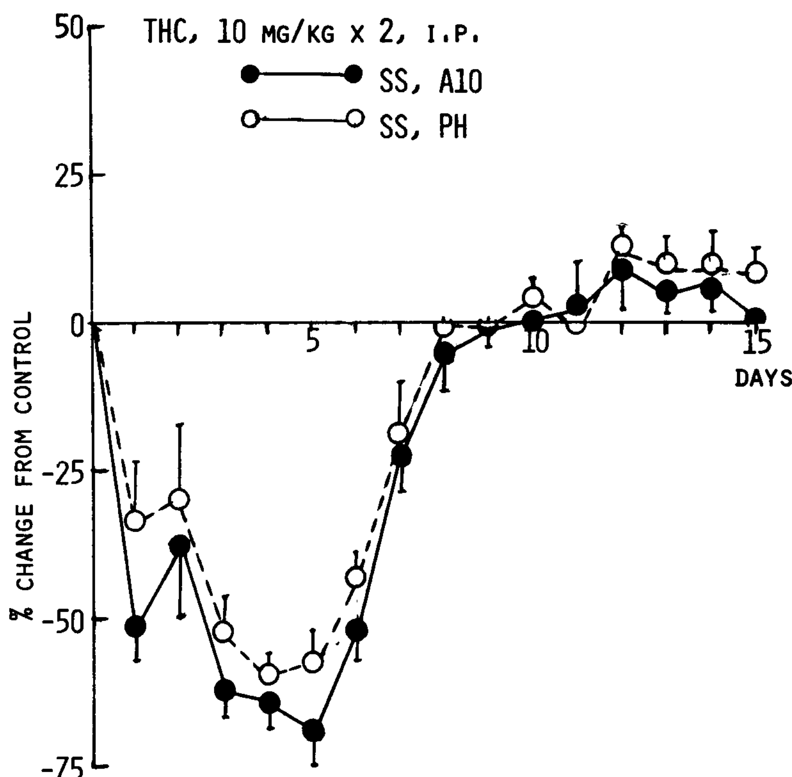


FIG. 13. Development of tolerance to depressant effects of Δ^9-THC following two repeated daily doses (10 mg/kg; one dose before the session and another 8 hours later).

barbiturate anesthesia. On the other hand, a behavior like SMA does not need handling of treated rats to manifest its stimulant phase.

This experiment demonstrated two patterns of behavioral effects of Δ^9-THC in rats. In one type, there was a gradual drug-induced depression that persisted for a prolonged period. Decrease in SS responding in nonhandled, nonaroused rats, lowering of rectal temperature, and potentiation of barbiturate-induced sleep are examples. These effects may be due to a nonspecific generalized depression of the central nervous system. In contrast, another type is a triphasic effect consisting of an initial depression followed by stimulation towards or above the baseline level and subsequent depression. This pattern is observed in SS behavior in aroused (handled) PH and A10 rats and SMA. Such depression-stimulation effects can be further substantiated by respective antagonism and potentiation by stimulants e.g. amphetamine and cocaine injected at 0 or 80 min post-THC in this experiment, and can probably explain two mutually antagonistic effects of cannabis, namely potentiation of barbiturate anesthesia and enhancement of amphetamine effects (for review see Pradhan and Bailey, 1972). Such triphasic behavioral pattern can be correlated to the changes in the levels of neurotransmitters, particularly those of DA and 5-HT in discrete brain areas.

Yagiela *et al.* (1974) who studied the effects of Δ^9-THC on hypothalamic and brainstem biogenic amine metabolism indicated various inconsistencies of results in a number of similar studies, and failed to show any time-related correlation between hypothermic effects

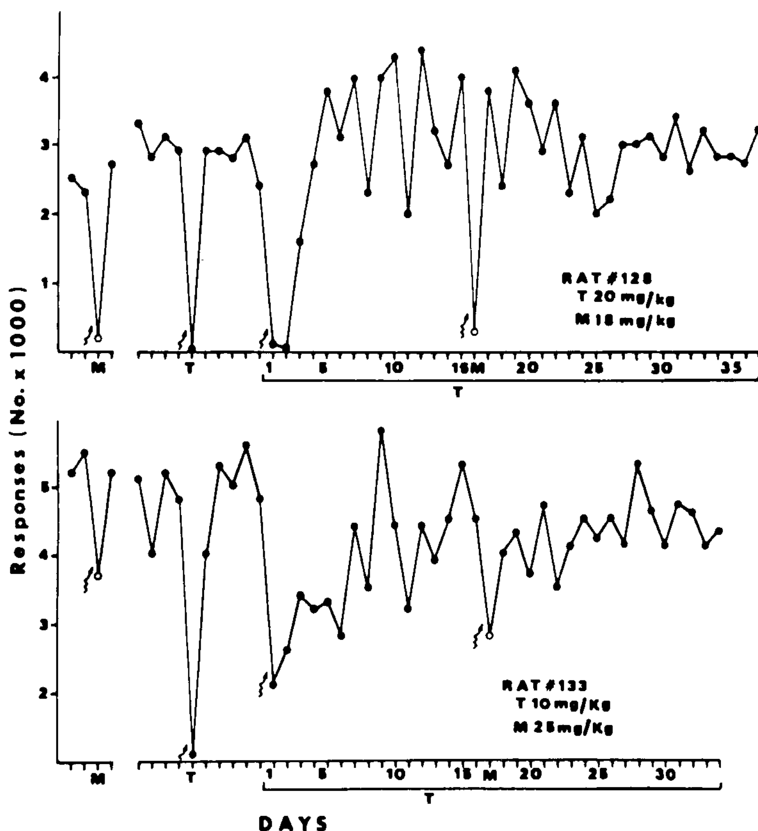


FIG. 14. Lack of cross-tolerance to mescaline (M) in two Δ^9-THC (T)-tolerant rats. Test doses of M and T were administered after 2 weeks interval. After 5 days, T was injected daily until tolerance was developed after 5–9 injections. An injection of M at this stage caused an usual decrease of SS responding.

of Δ^9-THC and induced changes in the brain amine metabolism. Our study, however, demonstrated time-related changes in the levels of DA and 5-HT in discrete brain areas that could be correlated with some behavioral (SS in aroused subjects and SMA) changes, but not with the hypothermic and other effects in Δ^9-THC-treated animals.

The present experiment shows that Δ^9-THC depresses the rewarding SS behavior (whatever motivational component it may involve) only initially and temporarily, in aroused animals. On repeated administration, this depressant effect disappeared rapidly indicating that in our experimental model a motivational depressant effect will be less likely to develop during chronic use.

Tolerance has been shown to develop rapidly to many of the effects of the cannabinoids in various species of animals (Pradhan, 1977; McMillan *et al.*, 1971; Dewey *et al.*, 1976). The present work on Δ^9-THC effect on SS in rats adds to this list. Lack of cross-tolerance to mescaline demonstrated in SS behavior supports the observation of Silva *et al.* (1968) and McMillan *et al.* (1971).

REFERENCES

ANSELL, G. B. and BEESON, M. F., A rapid and sensitive procedure for the combined assay of noradrenaline, dopamine and serotonin in a single brain sample. *Analyt. Biochem.* **23**,196–206 (1968).

BAILEY, P. T. and PRADHAN, S. N., Effects of Δ^9-tetrahydrocannabinol and mescaline on self-stimulation, *Life Sci.* **11**,831–838 (1972).

CHANG, C. C., A sensitive method for spectrophotofluorometric assay of catecholamines. *Int. J. Neuropharmacol.* **13**,643–649 (1964).

COX, R. H., JR. and PERHACH, J. L., JR., A sensitive, rapid and simple method for the simultaneous spectrophotofluorometric determinations of norepinephrine, dopamine, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in discrete areas of brain. *J. Neurochem.* **20**,1777–1780 (1973).

DAHLSTRÖM, A. and FUXE, K., Evidence for the existence of monoamine-containing neurones in the central nervous system. *Acta. physiol. scand. Suppl.* **62**,232, 1–55 (1965).

DEWEY, W. L., MARTIN, B. R. and HARRIS, L. S., Chronic effects of Δ^9-THC in animals: Tolerance and biochemical changes. In: M. C. Braude and S. Szara (Eds.). "The Pharmacology of Marihuana," Vol. 2. Raven Press, New York, pp. 585–594, 1976.

HALL, R. D., BLOOM, F. E. and OLDS, J., Neuronal and neurochemical substrates of reinforcement. *Neurosciences Research Program Bulletin*, **15**(2),133–313 (1977).

HART, B. L., The rat brain in section: a stereotaxic atlas. "Experimental Neuropsychology," S. Coopersmith (Ed.), W. H. Freeman Co., San Francisco, pp. 93–102, 1969.

KOLANSKY, H., and MOORE, W. T., Toxic effects of chronic marihuana use. *J.A.M.A.* **222**,35–41 (1972).

KÖNIG, J. F. R. and KLIPPEL, R. A., "The Rat Brain: a stereotaxic atlas of the forebrain and lower parts of the brainstem," The Williams and Wilkins Company, Baltimore, 1963.

MAICKEL, R. P., COX, R. H., JR., SAILLANT, J. and MILLER, F. P., A method for the determination of serotonin and norepinephrine in discrete areas of rat brain. *Int. J. Neuropharmacol.* **7**,275–281 (1968).

MARCUS, A. M., KLONOFF, H., and LOWE, M., Psychiatric status of the marihuana user. *Can. Psychiat. Assoc.* **19**,31–39 (1974).

MCGLOTHIN, W. H. and WEST, L. J., The marihuana problem: an overview. *Am. J. Psychiat.* **125**,370–378 (1968).

MCMILLAN, D. E., DEWEY, W. L. and HARRIS, L. S., Characteristics of tetrahydrocannabinol tolerance. *Ann. N.Y. Acad. Sci.* **191**,83–99 (1971).

MENDELSON, J. H., KUEHNLE, J. C., GREENBERG, I. and MELLO, N. K., The effects of marihuana use on human operant behavior: Individual data. In M. C. Braude and S. Szara (Eds.) "The Pharmacology of Marihuana," Vol. 2, Raven Press, New York, pp. 643–653, 1976.

NIMITKIPAISAN, Y., BOSE, S., KUMAR, R. and PRADHAN, S. N., Effects of L-dopa on self-stimulation and brain biogenic amines in rats. *Neuropharmacology* **16**,657–661 (1977).

OLDS, J., Self-stimulation of the brain. *Science* **127**,315–324 (1958).

OLDS, J., Hypothalamic substrates of reward. *Physiol. Rev.* **42**,554–604 (1962).

PRADHAN, S., ROY, S. N. and PRADHAN, S. N., Correlation of behavioral and neurochemical effects of acute administration of cocaine in rats. *Life Sci.* **22**,1737–1744 (1978).

PRADHAN, S. N., Balances among central neurotransmitters in self-stimulation behavior. In E. F. Domino and J. M. Davis (Eds.) "Neurotransmitter Balances and Behavior." Edward Brothers, Inc., Ann Arbor, Michigan, pp. 75–97, 1975.

PRADHAN, S. N., Balance of Central neurotransmitter actions in self-stimulation behavior. In A. Wauquier and E. T. Rolls (Eds.). "Brain-stimulation reward," North-Holland Publishing Co., Amsterdam, pp. 171–185, 1976.

PRADHAN, S. N., Marijuana. In S. N. Pradhan and S. N. Dutta (Eds.). "Drug Abuse, clinical and basic aspects." The C. V. Mosby Co., St. Louis, pp. 148–173, 1977.

PRADHAN, S. N. and BAILEY, P. T., Behavioral effects of marijuana and its derivatives. In J. M. Singh, L. H. Miller, and H. Lal (Eds.). Futura Publishing Co., Inc., Mount Kisco, New York, pp. 17–29, 1972.

PRADHAN, S. N. and BOWLING, C., Effects of nicotine on self-stimulation in rats. *J. Pharmacol. exp. Ther.* **176**,229–243 (1971).

ROBBINS, P. R. and TANCK, R. H., Psychological correlates of marihuana use: an exploratory study. *Psychol. Rep.* **33**,703–704 (1973).

ROLL, S. K., Intracranial self-stimulation and wakefulness: effect of manipulating ambient brain catecholamines. *Science*, **168**,1370–1372 (1970).

SHEA, P. A. and APRISON, M. H., An enzymatic method for measuring picomole quantities of acetylcholine and choline in CNS tissue. *Analyt. Biochem.* **56**,165–177 (1977).

SILVA, M., TERESA, A. and CARLINI, E. A., Lack of cross-tolerance in rats among (−) Δ^9-trans-tetrahydrocannabinol (Δ^9-THC), cannabis extract, mescaline and lysergic acid diethylamide (LSD-25). *Psychopharmacologia (Berl)* **13**,332–340 (1968).

SPANO, P. F. and NEFF, N. H., Procedure for the simultaneous determination of dopamine, 3-methoxy-4-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid in brain. *Analyt. Biochem.* **42**,113–118 (1971).

STEIN, L., Self-stimulation of the brain and the central stimulant action of amphetamine. *Fedn. Proc. Fedn. Am. Socs. Exp. Biol.* **23**,836–850 (1964).

WILLIAMS, E. G., HIMMELSBACH, C. K., WIKLER, A., RUBLE, D. C. and LLOYD, B. J., Studies on marihuana and pyrahexyl compound. *Public Health Rep.* **61**,1059–1083 (1946).

YAGIELA, J. A., MCCARTHY, K. D. and GIBB, W., The effect of hypothermic doses of 1-Δ^9-tetrahydrocannabinol on biogenic amine metabolism in selected parts of the rat brain. *Life Sci.* **14**,2367–2378 (1974).

CANNABIS AND THE DEVELOPMENT OF TOLERANCE

LEO E. HOLLISTER

Professor of Medicine, Psychiatry and Pharmacology, Stanford University School of Medicine and the Veterans Administration Hospital, Palo Alto, California 94304

TOLERANCE to cannabis has long been suspected to occur during its continued use. Narrative accounts indicate that chronic users of the drug either show very little effect from moderate doses or require very large doses to produce the characteristic intoxication. A pioneer study of subchronic administration of cannabis and synhexyl suggested at best some degree of tolerance to the euphoriant actions (Williams *et al*., 1946). Yet it has only been in the past few years that tolerance to cannabis has been clearly documented experimentally.

TOLERANCE IN MAN

The demonstration of tolerance in man was delayed by ethical restrictions on the amount of exposure permissible to human subjects. For instance, in an early study of ours, we were able to expose subjects only to a test oral dose of 20 mg of delta-9-tetrahydrocannabinol (THC) and then gave the same doses or placebos repeated at bedtime for four more days followed by the same THC dose as a challenge on the fifth day. Using such small doses and relatively infrequent intervals, it was impossible to show tolerance to the psychic effects of the drug, although tolerance to the tachycardia and dizziness produced by the drug was evident (Hollister and Tinklenberg, 1973).

Other early studies likewise suggested tolerance without definite proof. Tolerance to both tachycardia and "high" was reported following 21 days of consecutive smoking of only one cigarette a day by experienced smokers. It was possible that these subjects may have already been tolerant to the drug (Dornbush *et al*., 1972). Another study, in which subjects smoked a marihuana cigarette containing 14 mg of THC for 22 days, revealed a progressive decline in the increase of pulse rate following smoking, in the increase in alpha rhythm on the electroencephalogram, and in the degree of decrement in performance of short-term memory and reaction time tasks (Fink *et al*., 1976).

A number of other early studies provided less evidence of tolerance. Little evidence of tolerance to clinical effects of cannabis was found from daily smoking of marihuana cigarettes over periods of 10 and 28 days (Renault *et al*., 1974; Frank *et al*., 1976). Free choice

smoking of marihuana cigarettes for 21 days also provided little evidence to support the concept of tolerance in man (Mendelson *et al.*, 1974). Meanwhile, substantial evidence had accumulated that tolerance could be shown in various animal species, especially with high doses of THC given for prolonged periods.

Definite evidence of tolerance to the effects of THC in man was adduced only when it became permissible to use comparably large doses over longer periods of time. Subjects in one 30-day study were given high oral doses (70 to 210 mg/day) of THC around the clock. Tachycardia actually became bradycardia and a progressive loss of "high" was noted. Interestingly, the high was greater in the morning than later in the day, despite the round the clock administration of the drug. This variation suggests that perhaps absorption of orally administered drug during the night was delayed, so that more THC was available in the morning hours than during the rest of the day (Jones and Benowitz, 1976). Similar tolerance to marihuana smoking was observed in a 64-day study in which at least one cigarette daily had to be smoked with smoking as desired later in the same day (Nowlan and Cohen, 1977). Additionally, in this study tolerance developed to the respiratory depressant effect of THC (Bellville *et al.*, 1976).

The pattern that has emerged in man, therefore, is that tolerance is not a problem when doses are small, or infrequent, or where the pattern of use of the drug is not prolonged. Tolerance only becomes a major factor with high, sustained and prolonged use of the drug. It is interesting that no study in man or animals ever revealed any evidence for "reverse tolerance" or sensitization, such as had been reported in an early, rather naive clinical study of marihuana (Weil *et al.*, 1968).

TOLERANCE IN ANIMALS TO VARIOUS ACTIONS OF THC

Cannabis extract in rats produced tolerance to the reduction in rope climbing and bar pressing behavior during two weeks of treatment (Carlini, 1968). Single dose tolerance to the hypothermic effect of 10 mg/kg of THC given intraperitoneally in the rat could be shown (Lomax, 1971). Similar single dose tolerance to the hypothermic effect of THC was found in mice, as well as to depression of spontaneous motor activity. Tolerance to depressed intestinal motility required 4 doses rather than one (Anderson *et al.*, 1975). Further evidence of single dose tolerance to hypothermia in mice was found not only for THC but also for its double-bond isomer, delta-8-THC. No tolerance developed to the aggression-attenuating effect of either isomer of THC, despite repeated doses (ten Ham and de Jong, 1974). Scheduled behavior in pigeons became tolerant to doses of 1.8 to 5.6 mg/kg given intramuscularly for 7 days (McMillan *et al.*, 1970). In the dog, tolerance was demonstrated for ataxia and general depression, but not for the effects of THC on heart rate, respiration and body temperature (Kaymakcalan, 1973). Rates of tolerance development differ. In rats, tolerance to the effects of THC on body weight and body temperature were evident within 1 to 2 weeks of treatment with 10 mg/kg intraperitoneal doses of THC, but tolerance to the negative chronotropic action and the hypotensive effect of THC developed only with 28 days of treatment (Adams *et al.*, 1976).

From these studies one might conclude that tolerance may develop eventually to most actions of THC. A few exceptions have been noted. No tolerance was observed to decreased spontaneous motor activity on the rat in a study that showed tolerance to many

other effects (Adams *et al.*, 1976). Tolerance could not be demonstrated to the effects of THC on a discrimination task performed by chimpanzees. Doses of 1 mg/kg were given for a 21-day course and 4 mg/kg for a 42-day course. It is possible that these doses were not large enough, although they would be substantial for man (Ferraro and Grilly, 1973). Neither mice nor hamsters became tolerant to reduction of aggressivity by THC, even in doses as large as 50 mg/kg of delta-8-THC given for 30 days (ten Ham and van Noordwijk, 1973).

In summary, tolerance to the effect of cannabis and its active component, THC, has been demonstrated for a variety of behaviors or bodily systems in a variety of animal species. Some effects of THC are more resistant to development of tolerance than are others, although it can not be said definitely that any are totally impervious to becoming tolerant. Rates of tolerance will also vary between different effects; some show single dose tolerance.

MECHANISM OF TOLERANCE

Evidence for metabolic tolerance for THC is most compelling. Early on, studies in man revealed that the disappearance of radiolabeled THC from plasma was twice as fast in highly experienced users of the drug as in naive subjects. This difference was also consistent with diminished clinical effects of the drug in those with prior experience (Lemberger *et al.*, 1971). Rats given 10 mg/kg of THC intraperitoneally every 12 hours for 5 days showed a progressively shorter depressant effect from the drug. Animals sacrificed at intervals during treatment and examined for the distribution of metabolites of THC in brain showed a progressive shift from unchanged THC and the active THC metabolite, 11-hydroxy-THC, to more polar and presumably inactive metabolites (Magour *et al.*, 1977). Pretreatment of rats with proadifen (SKF-525A), a microsomal enzyme inhibitor, enhanced the impairment by THC of conditioned tasks, while pretreatment with phenobarbital, an inducer of microsomal enzymes, enhanced development of tolerance (Davis and Borgen, 1975). Thus, increasing the metabolism of THC promotes tolerance.

Pharmacodynamic tolerance is much more difficult to demonstrate. Tolerance to the anticonvulsant effect of THC and cannabidiol was cross tolerant with phenytoin and phenobarbital, although the drugs differed in their effects on hexobarbital sleeping time (Karler *et al.*, 1974). These results were interpreted as suggesting that the tolerance to the anticonvulsant actions of cannabinoids is due to adaptation of the central nervous system. Tolerance to depression of mouse brain homogenate respiration could be demonstrated after 7 daily injections of 50 mg/kg of THC. Radiolabeled drug was given on the final injection, after tolerance had been established. No change in distribution of the radiolabeled drug was noted in tolerant and control animals, suggesting that tolerance was cellular (Nazar *et al.*, 1974). The evidence to support that conclusion is weak, as the label might have represented inactive metabolites.

Tolerance based on prior learning, that is, behavioral tolerance, is extremely difficult to demonstrate in animals and the evidence is confusing (Manning, 1976).

CROSS-TOLERANCE

THC has effects which in man somewhat resemble those of hallucinogens and strongly resemble those of alcohol, while in animals it slightly resembles morphine. No cross-

tolerance to mescaline or lysergide (LSD) could be shown in rats (Silva *et al.*, 1968). Rats tolerant to the effects of THC were also tolerant to ethyl alcohol, but when the situation was reversed, less tolerance to THC was seen in alcohol-tolerant animals (Newman *et al.*, 1972). Perhaps this difference in sequential tolerance is why THC has never become established as a treatment for alcohol withdrawal, despite some early clinical trials that suggested a favorable effect. Cross tolerance between THC and morphine has been shown in rats using customary tests of analgesia (Kaymakcalan, 1973).

PHYSICAL DEPENDENCE ON THC

Evidence both from animals and man indicates that physical dependence can be induced by abuse of THC. All monkeys given automatic injections of doses of 0.1 to 0.4 mg/kg of THC showed abstinence signs when withdrawn. When monkeys were allowed to self-administer the drug for 3 to 8 weeks, the majority had an abstinence syndrome when the drug was abruptly discontinued. The syndrome appeared approximately 12 hours after the last administration and lasted about 5 days. It was characterized by irritability, aggressivity, tremors, yawning, photophobia, piloerection and penile erections (Kaymakcalan, 1973).

In man, a somewhat similar, though mild, withdrawal reaction was uncovered after abrupt cessation of doses of 30 mg of THC given every 4 hours orally for 10 to 20 days. Subjects became irritable, had sleep disturbances, and had decreased appetite. Nausea, vomiting and occasionally diarrhoea were encountered. Sweating, salivation and tremors were autonomic signs (Jones and Benowitz, 1976).

CONCLUSIONS

Cannabis would have been an exceptional centrally acting drug if tolerance/dependence were not one of its properties. The fact that tolerance was not strongly recognized as an effect of the drug until fairly recently was due to the narrative nature of previous accounts of tolerance in man and the lack of systematic animal experimentation. Tolerance has now been proven for most of the actions of THC. It develops at varying rates for different actions, but it is rapidly reversible. Both in animals and in man, large doses of THC are required over long periods of time for tolerance to develop. As most social use of the drug does not meet these requirements, neither tolerance nor dependence have been major issues in its social use. We must consider, however, that the widespread social use of this drug in Western societies is a rather recent development. With further experience, and with greater social acceptance of the drug, these issues might become more serious.

REFERENCES

Adams, M. D., Chait, L. D. and Earnhardt, J. T., Tolerance to the cardiovascular effects of delta-9-tetrahydrocannabinol in the rat. *Brit. J. Pharmacol.* **56**,43–48 (1976).

Anderson, P. F., Jackson, D. M., Chesher, G. B. and Malor, R., Tolerance to the effects of delta-9-tetrahydrocannabinol in mice on intestinal motility, temperature and locomotor activity. *Psychopharmacologia* **43**,31–36 (1975).

Bellville, J. W., Gasser, J. C., Miyake, T. and Aqlen, K., Tolerance to the respiratory effects of marijuana in man. *J. Pharmacol. Exp. Ther.* **197**,326–331 (1976).

CARLINI, E. A., Tolerance to chronic administration of cannabis sativa (marihuana) in rats. *Pharmacology* **1**,135–142 (1968).

DAVIS, W. M. and BORGEN, L. A., Tolerance development to the effect of delta-9-tetrahydrocannabinol on conditioned behavior: role of treatment interval and influence of microsomal metabolism. *Arch. int. Pharmacodyn.* **213**,97–112 (1975).

DORNBUSH, R. L., CLARE, G., ZAKS, A., CROWN, P., VOLAVKA, J. and FINK, M., Twenty-one day administration of marihuana in male volunteers, in Lewis, M. F., editor: "Current Research in Marihuana," Academic Press, New York, pp. 115–127, 1972.

FERRARO, D. P. and GRILLY, D. M., Lack of tolerance to delta-9-tetrahydrocannabinol in chimpanzees. *Science* **179**,490–492 (1973).

FINK, M., VOLAVKA, J., PANAYIOTOPOULOS, C. P., and STEFANIS, C., Quantitative EEG studies of marihuana, delta-9-THC and hashish in man, in Braude, M. and Szara, S., editors: "Pharmacology of Marihuana," Raven Press, New York, pp. 383–392, 1976.

FRANK, I. M., LESSIN, P. J., TYRRELL, E. D., HAHN, P. M. and SZARA, S., Acute and cumulative effects of marihuana smoking in hospitalized subjects: A 36-day study, in Braude, M. and Szara, S., editors: "Pharmacology of Marihuana," Raven Press, New York, pp. 673–680, 1976.

HOLLISTER, L. E. and TINKLENBERG, J. R., Subchronic oral doses of marihuana extract. *Psychopharmacologia* **29**,247–252 (1973).

JONES, R. and BENOWITZ, N., The 30-day trip: Clinical studies of cannabis tolerance and dependence, in Braude, M. and Szara, S., editors: "Pharmacology of Marihuana," Raven Press, New York, pp. 627–645, 1976.

KARLER, R., CELY, W. and TURKANIS, S. A., A study of the development of tolerance to an anticonvulsant effect of delta-9-tetrahydrocannabinol and cannabidiol. *Res. Commun. Chem. Path. Pharmacol.* **9**,23–39 (1974).

KAYMAKCALAN, S., Tolerance to and dependence on cannabis. *Bull. Narc.* **25**,39–47 (1973).

LEMBERGER, L., TAMARKIN, N. R., AXELROD, J. and KOPIN, I. J., Delta-9-tetrahydrocannabinol: metabolism and disposition in longterm marihuana smokers. *Science* **173**,72–74 (1971).

LOMAX, P., Acute tolerance to the hypothermic effect of marihuana in the rat. *Res. Commun. Chem. Path. Pharmacol.* **2**,159–167 (1971).

MCMILLAN, D. E., HARRIS, L. S., FRANKENHEIM, J. M. and KENNEDY, J. S., 1-delta-9-trans-tetrahydrocannabinol in pigeons: tolerance to the behavioral effects. *Science* **169**,501–503 (1970).

MAGOUR, S., COPER, H. and FÄHNDRICH, C., Is tolerance to delta-9-THC cellular or metabolic? The subcellular distribution of delta-9-tetrahydrocannabinol and its metabolites in brains of tolerant and non-tolerant rats. *Psychopharmacology* **51**,141–145 (1977).

MANNING, F. J., Role of experience in acquisition and loss of tolerance to the effect of delta-9-THC on spaced responding. *Pharm. Biochem. Behav.* **5**,269–273 (1976).

MENDELSON, J., ROSSI, A. and MEYER, R., editors: "The Use of Marihuana, Psychological and Physiological Inquiry," Plenum Press, New York, 1974.

NAZAR, B. L., HARCLERODE, J., ROTH, R. I. and BUTLER, R. C., Acquisition of tolerance to delta-9-THC as measured by the response of a cellular function. *Life Sci.* **14**,2513–2520 (1974).

NEWMAN, L. M., LUTZ, M. P., GOULD, M. H. and DOMINO, E. F., Delta-9-tetrahydrocannabinol and ethyl alcohol: evidence for cross-tolerance in the rat. *Science* **175**,1022–1023 (1972).

NOWLAN, R. and COHEN, S., Tolerance to marijuana: Heart rate and subjective "high". *Clin. Pharmacol. Ther.* **22**,550–556 (1977).

RENAULT, P. F., SCHUSTER, C. R., FREEDMAN, D. X., SIKIC, B., NEBEL DE MELLO, D. and HALARIS, A., Repeat administration of marihuana smoke to humans. *Arch. Gen. Psychiatry* **31**,95–102 (1974).

SILVA, M. T. A., CARLINI, E. A., CLAUSSEN, U., *et al.*, Lack of cross-tolerance in rats among $(-)\Delta^9$-trans-tetrahydrocannabinol (Δ^9-THC), cannabis extract, mescaline and lysergic acid diethylamide (LSD-25). *Psychopharmacologia* **13**,332–340 (1968).

TEN HAM, M. and DE JONG, Y., Tolerance to the hypothermic and aggression-attenuating effect of delta-8 and delta-9-tetrahydrocannabinol in mice. *Eur. J. Pharmacol.* **28**,144–148 (1974).

TEN HAM, M. and VAN NOORDWIJK, J., Lack of tolerance to the effect of two tetrahydrocannabinols on aggressiveness. *Psychopharmacologia* **29**,171–176 (1973).

WEIL, A. T., ZINBERG, N. E., NELSON, J. M., Clinical and psychological effects of marihuana in man. *Science* **162**,1234–1242 (1968).

WILLIAMS, E. G., HIMMELSBACH, C. K., WIKLER, A., RUBLE, D. C. and LLOYD, B. J., Studies in marihuana and pyrahexyl compound. *Pub. Health Rep.* **61**,1059–1083 (1946).

PHARMACOLOGICAL SIMILARITIES AND INTERACTIONS BETWEEN CANNABIS AND OPIOIDS

ŞÜKRÜ KAYMAKÇALAN

Professor and Chairman, Dept. of Pharmacology, Ankara University Medical School, Sihhiye, Ankara, Turkey

ACCORDING to the definitions of the 1961 Single Convention, cannabis is a narcotic drug. In the past there has been some criticism of classifying cannabis in this way. But recent scientific findings, revived in this paper, reveal a wide range of similarities and interactions between cannabis and the opioids, so that the classification, though legislative in origin, is becoming pharmacologically reasonable.

The term of narcotic analgesic is used interchangeably with the term of opioid and the term opioid refers to any natural or synthetic drug that has morphine-like pharmacological actions (Jaffe and Martin, 1975).

Since 9-*trans*-delta-Tetrahydrocannabinol (THC) is generally accepted as the main psychoactive component of cannabis, comparison will be made between THC and morphine.

A distinct effect of morphine in naive subjects is sedation. With higher doses this central depressant effect may change to a general anesthesia (narcosis)-like situation which the term "narcotic" originated. We have observed this kind of picture in dogs and monkeys after i.v. injections of 5 mg/kg THC. As with morphine-narcosis, dogs and monkeys can easily be aroused from the stupor induced by THC.

Other most important pharmacological characteristics of morphine include analgesia, tolerance and dependence. As it will be discussed below, THC also shares all these properties of morphine.

THC AND ANALGESIA

We have observed the analgesic effect of THC on several species of laboratory animals using different test procedures. Some of these studies have been published previously (Kaymakçalan and Deneau 1971, 1972).

On the hot plate test THC produced a significant delay in reaction time of both mice and rats. Especially in the rat this test was very reproducible; we have estimated the ED_{50}

of the analgesic effect of THC as 10 mg/kg, 2 hr after s.c. administration. In mouse the analgesic effect of THC develops more slowly.

In mice and rats the analgesic effect of THC was also evident in the phenylquinone or acetic acid induced writhing tests. After i.p. injections of 1.3 mg/kg phenylquinone or 60 mg/kg acetic acid, the protective effect of THC was calculated as proposed by Hendershot and Forsaith (1959). With these agents 10 mg/kg THC s.c. produced 92.6% protection in rats (phenylquinone test—30 min after THC) and 41.7% protection in mice (acetic acid test—2 hr after THC).

To investigate the analgesic effect of THC on higher laboratory animals we have applied the phenylquinone or acetic acid tests to cats, dogs and monkeys. The i.p. injection of phenylquinone (2 mg/kg) or acetic acid (40 mg/kg) to these animals produce a distinct discomfort; they usually lie down, change their positions very often, and whine or cry. THC prevents or diminishes this phenylquinone or acetic acid induced restlessness and other manifestations of peritoneal irritation and abdominal pain. We have found that in cats, counting the numbers of vocalizations during 30 minutes after injection of phenylquinone or acetic acid and using the Hendershot and Forsaith formula, it is possible to quantitate the protective action of THC. In both tests THC 1 mg/kg, s.c., produced a protection of 68–70%.

Another quantitative test for the analgesic effect of THC was by stimulation electrodes implanted in the tooth of the dog. After i.v. injection of 1 mg/kg THC the pain threshold was raised more than 4 times in 30 min and reached its maximum in 1 hr (Kaymakçalan *et al.*, 1974).

The analgesic effect of THC in man was described first by Noyes *et al.* (1974, 1975a, 1975b) in modern literature. In cancer patients the pain relief after oral administration of THC was comparable with the effect of high doses of codeine; 10 mg THC being equal to 60 mg codeine and 20 mg THC to 120 mg codeine. However THC-induced analgesia in man can be influenced by several factors including the expectation of patient and the setting. The tranquilizing and sedative properties of THC may enhance its analgesic effect in some conditions. On the other hand, the increase in perception may intensify some kinds of pain.

THC AND TOLERANCE

We have noticed the development of tolerance to the several effects of THC, in laboratory animals. Usually one week of daily administration is enough for tolerance development; but in some experiments we found that a few injections, even a single administration of THC, may produce a detectable tolerance.

To investigate the duration of the tolerance to the analgesic effect of THC, the hot plate test was used. Rats were divided in 6 groups, each consisting of 10 female rats. On the first day THC 10 mg/kg was injected s.c. to five groups (A, B, C, D, E) and the solvent was injected to a control group (F). The second injection of THC 10 mg/kg, s.c., was administered to Groups A, B, C, D, and E on the 2nd, 4th, 8th, 16th and 32nd days respectively. For each group the difference from the control value was then expressed as a percentage of the difference found on the first day (maximum expected prolongation).

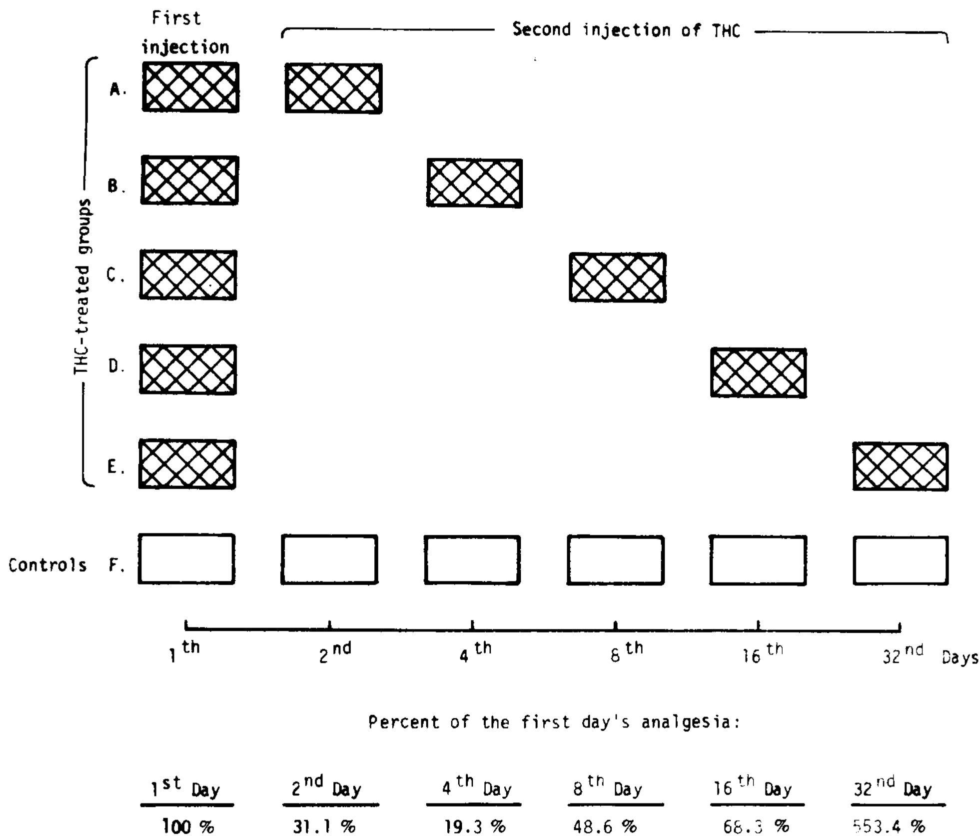


FIG. 1. Tolerance development to the analgesic effect of THC after single injection of THC (10 mg/kg) in rats on hot plate test. (See text).

The comparisons between the responses after various time intervals revealed that the analgesic effect of THC had been reduced considerably after the first injection indicating that tolerance has developed following the first application (Fig. 1). The data obtained in this study when plotted on semi-logarithmic paper show that the duration of tolerance to the analgesic effect of a single dose of THC lasts more than 1–2 months (Fig. 2). This situation is very similar to morphine tolerance. Indeed Cochin and Kornetsky had reported several years ago that the effects of a single injection of morphine can be detected in the rat for as long as twelve months, using the hot plate as a test of drug response (Cochin and Kornetsky, 1964).

THC produces a dose-dependent hypothermia in almost all species so far tested. We have observed this effect in monkey, dog, cat, rabbit and rat. During the chronic studies with THC in the rat which will be described below in connection with dependence, we followed the development of tolerance to hypothermia. While at the beginning 10 mg/kg of THC, s.c., produced a marked drop in body temperature, on the 15th day of experiments 40 mg/kg of THC, s.c., did not cause any significant hypothermia (Kaymakçalan *et al.*, 1977).

It is very well known that cannabis extracts produce ataxia in dogs. Besides ataxia,

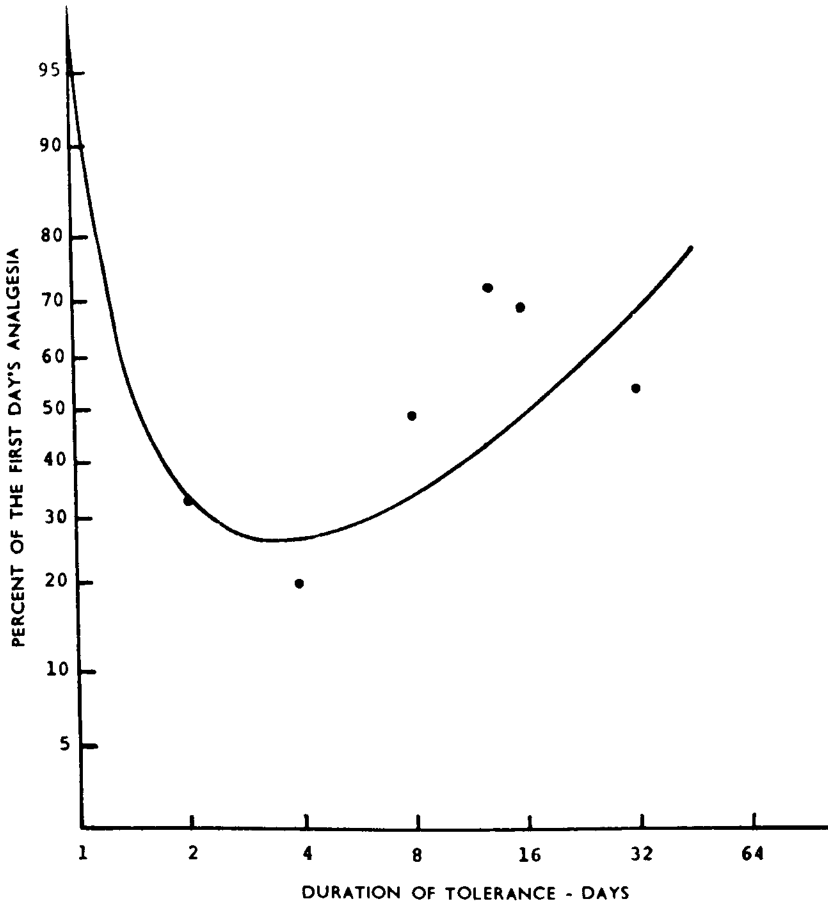


FIG. 2. Diagramatic representation of the duration of tolerance to the analgesic effect of the single injection of THC.

general depression or drowsiness, there are other easily observed signs of cannabis intoxication in the dog. Giving scores for degrees of ataxia and depression, it is possible to evaluate these reactions in a quantitative way. After daily administration of THC to dogs we have observed that, in 8 days, an almost complete tolerance developed to both ataxia and general depression (Kaymakçalan and Deneau, 1972).

The most characteristic effects of THC after a single administration in monkeys are ptosis of the eyelids, docility and loss of aggression. In chronic experiments tolerance to these effects developed in a few days. They reappeared after the dosage was increased, but then lasted only for a short period of time (Deneau and Kaymakçalan, 1971).

For a long time it was thought that cannabis produces no tolerance or dependence in man, an opinion stated in several textbooks. The simplest proof that tolerance develops to cannabis effects in man is the daily amount consumed by the chronic cannabis smokers in some countries. According to the estimates in the Report of a WHO Scientific Group (1971), the daily consumption of THC is about 5 to 10 mg in the recent wave of marihuana

use in North America. But in India and North Africa the average daily doses range from 13 to 66 mg, the maximum doses being between 200 and 700 mg. It is obvious that these huge amounts of cannabis cannot be consumed without tolerance development.

In recent clinical and laboratory investigations on young volunteers, after high doses of oral THC, tolerance development to the following effects of cannabis has been noted: mood changes, tachycardia, orthostatic hypotension, skin temperature decrease, body temperature increase, salivary flow decrease, intraocular pressure decrease, EEG slowing, EEG evoked potential alterations, sleep EEG changes, sleep time and quality, eye tracking, psychomotor task performance and ward behavior alterations (Jones *et al.*, 1976).

THC tolerance is not a phenomenon restricted to the mammalian species. It has been observed also in birds and in fish. In pigeons conditioned to obtain food, THC causes dose-dependent decreases in the rates of key pecking and tolerance to this effect develops after a week of daily injections of THC (Frankenheim *et al.*, 1970). It has been further demonstrated that a dose of THC, which completely disrupts food-reinforced responses in pigeons for many hours, can be increased more than 20-fold without disrupting this schedule-controlled behavior in tolerant birds (McMillan *et al.*, 1970). These authors point out that the pattern of the development of tolerance to THC is similar to that seen in the same kind of behavioral experiments with morphine and methadone in pigeons. Moreover the degree of behavioral tolerance obtained in the pigeon was more similar to morphine tolerance than barbiturate tolerance, which is weaker. In neonatal chicks THC decreases locomotor activity, inhibits escape from a heat stimulus and produces hypothermia. Tolerance to all these behavioral and physiological effects develops in approximately two weeks time (Abel, 1972). Marihuana extract and THC inhibit the spontaneous aggressive behavior of the male Siamese fighting fish (*Betta splendens*). However after 8–10 exposures the fishes develop tolerance to the drugs (Gonzales *et al.*, 1971).

One feature of the opiate tolerance is that tolerance does not develop to all effects. For example it is known that in opiate addicts constipation and miosis persist. Perhaps the same is true also for the THC tolerance, since tolerance may not develop to some effects of cannabis. In our study on the heart rate in rat we observed that in spite of daily administration of THC for 15 days, the bradycardiac effect did not diminish (Kaymakçalan and Sivil, 1974). Similarly in the experiments by Jones *et al.* (1976), there was no tolerance to the cannabis-related weight gain or to the falls in hematocrit and hemoglobin.

THC AND DEPENDENCE

Like all drugs of abuse, cannabis produces psychic dependence in man. However to show this phenomenon in self-administration studies in laboratory animals is not easy. There are two reports on this subject in the literature. The first one is related to our monkey studies (Kaymakçalan, 1972). We had shown that two monkeys during the withdrawal of chronic THC administration started and continued self-administration of THC. A third monkey switched to THC self-administration, following cocaine self-administration (see below). Similarly Pickens *et al.* (1973) reported self-administration of THC in two monkeys after substitution of THC for phencyclidine. It is known that monkeys like both cocaine and phenycyclidine very much and they self-administer these substances very eagerly.

Here we will discuss mainly the physical dependence on THC (or cannabis) both in laboratory animals and man.

DEPENDENCE IN LABORATORY ANIMALS

Until recently, animal studies showing the physical dependence on cannabis were rare and vague. However there were some reports suggesting that at least some symptoms of withdrawal have been observed in animals. For example Davis *et al.* (1972) noted tolerance development toward the locomotor-depressant property of THC in rats. During the post-drug period a slight but statistically significant elevation of motor activity occurred on the second withdrawal day. The effects of long-term administration and withdrawal of tetrahydrocannabinols on open-field behavior in rats have been studied by Sjöden *et al.* (1973). They reported that during the withdrawal period most open-field measures slowly returned to control levels, whereas the rate of grooming showed a manifold increase. Pirch *et al.* (1973) reported a reduction in cortical EEG voltage of rats after oral administration of cannabis extract. Following the discontinuation of cannabis treatment a rebound increase in EEG, which might represent a withdrawal phenomenon, was observed.

In our laboratory we have observed an opiate-like abstinence syndrome after administration of naloxone or following abrupt withdrawal of THC administration in rats chronically treated with high doses of THC (Kaymakçalan *et al.* 1977). In this study ten rats were injected s.c. with THC twice daily for 5 weeks in increasing doses. In the last 3 weeks they received 40 mg/kg THC in each administration. Ten control rats received the same amount of vehicle by the same route for the same period. The comparison of rectal temperatures of the first and fifteenth days showed that a very pronounced tolerance developed to the hypothermic effect of THC.

The administration of naloxone on the 22nd and 31st days precipitated an opiate-like abstinence syndrome. The termination of drug administration on the 35th day also produced a similar withdrawal syndrome. The most common signs of abstinence syndrome in THC-treated animals were teeth chattering, defecation, urination, dyspnea, and complete palpebral closure. Although the frequency of the incidence of the following signs were not high, they have been observed only in THC-treated animals: chewing, tremor of the head, escape behavior, jumping, eating of objects, biting of fingers, and sniffing.

During abstinence, an increased locomotor activity was recorded by an activity-meter. Similarly, the total amount of excreted feces and urine was higher in the THC group than in the controls. Both abstinence scores and increased motility exhibited the peak in the 48th h of withdrawal.

Our work with monkeys had also shown that THC can produce physical dependence and a morphine-like withdrawal syndrome is seen in monkeys. These monkey experiments were carried out in Dr. G. A. Deneau's laboratory in the Southern Research Institute, Birmingham, Alabama and the results were presented in two scientific meetings (Deneau and Kaymakçalan, 1971, Kaymakçalan, 1972). Here we will mention these experiments briefly.

Six rhesus monkeys have been prepared with intravenous catheters for self-administration of THC. The initial dose of THC was 0.1 mg/kg. During a period of 23 days, spontaneous self-administration of THC did not occur. Then the timer-controlled

injections started and monkeys received the drug automatically four times a day. Gradually increasing the dose of THC from 0.1 mg/kg to 0.2 mg/kg and to 0.4 mg/kg in 24 days, monkeys were injected with the highest dose for 12 days. At the end of this period of 36 days' THC administration, the automatic injections were discontinued and all monkeys showed abstinence signs. Two of the six monkeys then initiated and maintained self-administration of THC.

Following the first withdrawal of THC, one monkey was removed from the experiment for technical reasons (occlusion of the veins). For the remaining three monkeys which were not receiving THC, a cocaine solution was made available for self-administration. Two of them did not start to inject themselves with cocaine, but the third one initiated and maintained the cocaine self-administration. When cocaine was replaced by THC (0.4 mg/kg) in the drug reservoir of this animal, he continued to self-administer THC.

Three monkeys were allowed to self-administer THC for three to eight weeks after the prevention of self-administration; all of them presented abstinence signs for the second time. The rate of self-administration of THC showed individual as well as daily fluctuations, varying in 24 hours between 5 to 30 times, mostly during the day. Abrupt cessation of drug availability caused characteristic withdrawal symptoms. Abstinence signs generally appeared within 12 hours and lasted for approximately five days.

The general picture of a THC-induced abstinence syndrome consists of the following symptoms: there is hyperirritability and increase in aggressiveness; tremors and twitches in the muscles are easily observed; sometimes the whole body of the animal shakes. Among the autonomic disturbances yawning, photophobia, piloerection and erection of the penis are most common. Several ejaculations may follow masturbation in male animals. During the whole period anorexia is striking. Sitting in propped-up positions, eating faeces or other unusual things, biting and licking fingers, pulling hair are other signs which are considered to be related to behavioral and probably sensory changes. Some reactions like staring in circles, grasping as if catching flies and slapping the cage wall, suggest the presence of hallucinations. In three animals which were self-administering THC, pushing the button to receive the drug during the withdrawal period was repeatedly noticed.

Stadnicki *et al.* (1974) also described some withdrawal signs in monkeys following chronic oral administration of crude marihuana extract. The two monkeys that became tolerant after 50 daily treatments with THC 37.5 mg/kg responded to termination of treatment with withdrawal signs manifested by increased aggressiveness. One of the two exhibited hallucinations and increased periods of EEG desynchronization.

DEPENDENCE IN MAN

The development of physical dependence on THC in laboratory animals might not be applicable to man. It has been reported that marijuana smokers can easily give up marijuana without showing any abstinence syndrome. However, this may not be true for users of hashish or other more potent cannabis preparations. As pointed out in the Shafer Commission's report, "important distinctions must be made between the daily (moderate and heavy) American marihuana user and the very heavy hashish or charas users in other parts of the world" (First Report of the National Commission on Marihuana and Drug Abuse, 1972, p. 40).

First we will discuss some reports on the physical dependence in cannabis users in the different parts of the world and then we will mention experimental studies in human volunteers.

DEPENDENCE IN CANNABIS USERS

During the second World War, the withdrawal symptoms had been observed in cannabis users among Indian soldiers. The abstinence syndrome of nine ganja smokers has been described by Fraser (1949). These soldiers being unable to obtain cannabis during military service showed hyperirritability, acute psychosis, and masturbated almost continuously. At times most of them appeared to be visually and aurally hallucinated, and the hallucinations were terrifying. According to Kielholz and Ladewig (1970), some hashish smokers of the Middle East countries who have taken hashish over a long period show withdrawal symptoms such as sweating, disturbed sleep, fine tremors, hypotonia, anxiety, and depression lasting for 5 to 7 days.

Bensusan (1971) from South Africa reported marijuana abstinence symptoms in three males and two females, aged 19 to 29 years. The main signs of withdrawal were anxiety, restlessness coupled with acute abdominal cramps, nausea, sweating, increased pulse rate but no rise in temperature, low blood pressure, and muscular aches. The symptoms persisted from 1 to 3 days. The disappearance of the symptoms coincided with the arrival of a new supply of marijuana. The author had two other similar cases and concluded that "these seven patients had in fact been experiencing acute physical withdrawal symptoms from the smoking of what is recognized in our country as a more potent type of cannabis than usually encountered in Europe or America".

Another article from South Africa (Morley *et al.*, 1973) indicates that physical dependence to dagga (cannabis) is not very rare. Among 150 dagga users, 14 subjects reported withdrawal symptoms. The commonest symptom was irritability, which came on within a few hours of cessation of use and lasted from 2 hours up to 1 month in different subjects. One subject reported general malaise which came on 2 hours after stopping and lasted 3 to 6 days. Four of the subjects reported an intense craving for the drug which lasted up to 3 months after cessation of use. Three subjects felt depressed after giving up, and one of these experienced suicidal thoughts.

Miras (1972) from Greece states that "there is definitely a dependence risk, although much less serious than with opiates. The chronic hashish smoker is usually quiet, lazy, slow-going, a coward, seeking to avoid trouble. But in abstinence he becomes excited and dangerously aggressive. Later come tremor, depression, and loss of weight. In prison, withdrawal is easier than with other drugs, but as soon as they leave, they seek cannabis again".

Even in some parts of the U.S.A., if the recent trends on the escalation to the more potent forms of cannabis continue, one would expect to see some cannabis users who became physically dependent. As a matter of fact it has already been reported from California that three cases who have withdrawn from the prolonged use of marihuana have lapsed into a manic-depressive symptoms (Teitel, 1977).

EXPERIMENTAL DEPENDENCE IN MAN

Experiments on volunteers made by Jones and Benowitz (1976) and Jones *et al.* (1976) leave little doubt that cannabis produces physical dependence when it is administered at short intervals and in high doses. THC was administered orally every 4 hr in increasing doses. The fixed maximum dose of 180–210 mg/day continued for 11–21 days, followed by an abrupt switch to placebo. Then the patients exhibited the following signs and symptoms of abstinence: mood changes, disturbed sleep, decreased appetite, restlessness, perspiration, chills, feverish feeling, nausea, abdominal distress, tremulousness, hyperactivity, hiccups (rare) nasal congestion (rare), weight loss, hemoconcentration, salivation, tremor, loose bowel movements, body temperature increase, sleep EEG eye movement rebound, waking EEG changes, and intraocular pressure increase.

OTHER SIMILARITIES BETWEEN THC AND MORPHINE

The pharmacological similarities of THC to morphine are not limited to analgesia, tolerance and dependence. From the toxicologic and therapeutic point of view the other two most important effects of opioids are respiratory depression and cough suppression. THC also has these properties. In our experiments on monkeys and dogs, especially with high doses of THC, the decrease in the number and amplitude of respiratory movements was striking, and it has been shown that in anesthetized cats THC has an antitussive effect similar to codeine (Gordon *et al.*, 1976).

It is known that morphine produces bradycardia in many species, including rat (Kaymakçalan and Kayan, 1967). We have observed that THC also diminishes heart rate in monkey, dog, cat and rat.

In a review article on the animal pharmacology of marihuana, the common pharmacological effects of cannabis and morphine were listed as follows: decreased spontaneous activity; decreased lever-pressing for reward; sedation; raised threshold of EEG behavioral arousal; depressed polysynaptic reflexes; analgesia; hypothermia; hypotension; respiratory depression; inhibition of gastrointestinal motility; depressed thyroid function; decreased brain norepinephrine levels and antagonism of peripheral effects of 5-HT (Lomax, 1971). Based on this list, Lomax commented that "while such a duplication of effects does not imply that the sites and mechanisms of action of marihuana and morphine are the same, one cannot fail to be impressed by the similarities between the two" (Lomax, 1971). As another similarity, dose-related increases in newly synthesized dopamine and norepinephrine in mice brain due to both THC and morphine have been reported recently (Bloom and Dewey, 1978).

INTERACTIONS BETWEEN THC AND MORPHINE OR NALOXONE

Several kinds of interactions have been observed between THC and morphine or naloxone. These include the potentiation of morphine by THC, cross-tolerance between THC

and morphine, the effect of naloxone on the acute or chronic effects of THC and as effect of THC in morphine-dependent animals.

On the hot plate test we have checked the interactions of analgesic effects of THC and morphine sulfate (MS) in rats. Four group rats (A, B, C, and D) were used. Each group consisted of 8 female rats. First two group rats (A and B) received 5 mg/kg THC, s.c., and the other two groups (C and D) got the same amount of solvent by the same route. One hour later groups B and C were injected with MS 4.5 mg/kg, s.c., and the other two groups rats A and D received saline. The degrees of analgesia were calculated at the end of each hour. It was found that 2 or 3 hours after injection of THC the analgesic effect of the combination of THC and MS (group B) was greater than the algebraic sum of the analgesic effects of THC alone (group A) or MS alone (group C). This was clear indication of the potentiation of morphine analgesia by THC (Kaymakçalan and Deneau, 1972). Similar results have been obtained by Buxbaum *et al.* (1969). These authors have reported that in both mouse and rat, a combination of low doses of MS and THC produced greater than additive effects in analgesia test. Furthermore they observed that in the mouse THC potentiated the Straub tail and the increased locomotor activity produced by morphine.

In our laboratory effects of THC on locomotor stimulating action of morphine were also investigated in mice. In the first series of experiments, the pretreatment of mice by THC in doses up to 20 mg/kg have been found to potentiate dose-dependently the morphine-induced hyperactivity. In the second series of experiments the dose-response curve of morphine for motor activity has been found to shift to the left by the pretreatment of mice with 10 mg/kg of THC. These results have been interpreted as a synergism between morphine and THC (Ayhan *et al.*, 1978).

Since THC produces a general sedation in most species we were interested to see the effect of THC on the morphine-induced excitation (delirium) in the cat. At the beginning we were expecting to reduce morphine excitation by THC. But the results were completely contrary to our expectation; THC potentiated morphine effects in the cat (Kaymakçalan and Deneau, 1972).

A cross-tolerance between THC and morphine has been observed on hot-plate analgesic test in rats (Kaymakçalan and Deneau, 1972). Ten female rats were injected with THC 10 mg/kg, s.c., daily for 6 days. Ten control rats received daily injections of solvent for the same period. On the sixth day there was almost complete loss of analgesic effect of THC (Fig. 3). On the seventh day, both groups received MS 9 mg/kg, s.c.. The analgesic effect of morphine was significantly less in THC-tolerant animals than in controls (Fig. 4).

McMillan *et al.* (1971) obtained similar results in mice. A seven-day pretreatment of mice with 10 mg/kg THC lessened the inhibition of tail-flick response produced by morphine, almost as much as does the development of tolerance to morphine over the same seven-day period. They stated " it is very tempting to interpret these data on the basis of cross-tolerance between THC and morphine". However Bloom and Dewey (1978) reported an asymmetric cross-tolerance between THC and morphine in mice. Using the tail-flick method as the analgesia test, morphine-tolerant mice were also tolerant to THC; but THC-tolerant mice were not cross-tolerant to morphine. On the other hand in hypothermia test, THC-tolerant mice were tolerant to morphine; but morphine-tolerant mice did not show tolerance to THC. To explain the discrepancies in these experiments several factors can be considered: the differences in the species (rat *vs.* mouse), in technique used (hot plate *vs.* tail-flick), in the route of administration (s.c. *vs.* i.p.) and the time of observation after administration of drug (30 min *vs.* 2 hr).

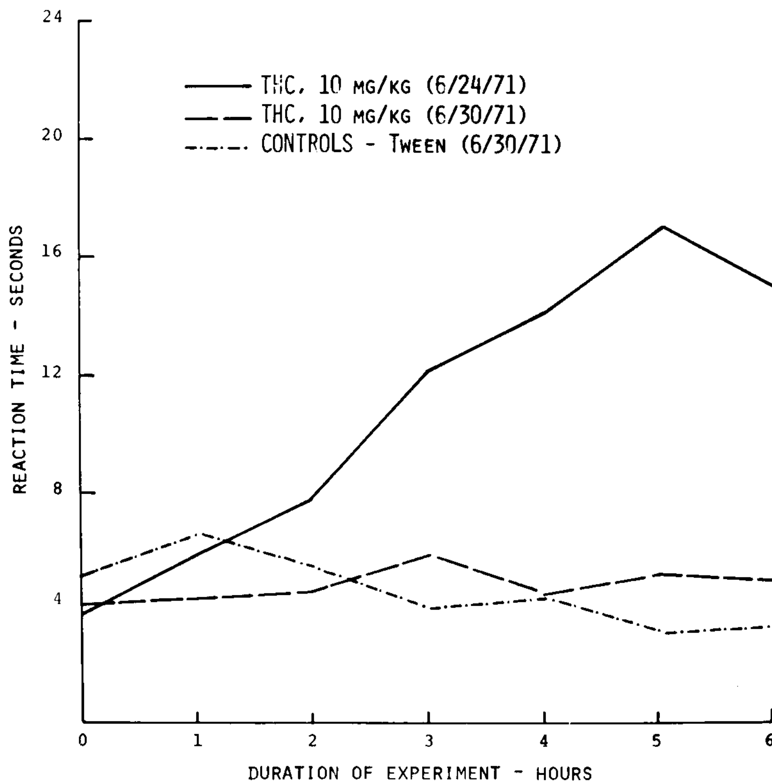


FIG. 3. Tolerance development to the analgesic effect of THC (10 mg/kg) in six days. (Hot-plate test).

It is interesting that naloxone, believed to be a pure antagonist to narcotic analgesics, shows also antagonism to some effects of THC or cannabinoids. Wilson and May (1975) found that in the hot plate test naloxone antagonized the analgesic effect of 11-hydroxy metabolite of Δ^8-tetrahydrocannabinol, which has been considered as the active metabolite in mice. Bloom and Dewey (1978) reported that in mice naloxone diminishes the hypothermic effect of THC as well as the increase in catecholamine accumulation in brain due to THC.

We had mentioned earlier that in rats chronically treated with THC, naloxone caused an opiate-like withdrawal syndrome (Kaymakçalan *et al.*, 1977). These results were in agreement with the report of Hirschhorn and Rosecrans (1974), who treated rats for a five-week period with i.p. doses of THC ranging from 8 mg/kg to 32 mg/kg. After naloxone administration the rats exhibited withdrawal symptoms including diarrhoea, teeth chattering, wet dog shakes, salivation, ptosis, and weight loss.

The precipitation of an opiate-like abstinence syndrome by naloxone in THC-dependent rats suggests the presence of some common features in the development of dependence on both THC and opioids. There is other experimental evidence supporting this view. For example Fernandes and Hill (1974), using naloxone-induced jumping as a criterion for the intensity of physical dependence in rats and mice, concluded that chronic simultaneous application of THC and morphine produced an enhancement of physical dependence. On the other hand Hine *et al.* (1975a) have reported that acute administration of THC before

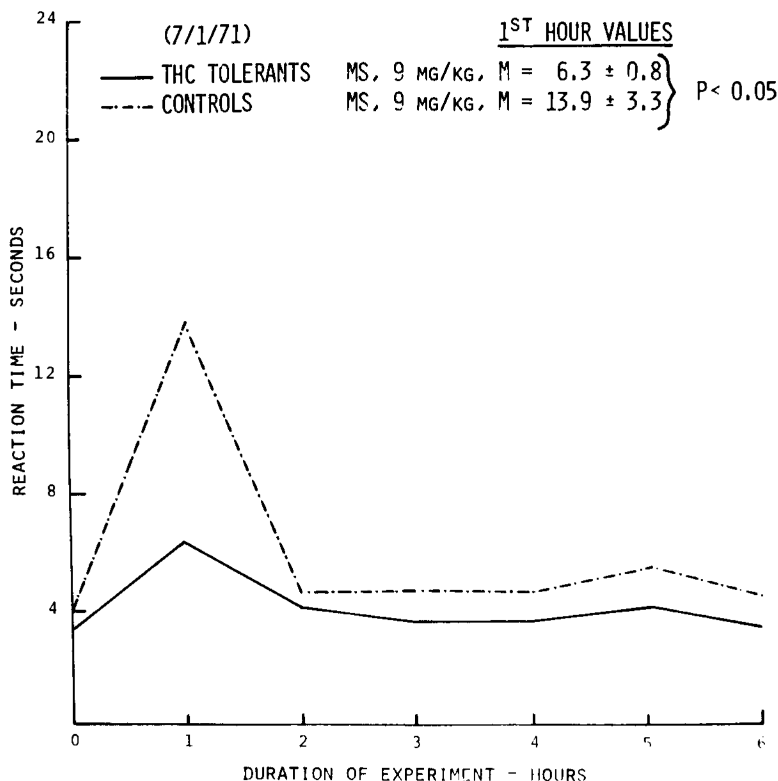


FIG. 4. The analgesic effect of morphine (9 mg/kg) on THC-tolerant and non-tolerant rats. (Hot-plate test).

naloxone challenge in morphine-dependent rats significantly reduces and in some cases blocks precipitated abstinence signs. Similarly, in methadone-dependent rats some signs of naloxone-precipitated abstinence were blocked by the pretreatment with THC (Hine *et al.*, 1975b). In morphine-dependent mice THC also inhibited naloxone-induced withdrawal symptoms (Bhargava, 1976). Finally, the results obtained from whole animals have been confirmed *in vitro* and in isolated ileum from morphine-dependent guinea pig THC antagonized the naloxone-induced contractions (Frederickson and Aiken, 1975).

CONCLUSION

There is considerable theoretical interest in the wide range of similarities and interaction between cannabis and the opioids. It is difficult to explain them as being a result of action on the same receptors, because of the great differences between their chemical structures. However, THC being a very liposoluble substance might have a great affinity for the cell surface which contains the opioid receptors. Thus THC might change the opioid receptor configuration causing more easy (or more difficult) accessibility of opioids or opiate-like endogenous peptides to the receptors. These configurational changes might also facilitate the releases of exogenous or endogenous morphine-like substances from the receptors. This speculation could explain the potentiation of morphine by THC, the cross-tolerance

between two substances, and the effect of naloxone on THC, and should be susceptible to experimental test. However it is clear that there is a need to study the interactions between cannabinoids and endorphines or enkephalins.

LITERATURE

1. ABEL, E. L., Studies of tolerance to 1-Δ^9-tetrahydrocannabinol (Δ^9-THC) in neonatal chicks. *Fed. Proc.* **31**,505 Abs. (1972).
2. AYHAN, I. H., KAYMAKÇALAN, Ş. and TULUNAY, F. C., Interaction between Δ^9-tetrahydrocannabinol and morphine on the motor activity of mice. Submitted for publication.
3. BENSUSAN, A. D., Marihuana withdrawal symptoms. *Brit. Med. J.* **2**,112 (1971).
4. BHARGAVA, H. N., Inhibition of naloxone-induced withdrawal in morphine dependent mice by 1-trans-Δ^9-tetrahydrocannabinol. *Eur. J. Pharmacol.* **36**,259 (1976).
5. BLOOM, A. S. and DEWEY, W. L., A comparison of some pharmacological actions of morphine and Δ^9-tetrahydrocannabinol in the mouse. *Psychopharmacology.* **57**,243 (1978).
6. BUXBAUM, D., SANDERS-BUSH, E. and EFRON, D. H., Analgesic activity of tetrahydrocannabinol (THC) in rat and mouse. *Fed. Proc.* **28**,735 (1969).
7. COCHIN, J. and KORNETSKY, C., Development and loss of tolerance to morphine in the rat after single and multiple injections. *J. Pharmacol. exptl. Therap.* **145**,1 (1964).
8. DAVIS, W. M., MORETON, J. E., KING, W. T. and PACE, H. B., Marihuana on locomotor activity: biphasic effect and tolerance development. *Res. Commun. Chem. Pathol. Pharmacol.* 3,29 (1972).
9. DENEAU, G. A. and KAYMAKÇALAN, Ş., Physiological and psychological dependence to synthetic Δ^9-tetrahydrocannabinol (THC) in rhesus monkeys. *The Pharmacologist* **13**,246 (1971).
10. FERNANDES, M. and HILL, R., Morphine-cannabinoid interactions in rats and mice. *Naunyn Schmiedebergs Arch. Pharmacol.* (Suppl) **282**,R 19 (1974).
11. First Report of the National Commission on Marihuana: and Drug Abuse. Marihuana: A Signal of Misunderstanding. Washington D.C.: U.S. Government Printing Office (1972).
12. FRANKHEIM, J. M., MCMILLAN, D. E. and HARRIS, L. S., Effects of 1-Δ^9-and 1-Δ^8-trans-tetrahydrocannabinol on schedule-controlled behaviour of the pigeon. *Fed. Proc.* **29**,619 (1970).
13. FRASER, J. D., Withdrawal symptoms in cannabis-indica addicts. *Lancet* **2**,747 (1949).
14. FREDERICKSON, R. C. A. and AIKEN, J. W., Naloxone-induced withdrawal and the effects of naloxone on isolated ileum from morphine-dependent guinea-pig: antagonism by Δ^9-tetrahydrocannabinol. *Sixth International Pharmacological Congress, Abstract No. 638, Helsinki* (1975).
15. GONZALEZ, S. C., MATSUDO, V. K. R. and CARLINI, E. A., Effects of marihuana compounds on the fighting behavior of siamese fighting fish (Betta splendens). *Pharmacology* **6**,186 (1971).
16. GORDON, R., GARDON, R. J. and SOFIA, R. D., Antitussive activity of some naturally occurring cannabinoids in anesthetized cats. *Europ. J. Pharmacol.* **35**,309 (1976).
17. HENDERSHOT, L. S. and FORSAITH, J., Antagonism of the frequency of phenilquinone-induced writhing in the mouse by weak analgesics and nonanalgesics. *J. Pharmacol. exptl. Therap.* **125**,237 (1959).
18. HINE, B., FRIEDMAN, E., TORRELIO, M. and GERSHON, S., Morphine-dependent rats: blockade of precipitated abstinence by tetrahydrocannabinol. *Science* **187**,443 (1975a).
19. HINE, B., TORRELIO, M. and GERSHON, S.: Attenuation of precipitated abstinence in methadone-dependent rats by Δ^9-THC. *Psychopharmacol. Commun.* **1**,275 (1975b).
20. HIRSCHHORN, I. D. and ROSENCRANS, J. A., Morphine and Δ^9-tetrahydrocannabinol: tolerance to the stimulus effects. *Psychopharmacologia (Berl.)* **36**,243 (1974).
21. International Narcotics Control Board: The genesis of international control of cannabis–1912 to 1978. *Document: E/INCB/W.222*, 12 May (1978).
22. JAFFE, J. H. and MARTIN, W. R., Narcotic analgesics and antagonists. In "The Pharmacological Basis of Therapeutics." Editors: L. S. Goodman and A. Gilman. Fifth edition, pp. 245–283. Macmillan Publishing Co., Inc., New York, 1975.
23. JONES, R. T. and BENOWITZ, N., The 30-day trip-clinical studies of cannabis tolerance and dependence. In "The pharmacology of marihuana," M. C. Braude and S. Szara, eds., pp. 627–642. Raven Press, New York, 1976.
24. JONES, R. T., BENOWITZ, N. and BACHMAN, J., Clinical studies of cannabis tolerance and dependence. *Ann. N.Y. Acad. Sci.* **282**,221 (1976).
25. KAYMAKÇALAN, Ş. and DENEAU, G. A., Some pharmacological effects of synthetic Δ^9-tetrahydrocannabinol (THC). *The Pharmacologist* **13**,247 (1971).
26. KAYMAKÇALAN, Ş. and DENEAU, G. A., Some pharmacological properties of synthetic Δ^9-tetrahydrocannabinol (THC). *Acta Medica Turcica, Supplementum* **1**, (1972).

27. KAYMAKÇALAN, Ş., Physiological and psychological dependence on THC in rhesus monkeys. In "Cannabis and its derivatives," pp. 142–146. Edited by W. D. M. Paton and June Crown, Oxford University Press, London, 1972.
28. KAYMAKÇALAN, Ş. and SIVIL, Ş., Lack of tolerance to the bradycardic effect of Δ^9-trans-tetrahydrocannabinol in rats. *Pharmacology* **12**,290 (1974).
29. KAYMAKÇALAN, Ş., TÜRKER, R. K. and TÜRKER, M. N., Analgesic effect of Δ^9-tetrahydrocannabinol in the dog. *Psychopharmacologia (Berl.)* **35**,123 (1974).
30. KAYMAKÇALAN, Ş. and KAYAN, S., The effects of morphine on the heart rate in normal or morphine-tolerant rats. *New Instanbul Contr. Clin. S.* **9**,99 (1967).
31. KAYMAKÇALAN, Ş., AYHAN, I. H. and TULUNAY, F. C., Naloxone-induced or postwithdrawal abstinence signs in Δ^9-tetrahydrocannabinol treated rats. *Psychopharmacology* **55**,243 (1977).
32. KIELHOLZ, P. and LADEWIG, G., Über Drogenabhangigkeit bei Jugendlichen, *Dtsh. med. Wshr.* **95**,101 (1970).
33. LOMAX, P., Animal pharmacology of marihuana. *Proc. West. Pharmacol. Soc.* **14**,10 (1971).
34. MCMILLAN, D. E., HARRIS, L. S., FRANKENHEIM, J. M. and KENNEDY, J. S., 1-Δ^9-tetrahydrocannabinol in pigeons: Tolerance to the behavioral effects. *Science.* **169**,501 (1970).
35. MCMILLAN, D. E., DEWEY, W. L. and HARRIS, L. S., Characteristics of tetrahydrocannabinol tolerance. *Ann. N.Y. Acad. Sci.* **19**,83 (1971).
36. MIRAS, C. J., Studies on the effects of chronic cannabis administration to man. In W. D. M. Paton and J. Crown (eds.) "Cannabis and Its Derivatives-Pharmacology and Experimental Psychology." pp. 150–153. Oxford Univ. Press, London, 1972.
37. MORLEY, J. E., LOGIE, P. and BENSUSAN, A. D., The subjective effects of dagga. *S. African Med. J.* **47**,1145 (1973).
38. NOYES, R., Jr. and BARAM, D. A., Cannabis analgesia. *Comprehensive Psychiat.* **15**,531 (1974).
39. NOYES, R., Jr., BRUNK, S. F., BARAM, D. A. and CARTER, A., Analgesic effect of delta-9-tetrahydrocannabinol. *J. Clin. Pharmacol.* **15**,139 (1975a).
40. NOYES, R., Jr., BRUNK S. F., AVERY, D. H. and CARTER, A., The analgesic properties of delta-9-tetrahydrocannabinol and codeine. *Clin. Pharmacol. Therap.* **18**,84 (1975b).
41. PICKENS, R., THOMPSON, T. and MUCHOW, D. C., Cannabis and phencyclidine self-administration by animals. In "Bayer-Symposium IV, Psychic Dependence." Editors: L. Goldberg and F. Hoffmeister, pp. 78–86. Springer-Verlag, 1973.
42. PIRCH, J. H., OSTERHOLM, K. C., COHN, R. A. and BARRATT, E. S., Studies on EEG tolerance to marijuana in the rat. *Arch. int. Pharmacodyn.* **203**,213 (1973).
43. Report of a WHO scientific group.: The use of cannabis, *Wld. Hlth. Org. tech. Rep. Ser. No. 478, Geneva* (1971).
44. SJÖDEN, P. O., JARBE, T. U. C. and HENRIKSON, B. G., Effects of long-term administration and withdrawal of tetrahydrocannabinol (Δ^8-THC and Δ^9-THC) on open field behavior in rats. *Pharmacol. Biochem. Behav.* **1**,243 (1973).
45. STADNICKI, S. W., SCHAEPPI, U., ROSENKRANTZ, H. and BRAUDE, M. C., Crude marihuana extract: EEG and behavioral effects of chronic oral administration in rhesus monkeys. *Psychopharmacologia (Berl.)* **37**,225 (1974).
46. TEITEL, B., Observations on marihuana withdrawal. *Am. J. Psychiatry.* **134**,587 (1977).
47. WILSON, R. S. and MAY, E. L., Analgesic properties of the tetrahydrocannabinols, their metabolites, and analogs. *J. Med. Chem.* **18**,700 (1975).

Δ^9-TETRAHYDROCANNABINOL AND THE QUASI MORPHINE WITHDRAWAL SYNDROME

G. B. CHESHER, S. G. ZALUZNY, D. M. JACKSON
and R. MALOR

Department of Pharmacology, University of Sydney, N.S.W. 2006, Australia.

CLINICAL reports of the effect of cannabis in reducing the severity of the withdrawal syndrome associated with opiate dependence were published late last century[4, 18]. More recently, further reports of this effect of cannabis and of the synthetic cannabinoid pyrahexyl, have been reported[1, 23]. However, experimental studies in opiate dependent animals were not made until those of Hine and his colleagues[13, 15] and by Bhargava[2]. These authors reported that Δ^9-tetrahydrocannabinol (THC) suppressed the signs of the naloxone precipitated withdrawal in opiate dependent rats and mice. These studies encouraged us to examine further the interaction between THC and the syndrome associated with narcotic withdrawal. For this purpose we decided to utilise the quasi-morphine withdrawal syndrome (QMWS), a model for the narcotic withdrawal syndrome described by Collier *et al.* in 1974[9], as this provides the opportunity of comparing the effects of THC with morphine (and other drugs) in narcotic naive animals.

The QMWS is produced by potent phosphodiesterase inhibitors and has been shown by Collier *et al.*[9] to be remarkably similar to that of the true morphine withdrawal. Like the true morphine withdrawal, the QMWS is suppressed stereospecifically by the narcotics with an order of potency that parallels their clinical efficacy. The rationale of the QMWS rests upon (a) the observation that the opiates interact with the cyclic nucleotides[11, 21] and (b) the hypothesis that the narcotic withdrawal syndrome is due to an increased activity of a neuronal cyclic AMP mechanism which does not necessarily result from the inhibition of phosphodiesterase[7, 10, 12].

In the present studies we compared the effect of morphine in suppressing the QMWS with that of THC, haloperidol (a dopamine receptor blocking drug) and chlordiazepoxide (a benzodiazepine sedative-hypnotic). The QMWS was observed before and after the administration of the narcotic antagonist naloxone. The doses for all of these drugs were chosen (by preliminary experiments) to produce approximately equivalent degrees of sedation.

METHODS

Male Sprague-Dawley rats (200–350 g) were used. Rats were observed for signs of the QMWS for two 15 minute periods, beginning 45 and 60 minutes after the administration of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) (15 mg/kg s.c.). Immediately after the first observation period, naloxone (3 mg/kg s.c.) was administered. The interacting drugs were administered at the following times: THC (1.25, 2.5, 5.0 or 10.0 mg/kg i.p.) or its vehicle as control was given immediately after IBMX, morphine (5.0 or 10.0 mg/kg), haloperidol (0.1, 0.2, 0.4 or 0.8 mg/kg), or chlordiazepoxide (5.0 or 10.0 mg/kg) were administered by intraperitoneal injection 30 minutes after IBMX. Control rats for these dosage groups were dosed intraperitoneally with an equivalent volume of saline. Control rats for the effect of IBMX were dosed with saline subcutaneously. Thus, there were seven dosage groups:

I. IBMX plus THC (4 dosage sub-groups, $n = 80$)
II. IBMX plus THC vehicle ($n = 40$)
III. IBMX plus morphine (2 dosage sub-groups, $n = 40$)
IV. IBMX plus haloperidol (4 dosage sub-groups, $n = 80$)
V. IBMX plus chlordiazepoxide (2 dosage sub-groups, $n = 40$)
VI. IBMX plus saline (controls for sub-groups III–V, $n = 40$; and sub-group VII, $n = 29$)
VII. saline plus saline ($n = 29$).

EXPERIMENTAL PROCEDURE

Four rats only were used for each experiment. The results presented here represent the accumulated data obtained from 378 rats. Each experiment was conducted over a period of 75 minutes according to the following design:

Time 0 (*t*0)	IBMX or saline administered THC groups dosed All rats placed into individual wire cages (19 cm diameter × 19 cm high)
30 min	Morphine, haloperidol or chlordiazepoxide administered
45 min	First 15 min observation period begins
50 min	Cages struck to observe sensitivity to noise
55 min	Rats individually handled to record squeak on touch, jump on touch, salivation, aggressiveness or ptosis
60 min	Naloxone administered, and the second 15 min observation period begins
70 min	Rats individually handled (as at *t*55)
75 min	Experiment terminated

SCORING METHODS:

Animals were observed for a total of 20 QMWS signs: **Rearing*, the animal stands on its back legs with its front legs off the ground; **Agitation and grooming*, exploratory movements, scratching and grooming; **Piloerection*, characterised by the fur standing on end

for most of the observation period; *Ptosis*, drooping of the upper eyelid (handling of the animal does not cause a change in the ptosis); **Wet dog shakes*, violent shaking movement of the head and body; **Head shakes*, violent shaking movements involving only the head and forelimbs and not the body; **Jumping*, the animal leaps to the edge of the cage or out of it; *Squeak on touch*, the animal squeaks when touched or handled; **Squeak*, a spontaneous squeak during which the animal is generally motionless; *Jump on touch*, touching the animal causes it to jump; **Penis licking*, the animal sits on its back legs and licks the penis; **Chewing*, the animal makes chewing movements as if it were eating; **Paw licks*, the animal assumes a grooming position but only licks its paws; **Paw shakes*, a rapid foot shake; *Diarrhoea*, soft liquid stools or six or more faecal boli are passed during the observation period; *Sensitive to noise*, noise made by striking the cage with a bar which causes the animal to vocalise, jump or both; **Teeth chattering*, rapid and audible chattering of the teeth; **Rapid respiration*, seen as rapid movements of the rib cage; *Salivation*, an abnormal volume of saliva around the outside of the mouth; *Aggressiveness*, biting and attacking on handling.

Signs marked with an asterisk were scored as 1 each time they occurred whilst others were recorded as being present (1) or absent (0). Ptosis and piloerection were scored between 0 (sign absent) and 3 (sign most intense). Scores for each sign were totalled for each of the two 15-minute observation periods (pre- and post-naloxone). Because of the subjectivity of the procedure it was performed blind by one of us (SZ) in that the assessor was unaware of the treatment regimen.

DRUGS:

3-isobutyl-1-methylxanthine (IBMX, Sigma); naloxone hydrochloride (Endo†); morphine hydrochloride (McFarlan Smith†); haloperidol ("Serenance"®) ampoules, Searle†); chlordiazepoxide hydrochloride (Roche†); (−)-trans-Δ^9-tetrahydrocannabinol (THC, National Institute on Drug Abuse†). Haloperidol ampoules were diluted with saline immediately before use. IBMX was dissolved in saline with the aid of heat. THC was dissolved in propylene glycol and diluted to produce in all cases a mixture containing THC, 10% propylene glycol, 1% tween 80 in saline (0.9%). This was prepared freshly each day. All other drugs were dissolved in or diluted with distilled water. An injection volume of 1.0 ml/kg was used for all drugs excepting IBMX (10 ml/kg).

STATISTICS AND DATA ANALYSIS:

The data were analysed in three ways.

a. Number of animals exhibiting each sign. For the comparison of the signs produced by IBMX- with those exhibited by saline-dosed control rats, the number of animals exhibiting any particular sign was calculated, expressed as a percentage of the total animals and statistical comparisons made between groups by the Chi^2 test (see Table 1).

b. The mean withdrawal score. For each dosage group the scores recorded during each assessment period were added to give a total withdrawal score. The scores for each animal

† We gratefully acknowledge these companies and NIDA for generous gifts of these drugs.

TABLE 1. SIGNS OBSERVED IN RATS AFTER IBMX (OR SALINE AS A CONTROL) BEFORE AND AFTER NALOXONE ADMINISTRATION IN ANIMALS PRETREATED WITH SALINE. THE DATA ARE EXPRESSED AS THE PERCENTAGE OF ANIMALS EXHIBITING EACH SIGN. RESULTS ABOVE THE DOTTED LINE WERE SIGNIFICANTLY AFFECTED BY IBMX.

	Saline		IBMX	
Sign	Pre-Naloxone $n = 29$ (*a*)	Post-Naloxone $n = 29$ (*b*)	Pre-Naloxone Saline $n = 28$* (*c*)	Post-Naloxone Saline $n = 28$ (*d*)
Rearing	38	41	65	90
Agitation and grooming	55	62	85	40
Piloerection	0	0	78	75
Ptosis	3	0	83	85
Wet dog shakes	3	0	83	85
Headshakes	41	8	98	93
Jumping	0	0	23	55
Squeak on touch	14	14	28	20
Squeak	0	0	3	3
Jump on touch	3	0	0	0
Penis licking	3	7	10	18
Chewing	17	10	10	13
Paw shakes	0	3	10	13
Paw licks	3	24	10	5
Diarrhoea	3	3	3	0
Sensitive to noise	0	3	10	0
Teeth chattering	3	6	0	3
Rapid respiration	0	0	0	8
Salivation	0	0	0	0
Aggressiveness	0	0	0	0

* 29 animals were dosed and one died during the experiment.

were transformed ($y = \sqrt{x} + \frac{1}{2}$)[22] and analysed by one-way analysis of variance. Where F ratios were significant, individual dosage groups were submitted to Student's *t*-test (two-tailed) to examine the differences in mean scores.

c. The total number of signs exhibited. The mean withdrawal score provides a measure of total withdrawal activity and could theoretically be generated by an intensive occurrence of only one or two signs. For this reason the results were also analysed to determine the presence (1) or absence (0) of each of the signs. These values were summed and the mean value expressed as mean number of signs exhibited. Differences between groups were analysed by Student's *t*-test (two-tailed).

RESULTS

1. THE QUASI-MORPHINE WITHDRAWAL SYNDROME

A comparison of the behavioural signs exhibited by rats dosed with IBMX with those dosed saline is summarised in Table 1. IBMX-dosed rats displayed a significant increase in

the incidence of seven signs: rearing ($p < 0.05$), agitation and grooming ($p < 0.02$), piloerection ($p < 0.01$), ptosis ($p < 0.001$), wet dog shakes ($p < 0.001$), headshakes ($p < 0.001$) and jumping ($p < 0.02$). Naloxone was without marked effect on the exhibition of the signs although a significant increase was recorded in rearing ($p < 0.05$) and jumping ($p < 0.01$), while a significant decrease ($p < 0.05$) was noted in agitation and grooming.

2. THE EFFECT OF MORPHINE ON THE QUASI-MORPHINE WITHDRAWAL SYNDROME

Morphine significantly suppressed the QMWS as expressed by the mean withdrawal score (Fig. 1a) and the total number of signs exhibited (Fig. 1b). Although only two dose levels were employed this effect suggested dose dependency. Six signs were significantly reduced (when compared with saline controls) by both doses of morphine. These were, agitation and grooming ($p < 0.001$), rearing ($p < 0.001$), wet dog shakes ($p < 0.001$), headshakes ($p < 0.001$), ptosis ($p < 0.02$) and piloerection ($p < 0.05$, only 10.0 mg/kg dose).

Assessment in the post-naloxone period indicated that the narcotic antagonist had reversed the attenuating effect of morphine on these individual signs, the mean withdrawal score and the total number of signs exhibited (Fig. 1a, b). There was, however, still a significant ($p < 0.02$) reduction in ptosis when compared with the controls and the score for agitation and grooming was significantly higher than for the controls ($p < 0.001$) in the 5 mg/kg group.

3. THE EFFECT OF THC ON THE QUASI-MORPHINE WITHDRAWAL SYNDROME

THC significantly and dose-dependently attenuated the QMWS when assessed as the mean withdrawal score (Fig. 2a). However, the assessment of the mean number of signs (Fig. 2b) showed no clear evidence for antagonism. Examination of the signs affected by THC indicated a significant decrease in the occurrence of five signs; agitation and grooming ($p < 0.001$ all doses), wet dog shakes ($p < 0.001$ all doses), headshakes ($p < 0.001$, all doses except 1.25 mg/kg), rearing ($p < 0.02$, 10.0 mg/kg) and ptosis ($p < 0.001$ for 1.25 mg/kg and $p < 0.02$ for 10.0 mg/kg). However, significant increase in the incidence of four signs was noted in the THC treated animals. These were squeak on touch ($p < 0.05$ all doses), spontaneous squeaking ($p < 0.05$ all doses), sensitivity to noise ($p < 0.05$ all doses) and jump on touch ($p < 0.02$, for 10.0 mg/kg). These signs were increased (when compared with saline controls) only in those animals that had received THC, are characteristic of the effects of THC in rats[17]. We felt justified therefore in reanalysing the data after the exclusion of the contribution of these four signs to the various scores because they would unfairly bias the interpretation of the IBMX-THC interaction. The exclusion of these data did not change the general picture of the antagonism of the QMWS by THC when analysed as the mean withdrawal score (Fig. 3a). These findings suggested that the signs that were increased in occurrence by THC itself did not contribute greatly to the mean withdrawal score. Furthermore, this reanalysis showed that THC produced a significant, dose dependent decrease in the mean number of signs exhibited (Fig. 3b).

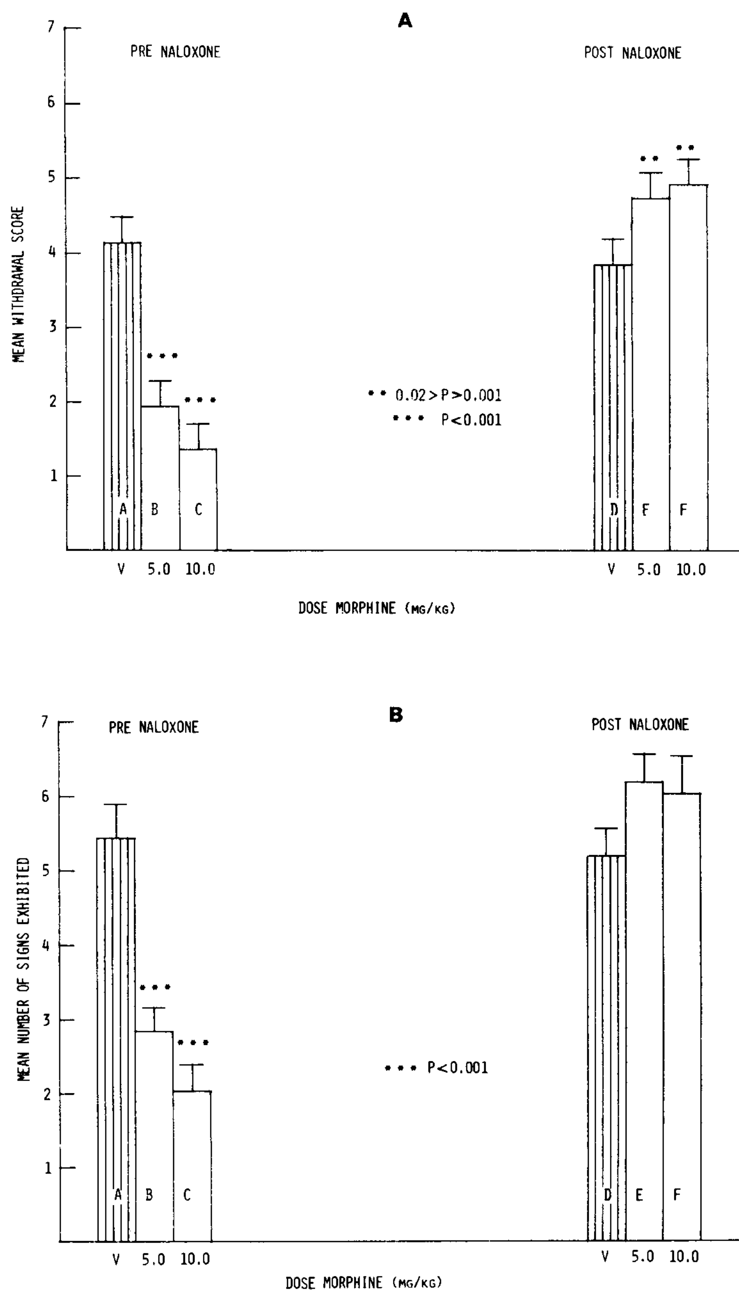


FIG. 1. The effect of pretreatment with morphine (5 or 10 mg/kg) or saline (V, as a control), and naloxone on the QMWS-induced by administration of IBMX to rats ($n = 20$ for each morphine group and 40 for saline controls). The vertical bars are the SEM, and the letters within each column are for statistical comparisons. The columns represent either: graph A, the mean withdrawal score or graph B, the mean number of signs exhibited. The stars on the graphs represent significance levels for statistical comparisons between the morphine-treated groups and the appropriate vehicle treated controls.

Statistical comparisons. Graph A. A compared to D, $p > 0.05$, BE and CF, both $p < 0.001$. Graph B. AD, $p > 0.05$, BE, CF, both $p < 0.001$.

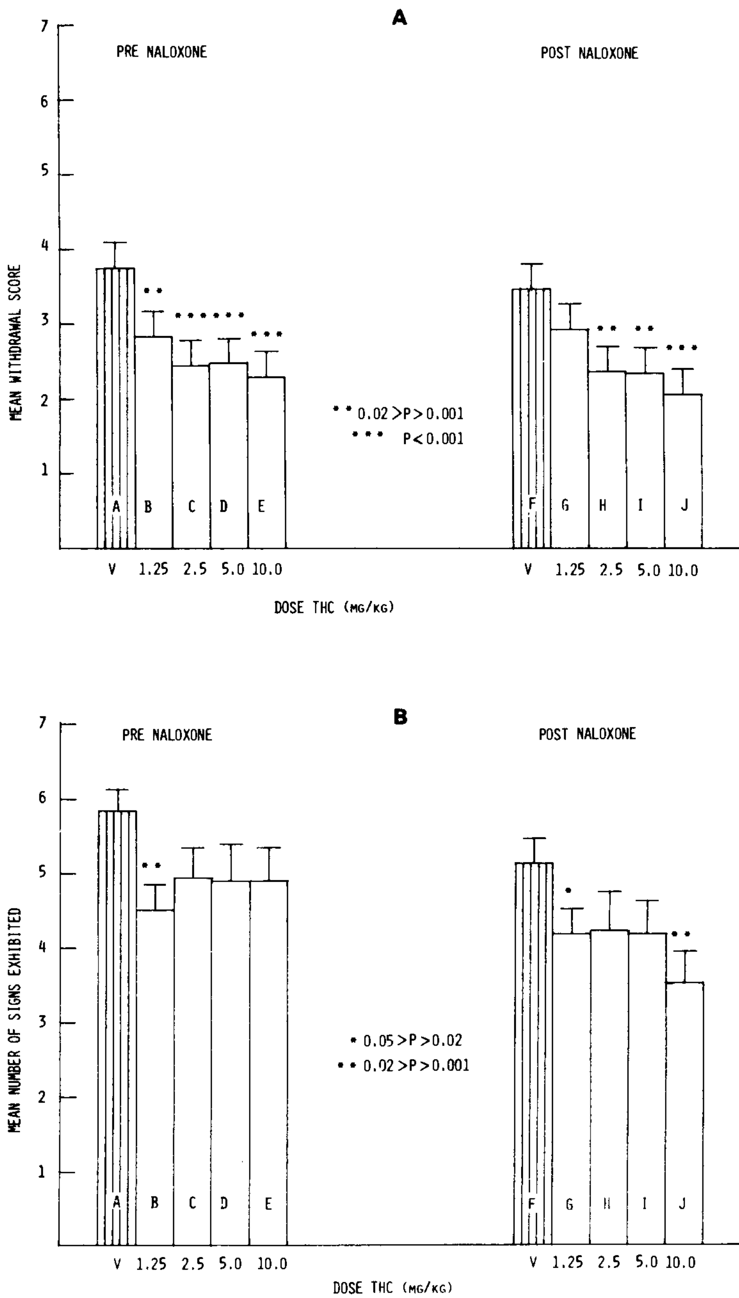


FIG. 2. The effect of pretreatment with THC (1.25, 2.5, 5 or 10 mg/kg) or vehicle (V, as a control), and naloxone in the QMWS-induced by the administration of IBMX to rats ($n = 20$ for each THC group and 40 for vehicle group). The vertical bars are the SEM, and the letters underneath each column are for statistical comparisons. The columns represent either: Graph A, the mean withdrawal score or graph B, the mean number of signs exhibited. The stars on the graphs represent significance levels for statistical comparisons between the THC-treated groups and the appropriate controls.

Statistical comparison. Graph A. AF, BG, CH, DI, EJ, all $p > 0.05$. Graph B. AF, BG, CH, DI, EJ, all $p > 0.05$.

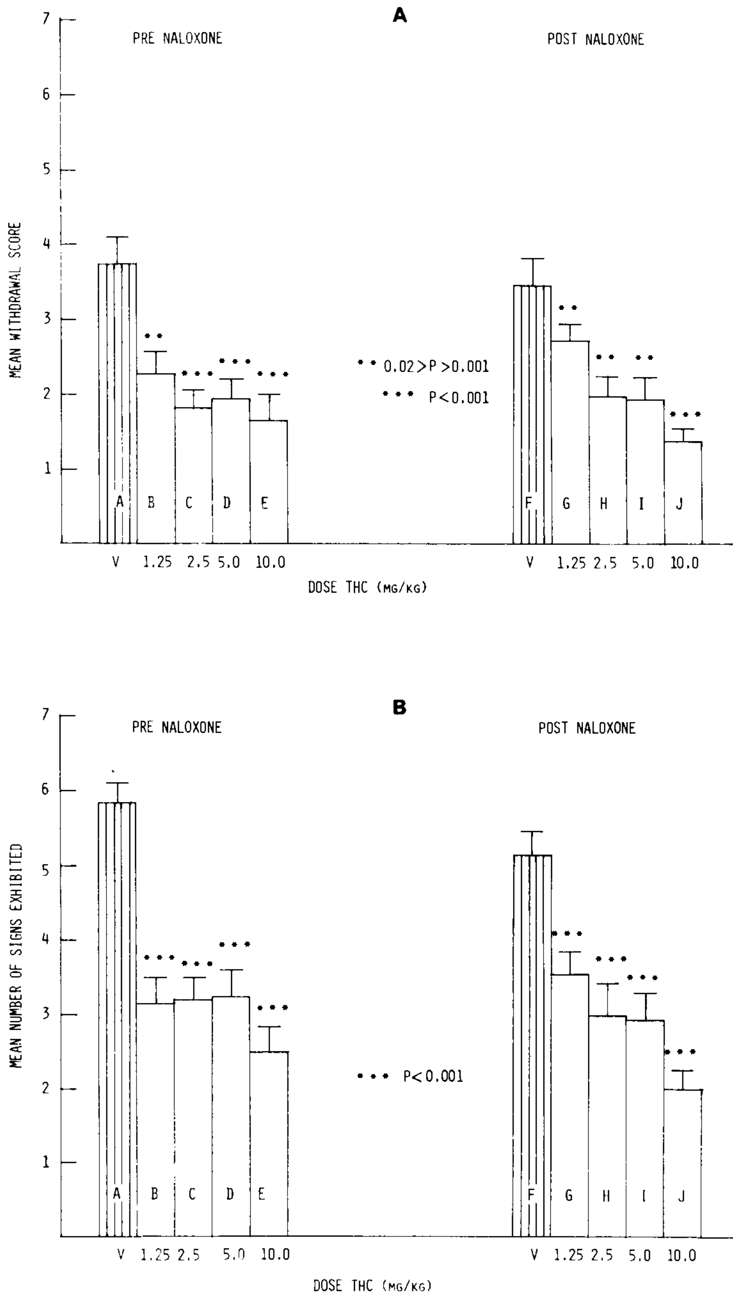


FIG. 3. The effect of pretreatment with THC (1.25, 2.5, 5 or 10 mg/kg) or vehicle (V, as a control), and naloxone, on the QMWS-induced by administration of IBMX to rats ($n = 20$ for each THC group and 40 for vehicle group). These data exclude the contribution of 4 QMWS signs (see text). The vertical bars are the SEM, and the letters underneath each column are for statistical comparisons. The columns represent either: graph A, the mean withdrawal score; or graph B, the mean number of signs exhibited. The stars on the graphs represent significance levels for statistical comparisons between the morphine-treated groups and the appropriate vehicle treated controls.

Statistical comparisons. Graphs A and B. AF, BG, CH, DI and EJ, all $p > 0.05$.

Naloxone was without significant effect on the mean withdrawal score and the total number of signs exhibited. The signs that were attenuated by THC (viz. agitation and grooming, rearing, headshakes, ptosis and wet dog shakes) were unaffected by naloxone.

4. THE EFFECT OF HALOPERIDOL ON THE QUASI-MORPHINE WITHDRAWAL SYNDROME

Only in the higher doses (0.4 and 0.8 mg/kg) did haloperidol exert any significant effect on the mean withdrawal score (Fig. 4a) and it was without effect at any dose level on the mean number of signs exhibited (Fig. 4b). Significant reductions were recorded for wet dog shakes at the 0.4 mg/kg dose ($p < 0.02$) and 0.8 mg/kg ($p < 0.001$), headshakes at the 0.8 mg/kg dose ($p < 0.05$) and agitation and grooming at the 0.4 mg/kg dose ($p < 0.05$). These changes were not altered consistently by naloxone. However, the incidence of rearing, which was not significantly reduced by haloperidol in the pre-naloxone period, was significantly lower than that of the controls in the post-naloxone period for all of the dosage levels except 0.2 mg/kg.

5. THE EFFECT OF CHLORDIAZEPOXIDE ON THE QUASI-MORPHINE WITHDRAWAL SYNDROME

At the dose levels tested chlordiazepoxide produced no significant effect on either the mean withdrawal score (Fig. 5a) or on the mean number of signs exhibited (Fig. 5b).

DISCUSSION

These results confirm the findings of Collier *et al.*[8, 9] that the phosphodiesterase inhibitor IBMX induces in the rat a behavioural syndrome that is similar to that of the true morphine withdrawal syndrome. Morphine was able to antagonise the exhibition of the QMWS, an effect that was reversed by the narcotic antagonist naloxone.

The QMWS was also antagonised by THC in a dose dependent manner and the signs suppressed by this drug (with the exception of piloerection) were the same as those affected by morphine. Furthermore, it is of interest to note that the quantitative estimates for both the mean withdrawal scores (Figs. 1a and 3a) and the total number of signs (Figs. 1b and 3b) for morphine and THC (after the exclusion of the THC-specific signs) were remarkably similar. In our hands the intensity of the QMWS did not change markedly after the administration of naloxone, therefore the pre-naloxone scores provide a meaningful comparison of the efficacy of morphine and THC. In this model therefore, morphine and THC show a similar potency on a mg/kg basis. Nevertheless, whilst the morphine suppression of the QMWS was convincingly antagonised by naloxone, the effect of THC was unchanged by the narcotic antagonist. It seems therefore that the site of action of THC is probably not at the same site as that of morphine in the antagonism of the QMWS.

Morphine and THC share also the property of analgesia[3, 5, 6] and in this parameter, as in the QMWS, the effect of THC is not affected by the narcotic antagonists[6]. In a recent study, the effect of naloxone on the morphine- and THC-attenuation of the formic acid

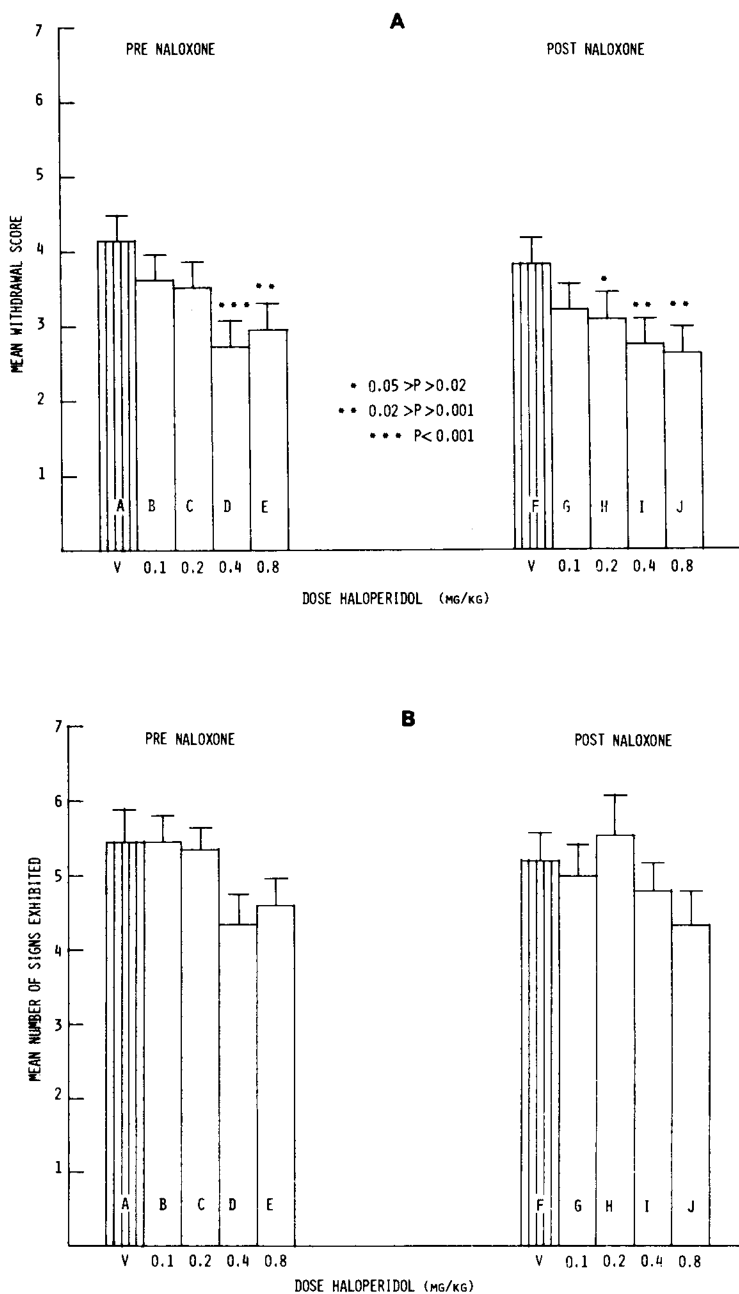


FIG. 4. The effect of pretreatment with haloperidol (0.1, 0.2, 0.4 or 0.8 mg/kg) or vehicle (V, as a control), and naloxone on the QMWS-induced by the administration of IBMX to rats ($n = 20$ for each haloperidol group and 40 for vehicle group). The vertical bars are the SEM, and the letters underneath each column are for statistical comparisons. The columns represent either: graph A, the mean withdrawal score, or graph B, the mean number of signs exhibited. The stars on the graphs represent significance levels for statistical comparisons between the haloperidol-treated groups and the appropriate vehicle control.

Statistical comparisons. Graphs A and B. AF, BG, CH, DI and EJ, all $p > 0.05$.

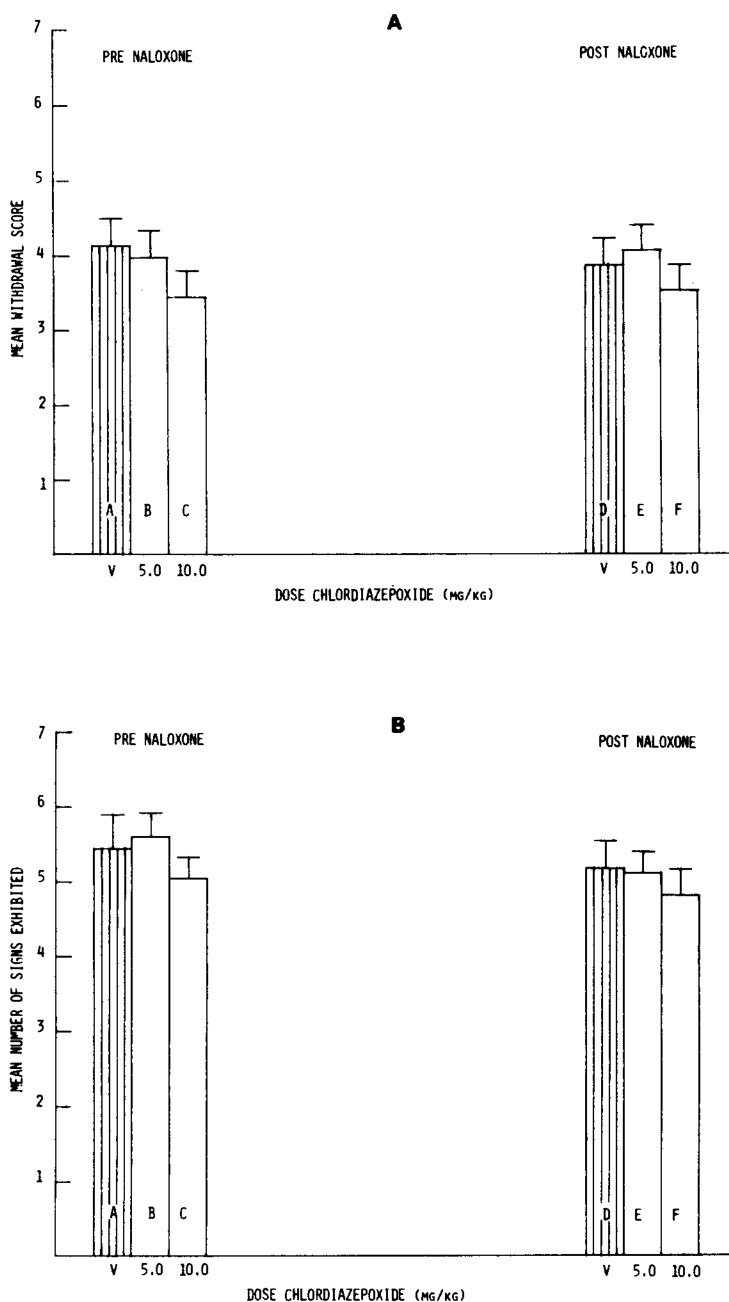


FIG. 5. The effect of pretreatment with chlordiazepoxide (5 or 10 mg/kg) or vehicle (V, as a control), and naloxone on the QMWS-induced by the administration of IBMX to rats ($n = 20$ for each chlordiazepoxide group and 40 for vehicle group). The vertical bars are the SEM, and the letters underneath each column are for statistical comparisons. The columns represent either: graph A, the mean withdrawal score or graph B, the mean number of signs exhibited. The stars on the graphs represent significance levels for statistical comparisons between the chlordiazepoxide-treated groups and the appropriate vehicle control.

Statistical comparisons for both graphs A and B, AD, BE and CF, all $p > 0.05$.

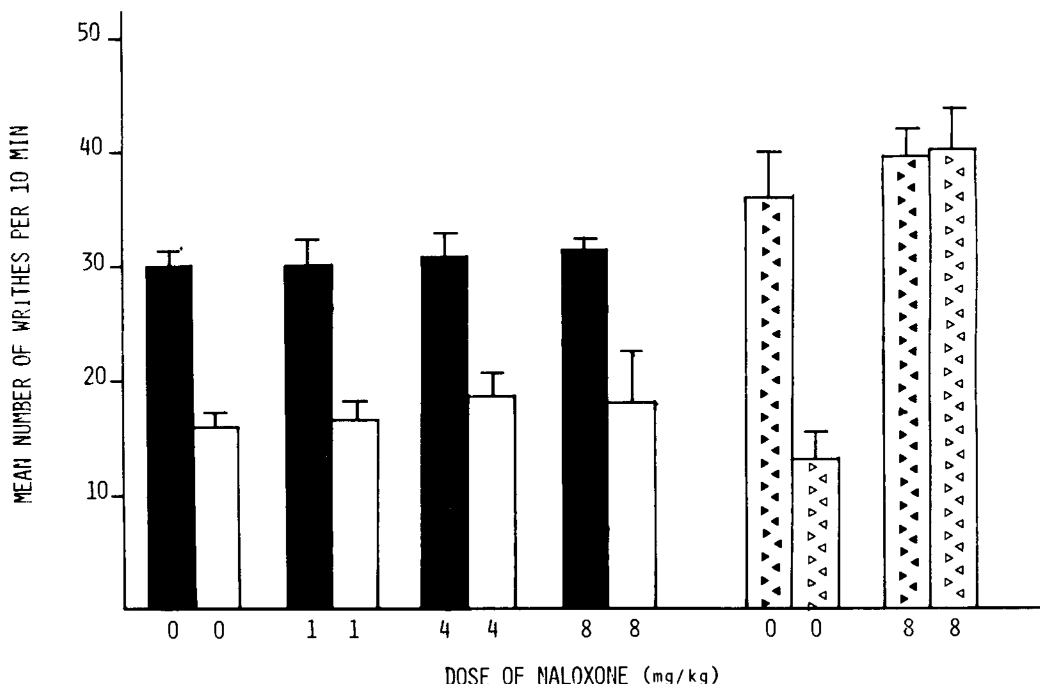


FIG. 6. The effect of naloxone (or saline control) upon THC- and morphine-antagonism of formic acid-induced writhing in mice. 2.0 mg THC/kg (or vehicle control) was administered (i.p.) 1 h prior to administration of the formic acid. 1.0 mg morphine/kg (or vehicle control) was administered (s.c.) 30 min prior to administration of the formic acid. Naloxone (1.0, 4.0 or 8.0 mg/kg or saline control) was administered (i.v.) 5 min after the administration of the formic acid. The data is expressed as the mean number of writhes/mouse/10 min, the vertical bars are the s.e.m. and the numbers in the columns represent the number of times the experiment was repeated. Filled columns and triangles, vehicle-treated; open columns and triangles, morphine-treated.

induced abdominal constriction response in mice was examined[20]. Naloxone (1.0, 4.0 or 8.0 mg/kg^{-1}) administered intravenously was without effect on the analgesic activity of THC (2 mg/kg^{-1}) but abolished completely the response to morphine (1.0 mg/kg^{-1}) (Fig. 6).

Depending on the dosage, THC is capable of producing sedation and for this reason we included chlordiazepoxide in dosage levels that produced sedation of approximately equivalent intensity as that produced in these studies by THC. That chlordiazepoxide was without effect on the QMWS suggests that the effect of THC reported here was not due to sedation alone.

Haloperidol, a potent and fairly specific dopamine receptor antagonist[1a], has been reported to be effective in attenuating some of the signs of the true narcotic withdrawal syndrome. For example, Hine and Gershon[16] have reported the alleviation of wet dog shakes and escapes in morphine dependent rats, and Puri and Lal[19] have found that in doses as high as 2.5 mg/kg^{-1} haloperidol blocked aggressive behaviour and decreased rearing in morphine dependent rats. The present results obtained with haloperidol, although showing only a moderate reduction in the intensity of the QMWS, were therefore not surprising.

CONCLUSION

The present results show that THC is able to attenuate considerably the quasi morphine withdrawal syndrome in rats. This activity was generally unchanged by the administration of the narcotic antagonist, naloxone, and is presumably therefore not dependent upon the opiate receptor. Furthermore, the effect is probably not mediated via dopaminergic mechanisms because the specific and potent dopamine receptor antagonist haloperidol was much less effective than THC. Finally, it is unlikely that sedation alone could account for the effectiveness of THC as approximately equisedative doses of chlordiazepoxide were without effect.

REFERENCES

1. ALLENTUCK, S. and BOWMAN, K. M., The psychiatric aspects of marihuana intoxication. *Amer. J. Psychiat.* **99**,250 (1942).
1a. ANDÉN, N.-E., BUTCHER, S. G., CORRODI, H., FUXE, K. and UNGERSTEDT, U., Receptor activity and turnover of dopamine and noradrenaline after neuroleptics. *Eur. J. Pharmacol.* **11**,303 (1970).
2. BHARGAVA, H. N., Inhibition of naloxone-induced withdrawal in morphine dependent mice by 1-trans-Δ^9-tetrahydrocannabinol. *Eur. J. Pharmacol.* **36**,259 (1976).
3. BICHER, H. I. and MECHOULAM, R., Pharmacological effects of two active constituents of marihuana. *Archs. int. Pharmacodyn. Ther.* **172**,24 (1968).
4. BIRCH, E. A., The use of indian hemp in the treatment of chronic chloral and chronic opium poisoning. *Lancet* **1**,625 (1889).
5. BUXBAUM, D. M., Analgesic activity of Δ^9-tetrahydrocannabinol in the rat and mouse. *Psychopharmacologia (Berl.)* **25**,275 (1972).
6. CHESHER, G. B., DAHL, C., EVERINGHAM, M., JACKSON, D. M., MARCHANT-WILLIAMS, H. and STARMER, G. A., The effect of cannabinoids on intestinal motility and their antinociceptive effect in mice. *Brit. J. Pharmacol.* **49**,588 (1973).
7. COLLIER, H. O. J. and FRANCIS, D. L., Morphine abstinence is associated with increased brain cyclic AMP. *Nature* **255**,159 (1975).
8. COLLIER, H. O. J. and FRANCIS, D. L., Stereospecific suppression by opiates of the quasi-morphine abstinence syndrome elicited by 3-isobutyl-1-methylxanthine (IBMX). *Brit. J. Pharmacol.* **56**,382P (1976).
9. COLLIER, H. O., FRANCIS, D. L., HENDERSON, G. and SCHNEIDER, C., Quasimorphine-abstinence syndrome. *Nature* **249**,471 (1974).
10. COLLIER, H. O. J., FRANCIS, D. L., MCDONALD-GIBSON, W. J., ROY, A. C. and SAEED, S. A., Prostaglandins, cyclic AMP and the mechanism of opiate dependence. *Life Sci.* **17**,85 (1975).
11. COLLIER, H. O. J., and ROY, A. C., Morphine-like drugs inhibit the stimulation by E prostaglandins of cyclic AMP formation by rat brain homogenate. *Nature (London)* **248**,24 (1974).
12. FRANCIS, D. L., ROY, A. C. and COLLIER, H. O. J., Morphine abstinence and quasi-abstinence effects after phosphodiesterase inhibitors and naloxone. *Life Sci.* **16**,1901 (1975).
13. HINE, B., FRIEDMAN, E., TORRELIO, M. and GERSHON, S., Morphine-dependent rats: blockade of precipitated abstinence by tetrahydrocannabinol. *Science* **187**,443 (1975).
14. HINE, B., FRIEDMAN, E., TORRELIO, M. and GERSHON, S., Tetrahydrocannabinol attenuated abstinence and induced rotation in morphine-dependent rats: possible involvement of dopamine. *Neuropharmacol.* **14**,607 (1975).
15. HINE, B., TORRELIO, M. and GERSHON, S., Attenuation of precipitated abstinence in methadone dependent rats by Δ^9-THC. *Psychopharmacol. Commun.* **1**,275 (1975).
16. HINE, B. and GERSHON, S., Haloperidol attenuation of morphine-abstinence: synergistic effect of acute lithium administration. *J. Pharm. Pharmacol.* **29**,238 (1977).
17. JÄRBE, T. U. C. and HENRIKSSON, B. G., Vocalization: a characteristic cannabis-induced behavior in the rat. *Physiol. Psychol.* **1**,351 (1973).
18. MATTISON, J. B., Cannabis Indica as an anodyne and hypnotic. *St. Louis Med. Surg. J.* **61**,266 (1891).
19. PURI, S. K. and LAL, H., Effect of dopaminergic stimulation or blockade on morphine-withdrawal aggression. *Psychopharmacologia (Berl.)* **32**,113 (1973).
20. SANDERS, J., JACKSON, D. M. and STARMER, G. A., Interactions among the cannabinoids in the antagonism of the abdominal constriction response in the mouse. *Psychopharmacology* (in press).

21. SHARMA, S. K., NIRENBERG, M. and KLEE, W. A., Morphine receptors as regulators of adenylate cyclase activity. *Proc. Nat. Acad. Sci. (USA)* **72**,590 (1975).
22. STEEL, R. G. D. and TORRIE, J. H., "Principles and procedures of statistics." McGraw-Hill Book Co., N.Y., 1960.
23. THOMPSON, L. J. and PROCTOR, R. C., Pyrahexyl in the treatment of alcoholic and drug withdrawal conditions. *N. Carolina Med. J.* **14**,520 (1953).

CANNABIS AND EPILEPSY

RALPH KARLER and STUART A. TURKANIS

Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah 84132.

Summary. Anticonvulsant activity is widespread among the cannabinoids, including major constituents of marihuana, synthetic congeners and metabolites of Δ^9-tetrahydrocannabinol (Δ^9-THC). A potential therapeutic value of these anticonvulsants became a reality with the recognition that some are devoid of the characteristic psychotoxicity of marihuana and that some have a protective index (P.I.: TD_{50}/ED_{50}) greater than one, which is an expression of a selectivity of anticonvulsant action relative to neurotoxicity. Of the non-psychoactive agents, cannabidiol (CBD) has the most favorable P.I. The anticonvulsant activity is not only widespread but, in mice, it varies greatly, and the differences in potency between stereoisomers are suggestive evidence for the existence of specific receptors. Marked species differences in the potency of CBD and Δ^9-THC were also noted; these results contrasted with those of phenytoin (PTN) and phenobarbital (PB), which have essentially identical potency in rat, mouse and frog. Furthermore, a comparison of the pharmacokinetics of CBD and PTN in brain with their pharmacodynamics revealed that the peak brain concentrations are identical, which implies that they may have comparable intrinsic activity, despite a large disparity in their ED_{50}s. The pharmacokinetics of CBD and PTN correlate well with their pharmacodynamics. Such is not the case for Δ^9-THC, which may derive its anticonvulsant activity from a metabolite. The anticonvulsant effects of CBD were recorded in a variety of electro- and chemoshock tests; in these tests, CBD resembles PTN rather than ethosuximide (ETS). Tolerance to antiseizure effects was evaluated in the different electroshock tests and it was found that tolerance is not a prominent characteristic of either CBD or PTN. The electrophysiological actions of CBD were compared with those of PTN and ETS on focal epileptic potentials, on posttetanic potentiation (PTP) and on after-discharges (ADs) elicited by electrically induced limbic seizures. CBD, like PTN, failed to suppress ETS-sensitive, cobalt-induced epileptic foci in rats. In contrast, Δ^9-THC enhanced focal epileptic potentials and seizure activity, suggesting that this drug may be contraindicated in epileptics. CBD resembled PTN by decreasing PTP in isolated bullfrog ganglia; Δ^9-THC had no effect, but both its mono- and dihydroxylated metabolites were potent inhibitors of PTP. Finally, CBD's effects on the AD associated with limbic seizures were unique, compared with those of either PTN or ETS. CBD elevated AD threshold and depressed AD duration and amplitude, PTN only elevated threshold and ETS decreased all three AD parameters. From the results of the electrophysiological studies, the mechanism of action of CBD and PTN may involve a depression of seizure spread (PTP) and the generation of seizure activity (ADs), rather than a depression of focal epileptic discharges. Their specific effects, however, on the epileptic AD are not the same. CBD's unique anticonvulsant properties and its relative lack of known toxicity combine to enhance its potential as an antiepileptic drug.

INTRODUCTION

THE historical, and primarily anecdotal, accounts of the antiseizure activity of marihuana (O'Shaughnessy, 1842; Loewe and Goodman, 1947; Davis and Ramsey, 1949) provided the original impetus for our examination of the anticonvulsant properties of cannabinoids, while the advent of chemically defined and relatively pure cannabinoids made their

systematic, pharmacological characterization feasible (Gaoni and Mechoulam, 1964). The initial investigations yielded two signal discoveries: The first was the recognition that cannabidiol (CBD), which lacks both the psycho- and cardiotoxicity of Δ^9-tetrahydrocannabinol (Δ^9-THC) (Hollister, 1973, 1974; Perez-Reyes *et al.*, 1973), has anticonvulsant activity (Izquierdo and Tannhauser, 1973; Karler *et al.*, 1973). The second important discovery was that the protective index (P.I.: motor toxic $dose_{50}$/anticonvulsant $dose_{50}$) varies greatly among the different cannabinoids (Karler *et al.*, 1974a,d). This finding is particularly significant, for it demonstrates that anticonvulsant activity is, at least partially, separable from neurotoxicity. In other words, there is a degree of selectivity for anticonvulsant activity among the cannabinoids, and, in fact, the selectivity in the case of CBD is equal to that of the clinically useful antiepileptic phenobarbital (PB) (Karler *et al.*, 1974a,d; Karler and Turkanis, 1976a).

There have been numerous reports of cannabinoid anticonvulsant effects (Garriott *et al.*, 1968; Sofia *et al.*, 1971, 1976; Consroe and Man, 1973; Corcoran *et al.*, 1973; Karler *et al.*, 1974a,b,c,d; Turkanis *et al.*, 1974; Carlini *et al.*, 1975; Wada *et al.*, 1975; Consroe *et al.*, 1976; Karler and Turkanis, 1976a,b; Consroe and Wolkin, 1977a,b), and the consensus of these studies is that the spectrum of their effects resembles that of phenytoin (PTN) (Turkanis *et al.*, 1974; Karler and Turkanis, 1976a,b); therefore the results of the preclinical studies suggest that the cannabinoids are potentially useful in the treatment of the epilepsies responsive to PTN therapy, and, because of its relative lack of toxicity, the cannabinoid with the greatest potential as an antiepileptic is CBD (Karler *et al.*, 1973; Karler and Turkanis, 1976a,b; Consroe and Wolkin, 1977a).

What follows, then, is an account of the results of our CBD studies, beginning with some of the initial experiments and concluding with our present attempts to describe the mechanism of action of this agent. In all our investigations, we have continued to compare CBD, not only to such prototype drugs as PTN and ethosuximide (ETS), but also to Δ^9-THC, which, despite its psychoactivity, has been included for two essential reasons: First, it serves as a reference cannabinoid; and secondly, the resulting descriptive information adds to our general knowledge of the drug's CNS effects.

METHODS

PREPARATION AND ADMINISTRATION OF DRUGS

In-vivo studies

Ultrasound was used to disperse the cannabinoids and PTN in isotonic saline solution with Tween 80 (Turkanis *et al.*, 1974). PB, ETS and pentylenetetrazol (PTZ) were dissolved in an isotonic saline-Tween 80 solution; drug and vehicle preparations contained a final concentration of 3% Tween 80. For rats the injection volume, with the exception of ETS, was 0.1 ml/100 g body wt; in the ETS experiments, 0.2 ml/100 g body wt was used. For mice and frogs the injection volume was 0.1 ml/20 g body wt. Drugs were administered i.p. in mice and rats and into the ventral lymph sac of frogs.

In-vitro studies

Drugs were dispersed in Ringer solution by the use of a nonionic surfactant, Pluronic F68 (Turkanis and Karler, 1975, 1976). The final concentration of Pluronic F68 in the bathing medium was 1.6 μM.

ANTISEIZURE EVALUATION

Experimental animals

The studies were carried out on approximately 4-week-old male Charles River mice (ICR) and Sprague-Dawley rats and on both female and male *Rana pipiens*. Body wt was 15–25 g for mice, 75–95 g for rats and 20–40 g for frogs.

Anticonvulsant experiments

Anticonvulsant activity was measured against electrically caused convulsions in the following tests: maximal electroshock (MES), MES threshold (MEST), 6-Hz-electroshock threshold (6-Hz-EST) and 60-Hz-EST (Swinyard, 1969; Karler *et al.*, 1974d). Stimuli were applied by means of conventional corneal electrodes. The end-points were abolition of hind-limb extension for the MES, hind-limb extension (a maximal seizure) for the MEST and front-limb and jaw clonus (a minimal seizure) for both EST tests (Swinyard, 1969; Karler *et al.*, 1974d).

Motor-toxicity experiments

Minimal neurotoxicity was determined by a bar-walk test. If an animal was unable to walk a steel rod in 3 attempts within a 2-min period, it was considered to be toxic. The rod was 100 × 6.3 cm for rats and 49 × 1.3 cm for mice.

Experimental design and data analysis for anticonvulsant and motor-toxicity studies

Each dose-, current- and voltage-effect curve has 3–6 points; each point represents the results obtained from 12–20 mice or 8–12 rats. Individual curves were determined at each drug's peak-effect time; median-effective values and their 95% confidence limits were calculated by probit analysis (Finney, 1971).

COBALT EPILEPSY (RATS WITH CHRONICALLY IMPLANTED ELECTRODES)

Male Sprague-Dawley rats (140–170 g) were used in these studies. To mount stainless-steel screw electrodes (0–80, 1.6 mm) in the cranium, rats were anesthetized with sodium

pentobarbital (40 mg/kg, i.p.). Recording electrodes were stereotaxically positioned over both frontal and parietal cortices. All electrodes were attached to a multi-pin connector, which was used with dental acrylic to form a conventional pedestal on each rat's head.

To induce an epileptic focus, a piece of cobalt wire, 1 mm in diameter and 1.5 mm in length, was positioned on the dura at the time of electrode implantation (Dow *et al.*, 1962; Colasanti *et al.*, 1974). The epileptic focus subsequently developed within 6–12 days; focal potentials had clearly recognizable shapes and amplitudes in the electrocortiocogram (Dow *et al.*, 1973).

During an experiment, the conscious, unrestrained rat was kept in a clear acrylic cage. Electrocorticograms were recorded differentially against a grounded reference electrode by high impedance, capacitance-coupled preamplifiers and a polygraph. Although electrical activity was measured from each frontal and parietal electrode simultaneously, focal epileptic potentials were directly recorded from the primary focus, which developed at the site of cobalt implantation in the left parietal cortex. Control electrocorticograms were recorded for 1 hr; then drug was administered, after which, recordings were made for an additional 2 hr.

LIMBIC SEIZURES (RATS WITH CHRONICALLY IMPLANTED ELECTRODES)

Electrodes were implanted stereotaxically in male Sprague-Dawley rats (250–280 g) that were anesthetized with 40 mg/kg sodium pentobarbital, i.p. Stainless-steel electrodes were bilaterally and symmetrically positioned in the ventral aspect of the subicula and the dorsal hippocampi; the hippocampal and right subiculum electrodes were monopolar. A pair of electrodes in the left subiculum was used for electrical stimulation; one of these was also used for recording. In addition, recording electrodes (stainless-steel screws, 0–80, 1.6 mm) were placed on each frontal cortex. Electrodes were attached to a multi-pin connector, which was fastened to the rat's head with dental acrylic.

During each experiment, the conscious, unrestrained rat was kept in a clear acrylic cage. After-discharges (ADs) and convulsions were evoked by a constant-current stimulator, and ADs were recorded simultaneously from the 6 areas of the brain with a polygraph. Two weeks after surgery, the rats were subjected to a modified kindling procedure involving repetitive electrical stimulation, so that each animal yielded relatively reproducible ADs and seizures (Goddard *et al.*, 1969; Racine, 1972). ADs were produced in the drug studies at hourly intervals by stimulating with a 2-sec, 15-Hz train of 2-msec, bipolar, rectangular pulses. AD parameters known to reflect anticonvulsant activity were determined: the threshold, the duration and the maximum amplitude (Ajmone Marsan, 1972). Maximum change in the AD parameters during a 6-hr drug-test period were expressed as a percentage of the average response during initial vehicle control.

An AD produced by subicular stimulation was usually accompanied by a minimal motor seizure, and, when a drug reduced or abolished the AD, the convulsive behavior was generally abolished or decreased in severity. A minimum of 7 days was allowed between drug administrations to a single rat in order to decrease the possibility of drug interactions.

POSTTETANIC POTENTIATION (PTP)

The PTP studies were carried out with the isolated paravertebral ganglia X of the bullfrog, *Rana catesbeiana* (Libet *et al.*, 1968). We described the technics and experimental design of these experiments elsewhere (Turkanis and Karler, 1975, 1976).

DRUG EXTRACTION AND ANALYSIS

For Δ^9-THC, brains were extracted twice with diethyl ether (15 ml/0.5 g); for CBD, tissues were extracted with methanol. All extracts were concentrated and then subjected to thin-layer chromatography on Eastman silica gel chromatograms (without fluorescent indicator), which were developed with a solvent mixture of hexane-acetone (5 : 2). Developed chromatograms were cut into 1-cm horizontal strips that were each counted by liquid scintillation, and known standards of Δ^9-THC, the 11-hydroxy metabolite and CBD were used to identify their positions on the chromatograms. PTN was extracted, separated by high-pressure liquid chromatography and quantitated by the procedure of Soldin and Hill (1976).

RESULTS

ANTICONVULSANT ACTIVITY OF NATURALLY OCCURRING CANNABINOID DERIVATIVES IN THE MES TEST (MICE)

The results shown in Table 1 illustrate several points: First, anticonvulsant activity is widespread among various cannabinoids and their derivatives, for most of the compounds tested were anticonvulsant in the MES test. Secondly, anticonvulsant activity is clearly separable from the psychotoxicity characteristically associated with marihuana or Δ^9-THC; for example, CBD, CBN (Hollister, 1973, 1974; Perez-Reyes *et al.*, 1973) and dimethylheptylpyran (DMHP) (Lemberger *et al.*, 1974) have little or no psychic effect in humans. Indeed, relatively massive doses of intravenous CBD appear to be totally free of psychotoxicity. Thirdly, all three of the metabolites of Δ^9-THC—that is, 11-OH-Δ^9-THC, 8α- and 8β-OH-Δ^9-THC—are anticonvulsant. Furthermore, the principal metabolite of Δ^9-THC, the 11-OH derivative, is about one order of magnitude more potent than is the parent drug, a factor which immediately suggests that the principal metabolite may contribute to, or even account for, the anticonvulsant activity of Δ^9-THC.

SPECIES DIFFERENCES IN ANTICONVULSANT POTENCIES

The data in Table 2 demonstrate that there are dramatic species differences in the anticonvulsant activity of the cannabinoids: for example, Δ^9-THC is much more potent in the rat than it is in the mouse; a similar species difference may be seen in CBD, but to a lesser degree. The most striking of these differences is illustrated by the frog, which is,

TABLE 1. ANTICONVULSANT ACTIVITY OF NATURALLY OCCURRING CANNABINOIDS AND DERIVATIVES IN THE MES TEST (MICE)

	ED_{50} or maximum dose* (mg/kg)	Anticonvulsant activity
CBD	120	+
Δ^9-THC	100	+
11-OH-Δ^9-THC	14	+
8α-OH-Δ^9-THC	100*	0
8β-OH-Δ^9-THC	100*	+
Δ^9-THC acid A	200*	+
Δ^9-THC acid B	400*	+
Δ^8-THC	80	+
9-nor-Δ^8-THC	100*	+
CBN	230	+
9-nor-9α-OH-hexahydro CBN	100*	+
9-nor-9β-OH-hexahydro CBN	100*	+
DMHP	13	+
d,l-threo-DMHP	7	+
d,l-erythro-DMHP	24	+
Cannabichromene	500*	0
Olivetol	500*	0

ED_{50} values were calculated from dose-response data; the starred (*) values represent an assessment of anticonvulsant activity in the absence of ED_{50} data based on the maximum dose tested.

compared with the mouse and rat, exquisitely sensitive to the anticonvulsant activity of the cannabinoids. This sensitivity, however, does not extend to all anticonvulsant drugs, for the activity of PTN and PB is approximately the same in the frog as it is in the rat and mouse.

TABLE 2. SPECIES DIFFERENCES IN DRUG POTENCIES IN THE MES TEST

Drug	Species: Mouse	Rat	Frog
	Anticonvulsant $Dose_{50}$ (mg/kg)*		
Δ^9-THC	100	5	0.1
CBD	120	50	0.1
PTN	9	5	10
PB	12	12	8

* Values were calculated from dose-response data.

RELATIVE PROTECTIVE INDICES OF THE CANNABINOIDS

The data in Table 3 reveal that the P.I.s vary among the cannabinoids, and some of these variations have been reported in a previous publication to be significantly different (Karler

et al., 1974a,d). In the mouse, drugs such as DMHP and Δ^9-THC are more potent relative to motor toxicity than to anticonvulsant activity. CBD, on the other hand, has a P.I. greater than one, indicating that anticonvulsant activity is more prominent than motor toxicity in this cannabinoid; and the same situation obtains for the two prototype drugs, PTN and PB. Indeed, several of the cannabinoids, including CBD have P.I.s comparable to that of PB but less than that of PTN.

TABLE 3. PROTECTIVE INDICES IN MOUSE AND RAT

Drug	Protective index	
	Mouse* (TD_{50}/ED_{50})	Rat† (TD_{50}/ED_{50})
PTN	6.0	5
Δ^8-THC	1.8	
11-OH-Δ^9-THC	1.6	
PB	1.5	
CBD	1.5	60
CBN	1.0	
Δ^9-THC	0.8	2
DMHP	0.4	

* Motor toxic activity (TD_{50}) measured in a bar-walk test (Karler *et al.*, 1974); anticonvulsant activity (ED_{50}), in a MES test.

† Motor toxic activity (TD_{50}) measured in a bar-walk test; anticonvulsant activity (ED_{50}) represents the mean dose required to elevate minimal seizure threshold 30–50% in electrically induced limbic seizures.

In the rat, the P.I.s were calculated from anticonvulsant activities in a seizure-threshold test, rather than in the MES test used for the mouse data. The ED_{50}s for CBD and Δ^9-THC were about equal in this test (3 mg/kg), but there was an enormous difference in the TD_{50}s of CBD (173 mg/kg) and Δ^9-THC (6.6 mg/kg). The TD_{50}s for CBD were approximately equivalent in both the rat and the mouse; that, however, is not the case for Δ^9-THC, which is about one order of magnitude (6.6 vs. 100 mg/kg) more potent in the rat than it is in the mouse, a further illustration that species differences exist not only in the anticonvulsant activity but also in the motor toxicity of the cannabinoids. Because of the difference in CBD's and Δ^9-THC's TD_{50}s in the rat, CBD has a much greater P.I., or margin of safety, in this particular test; moreover, CBD's P.I. is also greater than that of PTN.

Figure 1 graphically depicts some pharmacodynamic relationships between anticonvulsant and motor-toxic activity among various cannabinoids. In addition to those of CBD and Δ^9-THC, the results of representative drugs are shown to demonstrate that anticonvulsant activity and motor toxicity are separable effects. In this figure the anticonvulsant selectivity of CBD can be clearly seen, whereas in Δ^9-THC the two effects are quantitatively indistinguishable. The 8α- and 8β-OH stereoisomers of Δ^9-THC, on the other hand, have opposite properties: The 8α isomer is more selective in terms of motor toxicity; the 8β, like CBD, is a more selective anticonvulsant. Similarly, the 9-nor-Δ^8-THC compound appears to be more motor toxic relative to anticonvulsant activity than is its naturally occurring

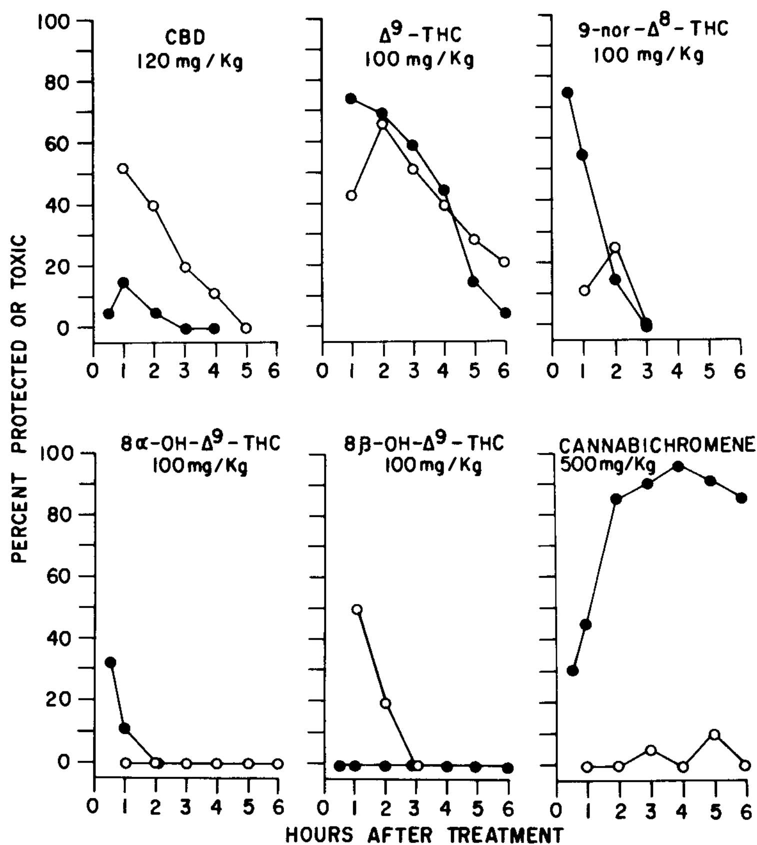


FIG. 1. Relationship between anticonvulsant activity and motor toxicity. Anticonvulsant activity (open circles) measured in an MES test; motor toxicity (closed circles) in a bar-walk test.

congener, Δ^8-THC (Table 3). Finally, cannabichromene, a major marihuana constituent, is a motor-toxic substance, yet it is structurally very different from the cannabinols. The severe motor toxicity elicited by the drug probably accounts for the apparent anticonvulsant effect exhibited by a few of the animals.

ANTICONVULSANT PROPERTIES OF THE CANNABINOIDS

Classically, anticonvulsant drugs are categorized relative to PTN or ETS, that is, PTN is the prototype antiepileptic in the treatment of grand mal-type epilepsy, but is ineffective against the absence-type seizure. The opposite, however, is true for ETS. Preclinically, these two drugs are pharmacologically distinguishable, because PTN selectively blocks seizures in the MES test, whereas anticonvulsant doses of ETS are inactive in this particular test. The activity of these drugs is again reversed in the PTZ-minimal seizure test where ETS is an effective anticonvulsant and PTN is inactive. PB, on the other hand, shares anticonvulsant properties with both PTN and ETS.

The data in Table 4 are the result of an attempt to identify similarities or dissimilarities between the cannabinoids and PTN and ETS. Because the cannabinoids are effective in the MES test, and because they are inactive in the PTZ-minimal seizure threshold test, they appear to have PTN-like, rather than ETS-like, properties in these tests.

TABLE 4. ANTICONVULSANT PROPERTIES OF THE CANNABINOIDS

Drug	Seizure test					
	Maximal		Threshold			
	MES	PTZ	MEST	60-Hz-EST	6-Hz-EST	PTZ
Δ^9-THC	+	+*	+	−	+	−
CBD	+	+	+	−	+	−
PTN	+	+	+	−	+	−
PB	+	+	+	+	+	+
ETS	−	+	§	§	§	+

* Limited efficacy, blocks tonic extension in only 50% of animals.
§ Effects have not been determined in our laboratory.

ANTICONVULSANT ACTIVITY AFTER SUBACUTE TREATMENT

The initial experiments in this study were designed to determine if tolerance develops to the anticonvulsant activity of the cannabinoids in the MES test; and, as can be seen in Table 5, subacute treatment did result in a decrease in sensitivity—or tolerance—to the cannabinoids; however, tolerance to PTN and PB also rapidly developed in this test. Subsequent to these preliminary observations, tolerance vis-à-vis several anticonvulsant effects was examined. For example, as shown in Table 5, in the MEST test, tolerance developed only to Δ^9-THC, whereas CBD, PTN and PB produced no change in sensitivity. The results in the 60-Hz-EST test were different still: tolerance failed to develop to either cannabinoid, but it did to PB; in contrast, the animals became sensitive to PTN in this test although, acutely, PTN is inactive in the 60-Hz-EST test (Table 4). Finally, in the

TABLE 5. CHANGES IN ANTICONVULSANT CANNABINOID ACTIVITY IN MICE AFTER SUBACUTE TREATMENT

Drug	Days of treatment	Sensitivity changes to drug after repeated treatment			
		MES	MEST	60-Hz-EST	6-Hz-EST
Δ^9-THC	3	−	−	0	+
CBD	4	−	0	0	+
PTN	4	−	0	+	0
PB	4	−	0	−	−

− = a decrease in sensitivity or tolerance
\+ = an increase in sensitivity
0 = no change in sensitivity

6-Hz-EST test, there was increased sensitivity to both Δ⁹-THC and CBD; on the other hand, there was no change in PTN's potency, but tolerance developed to PB. In summary, tolerance to CBD and PTN occurred in only one of the four tests, to Δ⁹-THC in two of the tests, and to PB in three of the tests. Tolerance to the anticonvulsant effects of CBD is not, therefore, a prominent feature of the drug.

ANTICONVULSANT ACTIVITY AND BRAIN-DRUG CONCENTRATIONS

The time courses of anticonvulsant activity and brain concentrations of CBD, Δ⁹-THC and PTN are shown in Fig. 2. The time courses of CBD and PTN resemble each other to

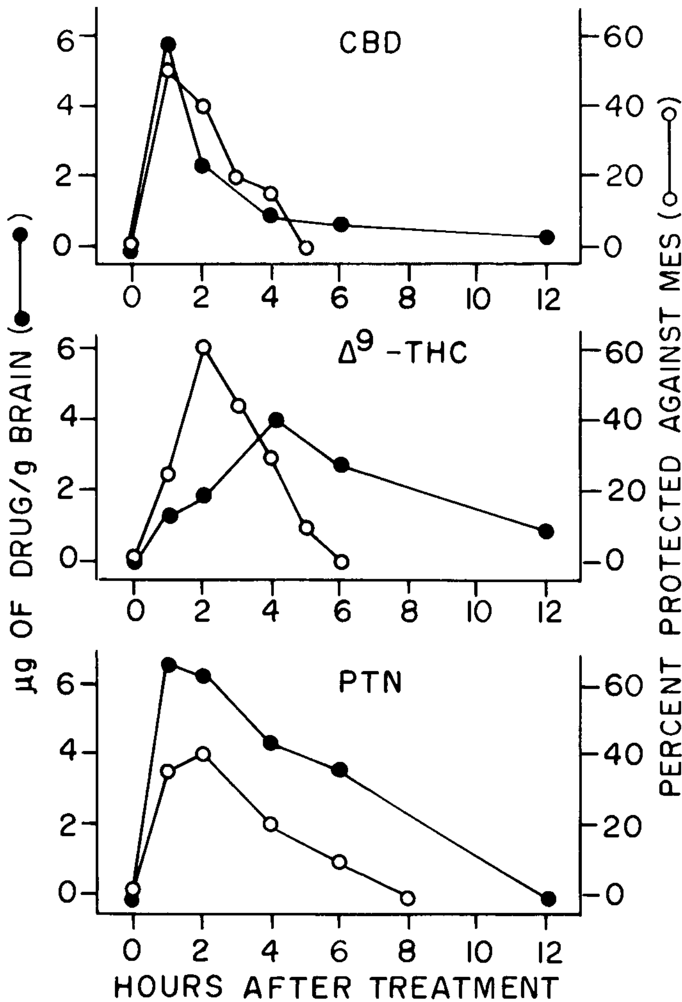


FIG. 2. Time courses of anticonvulsant activity and brain concentrations of CBD, Δ⁹-THC and PTN. Open circles represent activity in an MES test; closed circles, brain concentrations. All values were obtained after administration of ED_{50}s: CBD, 120 mg/kg; Δ⁹-THC, 100 mg/kg; PTN, 8 mg/kg.

the extent that anticonvulsant activity and brain-drug concentration are closely associated, a fact which suggests that CBD and PTN are directly responsible for their anticonvulsant effect. Such, however, is not the case for Δ^9-THC. This drug's time course in the brain does not correspond with anticonvulsant activity. The comparative pharmacokinetic results also indicate that the CBD and PTN ED_{50}s yield approximately equal brain concentrations, 6 μg/g brain, even though there is a potency difference of one order of magnitude between CBD and PTN. This discrepancy appears to be related to a difference in the fractional distribution of the dose in the brain.

The dissociation of Δ^9-THC's pharmacokinetic from its pharmacodynamic properties as seen in Fig. 2 was investigated further by determining the pharmacokinetics of the monohydroxylated metabolite fraction, as well as that of total tissue cannabinoids. In Fig. 3,

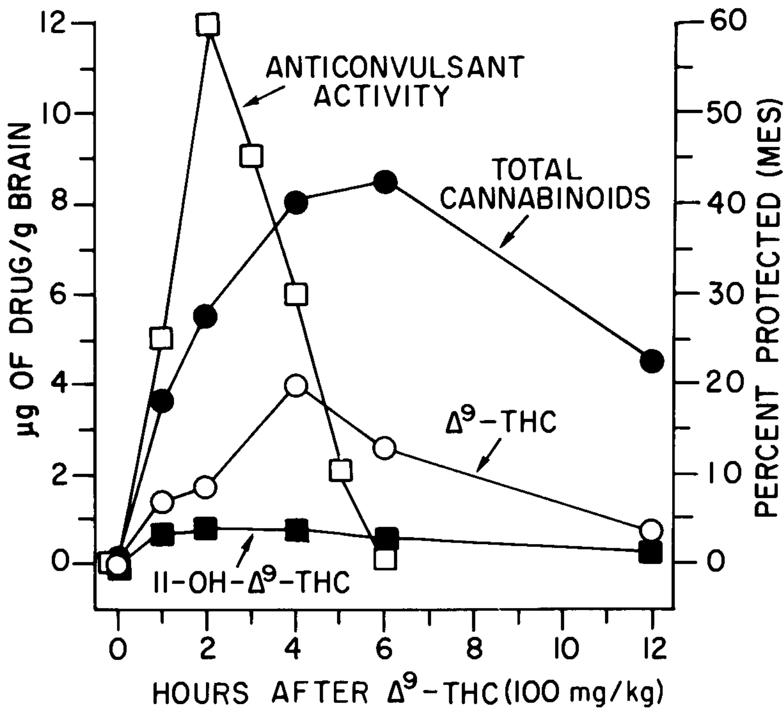


Fig. 3. Time courses of the brain concentrations of Δ^9-THC and its metabolites and anticonvulsant activity. Values obtained after administration of ^{3}H-Δ^9-THC, 100 mg/kg.

the monohydroxylated fraction consists largely of 11-OH-Δ^9-THC, the principal primary metabolite, and the total tissue cannabinoids is an analysis of total radioactivity. Like Δ^9-THC itself, neither of these correlates with anticonvulsant activity. Nevertheless, what is obvious from the data is that Δ^9-THC is very rapidly metabolized to substances other than the 11-OH product, because the other metabolites constitute more than half the total cannabinoid content in brain, even in the initial time periods. Whether a particular fraction of the "other" metabolites correlates with anticonvulsant activity remains to be determined.

CANNABINOID EFFECT ON PTP

With the exception of Δ^9-THC and PB, all the drugs tested (Table 6) depressed PTP in the isolated bullfrog ganglion. The failure of Δ^9-THC to depress PTP contrasts with the positive effect of CBD, although higher concentrations of the latter were tested. Comparable concentrations of Δ^9-THC could not be evaluated because they depressed the presynaptic potentials, which must remain relatively constant for a meaningful calculation of PTP; higher concentrations of PB were equally unuseful in determining PTP for the same reason. Despite the failure of Δ^9-THC to affect PTP, two of its metabolites, the mono- and dihydroxy derivatives, were not only effective, but were also much more potent than either CBD or PTN, the classical depressor of PTP (Turkanis and Karler, 1975, 1976).

TABLE 6. EFFECT OF DRUGS ON PTP IN ISOLATED BULLFROG GANGLIA

Drug	Effect on PTP
Δ^9-THC (25 μM)	0
11-OH-Δ^9-THC (0.1 μM)	–
8α,11-di-OH-Δ^9-THC (0.1 μM)	–
CBD (60 μM)	–
PTN (20 μM)	–
PB (30 μM)	0

– = a decrease in PTP
0 = no effect on PTP

INFLUENCE OF CANNABINOIDS ON A COBALT-INDUCED FOCAL EPILEPSY IN THE RAT

The dose-response curves demonstrating the effect of ETS, Δ^9-THC, PTN and CBD on the frequency of focal epileptic potentials are shown in Fig. 4. These interictal potentials were depressed by ETS but were unaffected by PTN and CBD, even in doses which were relatively high in terms of anticonvulsant activity in the MES test (Table 2). In contrast to the other drugs, Δ^9-THC enhanced the frequency of epileptic potentials; it must, therefore, selectively activate the focus, for it did not affect the frequency of potentials recorded at non-focal sites (Fig. 5). Associated with the enhanced focal potentials, Δ^9-THC either precipitated or exacerbated clonic seizures.

In addition, Δ^9-THC produced another excitatory effect recorded on the electrocorticogram, that is, intermittent, short, discrete, AD-like bursts. Representative samples of these are shown in Fig. 6. The bursts of activity are independent of the focus and would thus be the result of a generalized excitatory effect since they were not limited to the focal area. Indeed, they were detectable at all recording sites in the brain. Also seen in Fig. 6 is a comparable effect produced by Δ^9-THC's 11-OH metabolite, as well as by the known CNS stimulant, PTZ; however, in their excitatory properties, both these drugs differed from Δ^9-THC to the extent that neither activated the frequency of the epileptic-like focal potentials.

The comparative time courses of the AD-like bursts produced by Δ^9-THC and its

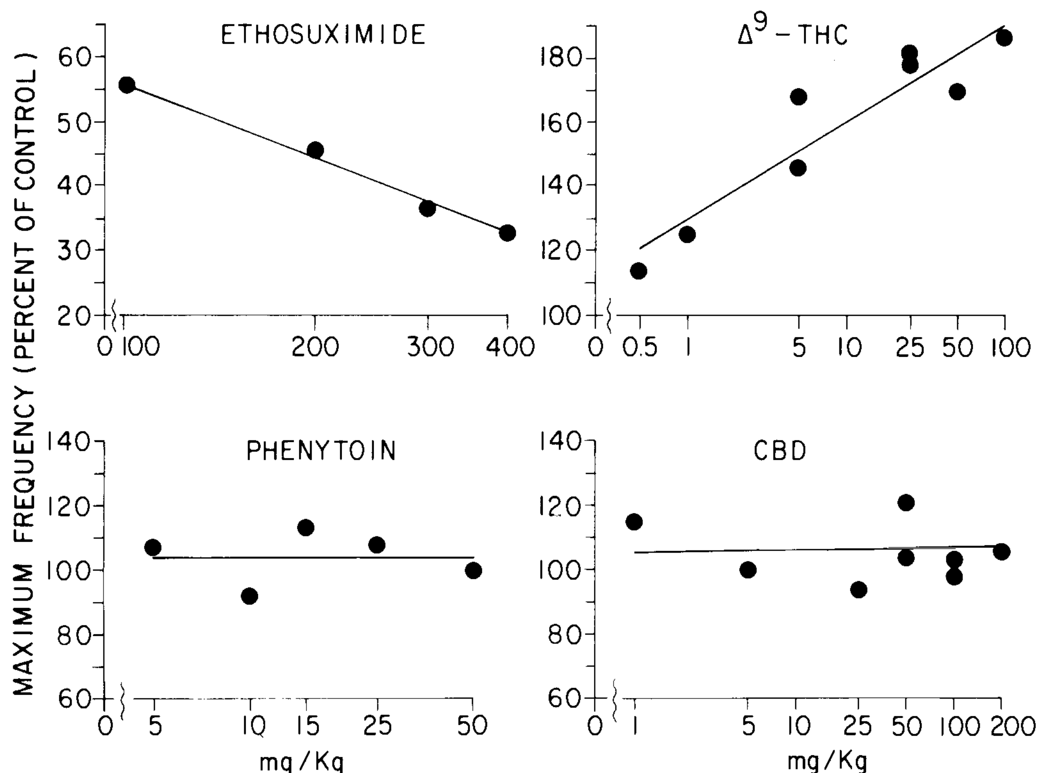


FIG. 4. Influence of ETS, PTN, Δ^9-THC and CBD on the frequency of focal epileptic potentials at a cobalt-caused focus in the left parietal cortex. Each point represents the results from one animal and is the maximum frequency expressed as a percentage of predrug control. The lines were calculated by least squares regression.

11-OH metabolite are shown in Fig. 7; PTZ response is included as a reference response from a recognized CNS stimulant. In all the experiments, the bursting effect of 11-OH-Δ^9-THC had a more rapid onset time than did that of Δ^9-THC, a phenomenon which suggests that this excitatory effect of Δ^9-THC may be caused by the metabolite formation from the parent compound.

INFLUENCE OF CANNABINOIDS ON ELECTRICALLY INDUCED LIMBIC SEIZURES

The data in Table 7 illustrate the effects of Δ^9-THC, CBD, ETS and PTN on the threshold, the duration and the amplitude of electrically induced ADs at the site of focal stimulation in the left subiculum. The dosages ranges for each drug were selected on the basis of the doses needed to produce antiseizure effects in this test. CBD affected all three of the electrical parameters: It increased threshold, it decreased AD duration and, finally, it reduced AD amplitude. In contrast, Δ^9-THC only elevated threshold and had no effect on either AD duration or amplitude. Furthermore, neither cannabinoid reduced the spread of AD from the site of focal stimulation to recording electrodes in the contralateral subicu-

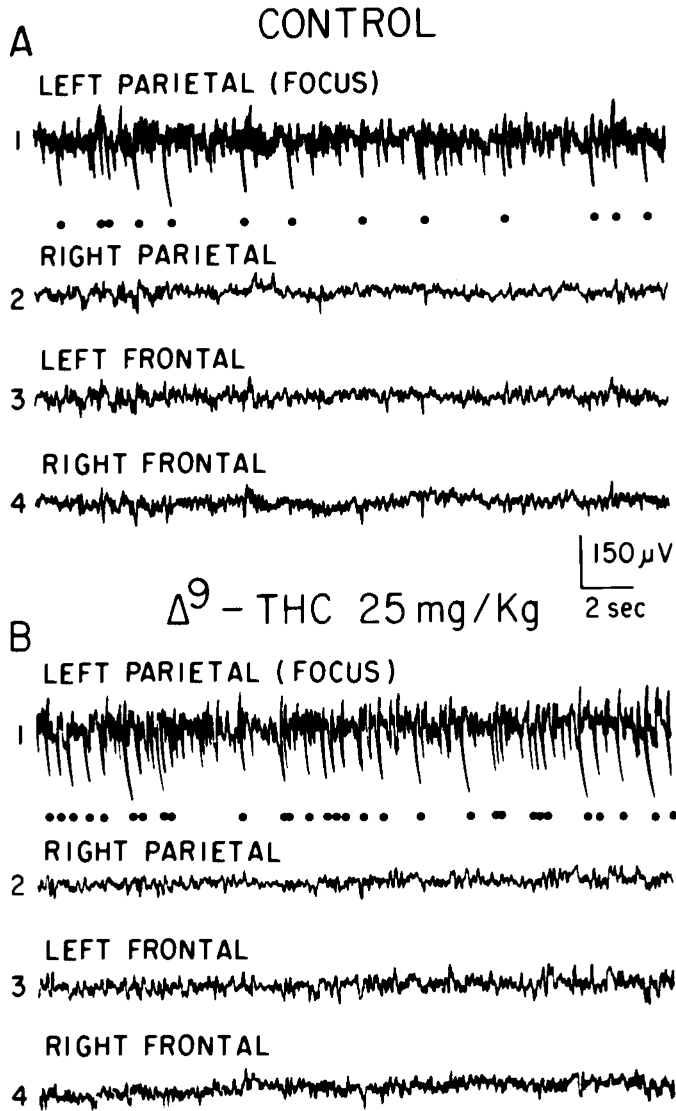


FIG. 5. Influence of Δ^9-THC on the electrocorticogram of an epileptic rat. The epileptic focus (left parietal recording) was induced by cobalt. The recordings were obtained 20 min after either vehicle or drug.

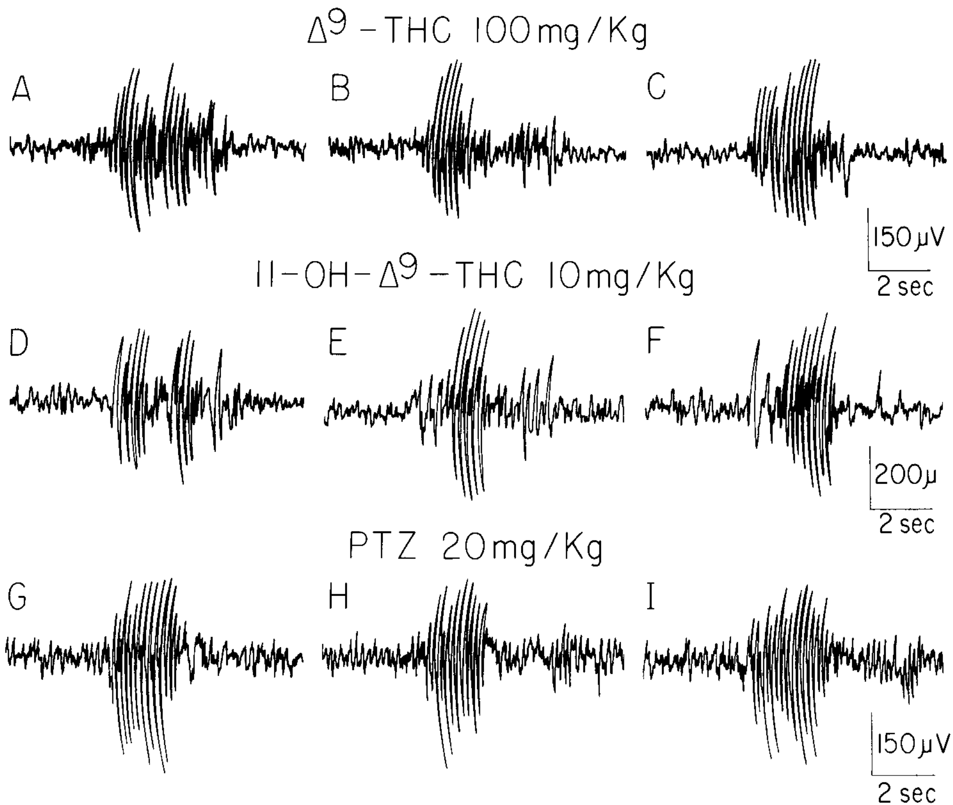


FIG. 6. AD-like bursts produced by Δ^9-THC, 11-OH-Δ^9-THC and PTZ. Responses A, B and C were recorded 81–90 min after Δ^9-THC; responses D, E and F, 15–60 min after 11-OH-Δ^9-THC; responses G, H and I, 16–20 min after PTZ.

lum, in the dorsal hippocampi or in the frontal cortices; therefore, cannabinoid anticonvulsant activity in this test appears to derive from electrophysiological effects on the generation of the AD at the site of focal stimulation. Unlike CBD, ETS lowered threshold and, despite this excitatory effect, was still anticonvulsant, a seeming paradox which can be

TABLE 7. ANTICONVULSANT EFFECTS OF Δ^9-THC AND CBD ON LEFT SUBICULUM AFTER-DISCHARGES

Treatment*	Anticonvulsant dosage range (mg/kg)	Means and ranges of maximum effects (expressed as percentage of internal vehicle control)		
		Threshold	Duration	Amplitude
Vehicle		99 (80–113)	97 (78–116)	103 (76–132)
CBD	0.3–3	150 (100–237)†	64 (32–119)†	61 (23–113)†
Δ^9-THC	0.3–3	136 (109–162)†	106 (75–129)	104 (66–138)
Ethosuximide	150–400	80 (60–104)†	49 (20–80)†	70 (38–86)†
Phenytoin	20–40	154 (75–204)†	111 (80–153)	103 (69–139)

* Nine experiments were carried out with each drug and the vehicle.

† Value is significantly different from vehicle control, as determined by the many-one rank statistic ($P < 0.05$) (Miller, 1966).

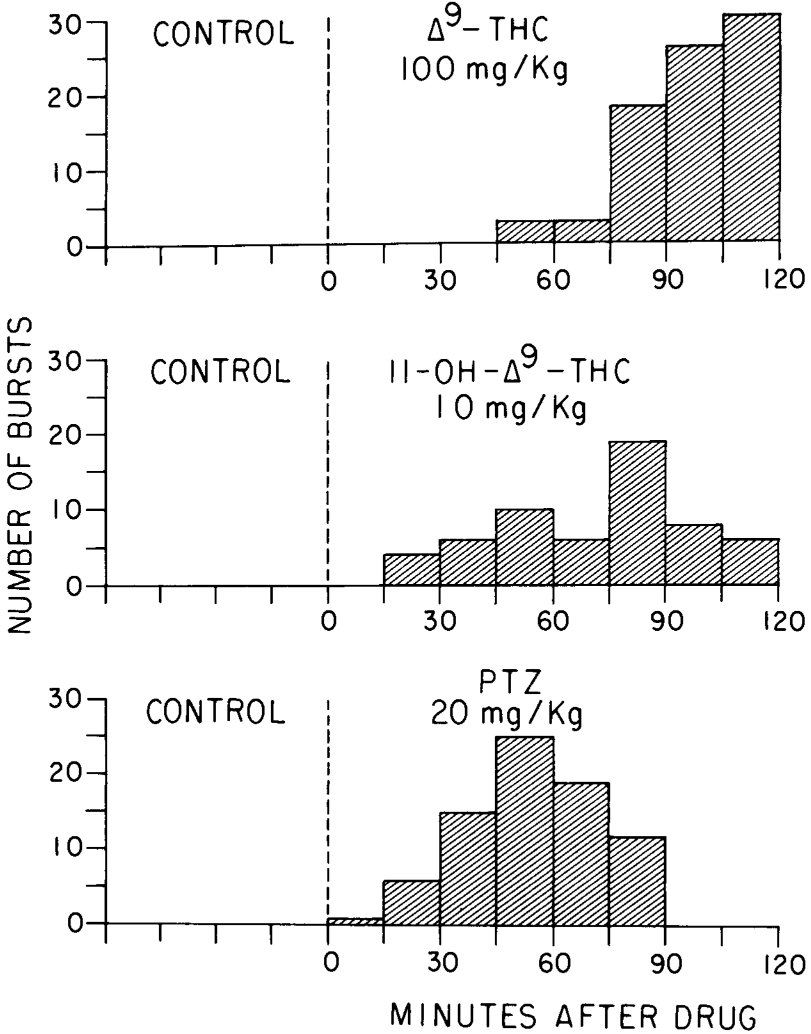


FIG. 7. Time course of AD-like bursts produced by Δ^9-THC, 11-OH-Δ^9-THC and PTZ in cobalt-epileptic rats. Vehicle was given 15 min after the beginning of the control period; drug, at the beginning of the 120-min test period; an additional dose of PTZ (20 mg/kg) was given after 45 min of the test period. The results shown were identical in eight Δ^9-THC, three 11-OH-Δ^9-THC and 5 PTZ experiments.

explained by its depressant effect on both AD duration and amplitude. On the other hand, PTN's anticonvulsant activity was mediated by an elevation in seizure threshold: There were no effects on either AD duration or amplitude at the site of stimulation, the left subiculum. A PTN anticonvulsant dose in this test was 20–40 mg/kg, which is higher than the 5–10 mg/kg effective dose in the MES test (Petty and Karler, 1965).

EXCITATORY EFFECTS OF Δ^9-THC AND PTN ON CORTICAL AFTER-DISCHARGES

The results in Fig. 8 were derived following stimulation of the left subiculum of animals treated with anticonvulsant doses of Δ^9-THC and PTN, and the subsequent recording of the cortical AD. The Δ^9-THC dose (10 mg/kg) was higher than those designated in Table 7 and was conspicuously toxic: animals vocalized, especially to touch, and they were severely ataxic, effects not noted at the lower anticonvulsant doses (Table 7). The significant increase in cortical AD duration seen in Fig. 8 appears to reflect excitatory properties, in spite of an elevation in threshold, which may account for the antiseizure effect. Once again, as in the case of Δ^9-THC, PTN produced both anticonvulsant and excitatory effects. CBD, like Δ^9-THC, was anticonvulsant in the 5–15 mg/kg dosage range and elevated seizure threshold. In contrast to PTN and Δ^9-THC, however, CBD never produced an excitatory effect on AD duration, even after massive doses (200 mg/kg).

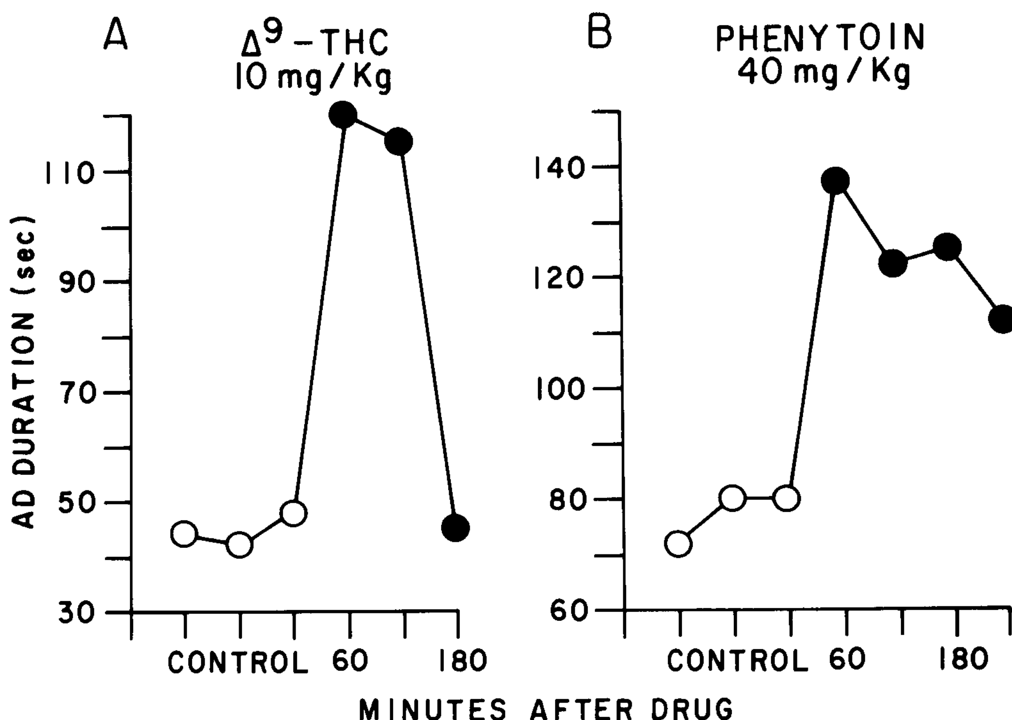


FIG. 8. Δ^9-THC- and PTN-caused prolongation of AD duration measured in the left frontal cortex. Data in A from one experiment, data in B from another.

DISCUSSION

Our tabular survey of numerous cannabinoids (Table 1) illustrates that anticonvulsant activity is a feature which is common to many of these drugs, and this finding has been strengthened by reports from other laboratories of additional anticonvulsant cannabinoids (Garriott *et al.*, 1968; Carlini *et al.*, 1975; Plotnikoff, 1976). A further and even more important conclusion to be derived from this comprehensive overview is that the distribution of anticonvulsant activity does not correlate with the psychotoxicity commonly associated with the cannabinoids (Hollister, 1973; Perez-Reyes *et al.*, 1973; Lemberger *et al.*, 1974; Lemberger and Rubin, 1976). CBD was the first compound in which a separation of psychic and anticonvulsant activity was recognized (Izquierdo and Tannhauser, 1973; Karler *et al.*, 1973), but other cannabinoids with little or no psychic activity have since been shown to be anticonvulsant: to wit, cannabinol (CBN) and DMHP in the mouse (Table 1), and 8α,11-dihydroxy-Δ^9-THC in the frog (Karler *et al.*, 1974c). The significance of such findings cannot be minimized, for any serious consideration of the cannabinoids as useful antiepileptics must be predicated on the separation of psychotoxicity from anticonvulsant activity.

Once it was established that psychotoxicity could be eliminated as an undesirable characteristic of anticonvulsant cannabinoids, then attention could be focussed on their selectivity vis-à-vis neurotoxicity. The question of selectivity is paramount in ascertaining the therapeutic potential of any anticonvulsant because, without exception, the single most serious limitation in the clinical usage of antiepileptics is their neurotoxicity. In our evaluation of anticonvulsant selectivity, neurotoxicity was assessed in terms of motor toxicity, a side effect commonly associated with all established antiepileptics (Woodbury and Fingl, 1975). The P.I. data in Table 3 represent a quantitative determination of selectivity and clearly indicate that in mice anticonvulsant activity is, at least partially, separable from motor toxicity. Of the non-psychoactive cannabinoids evaluated, CBD had the highest P.I., 1.5, which is equal to that of the clinically useful antiepileptic, PB. In a different way, the DMHP results also demonstrate that motor toxicity and anticonvulsant activity are indeed separable, for motor toxicity in this instance is more prominent than anticonvulsant activity, which is just the reverse of CBD's effects. In the rat studies, CBD again has a relatively high P.I. Taken together, these data lead us to conclude that motor toxicity is not a dominant feature of CBD's pharmacology, at least not in anticonvulsant doses.

Such demonstration of anticonvulsant selectivity relative to motor toxicity has greatly enhanced the clinical potential of the cannabinoids as antiepileptics, for although many CNS depressants can manifest anticonvulsant activity in doses that produce general depression, they are not useful in the treatment of epilepsy because of their non-selectivity of action. The recognition of cannabinoid selectivity for anticonvulsant activity, therefore, provides the experimental justification for a continued investigation of the antiseizure properties of these drugs.

While the question of selectivity is a compelling one, there are other useful and significant conclusions to be drawn from these extended cannabinoid studies. For example, the differences in potency, as evidenced by the differences in ED_{50} shown in Table 1, may be the result of variations in absorption, distribution or fate of the drugs; they may, however, also reflect differences in intrinsic activity related to the drug-receptor interaction, assuming the existence of specific receptors. In the case of the diastereomers of

DMHP and of 8-OH-Δ^9-THC, the potency differences bespeak the importance of stereospecificity and further reinforce the argument for specific receptors.

Unlike PTN and PB, cannabinoid potencies are species variable. Both of the former drugs have remarkably similar potency in the mouse, rat and frog; in fact, the species similarity extends even to humans where the dose used in the treatment of epilepsy approximates that required to block seizures in the MES test (Millichap, 1972). Species differences in sensitivity to cannabinoid anticonvulsant activity emphasize one of the crucial problems in extrapolating from animal data to humans; that is, the anticonvulsant dose in humans will have to be determined empirically. Although the reason for these species differences is not clear, the pharmacokinetic data shown in Fig. 2 offer an explanation for the intra-species potency difference between the cannabinoids and PTN. A comparison of brain-drug concentrations after administration of ED_{50}s revealed that the peak brain concentrations of all three drugs were approximately equal, despite the vast disparity between the PTN and cannabinoid doses. In the mouse, this potency difference appears to be related to differences in the fractional dose taken up by the brain, rather than to differences in the intrinsic activity of the two drugs. Differences in drug distribution to the CNS cannot, however, explain the frog's responsiveness to the cannabinoids: An ED_{50} in this species results in peak cannabinoid concentrations of only 20–30 ng/g brain (Karler *et al.*, 1974c), compared with 6 μg/g mouse brain. The comparative data suggest that the frog's nervous system is much more sensitive to the anticonvulsant action than is that of the mouse.

The clinical usefulness of CBD could obviously be affected by tolerance, which has frequently been ascribed to some cannabinoid effects. The initial evaluation of whether tolerance developed to anticonvulsant activity was limited to a study of the effect in the MES test (Karler *et al.*, 1974b; Karler and Turkanis, 1976b) where tolerance indeed developed rapidly. The significance of these results must be questioned, however, because, under identical experimental conditions, tolerance to PTN and PB was also recorded (Karler *et al.*, 1974b; Karler and Turkanis, 1976b). This comparison to the prototype drugs was singularly meaningful because, clinically, tolerance to either of these agents is not known to occur (Buchthal and Lennox-Buchthal, 1972a,b).

Further investigations of tolerance in the MES test revealed that it was not of the metabolic type (Karler and Turkanis, 1976b), a finding which implied that tolerance may not develop to all the anticonvulsant properties of CBD, and, in fact, this was discovered to be the case (Table 5). The results of four different antiseizure tests indicated that tolerance developed in only one of these tests; tolerance, therefore, cannot be considered one of CBD's prominent features.

Traditionally, antiepileptics are classified according to two types: the PTN type and the ETS type. This classification is based on clinical application: the PTN types are effective against generalized tonic-clonic (grand mal) seizures, while the ETS types are effective against absence (petit mal) seizures (Millichap, 1972). The results of the various antiseizure tests shown in Table 4 indicate that CBD is a PTN-like drug, for it is effective in the MES test and ineffective against PTZ-caused minimal seizures. ETS-like drugs have the reverse anticonvulsant effects. The hazards in such an oversimplification of anticonvulsants is evident from the results of PB in the seizure tests. This drug shares anticonvulsant properties with both PTN and ETS, but, clinically, PB more closely resembles PTN (Millichap, 1972). Despite such limitations on the preclinical models of epilepsy, new anticonvulsants are generally compared with PTN and ETS.

The mechanism of action of PTN and ETS can be viewed in terms of anticonvulsant effects on spontaneously firing epileptic foci and on the spread of epileptic activity in the CNS (Kutt, 1974). ETS is presumably anticonvulsant by virtue of its alteration of the electrophysiological character of an epileptic focus. On the other hand, PTN is considered to have no measurable effect on epileptic foci; yet, it, too, is anticonvulsant, hypothetically by means of a depression of seizure spread (Morrell *et al.*, 1959; Louis *et al.*, 1968; Kutt, 1974). In order to study drug effects on epileptic foci, the cobalt model of epilepsy was selected, and PTP and electrically induced limbic seizures were used for evaluating effects on seizure spread.

These studies of cannabinoid electrophysiological effects on foci and spread served a twofold purpose: first, the results repeatedly demonstrated that, in relatively low doses, Δ^9-THC manifests direct CNS excitatory effects; and, secondly, the results provided insights into the mechanisms of anticonvulsant action of CBD in comparison with those of such established antiepileptics as PTN and ETS. Excitatory effects of Δ^9-THC have been described previously; briefly, in contrast to CBD, Δ^9-THC exacerbated PTZ-caused minimal seizures (Karler *et al.*, 1974d), prolonged electrically evoked hippocampal ADs (Feeney *et al.*, 1973), augmented photically evoked cortical ADs (Turkanis *et al.*, 1977) and produced convulsions in epileptic beagles (Feeney *et al.*, 1976) and in a unique strain of rabbits (Martin and Consroe, 1976). The results of our cobalt-epilepsy and limbic seizure studies add other dimensions to the excitatory effects already associated with Δ^9-THC. The present data, for example, demonstrate that Δ^9-THC can activate an epileptic focus and enhance associated seizure activity, which intimates that marihuana may exacerbate certain focal epilepsies and would, therefore, be contraindicated in epileptics. Although there is, in fact, a report in the literature linking the use of marihuana to the precipitation of grand-mal seizures in one patient (Keeler and Reifler, 1967), in another patient, marihuana smoking enhanced the antiepileptic activity of PB and PTN (Consroe *et al.*, 1975). The apparent disparity of these very limited clinical reports may actually reflect the complexity of Δ^9-THC's known effects in animals where the drug not only produces numerous excitatory effects, but also effectively blocks seizures in certain types of antiseizure tests (Tables 4 and 7). Whether the use of marihuana by epileptics will affect seizure control remains to be determined; nevertheless, epileptic patients should be made aware of a possible adverse reaction.

In addition to elucidating Δ^9-THC's excitatory effects, our electrophysiological experiments showed that CBD resembled PTN in both the cobalt epilepsy model and in the PTP studies. Neither drug affected focal epileptic potentials, in contrast to ETS; and both of them depressed PTP, which is a synaptic phenomenon that has been proposed as a mechanism for the propagation of epileptic activity in the CNS (Esplin, 1957). These results indicate that CBD's anticonvulsant activity may involve a mechanism similar to that of PTN; however, the results of the limbic seizure study demonstrate that CBD shares anticonvulsant properties not only with PTN but also with ETS: That is, CBD, like ETS, reduced the AD duration and amplitude and, like PTN, raised threshold. It would seem that CBD has a unique combination of anticonvulsant properties, at least relative to limbic seizures; and, because limbic seizures are thought to be closely associated with complex partial seizures (Chronister and White, 1975; Penry, 1975), CBD may be clinically useful in this form of epilepsy.

In summary, we have found that CBD meets all the requirements as a potentially useful drug in the treatment of epilepsy: The drug is devoid of the psychotoxicity of marihuana,

and it displays a favorable degree of anticonvulsant selectivity over such neurotoxic effects as motor toxicity. Furthermore, it appears to be essentially free of the CNS excitatory effects that are characteristic of most other anticonvulsants, including PTN and ETS. The results of the initial studies of CBD's antiseizure properties likened the drug to PTN rather than to ETS; but our comparative study of the electrophysiological effects on limbic seizures produced evidence of dissimilarities in the potential mechanisms of action of all three drugs. Because of the therapeutic failures and because of the toxicity associated with the currently used antiepileptics, the search for relatively non-toxic drugs with different mechanisms of action is an obvious goal in epilepsy research. Both the lack of toxicity and the anticonvulsant properties of CBD combine to enhance its therapeutic potential as an antiepileptic.

ACKNOWLEDGEMENT

This investigation was supported by USPHS Grants DA-00346 and GM-00153.

REFERENCES

AJMONE MARSAN, C., Focal electrical stimulation. "Experimental Models of Epilepsy" (D. P. Purpura, J. K. Penry, D. Tower, D. M. Woodbury and R. Walter, eds.). Raven Press, New York, pp. 147–172, 1972.

BUCHTHAL, F. and LENNOX-BUCHTHAL, M. A., Diphenylhydantoin: Relation of anticonvulsant effect to concentration in serum. "Antiepileptic Drugs" (D. M. Woodbury, J. K. Penry and R. P. Schmidt, eds.). Raven Press, New York, pp. 193–209, 1972a.

BUCHTHAL, F. and LENNOX-BUCHTHAL, M. A., Phenobarbital: Relation of serum concentration to control of seizures. "Antiepileptic Drugs" (D. M. Woodbury, J. K. Penry and R. P. Schmidt, eds.). Raven Press, New York, pp. 335–343 (1972b).

CARLINI, E. A., MECHOULAM, R. and LANDER, N., Anticonvulsant activity of four oxygenated cannabidiol derivatives. *Res. Commun. Chem. Pathol. Pharmacol.* **12**,1–15 (1975).

CHRONISTER, R. B. and WHITE, L. E., Jr., Limbic lobe morphology. An old approach to a new system. "Advances in Neurology, Vol. II, Complex partial seizures and their treatment" (J. K. Penry and D. D. Daly, eds.). Raven Press, New York, pp. 15–25, 1975.

COLASANTI, B. K., HARTMAN, E. R. and CRAIG, C. R., Electrocorticogram and behavioral correlates during the development of chronic cobalt experimental epilepsy in the rat. *Epilepsia* **15**,361–373 (1974).

CONSROE, P., JONES, B., LAIRD, H., II and REINKING, J., Anticonvulsant effects of delta-9-tetrahydrocannabinol. "The Therapeutic Potential of Marihuana" (S. Cohen and R. C. Stillman, eds.). Plenum Publishing Co., New York, pp. 363–382, 1976.

CONSROE, P. F., WOOD, G. C. and BUCHSBAUM, H., Anticonvulsant nature of marihuana smoking. *J. Amer. Med. Assoc.* **234**,306–307 (1975).

CONSROE, P. F. and MAN, D. P., Effects of Δ^8- and Δ^9-tetrahydrocannabinol on experimentally induced seizures. *Life Sci.* **13**,429–439 (1973).

CONSROE, P. and WOLKIN, A., Cannabidiol-antiepileptic drug comparisons and interactions in experimentally induced seizures in rats. *J. Pharmacol. Exp. Ther.* **201**,26–32 (1977a).

CONSROE, P. F. and WOLKIN, A. L., Anticonvulsant interaction of cannabidiol and ethosuximide in rats. *J. Pharm. Pharmacol.* **29**,500–501 (1977b).

CORCORAN, M. E., MCCAUGHRAN, J. A., Jr. and WADA, J. A., Acute antiepileptic effects of Δ^9-tetrahydrocannabinol in rats with kindled seizures. *Exp. Neurol.* **40**,471–483 (1973).

DAVIS, J. P. and RAMSEY, H. H., Antiepileptic action of marihuana-active substances. *Fed. Proc.* **8**,284–285 (1949).

DOW, R. C., FERNANDEZ-GUARDIOLA, A. and MANNI, E., The production of cobalt experimental epilepsy in the rat. *Electroencephalogr. Clin. Neurophysiol.* **14**,399–407 (1962).

DOW, R. C., FORFAR, J. C. MCQUEEN, J. K., The effects of some anticonvulsant drugs on cobalt-induced epilepsy. *Epilepsia* **14**,203–213 (1973).

ESPLIN, D. W., Effects of diphenylhydantoin on synaptic transmission in cat spinal cord and stellate ganglion. *J. Pharmacol. Exp. Ther.* **120**,301–323 (1957).

FEENEY, D. M., SPIKER, M. and WEISS, G. K., Marihuana and epilepsy: Activation of symptoms by delta-9-THC. "The Therapeutic Potential of Marihuana" (S. Cohen and R. C. Stillman, eds.). Plenum Publishing Co., New York, pp. 343–362, 1976.

FEENEY, D. M., WAGNER, H. R., MCNAMARA, M. C. and WEISS, G., Effects of tetrahydrocannabinol and hippocampal evoked after discharges in cats. *Exp. Neurol.* **41**,357–365 (1973).

FINNEY, D. I., "Probit Analysis," third edition. Cambridge University Press, Cambridge, 1971.

GAONI, Y. and MECHOULAM, R., Isolation, structure and partial synthesis of an active constituent of hashish. *J. Am. Chem. Soc.* **86**,1646 (1964).

GARRIOTT, J. C., FORNEY, R. B., HUGHES, F. W. and RICHARDS, A. B., Pharmacologic properties of some cannabis related compounds. *Arch. Int. Pharmacodyn. Ther.* **171**,425–434 (1968).

GODDARD, G. V., MCINTYRE, D. C. and LEECH, C. K., A permanent change in brain function resulting from daily electrical stimulation. *Exp. Neurol.* **25**,295–330 (1969).

HOLLISTER, L. E., Cannabidiol and cannabinol in man. *Experientia* **29**,825–826 (1973).

HOLLISTER, L. E., Structure-activity relationships in man of cannabis constituents, and homologs and metabolites of Δ^9-tetrahydrocannabinol. *Pharmacology* **11**,3–11 (1974).

IZQUIERDO, I. and TANNHAUSER, M., The effect of cannabidiol on maximal electroshock seizures in rats. *J. Pharm. Pharmacol.* **25**,916–917 (1973).

KARLER, R., CELY, W. and TURKANIS, S. A., The anticonvulsant activity of cannabidiol and cannabinol. *Life Sci.* **13**,1527–1531 (1973).

KARLER, R., CELY, W. and TURKANIS, S. A., A study of the relative anticonvulsant and toxic activities of Δ^9-tetrahydrocannabinol and its congeners. *Res. Commun. Chem. Pathol. Pharmacol.* 7,353–358 (1974a).

KARLER, R., CELY, W. and TURKANIS, S. A., A study of the development of tolerance to an anticonvulsant effect of Δ^9-tetrahydrocannabinol and cannabidiol. *Res. Commun. Chem. Pathol. Pharmacol.* **9**,23–39 (1974b).

KARLER, R., CELY, W. and TURKANIS, S. A., Anticonvulsant activity of Δ^9-tetrahydrocannabinol and its 11-hydroxy and 8α,11-dihydroxy metabolites in the frog. *Res. Commun. Chem. Pathol. Pharmacol.* **9**,441–452 (1974c).

KARLER, R., CELY, W. and TURKANIS, S. A., Anticonvulsant properties of Δ^9-tetrahydrocannabinol and other cannabinoids. *Life Sci.* **15**,931–947 (1974d).

KARLER, R. and TURKANIS, S. A., The antiepileptic potential of the cannabinoids. "The Therapeutic Potential of Marihuana" (S. Cohen and R. C. Stillman, eds.). Plenum Publishing Co., New York, pp. 383–397, 1976a.

KARLER, R. and TURKANIS, S. A., The development of tolerance and "reverse tolerance" to the anticonvulsant activity of Δ^9-tetrahydrocannabinol and cannabidiol. "The Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, pp. 299–311, 1976b.

KEELER, M. H. and REIFLER, C. B., Grand mal convulsions subsequent to marijuana use. *Dis. Nerv. Syst.* **28**,474–475 (1967).

KUTT, H., Mechanism of action of antiepileptic drugs. "Handbook of Clinical Neurology," Volume 15: "The Epilepsies" (P. J. Vinken and G. W. Bruyn, eds.). North-Holland Publishing Co., Amsterdam, pp. 621–663, 1974.

LEMBERGER, L., MCMAHON, R., ARCHER, R., MATSUMOTO, K. and ROWE, H., Pharmacologic effects and physiologic disposition of delta^{6a,10a}-dimethyltetrahydrocannabinol (DMHP) in man. *Clin. Pharmacol. Ther.* **15**:380–386 (1974).

LEMBERGER, L. and RUBIN, A., "Physiologic Disposition of Drugs of Abuse." Spectrum Publications, Inc., New York, pp. 269–310, 1976.

LIBET, B., CHICHIBU, S. and TOSAKA, T., Slow synaptic responses and excitability in sympathetic ganglia of the bullfrog. *J. Neurophysiol.* **31**,383–395 (1968).

LOEWE, S. and GOODMAN, L. S., Anticonvulsant action of marihuana active substances. *Fed. Proc.* **6**,352 (1947).

LOUIS, S., KUTT, H. and MCDOWELL, F., Intravenous diphenylhydantoin in experimental seizures. II. Effect on penicillin-induced seizures in the cat. *Arch. Neurol.* **18**,472–477 (1968).

MARTIN, P. and CONSROE, P., Cannabinoid induced behavioral convulsions in rabbits. *Science* **194**,965–967 (1976).

MILLICHAP, J. G., Drug treatment of convulsive disorders. *N. Engl. J. Med.* **286**,464–469 (1972).

MORREL, F., BRADLEY, W. and PTASHNE, M., Effects of drugs on discharge characteristics of chronic epileptogenic lesions. *Neurology (Minneap.)*, **9**,492–498 (1959).

O'SHAUGHNESSY, W. B., On the preparation of the indian hemp or gunjah (Cannabis indica): The effects on the animal system in health, and their utility in the treatment of tetanus and other convulsive diseases. *Transactions of the Medical and Physical Society of Bombay* **8**,421–461 (1842).

PENRY, J. K., Perspectives in complex partial seizures. "Advances in Neurology," Volume 11: "Complex Partial Seizures and Their Treatment" (J. K. Penry and D. D. Daly, eds.). Raven Press, New York, pp. 1–14, 1975.

PEREZ-REYES, M., TIMMONS, M. C., DAVIS, K. H. and Wall, M. E., A comparison of the pharmacological activity in man of intravenously administered Δ^9-tetrahydrocannabinol and cannabidiol. *Experientia* **29**,1368–1369 (1973).

PETTY, W. C. and KARLER, R., The influence of aging on the activity of anticonvulsant drugs. *J. Pharmacol. Exp. Ther.* **150**,443–448 (1965).

PLOTNIKOFF, N. P., New benzopyrans: anticonvulsant activities. "The Therapeutic Potential of Marihuana" (S. Cohen and R. C. Stillman, eds.). Plenum Publishing Co., New York, pp. 475–493, 1976.

RACINE, R. J., Modification of seizure activity by electrical stimulation: II, Motor seizure. *Electroencephalogr. Clin. Neurophysiol.* **3**,246 (1971).

SOFIA, R. D., SOLOMON, T. A. and BARRY, H., III, The anticonvulsant activity of Δ^9-tetrahydrocannabinol in mice. *Pharmacologist* **13**,246 (1971).

SOFIA, R. D., SOLOMON, T. A. and BARRY, H., III, Anticonvulsant activity of Δ^9-tetrahydrocannabinol compared with three other drugs. *Eur. J. Pharmacol.* **35**,7–16 (1976).

SOLDIN, S. J. and HILL, J. G., Rapid micromethod for measuring anticonvulsant drugs in serum by high-performance liquid chromatography. *Clin. Chem.* **22**,856–859 (1976).

SWINYARD, E. A., Laboratory evaluation of anticonvulsant drugs. *Epilepsia,* **10**,231–246 (1969).

TURKANIS, S. A., CELY, W., OLSEN, D. M. and KARLER, R., Anticonvulsant properties of cannabidiol. *Res. Commun. Chem. Pathol. Pharmacol.* **8**,231–246 (1974).

TURKANIS, S. A., CHIU, P., BORYS, H. K. and KARLER, R., Influence of Δ^9-tetrahydrocannabinol and cannabidiol on photically evoked after-discharge potentials. *Psychopharmacology* **52**,207–212 (1977).

TURKANIS, S. A. and KARLER, R., Influence of anticonvulsant cannabinoids on posttetanic potentiation at isolated bullfrog ganglia. *Life Sci.* **17**,569–578 (1975).

TURKANIS, S. A. and KARLER, R., The influence of Δ^9-tetrahydrocannabinol and its 11-hydroxy metabolite on posttetanic potentiation at bullfrog sympathetic ganglia. "The Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, 331–334, 1976.

WADA, J. A., OSAWA, T. and CORCORAN, M. E., Effects of tetrahydrocannabinols on kindled amygdaloid seizures and photogenic seizures in Senegalese baboons, *Papio papio. Epilepsia* **16**,439–448 (1975).

WOODBURY, D. M. and FINGL, E., Drugs effective in the therapy of the epilepsies. "The Pharmacological Basis of Therapeutics," 5th Edition (L. S. Goodman, A. Gilman, A. G. Gilman and G. B. Koelle, eds.). Macmillan Publishing Co., New York, pp. 201–226, 1975.

MARIHUANA AND EPILEPSY: PARADOXICAL ANTICONVULSANT AND CONVULSANT EFFECTS

DENNIS M. FEENEY

Department of Psychology, University of New Mexico, Albuquerque, New Mexico 87131, U.S.A.

Summary. The effects of marihuana and cannabinoids on natural and experimental models of epilepsy are reviewed. The psychoactive constituent of cannabis, Δ^9-tetrahydrocannabinol, paradoxically exerts both a convulsant and anti-convulsant action. For example, it provokes myoclonus, psychomotor and grand mal seizures in epileptic beagles; however, at comparable doses it blocks maximal electroshock seizures in rats. In contrast, cannabidiol only exerts the anticonvulsant effects and lacks convulsant and psychotropic action. The anticonvulsant action of cannabinoids may result from decreased neural excitability and suppression of post-tetanic potentiation. The convulsant action of Δ^9-tetrahydrocannabinol may result from a production of hypersynchronous neural discharge. Given these findings, epileptics should be discouraged from using marihuana since there is some risk of provoking seizures. However, because of anticonvulsant potency and lack of convulsant or psychotropic action, cannabidiol should receive clinical trials with epileptic humans as a test of its anticonvulsant effectiveness.

THERE are two separate and important issues regarding marihuana and epilepsy: First, what are the implications of marihuana smoking for epileptic patients; and second, are there constituents of marihuana which hold promise as anticonvulsants? The data bearing on these two questions present a paradox. The conclusion from the data reviewed below indicates that the psychoactive ingredient has a convulsant action, but that cannabinoids also exert an anticonvulsant effect.

The question of marihuana use by epileptics is of immediate importance since young epileptics are not awaiting approval of cannabis from the scientific community. A survey (Feeney, 1976a) indicated a substantial incidence of marihuana use among young epileptics. The population questioned were diagnosed as epileptic and 88% were taking prescribed drugs to control their seizures. The self reports of illegal drugs used are summarized in Table 1.

Thus, of a physician's young epileptic patients approximately one of three will use marihuana. Given the data outlined below indicating an exacerbation of epileptic symptoms by Δ^9-tetrahydrocannabinol (Δ^9-THC—a psychoactive constituent of marihuana), I would suggest that an active program be undertaken to counsel young epileptics about the dangers of activating seizures by drug abuse.

The experimental and clinical literature on the effects of crude marihuana and individual

TABLE 1. PERCENT OF EPILEPTICS REPORTING DRUG ABUSE

	Under age 30	Over age 30
Marihuana	29%	1%
Amphetamine	10%	0%
LSD	4%	0%
Barbiturate	2%	0%
Cocaine	2%	0%
Heroin	0%	0%

cannabinoids on epilepsy is reviewed below and divided into four major sections, crude marihuana, Δ^8- and Δ^9-THC, cannabidiol (CBD), and other cannabinoids. This organization seemed most straightforward, although some studies which use several compounds are mentioned under several headings. The earlier review of Δ^9-THC effects on epilepsy (Consroe, Jones, Laird, and Reinking, 1976) was extremely helpful. The final section on mechanisms is a speculative attempt to integrate the growing body of data.

CRUDE MARIHUANA

The effects of crude marihuana on any symptom or measure are pharmacologically difficult to interpret since the multiple constituents interact in complex fashion. For example, CBD can potentiate some effects of Δ^9-THC and suppress others (Karniol and Carlini, 1973). However, it is crude marihuana that is popular and so it is important to determine that effects of individual constituents are seen following consumption of the plant.

There are no studies of the effects of crude marihuana on existing epileptic symptoms in man, but survey and case report data are available. In the survey described above most of the epileptic subjects reported no effect of marihuana on their symptoms. However, one subject reported that marihuana decreased the frequency of attacks, and another reported it "caused" seizures. Two published case reports parallel these individual accounts in that one describes a reduction of seizure frequency and the other a precipitation of fits. The more recent report describes a young male with poorly controlled grand mal seizures occurring once a week (Consroe, Wood, and Buchsbaum, 1975). However, when the patient combined illegal marihuana consumption with his usual dosage of phenobarbital (PB) and diphenylhydantoin (DPH) his frequency of seizures was reduced. In contrast, an earlier case report (Keeler and Reifler, 1967) describes a young male epileptic who had been seizure free for six months. During a three-week period he smoked marihuana on seven occasions and had three grand mal convulsions, although the fits did not occur during or immediately after consumption.

Questionnaires and case reports of this type must be considered with caution since neither seizures nor drug consumption were witnessed by the authors. Additionally, given the legal status of the drug there may be a tendency not to report negative effects. But these case reports follow a pattern suggested in a few animal studies with Δ^9-THC. That is, with a low pre-drug baseline seizure frequency or intensity, seizures may be activated by

Δ^9-THC; whereas against a high pre-drug baseline seizures may be attenuated. Such an effect would not be unique to marihuana and epilepsy.

In only one study has the effect of smoking crude marihuana been tested on experimental epilepsy (Labrecque, Halle, Berthiaume, Morin, and Morin, 1978). Mongrel dogs inhaled the smoke of crude marihuana cigarettes via a tracheotomy tube. Acute and chronic preparations were examined and in both, epileptiform electroencephalogram (EEG) activity was induced by intravenous administration of 750,000 units/kg of sodium penicillin G (intravenous). This dosage produces high voltage synchronous waves in the EEG with an epileptiform appearance. Convulsions, i.e., seizures with motor manifestations, are not seen in this model at doses of penicillin up to 2 million units/kg. In acutes, penicillin was administered once after a single dose of marihuana (Δ^9-THC estimated at 1–2.5 mg/kg; other cannabinoids not reported) and for chronics, after 10 weeks of daily smoking. In the acute dogs the marihuana exacerbated EEG seizure activity and involuntary jerks (myoclonus). The effects were more dramatic after chronic marihuana smoking and two of the five animals displayed grand mal convulsions. The authors suggested that the neurotoxicity of penicillin may be enhanced by marihuana opening the blood brain barrier. This hypothesis has not been confirmed (Segal, Edelstein, and Lerer, 1978). It is more likely that the penicillin seizure activity is additive with epileptiform neural activity induced by marihuana.

This synergistic hypothesis is supported by the observations of epileptic symptoms in normals after ingestion of high doses of crude marihuana. "Popcorn convulsions"—apparently involuntary vertical jumping resembling a massive myoclonus—has been observed in normal rats after inhalation of smoke of crude marihuana (Rosenkrantz and Braude, 1974). These were observed in 50% of the animals after 6–9 exposures at Δ^9-THC doses estimated at 4.2 mg/kg, but were also observed at doses as low as 0.7 mg/kg Δ^9-THC. These responses were not seen in "placebo" controls exposed to the smoke of marihuana from which the cannabinoids had been removed. Similar massive myoclonic jerks have been observed in man following a single oral dose of 10 mg of hashish (Tassinari, Ambrosetto, and Gastaut, 1976). Also in man there is an increase of the late rhythmic component of the visual evoked response (Tassanari, Ambrosetto, and Gastaut, 1973). This latter effect is relevant to the analysis of the convulsant action of cannabinoids and is discussed below under Mechanisms. The observations of cannabis-evoked myoclonus in man and animals is important since myoclonus is often associated with epilepsy and since many convulsant drugs produce spontaneous and stimulus-evoked myoclonus prior to the appearance of a seizure. These involuntary jerks may be the motor manifestations of the synchronous firing of many motor system neurons and this marihuana induced synchrony may contribute to the drug's convulsant action.

Given the above results the early observations (Lowe and Goodman, 1947) that "natural charas tetrahydrocannabinol" blocked the hindlimb tonic extensor phase of maximal electroshock seizures, as does DPH, appears paradoxical. However, this has been repeatedly confirmed using various cannabinoids; and also the provocation of seizures and myoclonus using psychoactive cannabinoids has been replicated. Apparently the convulsant and anticonvulsant action involve different mechanisms. If the convulsant action of cannabis is related to the psychoactive action, as it appears to be, then marihuana use should be seriously discouraged among epileptics. However, if the anticonvulsant action is independent of the convulsant action, these may be pharmacologically separable and the anticonvulsant mechanism could be exploited for its therapeutic potential.

Δ^8- AND Δ^9-THC

These psychoactive constituents of marihuana have been studied in detail in normal animals by a number of authors. In normal beagle dogs, clonic-tonic convulsions are seen after single, very high dosages (3,000–9,000 mg/kg) of Δ^9-THC (Thompson, Rosenkrantz, Schaeppi, and Braude, 1973). Tremors and clonic convulsions appeared in 50% of rats treated daily with Δ^9-THC at 50 mg/kg (oral) for 70 days and in 12% at 10 mg/kg (Rosenkrantz, Sprague, Fleischman, and Braude, 1975). Epileptiform polyspike EEG activity has also been noted in rabbits, rats and cats after high systemic doses of Δ^9-THC administered to acute and chronic preparations (Lipparini, Scotti de Carolis and Longo, 1969; Pirch, Cohn, Barnes, and Barratt, 1972). Additionally, epileptiform EEG activity is evoked by intracerebral implants of small amounts of Δ^8- or Δ^9-THC (20–45 μg) into rabbit hippocampus or caudate nucleus (Segal, 1974). In the monkey, intravenous doses as low as 0.05 mg/kg produce epileptiform burst activity in the EEG and at 12.8 mg/kg clonic motor spasms are seen (Martinex, Stadnicki, and Schaeppi, 1972). These suggestions of a convulsant action of Δ^9-THC have been extended by studies of some natural models of epilepsy.

In studies of photogenic seizures in the naturally epileptic baboon, Δ^9-THC produces a slight reduction of the latency to generalized body myoclonus (Wada, Osawa, and Corcoran, 1975). Variable effects of Δ^9-THC on seizures is also reported with occasional suppression or enhancement (Meldrum, Fariello, Puil, Derouanx, and Naquet, 1974). Neither of these more recent studies could replicate the earlier brief report of elevated photoseizure threshold by Δ^9-THC (Killam and Killam, 1972).

We have examined the effects of Δ^9-THC on symptoms in the naturally epileptic beagle dog (Feeney, Spiker, and Weiss, 1976; Weiss, Feeney, and Spiker, 1976). In these animals, interictal focal spiking can be observed in the amygdala suggesting a temporal lobe origin of their "psychomotor" attacks (short periods of immobility and unresponsiveness accompanied by high voltage synchronous rhythms in amygdala and frontal cortex). Grand mal attacks are infrequent. We studied these animals using cortical and depth electrodes for chronic EEG recording which were indispensable for detecting the "psychomotor" seizures. The problem of detecting these seizures is similar to that of the seizures evoked by enkephalins and morphine: the motor manifestations may be quite minor—immobility or twitching (Frenk, Urca, and Liebeskind, 1978). In the epileptic beagle Δ^9-THC evoked myoclonus after a single dose at 5 mg/kg (oral) and myoclonus was also observed after 19 daily doses of 3–5 mg/kg. This suggests a lack of tolerance to the convulsant action of Δ^9-THC. Additionally, Δ^9-THC reliably evoked multiple, brief psychomotor seizures (see Fig. 1) and occasionally grand mal attacks.

In another natural model—rats selected for a strong susceptibility to audiogenic seizures—both Δ^8- and Δ^9-THC at dosages of 1–5 mg/kg (intravenous) significantly reduced seizure severity (Consroe, Man, Chin, and Picchioni, 1973). This reflex seizure may be of different origin than the temporal lobe seizures of the epileptic beagle which may explain the different action of Δ^9-THC; or it may represent a species difference. However, it may reflect the baseline effect mentioned above. In this, as in many animal model experiments, the rats displayed a near maximal seizure response before drug treatment and so there is no opportunity to observe a convulsant action.

In this regard it is interesting that a population of rabbits has been identified which display convulsions when administered Δ^9-THC at intravenous doses as low as 0.05 mg/kg

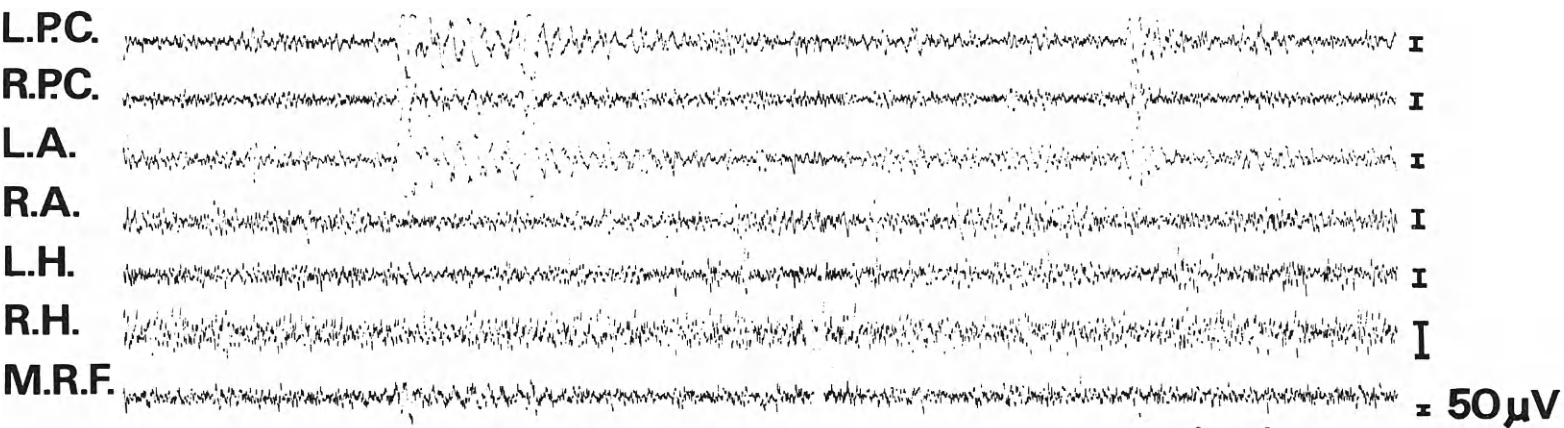


FIG. 1. "Psychomotor" seizure provoked in a naturally epileptic beagle dog by 5 mg/kg of Δ^9-THC (oral). Seizure activity is apparent in the left amygdala and left post-cruciate gyrus. The lateralization of this seizure indicates a THC activation of an existing epileptic focus. During these seizures the animal was immobile and unresponsive. Numerous psychomotor seizures and myoclonic jerks were observed between 30 and 180 minutes after drug treatment. Abbreviations: L.P.C., left post-cruciate gyrus; R.P.C., right post-cruciate gyrus; L.A., left-amygdala; R.A., right amygdala; L.H., left hippocampus; R.H., right hippocampus; M.R.F., mesencephalic reticular formation.

(Martin and Consroe, 1976). Spontaneous fits have never been observed in these animals. If these rabbits are like the epileptic beagle, then minor seizures with only an impairment of responsiveness may go undetected without surface and depth EEG recordings. Additionally, spontaneous major convulsions, lasting only a few minutes and occurring only a few times a month, may be difficult to detect without long-term 24-hour monitoring. One may reasonably speculate that these rabbits are naturally epileptic with a low spontaneous base rate and like the epileptic beagle, their fits are exacerbated by Δ^9-THC. These THC-evoked convulsions are readily blocked by standard anticonvulsants such as diazepam and DPH (Consroe, Martin, and Eisenstein, 1977). Convulsions are not seen in these rabbits after LSD, mescaline, or psilocybin and so the effect is not common to hallucinogins. Both Δ^8-THC and the psychoactive metabolite 11-OH-THC can evoke fits, but also the non-psychoactive cannabinoid, cannabinol (CBN), evokes some convulsions in these rabbits. Often the convulsant action of cannabinoids has been attributed to "stimulant" actions of this drug. If by stimulant is meant an increase in arousal and EEG desynchronization then this is not the mechanism in these rabbits since methamphetamine does not evoke seizures.

The work reviewed above with Δ^8- and Δ^9-THC has focused on apparently natural epilepsy, but more extensive work has been conducted with seizures and convulsions evoked by various experimental procedures. The best animal model of human cortical focal epilepsy is produced by injection of small amounts of alumina cream into the sensory/motor cortex of animals. Single neuron recordings in such foci indicate a very similar pattern of discharge as that reported for human cortical foci (Ward, 1969). In cats, the focus becomes increasingly active over 1–2 months, eventually producing grand mal convulsions which may be controlled by diazepam or barbiturate. This activity then gradually subsides and after six months practically no abnormal EEG activity or behavioral symptoms are seen (Gullotta, Feeney, and Gilmore, 1976). We have administered 1.5 mg/kg of Δ^9-THC (oral) to cats with foci in this quiescent stage and observed an activation of focal discharge as illustrated in Fig. 2. In this animal Δ^9-THC evoked focal activity was accompanied by localized somesthetic sensation presumably analagous to an aura. Similar results were seen in two other cats with quiescent foci. This activation of an alumina cream focus by Δ^9-THC is similar to the observation that Δ^8-THC enhances focal discharge induced by implantation of ouabain into rabbit hippocampus (Segal and Barak, 1972).

The effects of Δ^8- and Δ^9-THC on "kindled" convulsions has been extensively studied in rat, cat and baboon. This model is popular as the experimenter has direct control over the focal activity via implanted stimulating electrodes. So the threshold, duration and severity of the evoked seizure after-discharge (AD) may be measured. With repeated stimulation spaced over weeks, the threshold to AD decreases and seizures increase in severity. Thus one may also examine kindling time—the number of days of stimulation necessary to reach a generalized seizure. A drug increasing this time would provide a prophylactic effect.

In our experiments on the effects of Δ^9-THC on kindling in cats (Feeney, Wagner, McNamara, and Weiss, 1973) we noted that the drug produced a differential effect on threshold and AD duration which depended on stimulus strength. Using stable hippocampal AD we observed that Δ^9-THC (0.25–1.5 mg/kg, oral) reduced the probability of an AD indicating an increased threshold to brain stimulation. However, with stimulation well above threshold we observed a marked increase of variability of AD duration under Δ^9-THC compared to vehicle control. Under Δ^9-THC we observed the most severe as well

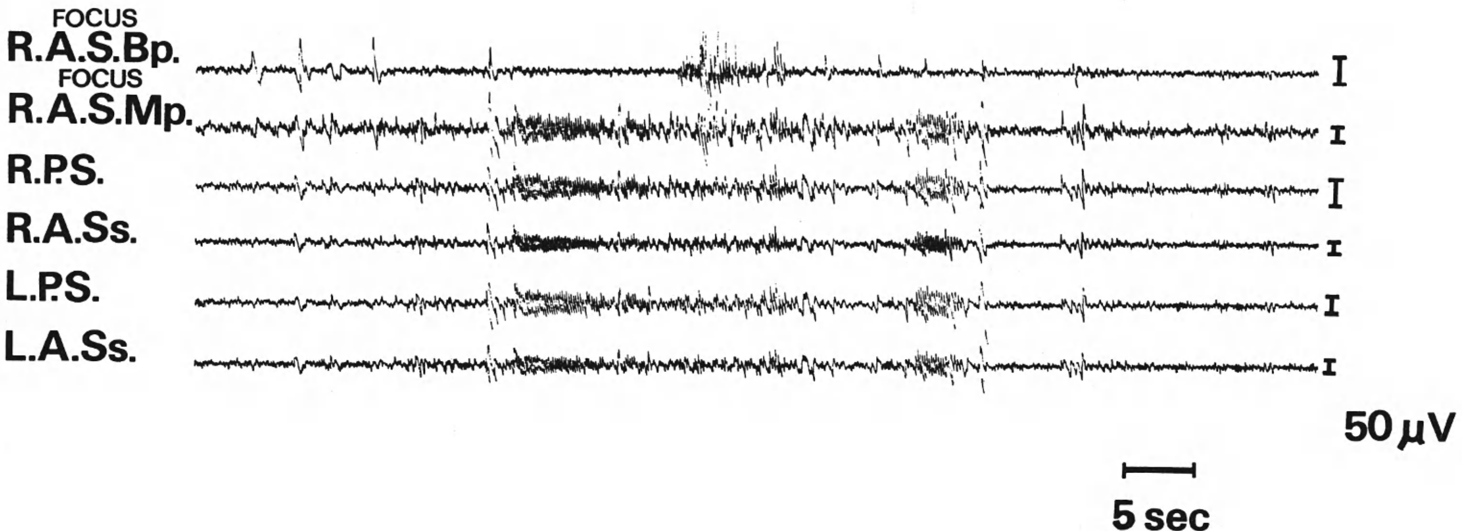


FIG. 2. Activation of quiescent alumina cream focus in cat sensory motor cortex by 1.5 mg/kg of Δ^9-THC (oral). This focus had been inactive for the previous month. Upper bipolar lead at the focus shows four large amplitude interictal spikes. The cat then scratched the left side of its face suggesting a somesthetic aura provoked by focal activity. Scratching produced artifact in the other monopolar leads. A "shower" of focal discharge then occurs, followed by small amplitude spikes and the cat scratches again. Abbreviations: R.A.S.Bp., right anterior sigmoid gyrus bipolar; R.A.S.Mp., right anterior sigmoid monopolar; R.P.S., right posterior sigmoid; R.A.Ss., right anterior suprasylvian; L.P.S., left posterior sigmoid; L.A.Ss., left anterior suprasylvian.

as mild convulsions and seizures. Once initiated, other processes may regulate the duration of a focal seizure and Δ^9-THC may differentially affect threshold and timing mechanisms.

In similar work, an effect apparently attributable to a Δ^8- and Δ^9-THC elevation of threshold has been reported (Wada, Wake, Sato, and Corcoran, 1975). They describe no effects on partially or fully developed amygdaloid kindled seizures, but a pronounced prophylactic effect if the drugs are administered early during kindling. However, in another report from this group (Corcoran, McCaughran, and Wada, 1978) they report no prophylactic effect on kindled amygdaloid convulsions in rats. In this latter study the authors note they used strong amygdaloid stimulation in contrast to the near threshold stimuli used in the previous experiment with cats. This differential Δ^9-THC effect dependent on stimulus strength is compatible with our previous observations mentioned above. These authors also noted a dramatic "rebound" effect with seizures enhanced on the drug-free test days following each high dose of Δ^9-THC (45 mg/kg intraperitoneal). Also rapid tolerance was displayed, being virtually complete after three doses as had been previously reported (Fried and McIntyre, 1973). In kindling studies on baboons, Δ^8- and Δ^9-THC reduced AD duration but presumably only stimulus intensities near threshold were employed (Wada *et al.*, 1975).

Convulsive shock in rodents has been a widely used model for examining anticonvulsant effects of Δ^8- and Δ^9-THC. Using maximal electroshock seizures (MES) several authors have reported that Δ^8- and Δ^9-THC block the tonic hindlimb extensor component (Chesher and Jackson, 1974; Consroe and Man, 1973; Fujimoto, 1972; Karler, Cely, and Turkanis, 1974a, 1974b, 1974c; McCaughran, Corcoran, and Wada, 1974). Only one study with Δ^8- and Δ^9-THC has reported no effect on MES (Dwivedi and Harbison, 1975). Δ^9-THC reportedly also elevates the threshold for 6 Hz electroshock seizures but has no effect on the 60 Hz threshold (Karler *et al.*, 1974a). In these tests in rodents the THC pattern of action is similar to that of DPH (Woodbury, 1969). However, when tested in hypothermic conditions (22°C) Δ^9-THC *lowered* the threshold for 60 Hz convulsions compared to undrugged controls (Karler *et al.*, 1974a). With as few as three doses tolerance develops to the Δ^9-THC increased MES threshold but not to the Δ^9-THC lowered 60 Hz threshold (Karler and Turkanis, 1976). This latter finding may again reflect the different mechanisms underlying the mixed anticonvulsant and convulsant actions of Δ^9-THC. Tolerance rapidly develops to the anticonvulsant effect but the convulsant mechanism is more persistent. In these convulsive shock tests with rodents, the effective dose varies greatly between species. In the rat the ED_{50} for MES with Δ^9-THC is approximately 4 mg/kg (Karler *et al.*, 1974a; Consroe and Man, 1973) although others estimate it at 58 mg/kg (McCaughran *et al.*, 1974) all using the intraperitoneal route. In mice the ED_{50} (i.p.) against MES is estimated at 100 mg/kg (Karler *et al.*, 1974a) and using the oral route it is greater than 200 mg/kg (Chesher and Jackson, 1974). A protective index (PI) has been calculated by several authors using ED_{50} for MES and a locomotor measure to estimate the toxic dose in half the animals—a TD_{50} (Karler *et al.*, 1974a; Consroe and Man, 1973). The $PI = TD_{50} \div ED_{50}$. In the rat the PI is estimated at 1.2–1.5 and in the mouse 0.8. This latter PI is quite low in comparison with the 7 for DPH or the 1–2 for trimethadione in the mouse (Swinyard, 1969).

The potential anticonvulsant effects of Δ^8- and Δ^9-THC have also been examined on both maximal and minimal seizures evoked by pentylenetetrazol (Metrazol—PTZ). With maximal PTZ seizures in the rat there may be some protection (Consroe and Man, 1973) but possibly only at very toxic doses (McCaugran *et al.*, 1974). In the mouse, Δ^9-THC

lowers threshold for minimal PTZ convulsions (Karler *et al.*, 1974a; Sofia, Solomon, and Barry, 1971) but others report protection (Dwivedi and Harbison, 1975) or in the rat, no effect (Consroe and Man, 1973). The reasons for the discrepancies in the PTZ data are not apparent.

CANNABIDIOL (CBD)

This constituent of cannabis has received considerable attention from animal research on anticonvulsant action. It lacks psychotropic effects in man (Hollister, 1973) but some hypnotic-like effects have been reported in rats (Monti, 1977). In our work with the epileptic beagle, in contrast to the convulsant action of Δ^9-THC, we did not observe an exacerbation of seizures or evocation of myoclonus with CBD at doses of 5 mg/kg (oral) (Feeney *et al.*, 1976; Weiss *et al.*, 1976). Similarly, in the rabbits which convulse to Δ^9-THC no convulsions are seen with intravenous doses of CBD as high as 20 mg/kg (Martin and Consroe, 1976). Thus CBD is neither psychoactive nor convulsant. However, there is a case report of an increase in interictal spike frequency in man following a single i.v. dose of CBD (Perez-Reyes and Wingfield, 1974).

The effects of CBD on experimental seizures have been examined by many authors using a variety of models. The seizures evoked in rabbits by Δ^9-THC can be blocked by concurrent administration of CBD (Consroe, Martin, and Eisenstein, 1977). However, the CBD blocking effect is absent if the CBD is given 30 min prior to the Δ^9-THC. This action is especially interesting since CBD, if given simultaneously with Δ^9-THC in man, blocks the usual psychological actions (Karnoil, Shirakawa, Kasinski, Pfeferman, and Carlini, 1974). On other more standard anticonvulsant tests, results indicate that CBD reaches its peak effect at 1–2 hours (Carlini, Mechoulam, and Lander, 1975; Consroe and Wolkin, 1977). In the MES test in rats, CBD blocked the hindlimb extensor phase and the ED_{50} was estimated at 12 mg/kg (oral) with a PI greater than 12 indicating a very potent anticonvulsant action (Consroe and Wolkin, 1977; Izquierdo, and Tannhauser, 1973). In the mouse no effect has been reported on MES after oral doses up to 200 mg/kg (Chesher and Jackson, 1974). However, several other studies (Carlini *et al.*, 1975; Karler *et al.*, 1973; Karler *et al.*, 1974a) have reported anticonvulsant action on this test with mice after intraperitoneal doses of 100–200 mg/kg. Using this model the PI is estimated at 1.5 which compares favorably to the value for trimethadione in mice (Karler *et al.*, 1974a).

In the audiogenic seizure test in rats, CBD blocks sound-evoked convulsions in rats rendered susceptible by barbiturate withdrawal (Carlini, Leite, Tannhauser, and Beardi, 1973). In another study of audiogenic seizures in spontaneously susceptible rats, CBD was found comparable to carbamazepine and Δ^9-THC, but more effective (lower dosage required) than DPH and trimethadione. CBD was less effective than clonazepam and Pb. In this test the ED_{50} for CBD was estimated at 17 mg/kg oral (Consroe and Wolkin, 1977).

A study comparing CBD with DPH, mysoline and Δ^9-THC indicated that CBD was the most efficacious in blocking hippocampal seizures evoked by afferent path stimulation (Izquierdo, Orzinger, and Berardi, 1975). The measure of anticonvulsant action was the increase in the number of electrical pulses to the fornix required to evoke an AD. These experiments were conducted in acute preparations and so were not kindled. An intraperitoneal dose of CBD at 3.5 mg/kg was as effective as 80 mg/kg DPH. These doses of DPH and CBD produced a similar learning impairment and so may be comparable in toxicity (Izquierdo and Nasello, 1973).

In summary, CBD lacks both psychoactive and convulsant action. Comparisons with other anticonvulsants indicate that CBD has an equivalent or more potent action at dose levels which produce little behavioral impairment. This cannabinoid appears to show considerable promise as an anticonvulsant and clinical trials should be initiated.

OTHER CANNABINOIDS

In man, the early work of Davis and Ramsey (1949) is often cited as indicating an anticonvulsant action of cannabinoids. They describe an "antiepileptic" action of 3 (1,2 dimethylheptyl) homologs of THC on five patients with mixed symptoms. The abstract notes that one of the patients "had prompt exacerbation of seizures" after administration of one of the homologs. This observation is in accord with the mixed anticonvulsant-convulsant effects of cannabis described above. Their work was prompted by the findings (Loewe and Goodman, 1947) that THC homologs 3-*n*-hexyl (parahexyl), 3-(1-methyloctyl) and 3-(1,2 dimethyl-heptyl) block the extensor MES component. The parahexyl effect has been replicated (Garriott, Forney, Hughs, and Richards, 1968).

A few other studies have examined the potential anticonvulsant actions of other cannabinoids on experimental seizure models, usually MES. Cannabinol has generally been found inactive or less effective than Δ^9-THC or CBD. Dimethylheptylpyran (DMHP) and 1-threo-DMHP and *d,l*-erythro-DMHP are effective against MES at very low dosages but are also quite toxic (Consroe and Wolkin, 1977; Karler, Cely, and Turkanis, 1974a). Work on several analogs of DMHP (SP-141, SP-143 and SP-145) have indicated a potent anticonvulsant action on MES, audiogenic and maximal PTZ seizures (Plotnikoff, 1976). Against MES, all of these compounds were more potent than DPH. Against audiogenic seizures in mice DMHP and SP-175, like DPH and Pb, did not exhibit tolerance.

In pursuing the anticonvulsant potential of CBD, four CBD derivatives have been tested against MES (Carlini *et al.*, 1975). The derivative CBD-aldehyde-diacetate is extremely toxic. Of the other compounds—9-hydroxy-CBD triacetate, 6-hydroxy-CBD-triacetate and 6-oxo-CBD diacetate—only the latter showed promise. In addition to protection against MES, which all the derivatives displayed, it produced less depression of spontaneous motor activity and so may be somewhat less toxic.

In summary, several cannabinoid derivatives and CBD show potential as anticonvulsant drugs. However, Δ^9-THC, a psychoactive constituent of marihuana, also displays a convulsant action. So, in returning to the questions posed at the beginning, we must counsel epileptics against marihuana use because there is some risk of provoking seizures by Δ^9-THC. Additionally, we must continue to explore the anticonvulsant potential of CBD and cannabinoid derivatives.

MECHANISMS

SEIZURE PROCESS

Before developing hypotheses regarding the anticonvulsant and convulsant actions of cannabinoids it may be helpful to review current conceptions of the transition from a normal brain state to a seizure. The basic process involves the progressive recruitment of

normal neurons into rhythmic and then high frequency bursting. With the overwhelming of inhibitory restraints the pauses between bursts disappear and are replaced by tonic high frequency firing and the seizure appears. The function and size of the population drawn into this activity pattern determine the clinical or behavioral characteristics of the fit. With a limited involvement of, for example, a somesthetic network, perhaps only a tactile aura would be experienced. With more extensive involvement perhaps conciousness or responsivity will be impaired and with widespread recruitment and motor system involvement a tonic-clonic convulsion appears. Petit mal attacks may show a different end point and dominant frequency with only massive rhythmic entrainment. However in all types of attacks, populations of neurons lose their normal input-output relations and instead respond to an exaggerated internal cadence. A prominant feature of epilepsy is its episodic nature, and the progression to attacks may be altered by drugs at any of a number of points: (1) firing threshold may be elevated; (2) inhibitory mechanisms may be strengthened; (3) synchronized firing may be antagonized by desynchrony; (4) post-tetanic potentiation—PTP may be suppressed (Spencer and Kandel, 1969; Woodbury, 1969).

ANTICONVULSANT MECHANISM OF CANNABINOIDS

Cannabinoids may block seizures by increasing the threshold for neuronal firing, and an increase of thresholds to brain stimulation has been demonstrated (Feeney *et al.*, 1973). Additionally, cannabinoids may block recruitment of neurons by suppressing PTP. In PTP the excitability of post-synaptic neurons is greatly facilitated after tonic firing of presynaptic afferents. Thus subsequent inputs have an exaggerated effect and could produce PTP in the next cells of a network. This may cascade, and with a loss of inhibitory restraints, culminate in a seizure. DPH suppresses PTP and may exert its anticonvulsant action in this fashion (Woodbury, 1969). In rat hippocampus, both Δ^9-THC and CBD suppress PTP (Izquierdo and Nasello, 1973; Weisz, Gunnell, and Vardaris, 1977). This occurs at intraperitoneal doses of 2–16 mg/kg which are anticonvulsant dosages on the MES test in this species.

CONVULSANT MECHANISM OF CANNABINOIDS

A prominent action of psychoactive and convulsant cannabinoids is that they induce hypersynchronous EEG activity. This activity has been described as "spike and waves" (Lipparini *et al.*, 1969), "polyspikes" (Masur and Kaazan, 1970; Stadnicki, Schaeppi, Rosenkrantz, and Braude, 1974), and "spindle-like" activity (Pirch *et al.*, 1972). The term spindle seems most accurate since this activity is very similar to the spindle waves of slow-wave-sleep. This THC evoked activity has a spindle shape with incrementing then decrementing sharp waves of 6–12 Hz. The same forebrain lesions which block spontaneous sleep spindles also block spindles induced by Δ^9-THC so they are of similar origin (Skinner, 1971). Spindling can be produced in rats by cannabis extract or by psychoactive cannabinoids and tolerance is not readily observed to this effect (Pirch *et al.*, 1972). After Δ^9-THC, spindles are observed in an awake animal (Lipparini *et al.*, 1969; Martinez *et al.*, 1972) and in the epileptic beagle are interspersed with myoclonus (see Fig. 1 in Feeney *et*

al., 1976). During sleep spindles there is rhythmic and synchronized bursting of cortical and thalamic cells. While amplified at cortex, spindles are of thalamic origin (Anderson and Andersson, 1968; Andersson and Manson, 1971) and are modulated by both brain stem and forebrain mechanisms. Reticular formation lesions enhance and basal forebrain lesions suppress spindles (Feeney, Gullotta, and Pittman, 1977). THC spindles may reflect a synchronizing influence of the basal forebrain and the continued wakefulness a dissociation of forebrain and brainstem controls. Spindle waves are conducive to seizures since their suppression by lesion (Feeney *et al.*, 1977) or stimulation (Wagner, Feeney, Gullotta, and Cote, 1975) will also suppress seizure activity. Additionally, morphine and enkephalins evoke spindle discharge prior to producing seizure activity (Frenck *et al.*, 1978).

Another manifestation of synchronous neuronal discharge is the visual evoked response-after-discharge (VER-AD). This response is apparently a sensory evoked spindle as it has a similar waveform and distribution and drugs affect both in a similar manner (Bigler, 1977). Additionally, basal forebrain lesions which block spindles also block the VER-AD (Feeney, unpublished observations). The effects of convulsant and anticonvulsant drugs on the VER-AD are well characterized. For example the convulsants bemegride, bicuculline, penicillin and PTZ all enhance VER-AD whereas the anticonvulsants diazepam, ethosuximide and trimethadione all suppress VER-AD (Bigler, 1977). The stimulants dextro- and methamphetamine also suppress the VER-AD (Bigler, 1977) and are effective anticonvulsants especially for patients whose fits occur predominantly in sleep (Livingston and Pauli, 1975). The influence of Δ^9-THC on the VER-AD has been examined in chronic rats. At 5 mg/kg, Δ^9-THC produces a convulsant-like enhancement of the VER-AD (Turkanis, Chiu, Borys, and Karler, 1977). Additionally, low doses of marihuana enhance the VER-AD in man (Tassinari *et al.*, 1973). Thus several measures suggest that Δ^9-THC has its convulsant action by producing hypersynchronous neuronal discharge. This would be an interesting subject for single unit investigation.

The mixed anticonvulsant and convulsant action of Δ^9-THC is a most unusual property. Which action would dominate may depend on the particular mechanism of seizure spread. In cases with rapidly evoked tonic discharge (perhaps like MES) utilizing PTP for recruitment of neurons, an anticonvulsant THC effect would be seen. With seizures of slower propagation, perhaps utilizing spatial summation for recruitment, a convulsant THC action may be observed because of the additive effect of drug induced hypersynchrony. In contrast to Δ^9-THC, CBD does not evoke spindles (Willinsky, Scotti de Carolis, and Longo, 1973) nor does it augment the VER-AD and in this latter respect is again similar to DPH (Turkanis *et al.*, 1977). Hypersynchrony may be an action peculiar to psychoactive cannabinoids.

Psychoactive cannabinoids possess some convulsant action which may be related to their induction of hypersynchronous thalamo-cortical discharge. Such activity of internal origin may interact with and modify sensory inputs and could be a source of the Δ^9-THC produced variability of experience which has been hypothesized as the motivation for human marihuana use (Feeney, 1976b). This neuronal activity appears in epochs and perhaps is related to the waxing and waning of psychic effects. In this regard it is noteworthy to quote an earlier review of the actions of cannabis in man: "... a good deal would be explained if part of its action ... allowed abnormal hypersynchrony and discharges to take place" (Paton and Pertwee, 1973, p. 329). The recent demonstrations of marihuana induced hypersynchronous EEG activity is very likely related to its convulsant action and perhaps may also account for some of its psychic effects.

REFERENCES

ANDERSON, P. and ANDERSSON, S., "Physiological basis of the alpha rhythm." Appleton-Century-Crofts, New York, 1968.

ANDERSON, S. A. and Manson, J. R., Rhythmic activity in the thalamus of the unanesthetized decorticate cat. *Electroencephalography and Clinical Neurophysiology*, **31**,21–34 (1971).

BIGLER, E. D., Neurophysiology, neuropharmacology and behavioral relationships of visual system evoked after discharges: A review. *Biobehavioral Reviews*, **1**,95–112 (1977).

CARLINI, E. A., LEITE, J. R., TANNHAUSER, M. and BERARDI, A. C., Cannabidiol and cannabis sativa extract protect mice and rats against convulsive agents. *Journal of Pharmacy and Pharmacology*, **25**,664–665 (1973).

CARLINI, E. A., MECHOULAM, R. and LANDER, N., Anticonvulsant activity of four oxgenated cannabidiol derivatives. *Research Communications in Chemical Pathology and Pharmacology*, **12**,1–15 (1975).

CHESHER, G. B. and JACKSON, D. M. Anticonvulsant effects of cannabinoids and cannabinoid interactions with phenytoin. *Psychopharmacologia*, **37**,255–264 (1974).

CONSROE, P., JONES, B., LAIRD, H. and REINKING, J. Anticonvulsant-convulsant effects of delta^9-tetrahydrocannabinol. In S. Cohen and R. C. Stillman (Eds.), "Therapeutic potential of marihuana." Plenum, New York, 363–382, 1976.

CONSROE, P. F. and MAN, D. P., Effects of delta^8- and delta^9-THC on experimentally induced seizures. *Life Sciences*, **13**,429–439 (1973).

CONSROE, P. F., MAN, D. P., CHIN, L. and PICCHIONI, A. L., Reduction of audiogenic seizure by delta^8- and delta^9-tetrahydrocannabinols. *Journal of Pharmacy and Pharmacology*, **25**,764–765 (1973).

CONSROE, P. F., MARTIN, P. and EISENSTEIN, D., Anticonvulsant drug antagonism of Δ^9-tetrahydrocannabinol induced seizures in rabbits. *Research Communications in Chemical Pathology and Pharmacology*, **16**,1–13 (1977).

CONSROE, P. F. and WOLKIN, A. Cannabidiol-antiepileptic drug comparisons and interactions in experimentally induced seizures in rats. *Journal of Pharmacology and Experimental Therapeutics*, **201**,26–32 (1977).

CONSROE, P. F., WOOD, G. C. and BUCHSBAUM, H. Anticonvulsant nature of marihuana smoking. *Journal of the American Medical Association*, **234**,306–307 (1975).

CORCORAN, M. E., MCCAUGHRAN, J. A. and WADA, J. A., Antiepileptic and prophylactic effects of tetrahydrocannabinols in amygdaloid kindled rats. *Epilepsia*, **19**,47–55 (1978).

DAVIS, J. P. and RAMSEY, H. H., Antiepileptic actions of marihuana-active substances. *Federation Proceedings*, **8**,284 (1949).

DWIVEDI, C. and HARBISON, R. D., Anticonvulsant activities of delta^8- and delta^9-tetrahydrocannabinol and uridine. *Toxicology and Applied Pharmacology*, **31**,452–458 (1975).

FEENEY, D. M., Marihuana use among epileptics. *Journal of American Medical Association*, **235**,1105 (1976a).

FEENEY, D. M., The marihuana window: A theory of cannabis use. *Behavioral Biology*, **18**,455–471 (1976b).

FEENEY, D. M., GULLOTTA, F. P. and PITTMAN, J. C., Slow-wave sleep and epilepsy: Rostral thalamus and basal forebrain lesions suppress spindles and seizures. *Experimental Neurology*, **56**,212–226 (1977).

FEENEY, D. M., SPIKER, M. and WEISS, G., Marihuana and epilepsy: Activation of symptoms by delta^9-THC. In S. Cohen and R. C. Stillman (Eds.), "Therapeutic potential of marihuana." Plenum, New York, 343–362, 1976.

FEENEY, D. M., WAGNER, H. R., MCNAMARA, M. C. and WEISS, G., Effects of tetrahydrocannabinol on hippocampal evoked after discharge in cats. *Experimental Neurology*, **31**,452–458 (1973).

FRENK, H., URCA, G. and LIEBESKIND, J. C., Epileptic properties of leucine and methionine-enkephalin: Comparison with morphine and reversibility by Naloxone. *Brain Research*, **147**,327–337 (1978).

FRIED, P. A. and MCINTYRE, D. C., Electrical and behavioral attenuation of the anti-convulsant properties of Δ^9-THC following chronic administrations. *Psychopharmacologia*, **31**,215–227 (1973).

FUJIMOTO, J. M., Modification of the effects of Δ^9-tetrahydrocannabinol by phenobarbital pretreatment in mice. *Toxicology and Applied Pharmacology*, **23**,623–634 (1972).

GARRIOTT, J. C., FORNEY, R. B., HUGHES, F. W. and RICHARDS, A. B., Pharmacological properties of some cannabis related compounds. *Archives Internationales de Pharmcodynamie et de therapie*, **171**,425–434 (1968).

GULLOTTA, F. P., FEENEY, D. M. and GILMORE, W., Effects of experimental epilepsy on the sexual behavior of cats. *Neuroscience Abstracts*, **2**,831 (1976).

HOLLISTER, L. E., Cannabidiol and cannabinol in man. *Experientia*, **29**,825–826 (1973).

IZQUIERDO, I. and NASELLO, A. G., Effects of cannabidiol and diphenylhydantoin on the hippocampus and on learning. *Psychopharmacologia*, **31**,167–175 (1973).

IZQUIERDO, I., ORSINGER, O. A. and BERARDI, A. C., Effect of cannabidiol and of other cannabis sativa compounds on hippocampal seizure discharges. *Psychopharmacologia*, **28**,95–102 (1973).

IZQUIERDO, I. and TANNHAUSER, M., The effect of cannabidiol on maximal electroshock seizures in rats. *Journal of Pharmacy and Pharmacology*, **25**,916–917 (1973).

KARLER, R., CELY, W. and TURKANIS, S. A., Anticonvulsant properties of delta^9-tetrahydrocannabinol and other cannabinoids. *Life Sciences*, **15**,931–947 (1974a).

KARLER, R., CELY, W. and TURKANIS, S. A., A study of the development of tolerance to an anticonvulsant effect of delta^9-tetrahydrocannabinol and cannabidiol. *Research Communications in Chemical Pathology and Pharmacology*, **9**,23–29 (1974b).

KARLER, R., CELY, W. and TURKANIS, S. A., A study of the relative anticonvulsant and toxic activities of delta^9-tetrahydrocannabinol and its congeners. *Research Communications in Chemical Pathology and Pharmacology*, **7**,353–358 (1974c).

KARLER, R. and TURKANIS, S. A., The development of tolerance and "reverse tolerance" to the anticonvulsant activity of Δ^9-tetrahydrocannabinol and cannabidiol. In M. C. Braude and S. Szara (Eds.), "The pharmacology of marihuana." Raven Press, New York, 299–311, 1976.

KARNIOL, I. B. and CARLINI, E. A., Pharmacological interaction between cannabidiol and Δ^9-tetrahydrocannabinol. *Psychopharmacologia*, **33**,53–70 (1973).

KARNIOL, G., SHIRAKAWA, I., KASINSKY, N., PFEFERMAN, A. and CARLINI, E. A., Cannabidiol interferes with the effects of Δ^9-tetrahydrocannabinol in man. *European Journal of Pharmacology*, **28**,172–177 (1974).

KEELER, M. H. and REIFLER, C. B., Grand mal convulsions subsequent to marijuana use. *Diseases of the Nervous System*, **28**,474–474 (1967).

KILLAM, K. F. and KILLAM, E. K., The action of tetrahydrocannabinol on EEG and photomyoclonic seizures in the baboon. *Fifth International Congress on Pharmacology*, 124 (1972).

LABRECQUE, G., HALLÉ, S., BERTHIAUME, A., MORIN, G. and MORIN, P. J., Potentiation of the epileptogenic effect of penicillin G by marihuana smoking. *Canadian Journal of Physiology and Pharmacology*, **56**,87–96 (1978).

LIPPARINI, F., SCOTTI DE CAROLIS, A. and LONGO, V. G., A neuropharmacological investigation of some trans-tetrahydrocannabinol derivatives. *Physiology and Behavior*, **4**,527–532 (1969).

LIVINGSTON, S. and PAULI, L. L., Dextroamphetamine for epilepsy. *Journal of American Medical Association*, **233**,278–279 (1975).

LOEWE, S. and GOODMAN, L. S., Anticonvulsant action of marihuana-active substances. *Federation Proceedings*, **6**,352 (1947).

MARTIN, P. and CONSROE, P., Cannabinoid induced behavioral convulsions in rabbits. *Science*, **194**,965–967 (1976).

MARTINEZ, J. L., STADNICKI, S. W. and SCHAEPPI, U. H., Δ^9-tetrahydrocannabinol: Effects on EEG and behavior of Rhesus monkeys. *Life Sciences*, **11**,643–651 (1972).

MASUR, J. and KHAZAN, N., Induction by cannabis sativa (marihuana) of rhythmic spike discharges overriding REM sleep electrocorticogram in the rat. *Life Sciences*, **9**(I), 1275–1280 (1970).

MCCAUGHRAN, J. A., CORCORAN, M. E. and WADA, J. A., Anticonvulsant activity of Δ^8- and Δ^9-tetrahydrocannabinol in rats. *Pharmacology, Biochemistry, and Behavior*, **2**,227–233 (1974).

MELDRUM, B. S., FARIELLO, R. G., PUIL, E. A., DEROUAUX, M. and NAQUET, R., Delta^9-tetrahydrocannabinol and epilepsy in the photosensitive baboon, *Papio papio*. *Epilepsia*, **15**,255–264 (1974).

MONTI, J., Hypnotic-like effects of cannabidiol in the rat. *Psychopharmacology*, **55**,263–265 (1977).

PATON, W. D. M. and PERTWEE, R. G., The actions of cannabis in man. In R. MECHOULAM (Ed.), "Marijuana." Academic Press, New York, 288–330, 1973.

PEREZ-REYES, M. and WINGFIELD, M., Cannabidiol and electroencephalographic epileptic activity. *Journal of American Medical Association*, **230**,1635 (1974).

PIRCH, J. H., COHN, R. A., BARNES, P. R. and BARRATT, E. S., Effects of acute and chronic administration of marijuana extract on the rat electrocorticogram. *Neuropharmacology*, **11**,231–240 (1972).

PLOTNICKOFF, N. P., New benzopyrans: Anticonvulsant activities. In S. Cohen and R. C. Stillman (Eds.), "The theraputic potential of marihuana." Plenum, New York, 1976.

ROSENKRANTZ, H. and BRAUDE, M. C., Acute, subacute and 23-day chronic marijuana inhalation toxicities in the rat. *Toxicology and Applied Pharmacology*, **28**,428–441 (1974).

ROSENKRANTZ, H., SPRAGUE, R. A., FLEISCHMAN, R. W. and BRAUDE, M. C., Oral delta^9-tetrahydrocannabinol toxicity in rats treated for periods up to six months. *Toxicology and Applied Pharmacology*, **32**,399–417 (1975).

SEGAL, M., Central implantation of cannabinoids: Induction of epileptiform discharges. *European Journal of Pharmacology*, **27**,40–45 (1974).

SEGAL, M. and BARAK, Y., Central excitatory effects of Δ^1- and Δ^6-tetrahydrocannabinol. *Brain Research*, **42**,547 (1972).

SEGAL, M., EDELSTEIN, E. L. and LERER, B., Interaction between delta^6-tetrahydrocannabinol (delta^6-THC) and lithium at the blood brain barrier in rats. *Experientia*, **15**,629 (1978).

SKINNER, J. E., Abolition of several forms of cortical synchronization during blockade of the inferior thalamic peduncle. *Electroencephalography and Clinical Neurophysiology*, **31**,211–221 (1971).

SOFIA, R. D., SOLOMON, T. A. and BARRY, H., The anticonvulsant activity of delta^1-tetrahydrocannabinol in mice. *Pharmacologist*, **13**,246 (1971).

SPENCER, W. A. and KANDEL, E. R., Synaptic inhibition in seizures. In H. H. Jasper, A. A. Ward, and A. Pope (Eds.), "Basic mechanisms of the epilepsies." Boston: Little, Brown and Co. 575–603, 1969.

STADNICKI, S. W., SCHAEPPI, U., ROSENKRANTZ, H. and BRAUDE, M. C., Delta^9-tetrahydrocannabinol: Subcortical spike bursts and motor manifestations in a Fischer rat treated orally for 109 days. *Life Sciences*, **14**,463–472 (1974).

SWINYARD, E. A., Laboratory evaluation of antiepileptic drugs. *Epilepsia*, **10**,107–119 (1969).

TASSINARI, C. A., AMBROSETTO, G. and GASTAUT, H., Effects of marijuana and Δ^9-THC at high doses in man. *Electroencephalography and Clinical Neurophysiology*, **34**,760 (1973).

TASSINARI, C. A., AMBROSETTO, G. and GASTAUT, H., The neuropsychiatric syndrome of Δ^9-THC and cannabis intoxication in naive subjects. In Braude, M. C. and Szara, S. (Eds.), "Pharmacology of marihuana" (Vol. 1). Raven Press, New York, 357–376, 1976.

THOMPSON, G. R., ROSENKRANTZ, H., SCHAEPPI, U. H., and BRAUDE, M. C., Comparisons of acute oral toxicity of cannabinoids in rats, dogs and monkeys. *Toxicology and Applied Pharmacology*, **25**,363–372 (1973).

TURKANIS, S. A., CHIU, P., BORYS, H. K. and KARLER, R., Influence of Δ^9-tetrahydrocannabinol and cannabidiol on photically evoked after-discharge potentials. *Psychopharmacologia*, **52**,207–212 (1977).

WADA, J. A., OSAWA, T. and CORCORAN, M. E., Effects of tetrahydrocannabinols on kindled amygdaloid seizures and photogenic seizures in Senegalese baboons, *Papio papio*. *Epilepsia*, **16**,439–448 (1975).

WADA, J. A., WAKE, A., SATO, M. and CORCORAN, M. E., Antiepileptic and prophylactic effects of tetrahydrocannabinols in amygdaloid kindled cats. *Epilepsia*, **16**,503–510 (1975).

WAGNER, H. R., FEENEY, D. M., GULLOTTA, F. P. and COTE, I. L., Suppression of cortical epileptiform activity by generalized and localized ECog desynchronization. *Electroencephalography and Clinical Neurophysiology*, **39**,499–506 (1975).

WARD, A. A., The epileptic neuron: Chronic foci in animals and man. In H. H. Jasper, A. A. Ward, and A. Pope (Eds.), "Basic mechanisms of the epilepsies." Little, Brown and Co., Boston, 263–288, 1969.

WEISS, G. K., Feeney, D. M. and SPIKER, M., Effect of Δ^9-THC on physical symptoms and EEG in the naturally epileptic beagle. *Federation Proceedings*, **35**,644 (1976).

WEISZ, D. J., GUNNELL, D. L. and VARDARIS, R. M., Effects of Δ^9-THC on frequency potentiation and recurrent inhibition in rat hippocampus. *Neuroscience Abstracts*, **3**,433 (1977).

WILLINSKY, M. D., SCOTTI DE CAROLIS, A. and LONGO, V. G., EEG and behavioral effects of natural, synthetic and biosynthetic cannabinoids. *Psychopharmacologia*, **31**,365–374 (1973).

WOODBURY, D. M., Mechanism of action of anticonvulsants. In H. H. Jasper, A. A. Ward and A. Pope (Eds.), "Mechanisms of the epilepsies." Little, Brown and Co., Boston, 647–681, 1969.

EFFECTS OF CANNABIS ON HUMAN EEG

P. ETEVENON

Charge de Recherches a l'INSERM. Section d'EEG Quantitative du S.H.U. de Sante Mentale et de Therapeutique du Centre Hospitalier Sainte-Anne, 1, rue Cabanis, F 75014 Paris

IN THEIR classification of psychotropic drugs, Delay and Deniker have classified cannabis and its derivatives among the "psychodysleptic" compounds (in the sub-group of "euphoriants") which deviate mental function.

Polygraphic EEG were recorded after Delta-9-tetrahydrocannabinol (5 to 30 mg) was administered orally to volunteers. A 10 mg dose produces quick shifts in vigilance levels with euphoric or dysphoric effects, arousal during the first two hours, then an increasing tendency to sleep in the last six hours.

Deniker, Boissier, Etevenon *et al.* (1974) have observed arousal EEG recordings (Fig. 1) with subsequent rapid shifts in vigilance between sedated states IA, IB, phase II of sleep and phase III of sleep (Fig. 2). Periods of rapid eye movements (Fig. 3) were associated with intense visual images similar to those in dream-like states.

The intoxication observed with cannabis has a greater sensory component than that of alcohol intoxication. The former also displays impairment of concentration and immediate memory and at times confusional states of consciousness, dysphoria and irritability, emotional lability (uncontrolled bursts of laughing or crying).

Low doses of THC induce specific psychophysiological and EEG effects which depend on the previous resting state and EEG baseline of the subjects. These observations were recently discussed by Koukkou and Lehmann (1976). Through quantitative EEG changes they were able to distinguish between body image distortions and visual hallucinations induced by THC (200 μg/kg per os). The differences included slower alpha and more theta during THC experiences, reminiscent of initial drowsiness EEG, and of EEG records from schizophrenic patients. "Subjects with a high susceptibility to THC-induced experience displayed resting spectra before and after THC with higher modal alpha frequencies (reminiscent of subjects with high neuroticism scores) than subjects with a low susceptibility."

Other EEG effects of cannabinoids have been previously reported (Low *et al.*, 1973; Rodin *et al.*, 1970; Roth *et al.*, 1973), such as the tendency to sleep-like EEG patterns, more closely related with confusional states when the doses of THC are increased (Pivik *et al.*, 1972; Tassinari, 1976). Tolerance to these effects have been observed in chronic smokers in Greece (Stefanis *et al.*, 1977).

The euphoric effect of cannabis has been correlated with concomitant tachycardia and

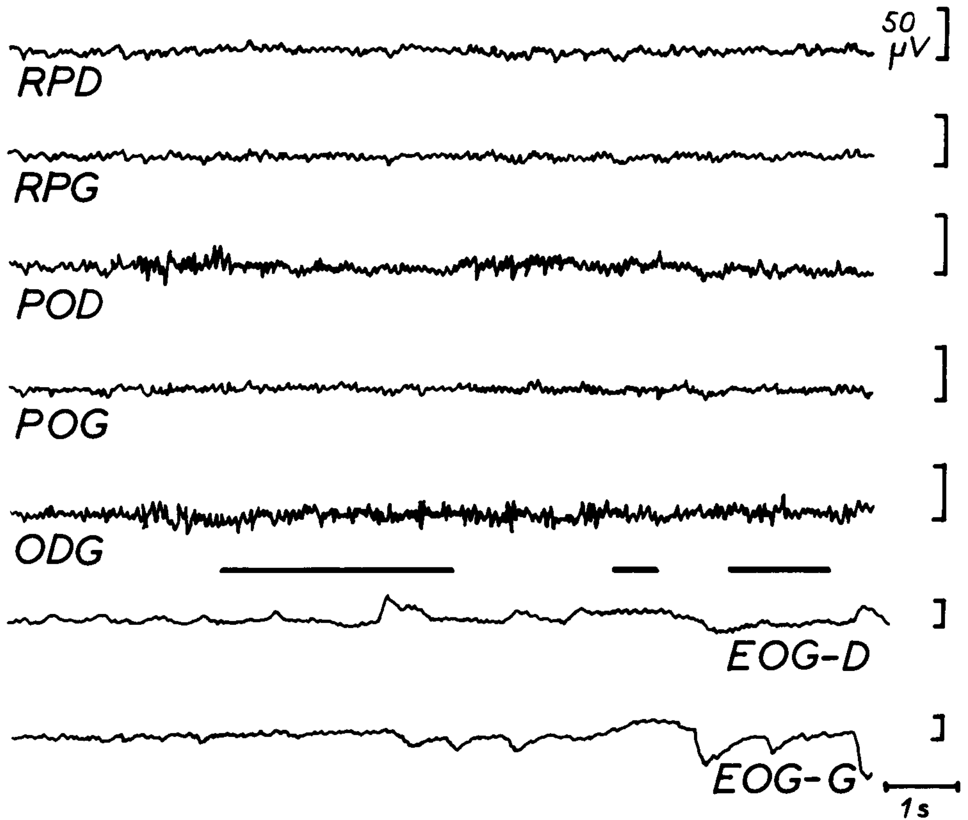


FIG. 1. Arousal reaction in a male volunteer, one hour after administration of THC (10 mg p.o. in sesame oil). EEG channels: right (RPD) and left (RPG) Rolando-parietal, right (POD) and left (POG) parieto-occipital. Two bottom channels: right (EOG-D) and left (EOG-G) horizontal electro-oculogram. From Deniker *et al.* (1974).

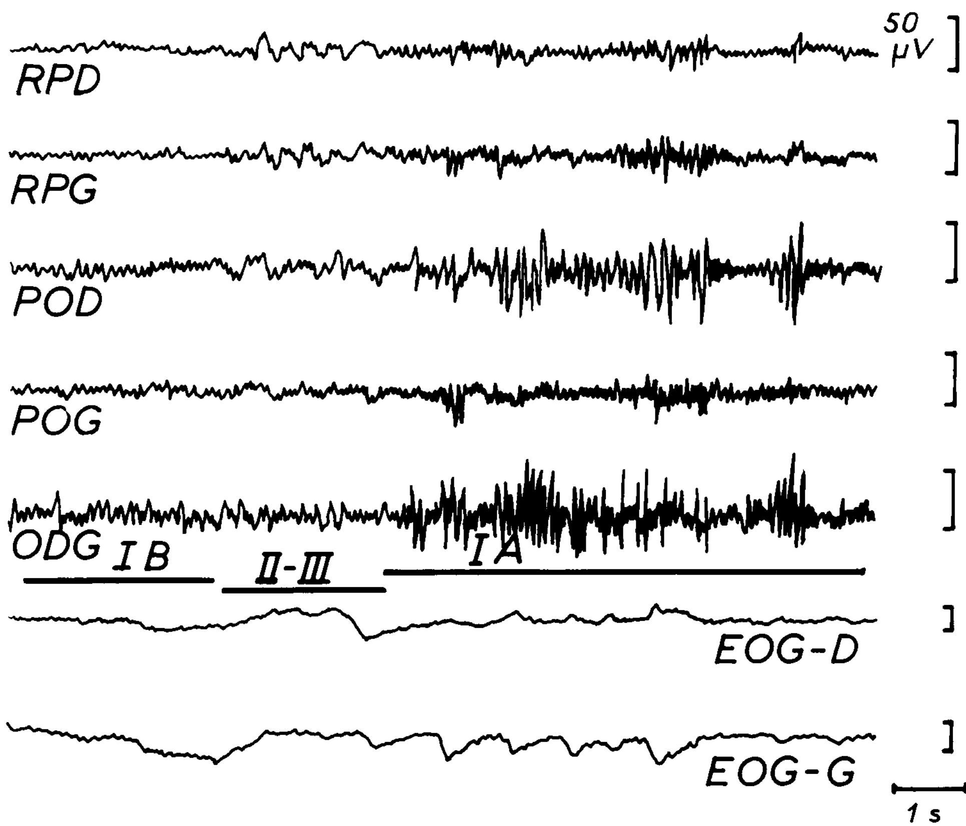


FIG. 2 Rapid shifts between IB-(II, III)-IA states of vigilance following THC administration. Same legends as in Fig. 1. From Deniker *et al.* (1974).

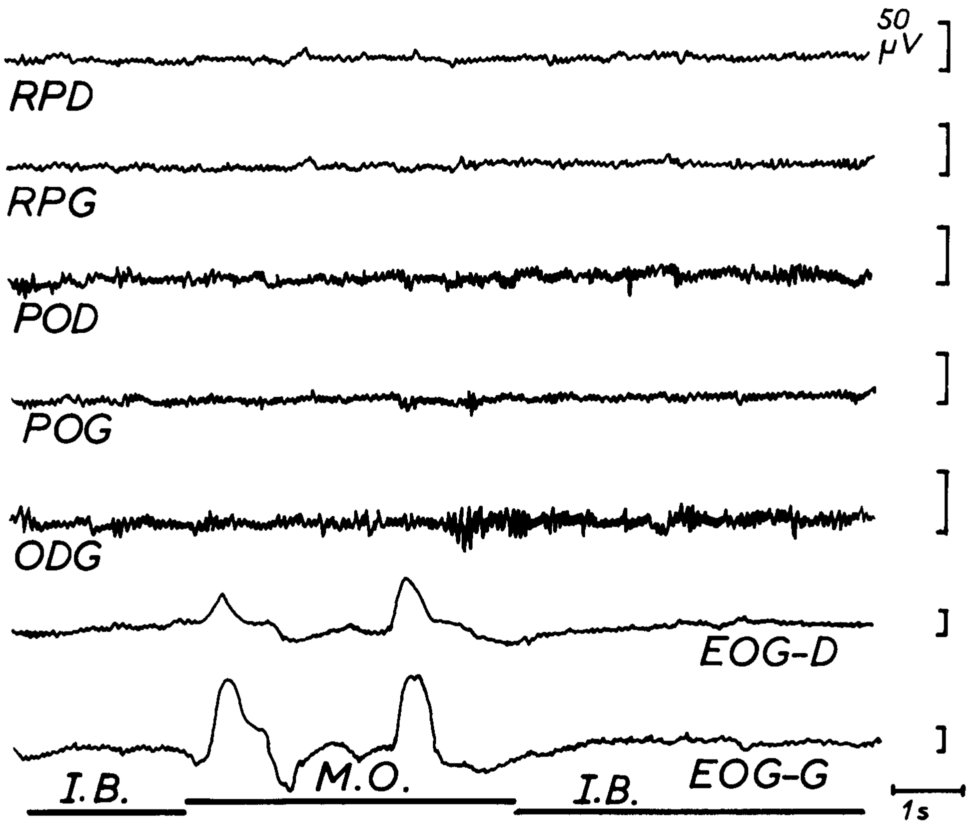


FIG. 3. Rapid eye movements period (M.O., ocular movement) between two states IB of deep sedation, associated with dreamlike visual imagery. Same legend as in Fig. 1. From Deniker *et al.* (1974).

compared with the euphoria of heroin administration (Fink *et al.*, 1972; Fink *et al.*, 1976; Volavka *et al.*, 1971).

Cannabidiol, which has some antiepileptic and sedative properties without the psychodysleptic and initial psychostimulant action of THC, has not been studied with electroencephalography. We have studied in man, with the same EEG method, an epoxide of caryophyllene, a very odorant compound present in some cannabis extracts. We observe only a short arousal reaction following the first olfactory exposure.

Additional studies of cannabinoids are indicated in the field of quantitative EEG analysis correlated with pharmacokinetics, biochemical monitoring, psychophysiological rating scales and behavioral studies.

REFERENCES

1. Deniker, P., Boissier, J. R., Etevenon, P., Ginestet, D., Peron-Magnan, P. and Verdeaux, G., Etude de pharmacologique clinique du Delta-9-têtrahydrocannabinol chez des sujets volontaires sains avec contrôle polygraphique. *Thérapie*, **29**,185–200 (1974).
2. Fink, M., Volavka, J., Dornbush, R., and Crown, P., Effects of THC-Delta-9, Marihuana and Hashish on EEG, Mood and Heart Rate of volunteers. *Psychopharmacologia*, **26**, (suppl.), 126 (1972).
3. Fink, M., Volavka, J., Panayiotopolis, C. P. and Stefanis, C., Quantitative EEG studies of marihuana, delta-nine-tetrahydrocannabinol, and hashish in man. In: M. C. Braude, S. Szara, Eds., "The Pharmacology of marihuana". Raven Press, New York, 383-391, 1976.
4. Koukkou, M. and Lehmann, D., Human EEG Spectra Before and During Cannabis Hallucinations. *Biological psychiatry*, **11** (6) 663–677 (1976).
5. Low, M. D., Klonoff, H. and Marcus, A., The neurophysiological basis of the marihuana experience. *Can. Med. Assoc. J.*, **108**,157-164 (1973).
6. Pivik, R. T., Zarcone, V., Dement, W. C. and Hollister, L. E., Delta-9-tetrahydrocannabinol and synhexyl effects on human sleep patterns. *Clin. Pharmacol. Ther.*, **13**,426-435 (1972).
7. Rodin, E. A., Domino, E. F. and Porzak, J. P., The marihuana-induced "social high". Neurological and electroencephalographic concomitants. *J. Am. Med. Assoc.*, **213**,1300–1302 (1970).
8. Roth, W. T., Galanter, M., Weingartner, H., Vaughan, T. B. and Wyatt, R. J., Marihuana and synthetic Delta-9-tetrahydrocannabinol: some effects on the auditory evoked response and background EEG in humans. *Biol. Psychiat.*, **6**,221-233 (1973).
9. Stefanis, C., Dornbush, R. and Fink, M., Hashish: studies of long-term use, Raven Press, N.Y., p. 195, 1977.
10. Tassinari, C. T., Reference Number 2762 in "Marihuana: an annotated bibliography", Macmillan, 1976.
11. Tassinari, C. A., Neuropsychotic syndrome of delta-9-tetrahydrocannabinol and cannabis intoxication in naive subjects. In "Pharmacology of marihuana" pp. 357–375, M. C. Braude and S. Szara Ed. Raven Press, New York, 1976.
12. Volavka, J., Dornbush, R., Feldstein, S., Zaks, G. C. A., Fink, M. and Freedman, A. M., Marihuana, EEG and Behavior. *Am. N.Y. Acad. Sci.*, **191**,206–215 (1971).

EFFECTS OF MARIHUANA ON PAIN AND VERBAL MEMORY: A SENSORY DECISION THEORY ANALYSIS

W. CRAWFORD CLARK, RAYMOND R. GOETZ, RICHARD H. MCCARTHY, BRENDA BEMPORAD and PHILLIP ZEIDENBERG

New York State Psychiatric Institute and Department of Psychiatry
College of Physicians and Surgeons, Columbia University
New York, N.Y. 10032, U.S.A.

Summary. In agreement with Klonoff *et al.* (1973), who used much lower doses, the results suggest that for moderately heavy smokers who averaged 8.9 cigarettes a day, only the retrieval mechanism and not the acquisition or storage components of memory were impaired. The clustering data indicate that this recall memory loss was due to impaired organizational processes. Our conclusions, based on heavy use by habitual smokers, differ from those of Abel (1971) who feels that only acquisition is adversely affected. The present findings also fail, in part, to support Dornbush and Kokkevi (1976) who argue that storage and not acquisition or retrieval processes are affected. However, their view that encoding for later retrieval is harmed by marihuana is strongly supported by our data. The conclusion that memory storage is not harmed holds only for the moderately heavy smokers. The extremely heavy smokers who averaged 13.7 cigarettes a day, showed a definite memory storage deficit, and perhaps an acquisition loss, in addition to the retrieval deficiency.

INTRODUCTION TO SENSORY DECISION THEORY

SIGNAL detection or sensory decision theory (SDT) has recently been proven to be an excellent procedure for the measurement of pain perception in the laboratory (Clark, 1978). Here we use it to test the hypothesis that marihuana possesses hypalgesic properties, and to investigate its effects on recognition memory. The SDT parameter d', or its nonparametric equivalent, $P(A)$, measures the subjects' ability to discriminate between physical stimuli of different intensities or, in the case of recognition memory, their ability to discriminate between old and new items. $P(A)$ reflects neurosensory function, and is relatively uninfluenced by psychological variables, such as expectation. Known analgesics such as morphine and nitrous oxide decrease $P(A)$, presumably because the amount of sensory information reaching higher centers is attenuated. The other SDT parameter, the report criterion, B, indexes the subject's response bias, that is, his tendency to report or to deny the presence of pain as a result of suggestion, attitude, or motivation. Recent work suggests that mood altering drugs such as diazepam may alter the criterion while leaving $P(A)$ relatively unchanged (Yang and Clark, 1978). A high criterion, few pain reports, usually indicates a stoical observer. However, when it is coupled with a decrease in $P(A)$ following

the administration of a drug, a high criterion is indicative of analgesia. In the instance of memory, a high criterion, few reports of "old, yes, I remember it" indicates a cautious observer, one who does not report something as remembered unless he is certain.

The major contribution of sensory decision theory is the dissection of the traditional sensation threshold into two components. The sensory component, $P(A)$, provides a relatively pure measure of discriminability between stimuli; $P(A)$ varies with the hit rate and false alarm rate. A hit is a positive response, e.g., "pain" or "old" following the presentation of a relatively high intensity stimulus or a to-be-remembered item, while a false alarm is a positive response to a relatively low intensity stimulus or a new item. Misses and correct rejections are the complements of hits and false alarms (Tables 1 and 2).

TABLE 1

	Response	
	"Pain" or "old"	"No pain" or "new"
Higher intensity stimulus or previously viewed item	Hit	Miss
Lower intensity stimulus or new item	False alarm	Correct rejection

Parametric sensory decision theory is based on a mathematical model which has been described in full in the instance of pain (Clark, 1974). McNicol (1972) has published an excellent introduction to the nonparametric model, a superior approach when the number of observations is small. A brief introduction is given here, using noxious thermal stimulation as the example; the transposition to a memory test is easily made. The two parameters of decision theory, discriminability and response bias, may be understood by viewing the response matrices of 4 subjects who differ in their ability to discriminate between high and low intensity stimuli, and who differ in where they locate their subjective criterion for reporting pain.

Subject S-1 in the upper left hand corner was presented 10 high and 10 low intensity stimuli in random order. When the high stimulus was present, he reported "pain" or "high" 9 times, and "hot" once. This yields a hit rate of 0.90. When the low intensity stimulus was present, S-1 reported "pain" or "high" 4 times. Thus, he obtained a false alarm rate of 0.40. S-1 is doing an excellent job of discriminating between the high and low intensity stimuli. This can be seen in the difference, 5, between the number of hits, 9, and the number of false affirmatives, 4. This difference is closely related to the decision theory measure of discriminability, $P(A)$. For a hit probability of 0.90 and a false alarm probability of 0.40, $P(A)$ equals 0.85. $P(A)$ represents areas under the receiver operating characteristic curve, which is a plot of the hit rate against the false alarm rate. $P(A)$ thus takes on values between 1.0, perfect discrimination, and 0.5, chance performance. A 2 arcsin square root $P(A)$ transformation is usually applied for statistical treatment of $P(A)$. These values run between 2.94, almost perfect discrimination, to 1.57, chance performance.

TABLE 2. RELATIONSHIPS BETWEEN HIT AND FALSE AFFIRMATIVE RATES AND VALUES OF $P(A)$, DISCRIMINABILITY AND B, REPORT CRITERION.

		Low criterion, *B* Response			High criterion, *B* Response	
		S-1			S-2	
		"Pain"	"Hot"		"Pain"	"Hot"
	10 high intensity stimuli	9	1	10 high intensity stimuli	6	4
High		(5)			(5)	
$P(A)$	10 low intensity stimuli	4	6	10 low intensity stimuli	1	9
	Total	13	7	Total	7	13
		S-3			S-4	
		"Pain"	"Hot"		"Pain"	"Hot"
	10 high intensity stimuli	7	3	10 high intensity stimuli	4	6
Low		(1)			(1)	
$P(A)$	10 low intensity stimuli	6	4	10 low intensity stimuli	3	7
	Total	13	7	Total	7	13

On the other hand, S-3 said "pain" to the high intensity stimulus 7 times, and "pain" to the low intensity stimulus 6 times, yielding a hit rate of 0.70 and a false affirmative rate of 0.60. Obviously this subject is doing a poor job of discriminating between the stimuli. He is almost as likely to report "pain" to the low intensity stimulus as to the high intensity stimulus. This poor performance is specified by the small difference, 1, between the number of hits and false alarms. The actual value of discriminability, $P(A)$, is 0.6. If the hit and false alarm rates equal each other, the subject fails to distinguish between the high and low stimulus and $P(A)$ equals 0.5. A low $P(A)$ could be caused either by a small physical difference between the high and low intensity stimuli, or by a sensory defect.

Note that S-2 has the same high ability to make discriminations as does S-1, for although the hit rate, 6, and the false affirmative rate, 1, are less, the difference between them is also 5 and $P(A)$ remains the same, 0.85. S-2 appears to be less sensitive because the hit rate is less, but he is not. Similarly, S-4 has the same poor discriminability as does S-3. Known analgesics such as morphine (Yang and Clark, 1978) and nitrous oxide (Clark and Yang, 1978) have been shown to decrease discriminability, $P(A)$, that is, to move a subject from the top row to the lower row. The discrimination measure does not depend upon the reports of "pain" and "hot." If the same hit and false alarm rates had been obtained when the subject had reported "high" and "low," the measure of discriminability would be identical. Thus, signal detection theory permits the determination of the integrity or

functional state of the pain system without any reference to the patient's subjective report of pain.

The other sensory decision theory index of subject performance is the subject's report criterion, L_x, or its nonparametric equivalent, B. The pain report criterion identifies the particular sensory experience along the sensory continuum above which the subject reports "painful." The criterion locus reflects the subject's attitude toward pain or the meaning which he attaches to the word, pain; it is influenced by non-sensory, that is, psychological, or attitudinal variables.

The total number of pain responses (see Table 2), that is, the sum of the hits and false alarms indicates the subject's response bias or criterion locus. S-1 said, "pain" 9 times when the high intensity stimulus was present and 4 times when the low intensity stimulus was present. Thus, hits plus false alarms yield a total of 13 pain responses. Such a subject has set a low or lax response criterion. The pain responses just pour out; he is willing to say "pain" even when he is uncertain. Subjects S-1 and S-3 with low pain criteria are "kvetches." Note that although S-1 and S-3 differ enormously in their ability to discriminate the stimuli, that is, $P(A)$ differs, they both share a similar low criterion. Discriminability and criterion or report bias are independent of each other. This independence is fundamental to decision theory and is its most important contribution to psychophysical measurement. In contrast to S-1 and S-3, S-2 has said "pain" only 6 times when the high intensity stimulus was present, and but once when the low intensity stimulus was present, for a total of 7. Such a subject has set a high or strict criterion; he says "pain" only when he is certain that the stimulus really hurt. S-4, the poor discriminator, also has emitted but 7 pain responses; subjects S-2 and S-4 with high pain criteria are both stoics.

The criterion is set by the subject, but the experimenter should be able to manipulate its location by suggestion. Clark (1969) convinced a group of subjects that they had received a potent analgesic: the number of pain reports sharply decreased, and the pain threshold increased, as it would if an analgesic had actually been administered. A sensory decision theory analysis, however, revealed that discriminability, d', had not been altered. The sole effect of the placebo was to raise the pain report criterion in accordance with the "social demand characteristics" of the experimental situation. Thus, in Table 2, the placebo failed to move the subjects from the top to the bottom row; it only shifted them from column 1 to column 2; they became more stoical.

INTRODUCTION TO PAIN STUDIES

Folklore surrounding the use of various preparations of *cannabis sativa* suggests that they possess analgesic properties. Some studies with animals support this belief. However, the dose levels used tend to be high, and it is difficult in animals to distinguish drug effects on pain sensation from those which merely influence motor responses. A more direct and valid approach is to study the effect of drugs on the verbal response to noxious stimulation in human volunteers. Very few such studies have been undertaken and these have yielded conflicting results.

A double blind study by Milstein, MacCannel, Karr and Clark (1975) showed a significant increase in pain tolerance among those who had smoked marihuana. Noyes, Brunk, Baram and Canter (1975) studied cancer patients who were given delta-9 tetrahydrocannabinol (THC) in 5, 10, 15 or 20 mg doses or placebo. Pain reduction was greater at

all THC level conditions than placebo, and was significant at the 15 and 20 mg levels. Wilson and May (1975) postulated that the analgesic activity of marihuana resides primarily in the 11-hydroxy metabolites. This was based on their observation that 9-nor derivatives (which cannot be transformed into 11-hydroxy metabolites) lacked significant analgesic activity. The difficulty with these studies is that the pain threshold is influenced by expectation. It has been demonstrated that subjects who believe that they have received an analgesic will show a raised pain threshold (Clark, 1969). The "double blind" control does not solve this problem, since subjects are able to distinguish between the placebo and the drug condition on the basis of subjective effects such as euphoria, clouded consciousness, etc.

Other investigators have failed to find THC-induced analgesia. For example, Harris (1976) was not able to confirm the analgesic effect of THC using standard analgesic test procedures. Hill, Schwin, Goodwin, and Powell (1974) studied the pain thresholds to cutaneous electrical stimulation in male volunteers by the method of limits. The dose was approximately 12 mg of THC. In contrast to those who reported analgesia or no effect, they found that the marihuana actually decreased the pain threshold, that is, made the subject more sensitive to painful stimulation. This result is consistent with reports of heightened sensitivity or "perceptual sharpening" produced in other sensory modalities by marihuana. Hill, *et al.* concluded that marihuana is unlikely to prove to be useful as an analgesic.

Their study although superior to most may be criticized on two grounds: first, the dose of THC may not have been high enough; and second, the psychophysical procedure used—like all such traditional procedures—yields a pain threshold which is a mixture of sensory and psychological or attitudinal variables. Thus, as Hill, *et al.* point out, the decrease in threshold which they found may have been caused, not by drug induced physiological changes, but by the subjects' expectation that pain sensitivity would be enhanced by marihuana.

According to sensory decision theory, if marihuana is an analgesic it should decrease discriminability, $P(A)$, and raise the pain report criterion, B. On the other hand, if it enhances pain perception the opposite effects may be expected.

INTRODUCTION TO MEMORY STUDIES

There is considerable evidence that human memory is adversely affected by smoking marihuana. However, there are a number of aspects of memory: acquisition (which involves attention and short-term memory), storage and retrieval. The acquisition rather than the retrieval phase is regarded as being most susceptible to marihuana effects in acute studies where words are to be recalled and recognized (Abel, 1971), but not in chronic studies (Rossi, *et al.*, 1977) where the subject has the yet simpler task of holding a digit in memory for less than 10 sec. The study by Abel is of interest because it represents the first application of sensory decision theory methodology to the effects of a drug on memory. He found that marihuana did not affect delayed free recall memory but did impair recognition memory (lower d'); it also increased the report of "old" (lower criterion, B). Abel concluded that marihuana does not interfere with the retrieval of information already stored in memory (the subjects learned the material before smoking the marihuana) and, hence, that the loss probably occurs in the acquisition stage.

In contrast, Dornbush and Kokkevi (1976) note that immediate recall is nearly perfect when there is no interpolated task, as with the Rossi, *et al.* study. They conclude from this fact that acquisition and retrieval processes are not affected by marihuana. However, when there is a delay between presentation and recall a memory loss occurs suggesting that storage and encoding for later retrieval are harmed by marihuana. Klonoff, *et al.* (1973) using an acute dose found impairment in complex tasks such as concept formation and immediate recall of meaningful material, but not in visual picture recognition or memory for musical notes. The loss in recall memory in combination with no loss in recognition memory suggests that retrieval rather than storage processes had been interfered with. The efficiency of storage and retrieval on a free recall task depends upon how well the subject has cognitively organized the to-be-remembered material. For example, Rosen and Lee (1976) demonstrated that alcohol interfered with the semantic (taxonomic) organization of the to-be-learned material which in turn disrupted storage and retrieval processes.

There apparently has been no attempt to partial out drug effects on the various components of memory in a group of heavy marihuana smokers. In the present study the effect of marihuana on attention and short term recognition memory was investigated by the lag-interval technique. This procedure is amenable to sensory decision theory analysis. Details concerning the application of sensory decision theory to recognition memory are described by Gordon and Clark (1974a; 1974b). The effect of marihuana on cognitive organization and storage was studied by a free recall task of taxonomic material. A comparison between recognition and recall memory performance was used to index the effect of marihuana on retrieval processes.

METHODS

CLINICAL PROCEDURES

The subjects in this study were primarily recruited for an investigation of the biological effects of marihuana. These studies have been reported elsewhere (Hembree, Nahas, Zeidenberg and Dryrenfurth, 1976; Hembree, *et al.*, 1979; Nahas, DeSoize, Armand, Hsu and Morishima, 1976). Male marihuana users between 18 and 30 years of age were recruited by advertisement in a local newspaper. All candidates were screened by a preliminary interview with a psychiatric nurse. Those felt to be suitable were further screened by a psychiatrist. Subjects using other drugs except for tobacco and moderate amounts of alcohol were excluded. Subjects who had never smoked marihuana or smoked occasionally were also excluded. All subjects who were taking psychotherapeutic drugs or who were in psychotherapy were likewise excluded from the study. Another reason for exclusion was recent virus infection such as hepatitis or mononucleosis. This would have interfered with the biological portion of the study. Thus a population of users was obtained who used marihuana and who did not use other drugs. The regularity of marihuana use among these subjects varied with their economic situation, and ranged from 1 to 2 cigarettes every few days to 6 cigarettes daily. Each subject was evaluated thoroughly with a physical examination and a complete battery of laboratory tests and procedures, and was found to be in good health prior to beginning the research. Subjects were admitted to the Drug Abuse Research Ward of New York State Psychiatric Institute and were restricted to the ward for the duration of the experiment. When the necessity arose for subjects to leave the hospital they were

accompanied by hospital staff members. Thus the possibility of use of other drugs throughout the experimental period was virtually negligible. The 14 subjects were paid for participating in the study.

The experiment was divided into three parts: "washout" period, 3–4 weeks; smoking period, 4 weeks; and post-smoking "washout" period, 3–4 weeks. At the end of the washout period subjects began smoking marihuana cigarettes. These were provided by the National Institute of Health and were standardized at 2% content of THC (20 mg. per cigarette). Smoking proceeded by increments of approximately one cigarette each day until ten cigarettes had been reached, then the subjects were at liberty to smoke as many cigarettes as they wished. The subjects smoked an average of 3.7, 10.4, 14.5 and 16.5 cigarettes per day during the first, second, third and fourth week of smoking. The total number of cigarettes smoked by each subject ranged from 151 to 343.

The subjects were administered each of the memory tests once a week and, on a different occasion, the pain test approximately once every two weeks. The subjects smoked one cigarette immediately before being tested.

PAIN PROCEDURES

A modified Hardy-Wolff Dolorimeter was used to present 20 thermal stimuli, in a random manner, at each of the following intensities: 0, 45, 90, 135, 180, 225, 270, 315, 360, and 405 mcal/sec^{-1}/cm^{-2}. The stimuli were 3.0 sec in duration (unless the subject withdrew sooner) and 2 cm in diameter. The subject responded from a 14 category scale (see Table 3) which ranged from "nothing" through various degrees of "warmth," "heat," "pain," and early withdrawals.

TABLE 3. RELATIONSHIP BETWEEN VALUES OF *B*, RESPONSE BIAS, AND RESPONSE CATEGORIES. A HIGH VALUE OF *B* OCCURS WHEN THERE ARE FEW REPORTS OF PAIN.

Values of *B*	14	13	12	11	10	9	8
Response categories	Nothing	Maybe something	Faint warmth	Warm	Hot	Very hot	Very faint pain
Values of *B*	7	6	5	4	3	2	1
Response categories	Faint pain	Pain	Very painful	Withdrawal times (sec)			
				2.66 3.00	2.32 2.65	2.00 2.31	1.99 ≥

MEMORY PROCEDURES

Recognition Memory. The subjects viewed 200 three-consonant trigrams presented at the rate of one every four seconds on a memory drum. Seventy-two of these trigrams were repeated once with different numbers of intervening items (lags): 0, 3, 6, 9, 12, 18, corresponding to time intervals of 4, 16, 28, 40, 52, and 76 seconds, before the trigram was

viewed again. Zero lag is a special case and represents a measure of alertness or attention. Each lag interval was represented by twelve repeated items. Thus, the complete list contained seventy-two "new" stimuli which were later repeated as seventy-two "old" stimuli and fifty-six "fillers," new stimuli which did not reappear. A new set of trigrams was used on each period of the study. The task of the subject was to report "old" or "new" to each trigram as it appeared, depending on whether they believed they had seen it previously. Practice trials were given with stimuli printed on index cards.

The *hit rate*, subject reports "old" to a previously presented item, and the *false alarm rate*, subject reports "old" to a new item, were treated by sensory decision theory techniques to yield indices of recognition memory, d', and report criterion, L_x. The hit rate was determined for each lag, since the separation between the first and second presentation is known; the false alarm rate cannot be identified with a particular lag and is based on the responses to all 128 new items on the list. The measure d' was used instead of $P(A)$, since they are equivalent when a binary decision, rather than a confidence rating procedure is used.

The subjects were tested on 8 periods one week apart: 1 pre-smoking, 4 while smoking, and 3 post-smoking.

Recall and Clustering. The subject was presented 24 words, each on an index card which he read out loud to insure that they had been attended to. Although the words were presented in random order, the list contained 6 words in each of 4 categories: clothing, professions, birds, and countries. The subject immediately recalled as many words as he could. Before the next trial the subject was rehearsed by a presentation of the words which he had failed to recall. There were 4 trials in each test period. The words were different on each of the 8 test sessions.

In addition to recall, the subjects' responses were examined for category clustering, that is for the amount of organization in the responses. When a category, or some other classification is recalled, the items are often recalled by class rather than by order of presentation. Bousfield (1953) called this phenomenon "clustering" and measured it by counting class repetitions. It has been demonstrated that subjects who cluster their material remember it better. Clustering reflects the degree of cognitive organization imposed on the material in order to aid retrieval of the information. The score is the mean number of words in each cluster. The number of words in a cluster, which can vary between 1 and 6, is determined from the following formula:

$$\text{Number of words per cluster} = \frac{\text{total words recalled}}{\text{transition points} + 1}$$

A transition point occurs each time a word from a category different from the previous word is encountered. For example, if the words had been perfectly organized so that all words in each category were grouped together, there would be but three transition points or four clusters with six words in each cluster.

RESULTS

PAIN RESULTS

Separate analyses of variance (period by dose level) were performed for discriminability and report bias at each of the 9 intensity pairs studied: 0–45, 45–90, etc. to

360–405 mcal sec^{-1} cm^{-2} (see Table 4). Since $P(A)$ is not a normally distributed variable, the data were subjected to a 2 arcsin square root $P(A)$ [2ARS − $P(A)$] transformation for statistical analysis. Zero discrimination or chance performance yields 2ARS − $P(A)$ equal to 1.57, while almost perfect discriminability yields a value of 2.94.

TABLE 4. MEAN DISCRIMINABILITY, 2 ARCSIN SQUARE ROOT $P(A)$, AND REPORT BIAS, B, IN A GROUP OF MARIHUANA SMOKERS ($n = 13$)

Stimulus intensity pair (mcal/sec^{-1}/cm^{-2})	2 arcsin square root $P(A)$				B			
	Pre	During wks 1, 2	During wks 3, 4	Post	Pre	During wks 1, 2	During wks 3, 4	Post
0–45	2.02	1.99	1.92	2.02	12.9	12.8	13.9	13.2
45–90	2.08	1.89	1.93	1.98	12.1	12.2	12.4	12.5
90–135	1.84	1.88	1.80	1.84	11.2	11.6	11.9	11.9
135–180	1.95	1.99	1.92	1.98	10.5	10.8	11.2	11.1
180–225	2.00	1.90	2.00	1.90	9.2	9.9	10.3	10.3
225–270	2.03	2.18	2.10	2.20	7.1	8.1	8.8	8.5
270–315	2.09	2.17	2.28	2.27	5.4	4.9	6.0	6.1
315–360	2.04	2.06	2.10	2.22	4.1	2.8	3.8	3.6
360–405	1.91	1.84	2.01	1.97	2.8	1.9	2.4	2.1

For 45 against 90 mcal/sec^{-1}/cm^{-2}, discriminability decreased during and after smoking, $F(3, 30) = 4.39$, $P < 0.025$, a finding consistent with the hypothesis that some slight analgesia had occurred. However, at higher (faint pain) intensities, 270 against 315 mcal/sec^{-1}/cm^{-2}, discriminability increased during and after smoking, $F(3, 30) = 3.73$, $P < 0.025$, a finding consistent with heightened sensitivity to pain. Although individually they failed to achieve significance, it is worth noting that thermal discriminability increased during and following marihuana smoking for all of the noxious intensities, 225 to 405 mcal/sec^{-1}/cm^{-2}. This increase in discriminability suggests that marihuana has interfered with the functioning of a pain inhibitory system.

The response bias measure, B, locates the rating scale criterion at which half of the responses are to the higher response categories and half are to the lower. The stimuli were rated on a 14 point scale (see Table 3) with a report of "nothing" equal to 14, and with a report of "very painful" coupled with a withdrawal in less than 1.99 sec equal to 1. Thus $B = 13.5$ means a high pain report criterion, few pain reports, since half of the responses consist of "nothing," and the remaining half are distributed amongst "maybe something," "faint warmth," etc. $B = 8.5$ means that half of the responses include a pain response ranging from very faint pain to very painful and withdrawal in less than 1.99 sec, while $B = 2.5$ represents a very low pain report criterion, since half of the responses involved a report of "very painful" and a withdrawal from the stimulus in less than 1.99 sec.

The three highest intensity pairs, 270 to 405 mcal/sec^{-1}/cm^{-2} reveal a very interesting pattern in pain report. B at 315 against 360 and 270 against 315 mcal/sec^{-1}/cm^{-2} revealed significant period by dose interactions, $F(6, 30) = 3.16$, $P < 0.025$ (see Table 4). At these painful intensities, during the first and second weeks of smoking, the pain report criterion decreased (more pain reports) particularly for the high dose group, to a level which was well below that of the pre-test period. Then during weeks 3 and 4 and in the post-test period the pain report criteria returned to their pre-test levels.

Significant but small increases in B were found during week 4 and post-test, 0 against 45 mcal/sec^{-1}/cm^{-2}, $F(3, 30) = 7.78$, $P < 0.001$; 90 against 135 mcal/sec^{-1}/cm^{-2}, $F(3, 30) = 3.95$, $P < 0.02$, and 135 against 180 mcal/sec^{-1}/cm^{-2}, $F(3, 30) = 3.02$, $P < 0.05$. However, these low stimulus intensities do not involve the report of pain, and hence are of little interest; the increase in the criterion means that there were significantly more reports of "nothing" and fewer reports of "maybe something," to the blank. Thus, there are fewer thermal "hallucinations," a finding which is opposite to that reported for tactile sensations.

MEMORY RESULTS

Recognition Memory. Values of d' for each lag interval in each test period appear in Table 5. A separate 2-factor, period by dose, analysis of variance was performed for each of the 6 lag intervals. Inspection of the table reveals that, as expected, 0-lag yielded the best memory and that there was a slight decrease in performance at the longest lag intervals. For the 0-lag interval there was a steady improvement in recognition memory over the experimental periods, $F(7, 84) = 2.78$, $P < 0.01$. No main effects for period or dose were found for lags 3 to 18. However, at lag 12 there was a significant period by dose interaction; for the pre-drug and early test period the low and high dose groups were equal in recognition memory, but in all of the post-drug periods the low dose users showed superior recognition memory $F(7, 84) = 2.11$, $P < 0.05$. The data averaged over all lag periods (Table 6) also revealed a significant period by dose interaction $F(7, 84) = 2.49$, $P < 0.02$. The interaction was caused by an increase in d' over periods by the low dose group, while the high dose group evidenced a decline. Thus, the low dose group improved slightly over time while the high dose group did not.

TABLE 5. RECOGNITION MEMORY (d')[a] FOR VARIOUS LAG INTERVALS

Lag	Pre	Week 1	Week 2	Week 3	Week 4	Post 1	Post 2	Post 3	*M*
0	2.1	2.3	2.3	2.4	2.4	2.6	2.6	2.4	2.4
3	1.6	1.9	1.6	1.8	1.8	1.8	1.6	1.7	1.7
6	1.5	1.7	1.7	1.8	1.7	1.8	1.6	1.5	1.7
9	1.4	1.8	1.6	1.5	1.7	1.7	1.6	1.5	1.6
12	1.5	1.6	1.6	1.7	1.7	1.8	1.8	1.6	1.7
18	1.2	1.4	1.5	1.5	1.3	1.5	1.4	1.3	1.4
M	1.5	1.7	1.7	1.7	1.7	1.8	1.7	1.6	1.7

a. A high value indicates superior memory.

TABLE 6. RECOGNITION MEMORY (d')[a] FOR HIGH AND LOW DOSE

Dose (groups)	Pre	Week 1	Week 2	Week 3	Week 4	Post 1	Post 2	Post 3
Low	1.4	1.7	1.8	1.8	1.8	2.0	2.0	1.9
High	1.6	1.8	1.5	1.6	1.6	1.6	1.3	1.3

a. A high value indicates superior memory.

Values of the likelihood ratio, L_x, were computed from hit and false affirmative rates. Since the likelihood ratio is distributed logarithmically, the data were normalized by a logarithmic transformation for analysis of variance. Separate 2-factor, period by dose, analyses of variance were performed at each of the 6 lag periods studied (Table 7). At all lags but lag 0, the subjects on the three post-test weeks raised their criterion, log L_x, for reporting a nonsense syllable as "old," $F(7, 84) \leq 2.59$, $P \leq 0.02$; averaged over all lags, $F(7, 84) = 5.77$, $P < 0.001$.

TABLE 7. REPORT CRITERION (log L_x) FOR VARIOUS LAG INTERVALS[a]

Lag	Pre	Week 1	Week 2	Week 3	Week 4	Post 1	Post 2	Post 3
0	−.19	−.40	−.26	−.27	−.26	−.28	−.19	−.27
3	−.00	−.38	−.06	−.09	−.09	.06	.16	−.04
6	−.01	−.19	−.07	−.10	−.03	.10	.19	.04
9	.03	−.19	−.04	.03	−.05	.11	.19	.09
12	.07	−.17	−.02	−.04	−.05	.00	.15	.04
18	.04	−.12	.06	.05	.06	.15	.22	.10
M	.08	−.17	.01	.02	.03	.11	.22	.09

a. A high value indicates a conservative report criterion, few reports of "old".

Recall Memory. The number of words recalled out of the 24 presented on each trial appear in Table 8. These data were treated by a 3-factor analysis of variance for period, trial and dose; the design was for repeated measures with subjects nested within dose level. There was a significant period effect, $F(6, 72) = 4.48$, $P < 0.001$. It is clear that smoking during weeks 1 and 2 decreased the number of words recalled on each trial. This was followed by recovery during weeks 3 and 4 which continued after smoking stopped. As expected, between each trial within a session, the number of words recalled increased, $F(3, 36) = 35.6$, $P < 0.001$. The low dose and high dose groups recalled an average of 18.3 and 16.9 words, respectively, but this difference did not reach significance.

TABLE 8. MEAN NUMBER OF WORDS RECALLED OUT OF 24

Trial	Pre	Week 1	Week 2	Week 3	Week 4	Post 1	Post 2	*M*
1	15.0	12.1	13.5	14.3	15.3	15.0	16.2	14.5
2	18.4	16.3	15.9	17.1	18.4	18.8	19.6	17.8
3	19.0	17.1	16.4	18.2	20.6	19.8	20.2	18.8
4	19.2	17.8	18.6	20.0	20.6	20.0	19.5	19.4
M	17.9	15.8	16.1	17.4	18.7	18.4	18.9	17.6

Clustering. The mean number of words in a cluster appear in Table 9. Analysis of variance revealed a significant period effect $F(6, 72) = 5.08$, $P < 0.001$. Over trials, the Tukey critical difference test indicated that clustering was significantly greater on the last week of smoking and during the two post-smoking weeks than during the pre-test and first smoking week, *C.D.* $(7, 72) = 0.67$, $P < 0.05$. A significant trial effect $F(3, 36) = 19.3$, $P < 0.001$ showed that clustering increased within each session, *C.D.* $(4, 36) = 0.80$, $P < 0.05$.

TABLE 9. MEAN NUMBER OF WORDS CLUSTERED INTO THE SAME CATEGORY

Trial	Pre	Week 1	Week 2	Week 3	Week 4	Post 1	Post 2	*M*
1	2.5	2.5	2.7	2.8	3.1	3.1	3.0	2.8
2	2.6	2.9	3.0	3.1	3.8	3.5	3.8	3.2
3	3.2	3.5	3.2	3.8	4.0	3.9	4.1	3.7
4	3.6	3.4	3.9	4.1	4.2	4.2	4.3	4.0
M	3.0	3.1	3.2	3.5	3.8	3.7	3.8	3.4

DISCUSSION

The increase in thermal discriminability, $P(A)$, found during the four weeks of smoking, and which extended for at least one week post-smoking, indicates that marihuana suppresses a pain inhibitory pathway. Such suppression also explains the decrease in the pain report criterion (more pain reports), during the first two weeks of smoking, but not the subsequent increase in the pain criterion. Messing and Lytle (1977) have summarized a large number of studies which support the hypothesis that reduction in brain or spinal cord serotonergic neurotransmission is associated with increased sensitivity to noxious stimulation. For example, the administration of *para*-chlorophenylalanine, a drug which reduces brain serotonin levels has been reported to produce algesia in humans (Sicuteri, Anselmi, and DelBianco, 1973). The report by Taylor and Fennessy (1978) that brain levels of serotonin are reduced in rats after 5 days of THC administration is congruent with our findings of increased discriminability and lowered criterion.

At the noxious thermal intensities there was a decrease in the pain report criterion, B, during the first two weeks of smoking. This pain enhancement effect was followed by a return to the pre-smoking pain level during weeks 3 and 4, and in the post-smoking period. The finding that marihuana smoking initially enhanced pain perception according to both of the sensory decision theory parameters ($P(A)$ and B) at the noxious stimulus intensities, agrees with the results of Hill, *et al.* (1974) who found that marihuana decreased the pain threshold. The two studies agree because both a decrease in the criterion B and a decrease in pain threshold reflect an increase in the number of pain reports. This finding is consistent with the "perceptual sharpening" reported in other sensory modalities, and with comments from our subjects that they never smoke marihuana while suffering a headache or toothache because it would increase the pain. We also noticed that the subjects did not smoke before being veni-punctured for blood samples, a procedure which they found very painful. The subsequent disappearance of the pain enhancement effect during weeks 3 and 4 of smoking indicates that tolerance to psychotropic effects had developed. By the third and fourth weeks the subjects were complaining that the cigarettes were no longer producing a " high ". In fact, several subjects believed we had substituted placebo for the marihuana cigarettes. It is reasonable to speculate that when tolerance to the psychotropic effect developed, the associated effect on the pain report criterion, namely, pain enhancement, disappeared. Jones and Benowitz (1976) have shown that prolonged administration of THC rapidly produces a dose-related tolerance.

It is of considerable interest that tolerance developed to the pain report criterion, a cognitive variable presumably mediated by complex neocortical and limbic system activ-

ity, but not to thermal discriminability, the neurosensory component which is mediated via spinal-thalamic tracts and sensory cortex. Other investigators have reported that various physiological response systems develop tolerance at different rates, and conclude that more than one pharmacodynamic mechanism must be involved (Ferraro, 1976). Obviously, a single mechanism, such as metabolic tolerance cannot explain our results.

The results of the present experiment differ from our earlier report (Zeidenberg, *et al.*, 1973) where an acute oral dose of 15 mg. of pure THC decreased the discriminability measure (interpreted as a hypalgesic effect) and had variable effects on the pain report criterion. This difference may be attributed to differences in the subjects' history, by route of administration and the pharmacology of the drug. In the earlier experiment, naive subjects were administered a large acute dose of THC. These subjects would not be expected to have developed tolerance to any of the many pharmacological actions of the drug. Hence, these subjects experienced marked psychotropic and analgesic responses to the drug.

Our finding that marihuana enhances pain agrees with Hill *et al.* (1974), but is at variance with most of the literature. As Harris (1976) points out, studies of the effect of marihuana on the pain threshold are very inconsistent. This inconsistency is probably due to the classical pain threshold measure which has been shown to be heavily influenced by nonsensory variables such as expectation and suggestion (Clark, 1969; Clark and Goodman, 1974). Thus, subject expectation could explain the increase in pain tolerance reported by Milstein *et al.* (1975); the use of the double-blind control is not sufficient, since psychotropic effects of marihuana permit the subject to "peek through the double-blind." The reputed analgesic effect of marihuana in patients suffering cancer pain (Noyes, *et al.*, 1975) could be due to placebo effects, to euphoria, or, in the instance of patients receiving chemotherapy, to increased well-being which results from the anti-emetic effect of marihuana (Borison, *et al.*, 1978).

The preponderance of evidence in this study suggests that marihuana lacks any hypalgesic activity, and in fact probably enhances the perception of pain.

Memory and Clustering. Recognition memory (lags 3 through 18) was not impaired by moderately heavy marihuana smoking. Thus the trigrams entered short-term memory (registration) and were stored for at least 76 seconds without loss. Since the subject is presented the to-be-remembered item again he does not have to retrieve it from memory, but merely make a match between the stimulus and the stored item. In contrast, recall memory, which requires active retrieval, was impaired during weeks 1 and 2 of smoking. Unimpaired recognition memory coupled with a loss in recall memory strongly suggests that moderately heavy marihuana smoking interferes with the memory retrieval mechanism, but not with either registration (input) or storage of the to-be-remembered material.

The poor recall memory agrees with the anecdotal reports and laboratory findings (Zeidenberg, *et al.*, 1973) that subjects under the influence of marihuana have difficulty maintaining or following a coherent train of thought.

Recall memory improved during weeks 3 and 4 of very heavy smoking, suggesting that tolerance had developed in the neural mechanisms subserving retrieval. Withdrawal did not interfere with performance. The amount of clustering, a measure of organization imposed on the to-be-remembered material, also increased during this period. This suggests that tolerance had developed in a pre-retrieval memory mechanism which organizes the material for recall. This argument is based on the fact that subjects who are told that they will be tested by recall show superior recall compared to subjects who are told they

will be tested by recognition, but are given an unexpected recall test. Cluster analysis demonstrates that the superior performance of the forewarned group is due to the specific organization they have imposed on the material. High motivation is known to increase cognitive processing of the material for subsequent recall; however, since marihuana intake may be expected to lower motivation, especially at the later stages of the study, we speculate that some mechanism not under direct conscious control has acquired tolerance permitting the words to be organized for more facile retrieval subsequently.

The only significant drug effect on recognition memory was found for lag 0. Since this measures the ability of the subject to recognize an item repeated 4 seconds later, and there are no intervening "distractor" items, lag 0 is more a measure of attention or alertness than memory. The poor performance during the pre-test period may be due to anxious preoccupation with the entire novel situation, or the higher values subsequently may be caused by an improved focus of attention induced by marihuana. The steady rise in the discriminability score from pre- to post-periods strongly suggests that experience rather than marihuana caused the improved attention.

The interaction between dose level and recognition memory measures, with the low dose group showing a marked improvement during the post-smoking periods, suggests that a high dose produces a more persistent deleterious effect on recognition memory.

The subjects set a low criterion for reporting "old" for all lag intervals but zero, during the 4 smoking weeks. This increased tendency to label a nonsense syllable as old, whether it was actually old or new, may reflect an attempt, either conscious or unconscious, to give the impression that marihuana does not impair memory by saying "old, yes, I remember it" even when they were in doubt. Clark and Greenberg (1971) found a similar effect, that is, a low report criterion for reporting "old" in subjects desperately trying to perform well on a memory task which they had been led to believe was a measure of their intelligence.

These results with respect to the criterion serve to illustrate an important aspect of the sensory decision theory approach. The lowered report criterion during the 4 weeks of smoking means that both hit and false affirmative rates were higher. Thus more old words appear to be identified correctly as old; from the traditional point of view the data obtained here would mistakenly indicate that marihuana had improved memory. In contrast, the sensory decision theory analysis makes it clear that memory has not been influenced by marihuana, since d' did not change.

The lack of an interaction between period and within session measures of recall and clustering had not been anticipated. It was thought that the decrease in motivation induced by smoking would manifest itself as a within-session memory and clustering deficit. Such a deterioration in performance has often been reported in acute studies of marihuana smoking. Apparently lack of motivation does not develop within a test session in the instance of chronic smoking. An interaction between drug and recognition lag times also had been expected in view of the findings that marihuana impairs short-term memory. Failure to find such an effect may be due to the fact that the lag intervals chosen did not extend to long enough intervals.

ACKNOWLEDGEMENT

We wish to thank Dr. Gabriel G. Nahas, M.D., Ph.D., who both enabled and encouraged us to study perceptual and cognitive function in his subject sample. We also

thank Malvin N. Janal and James R. Falk for assistance with the statistical analysis of the data. This study was supported in part by the General Research Support Branch, Division of Research Facilities and Resources, NIH Grant RR05650 to the New York State Psychiatric Institute.

REFERENCES

ABEL, E. L., Marihuana and memory: acquisition or retrieval. *Science*, **173**,1038 (1973).

BORISON, H. L., MCCARTHY, L. E. and LONDON, S. W., Cannabinoids and emesis. *New England Journal of Medicine*, **298**,1480-1481 (1978).

BOUSFIELD, W. A., The occurrence of clustering in the recall of randomly arranged associates. *Journal of General Psychology*, **49**,229-240 (1953).

CLARK, W. C., Sensory-decision theory analysis of the placebo effect on the criterion for pain and thermal sensitivity (d'). *Journal of Abnormal Psychology*, **74**,363-371 (1969).

CLARK, W. C., Pain sensitivity and the report of pain: An introduction to sensory decision theory. *Anesthesiology*, **40**,272-287 (1974).

CLARK, W. C., Measurement of pain in humans: Signal detection theory and pain. *Neurosciences Research Program Bulletin*, **16**,14-27 (1978).

CLARK, W. C. and GOODMAN, J. S., The effect of suggestion on d' and C_x for pain detection and pain tolerance. *Journal of Abnormal Psychology*, **83**,364-372 (1974).

CLARK, W. C. and GREENBERG, D. B., Effect of stress, knowledge of results, and proactive inhibition on verbal recognition memory (d') and response criterion (L_x). *Journal of Personality and Social Psychology*, **17**,42-47 (1971).

CLARK, W. C. and YANG, J. C., Naloxone reversal of nitrous oxide hypalgesia in man. International Association for the Study of Pain, Montreal, September, 1978.

DORNBUSH, R. L. and KOKKEVI, A., Acute effects of cannabis on cognitive, perceptual, and motor performance in chronic hashish users. In R. L. Dornbush *et al.* (Eds.), "Chronic Cannabis Use," *Annals of the New York Academy of Sciences*, **282**,313-322 (1976).

FERRARO, D. P., Preclinical chronic effects: unlearned and learned behavior. In R. C. Peterson (Ed.), "Marihuana Research Findings," U.S. Government Printing Office, Washington, D.C., pp. 118-127, 1976.

GORDON, S. and CLARK, W. C., Application of signal detection theory to prose recall and recognition in elderly and young adults. *Journal of Gerontology*, **29**,64-72 (1974a).

GORDON, S. and CLARK, W. C., Adult age differences in word and nonsense syllable recognition memory and response criterion. *Journal of Gerontology*, **29**,659-665 (1974b).

HARRIS, L. S., Analgesic and antitumor potential of the cannabinoids. In S. Cohen and R. C. Stillman (Eds.), "The Therapeutic Potential of Marihuana." Plenum, New York, pp. 299-312, 1976.

HEMBREE, W. C., NAHAS, G. G., ZEIDENBERG, P. and DYRENFURTH, I., Marihuana effects on the human testis. *Clinical Research*, **24**(3),272A (1976).

HEMBREE, W. C., III, NAHAS, G. G., ZEIDENBERG, P. and HUANG, H. F. S., Changes in human spermatozoa associated with high dose marihuana smoking. This volume. pp. 429-439 (1979).

HILL, S. Y., SCHWIN, R., GOODWIN, D. W. and POWELL, B. J., Marihuana and pain. *Journal of Pharmacology and Experimental Therapeutics* **188**,415-418 (1974).

JONES, R. T. and BENOWITZ, N., The 30-day trip: Clinical studies of cannabis tolerance and dependence. In M. C. Braude and S. Szara (Eds.), "Pharmacology of Marihuana." Raven Press, New York, pp. 627-642, 1976.

KLONOFF, H., LOW, M. and MARCUS, A., Neurophysiological effects of marijuana. *Canadian Medical Association Journal*, **108**,150-165 (1973).

MCNICOL, D., "A Primer of Signal Detection Theory," Allen & Unwin, London, 1972.

MESSING, R. B. and LYTLE, L. D., Serotonin-containing neurons: Their possible role in pain and analgesia. *Pain*, **4**,1-21 (1977).

MILSTEIN, S. L., MACCANNELL, K., KARR, G. and CLARK, S., Marihuana-produced changes in pain tolerance: Experienced and nonexperienced subjects. *International Pharmacopsychiatry* (*Basle*), **10**,177-182 (1975).

NAHAS, G. G., DESOIZE, B., ARMAND, J. P., HSU, J. T. and MORISHIMA, A., Natural cannabinoids: Apparent depression of nucleic acids and protein synthesis in cultured human lymphocytes. In M. C. Braude and S. Szara (Eds.), "Pharmacology of Marihuana," Raven Press, New York, pp. 177-186, 1976.

NOYES, R., BRUNK, S. F., BARAM, D. A. and CANTER, A., Analgesic effect of delta-9-tetrahydrocannabinol. *Journal of Clinical Pharmacology*, **15**,134-143 (1975).

ROSEN, L. J. and LEE, C. L., Acute and chronic effects of alcohol use on organizational processes in memory. *Journal of Abnormal Psychology*, **85**,309-317 (1976).

ROSSI, A. M., KUEHNLE, J. C. and MENDELSON, J. H., Effects of marihuana on reaction time and short-term memory in human volunteers. *Pharmacology Biochemistry & Behavior*, **6**,73-77 (1977).

SICUTERI, F., ANSELMI, B. and DELBIANCO, P. L., 5-hydroxytryptamine supersensitivity as a new theory of headache and central pain: a clinical pharmacological approach with *p*-chorophenylalanine. *Psychopharmacologia* (*Berl.*), **29**,347–356 (1973).

TAYLOR, D. A. and FENNESSY, M. R., Relationship between body temperature and brain monoamines during the development of tolerance to delta-9-tetrahydrocannabinol in the rat. *Psychopharmacology*, **56**,279–285 (1978).

WILSON, R. S. and MAY, E. L., Analgesic properties of the tetrahydrocannabinoids, their metabolites and analogs. *Journal of Medicinal Chemistry*, **18**,700–703 (1975).

YANG, J. C. and CLARK, W. C., Morphine and diazepam hypalgesia in man. International Association for the Study of Pain, Montreal, September, 1978.

ZEIDENBERG, P., CLARK, W. C., JAFFE, J., ANDERSON, S., CHIN, S. and MALITZ, S., Effect of oral administration of delta-9-tetrahydrocannabinol on memory, speech, and pain. *Comprehensive Psychiatry*, **14**,549–556 (1973).

RESIDUAL EFFECTS OF HIGH-DOSE CANNABIS TREATMENT ON LEARNING, MURICIDAL BEHAVIOR AND NEUROPHYSIOLOGICAL CORRELATES IN RATS

KEVIN O'BRIEN FEHR, HAROLD KALANT and GEORGE V. KNOX

Department of Pharmacology, University of Toronto

and

Addiction Research Foundation of Ontario, Toronto, Canada M5S 2S1

Summary. Immature male rats were given large oral doses of cannabis extract (THC dose, 20 mg/kg), ethanol (6 g/kg) or control solution daily, for six months. One month after the end of treatment, the cannabis and ethanol groups showed significantly impaired learning of a complex motor performance. In addition, a much higher proportion of the cannabis and ethanol groups than of controls showed muricidal behavior when tested at one and two months after the end of drug treatment. These animals also showed irregular spike-like waves in electrical recordings from the dorsal hippocampus. Pilot studies suggest a reduced number of dendritic spines in the hippocampus and cortex of the ethanol-treated animals, but not in the cannabis group.

In contrast, brief intra-uterine exposure to comparable doses of cannabis extract (THC 10 or 20 mg/kg) during the critical phase of nervous system development (days 8 to 12 of pregnancy) did not affect maze-learning ability of the offspring. However, the mothers were apparently able to distinguish drug-treated from control offspring, so that some subtle behavioral differences may exist.

IN AN earlier report[1] we described apparently permanent deficits in the learning of two types of task by rats which had previously received large doses of Δ^9-tetrahydrocannabinol (THC) in cannabis extract or of ethanol daily for six months. The tasks were food-reinforced maze-running, and treadmill pacing to avoid electric shock to the feet. The doses employed were 6 g/kg for ethanol and 20 mg/kg for THC (contained in marihuana extract), by gastric intubation; in the case of THC, one-quarter as great a total drug exposure (one-half the dose for one-half the length of time) had failed to produce residual impairment of maze-learning.

Because of the rather small number of animals used in those studies, and the large variances in scores on the treadmill test used in the last part of the work, we decided to repeat the study with improved techniques aimed at producing greater homogeneity of test scores. In addition, certain very preliminary electroencephalographic (EEG) findings, including abnormal spike-like waves in the hippocampal tracings obtained from two

cannabis-treated and two alcohol-treated rats in the earlier work, required fuller exploration.

Several authors[2–4] had reported that muricidal behavior appeared in previously non-muricidal rats during chronic treatment with intraperitoneal injection of cannabis preparations, and persisted for up to 20 days after the end of drug administration. However, this residual behavior might have represented prolonged withdrawal effects, or might have been caused by irritability resulting from the repeated i.p. injections. Moreover, these animals had begun to show muricidal behavior during the period of drug administration; persistence of the behavior after withdrawal could have represented a conditioned response rather than a residual neurological alteration after the end of the drug period. Therefore muricidal behavior was also examined in the animals used for the learning study, with the safeguard that the first test occurred long after the end of the drug administration period.

Finally, it was also of interest to examine the effects of intra-uterine exposure to cannabis on the subsequent learning ability of the offspring. There have been few studies of this type, especially at reasonably low doses. Gianutsos and Abbatiello[5] reported that the offspring of rats given subcutaneous injections of cannabis extract on days 8 to 11 (inclusive) of pregnancy made more errors than control offspring when tested in the Lashley III maze at 80 days of age. However, the offspring were reared by their own mothers, and the differences in performance might have been due to residual effects of cannabis on maternal behavior toward the young. Moreover, the THC content of the cannabis extract was not stated, so that the actual dose is unknown.

Other studies of the effects of prenatal exposure to cannabis on postnatal behavior, involving a variety of species, doses and experimental techniques[6–10], have yielded conflicting results. We therefore decided to replicate the study by Gianutsos and Abbatiello[5], but with a cannabis extract of known THC content and using the same food-motivated Hebb-Williams maze test as in our other studies[1].

EXPERIMENTAL

LEARNING EXPERIMENTS IN YOUNG ADULT RATS

Thirty male Wistar rats of 100 g body weight were caged individually, given free access to tap water, and assigned randomly to three equal groups: control, ethanol and cannabis. The cannabis group received daily gavage of 0.5 ml/kg of olive oil, containing marijuana extract in an amount that provided THC 20 mg/kg, cannabidiol 1.3 mg/kg and cannabinol 0.65 mg/kg. The ethanol group were given daily intubation of 25% (w/v) ethanol solution at an initial dose of 2 g of ethanol per kg, increasing gradually to 6 g/kg over a two-week period and then held constant. The controls received equal volumes of a sucrose solution equicaloric with the ethanol solution.

Each animal was allowed free access to laboratory rat chow until it reached a weight of 350 g. From this point on, its daily ration was reduced to about 20 g of chow, with minor upward or downward adjustments to hold body weight constant.

All drug and control intubations were carried on for six months, then discontinued abruptly. Thirty days later, training trials on the moving belt test were begun[11]. In this test, the animals must learn to walk on a narrow metal mesh belt which is driven at

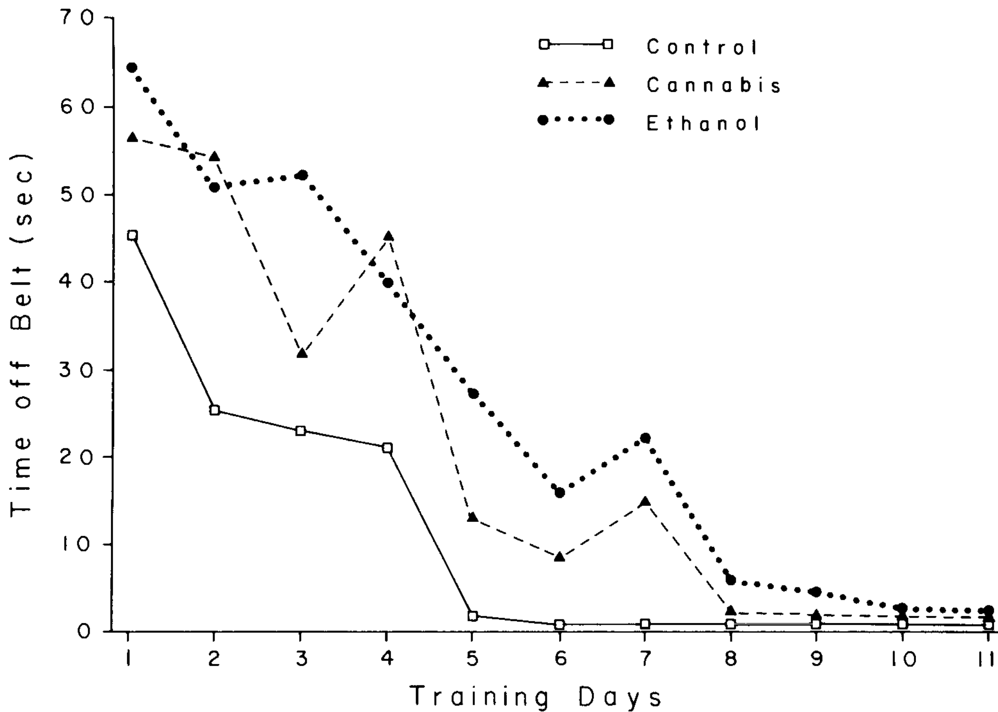


FIG. 1. Learning curves on the moving belt test for the control and drug treatment groups, beginning one month after the end of six months of daily gavage with sucrose solution (controls), cannabis extract in olive oil (THC 20 mg/kg), or ethanol 6 g/kg. Values shown are group mean scores for two successive trials per animal daily.

constant speed over a shock grid. Learning is shown by a progressive reduction in the time off belt, i.e. in the number of seconds in which foot-shock is being received, during a standard 2-min test run. Each animal received two 2-min runs daily for 15 days, or until it reached a criterion of not more than 1.2 sec off belt per run, on two consecutive days. If the rat failed to reach this criterion by the end of 15 days the trials were stopped and a learning time of 15 days was assigned.

The raw daily scores for the three groups on the moving belt test are shown in Fig. 1. Even on day 1 the controls had a lower score than the ethanol or cannabis groups. This is explained by the fact that each point represents the group mean for two test runs on the same day. The controls adapted rapidly to the moving belt even during the first trial, and improved significantly on the second, so that the mean of the two scores on day 1 was less than for the two former drug groups. Control animals took a learning time of 5.5 ± 1.1 days to reach criterion. Both drug groups took significantly longer, the cannabis rats taking 9.0 ± 2.4 days and the ethanol group 10.1 ± 3.3 days ($p < 0.01$ in both cases, by Dunnett's one-tailed t-test).

For further analysis the results were converted to a probit scale and subjected to analysis of variance and post-hoc comparisons by Dunnett's one-tailed t-test. This confirmed again that the animals in both chronic drug-treatment groups learned significantly more slowly than the controls.

MURICIDAL BEHAVIOR

The same animals were also tested on two occasions for their response to mice. The first test was carried out 30 days after the end of the chronic drug treatments, just before the start of the learning trials on the moving belt apparatus, and the second test was at 60 days after the last intubation. On each occasion, four hours after feeding, a mouse was introduced into the rat's cage and left there for up to 10 min. If no attack occurred, the mouse was removed. When attack occurred, it was typically directed to the back of the mouse's neck, resulting in almost instantaneous kill. Latency-to-attack was recorded, and was considered synonymous with latency-to-kill. The test was immediately repeated with a second mouse. The number of rats in each group that killed at least one of the two mice was recorded.

Thirty days after the end of drug treatment, 1 control rat, 4 ethanol rats and 6 cannabis rats killed at least one of two mice (Table 1). At 60 days, one previously muricidal ethanol rat did not kill, and one previously non-muricidal cannabis rat did. Fisher's chi-square test[12] showed that significantly more cannabis than control rats were muricidal at both 30 and 60 days ($p < 0.01$ in both cases). The ethanol group did not differ significantly from the controls or from the cannabis group at either time, although the difference from controls approached significance at 30 days ($p < 0.075$).

TABLE 1. KILLING LATENCIES OF MURICIDAL RATS

Rat	30 days post-drug		60 days post-drug	
	Trial 1	Trial 2	Trial 1	Trial 2
Control	(1/10 killers)			
208C	60*	15	13	71
Ethanol	(4/10 killers)			
226E	60	14	15	16
227E	no kill	120	no kill	no kill
233E	360	15	15	10
234E	60	15	13	13
Cannabis	(7/10 killers)			
211M	15	5	6	5
212M	480	25	10	4
213M	15	12	12	8
216M	12	10	15	10
217M	16	10	15	14
218M	no kill	no kill	60	15
219M	15	10	18	14

* Seconds

In most cases, the latency-to-attack decreased as the animals gained mouse-killing experience. Five of the six cannabis rats which were muricidal on the first test killed in less than 16 sec. All of the muricidal control and ethanol rats took at least 60 sec to attack on the first test.

Novakova *et al.*[13] reported that spontaneously muricidal rats learned an avoidance response more slowly than non-killers did. We therefore compared the mean number of

days to criterion on the moving belt test of all our non-muricidal rats (7.00 ± 0.53) with the mean of all the killers (10.00 ± 0.98). The latter took significantly longer than the former ($p < 0.005$ by Student's one-tailed t-test). Within each treatment group separately, muricidal rats also took more days to reach criterion than the non-muricidal ones did, but because of small n values the differences approached but did not attain statistical significance ($p < 0.075$ for ethanol and cannabis groups).

ELECTROPHYSIOLOGICAL AND HISTOLOGICAL STUDIES

On completion of the second muricide test, the rats were studied for EEG alterations. Three bipolar stainless-steel electrodes were implanted stereotaxically in the brain of each rat under pentobarbital anesthesia. The implantation sites included anterior cerebral cortex, dorsal hippocampus, anterior thalamic nucleus, septum, mammillary bodies, dorsomedial nucleus of the thalamus, amygdala and mesencephalic reticular formation. A different combination of three sites was used in each rat, so that each site was sampled at least three times in each treatment group.

Two weeks after recovery from the operation, EEG activity was recorded from each lead in the conscious unrestrained animals. Repeated recordings were made on five consecutive days. During the recording session, the animal was observed on a television monitor, and recordings were made only when the animal was awake but at rest. A total of 6 controls, 5 ethanol and 7 cannabis rats completed the recording sessions.

Figure 2 illustrates the typical cortical and dorsal hippocampal activity patterns recorded in the three groups. The most striking finding was the presence of irregular high-amplitude spike-like waves in the hippocampal tracings of the cannabis rats, and in both the cortical and hippocampal tracings of the alcohol rats. Occasional similar waves were seen sporadically in tracings from amygdala and medial septum in some of the ethanol animals. Other recordings appeared normal. There was a tendency toward slowing of the basic wave pattern in cortical and limbic system leads in both ethanol and cannabis animals, but this has not been analysed quantitatively.

HISTOLOGICAL PILOT STUDIES

Four months after the last drug or placebo administration, the surviving animals were anesthetized and the brains were perfused *in situ* with a 10% formalin-saline solution. No difference in gross weight of the brain was found; the mean weights were 2.31 ± 0.08 g for controls, 2.31 ± 0.04 g for the cannabis group and 2.28 ± 0.03 g for the ethanol group.

Coronal sections stained with hematoxylin and eosin showed no apparent group differences in either cell density of the cerebral cortex and hippocampus, or cortical thickness.

Companion tissue blocks were stained with Golgi's silver stain, as modified by Mehraein *et al.*[14]. Sections from the stained and dehydrated blocks were cut from an area corresponding to 3–4 mm posterior to the bregma. All slides were coded and assessed independently by three examiners who rated the abundance of dendritic spines in the cerebral cortex and hippocampus on a scale of 0-4. The results suggested a reduction in dendritic spines in the cortex of both drug groups, a reduction in the hippocampus of the ethanol

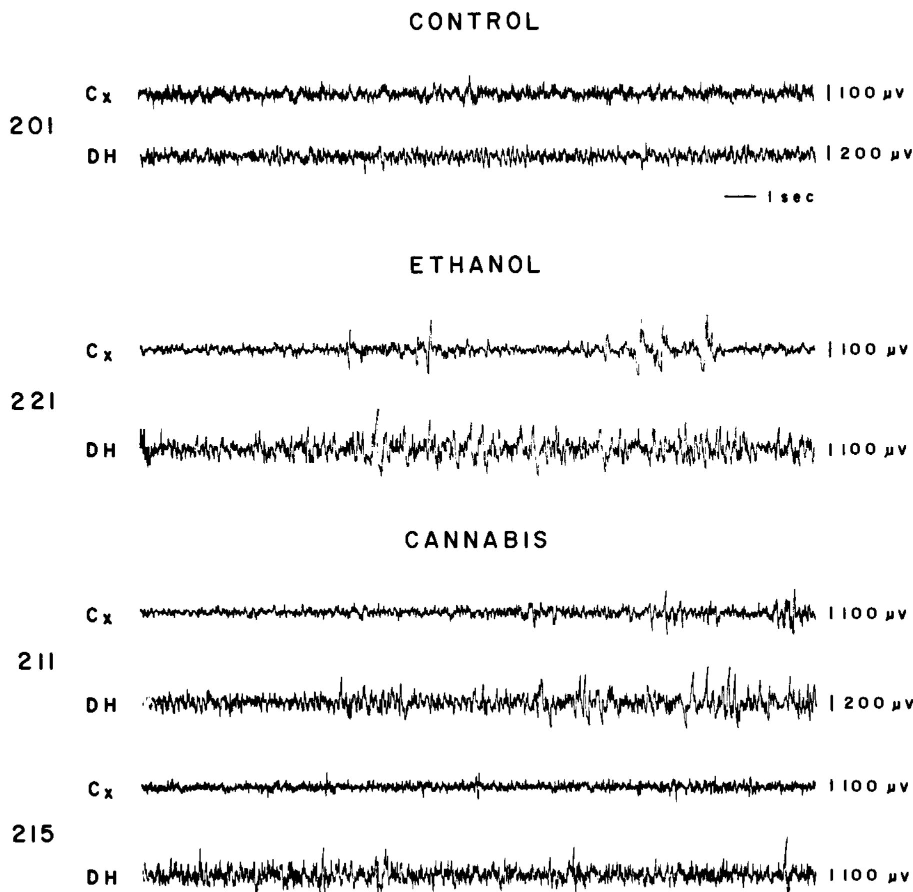


FIG. 2. Typical spontaneous electrical activity recorded via chronically implanted electrodes in cerebral cortex (Cx) and dorsal hippocampus (DH), in representative rats of the groups shown in Fig. 1. All recordings were made with the animals in the waking unrestrained state, but at rest. Calibrations as shown individually.

group, and an increase in the hippocampus of the cannabis group. These possible changes were not statistically significant.

PRENATAL EXPOSURE STUDIES

Twenty female Wistar rats, weighing 220–275 g, were obtained from the supplier in their sixth day of pregnancy. They were caged singly, and had free access to standard laboratory rat chow and water. From day 8 to day 12 of gestation, ten of the rats received daily gastric intubation of cannabis extract in a dose providing 10 mg of THC per kg, dissolved in 0.5 ml of olive oil per kg. The other ten received the same volume of olive oil alone.

The control mothers gained weight continuously and normally throughout their pregnancy. In contrast, the cannabis-treated rats did not gain any weight during the five-day drug treatment period, but subsequently gained at the same rate as the controls, so that they had not caught up to the controls at parturition. None of the offspring of the cannabis

group showed any gross externally visible structural anomalies at birth, and their birth weights (6.40 ± 0.27 g) were indistinguishable from those of the controls (6.45 ± 0.20 g).

Only litters born on day 22 were accepted for the study, to ensure that the drug and vehicle treatments had been carried out at the same stage of fetal development in all cases. There were nine such litters, four control and five cannabis-treated. One randomly-selected cannabis litter was excluded, and the remaining eight litters were randomly culled, if necessary, to six pups each, since litter size can affect maternal behavior toward the young[15]. This selection procedure yielded 19 cannabis and 18 control offspring. Two of the four litters in each treatment group were transferred for cross-fostering by mothers of the opposite treatment group, to control for treatment effects on maternal behavior toward the young. The remaining two litters in each treatment group were exchanged between the respective mothers for within-treatment fostering, to control for possible non-treatment-specific effects of separation from the biological mother.

At day 65, all the offspring were healthy and exhibited no obvious unusual behavior. The mean weight of the females was 165.8 ± 7.5 g, and of the males, 235.8 ± 9.3 g; there was no weight difference between cannabis-treated and control offspring. Training on the Hebb-Williams maze[16], as modified by Rabinovitch and Rosvold[17], was started on day 65. As in the earlier study[1], trained rats were then given at least nine runs on each of 12 maze problems arranged in order of increasing difficulty. The first run on each problem was considered a learning trial and was not scored. The next eight runs were scored for errors[1], and the cumulative error on the total 96 scored runs was recorded. In addition, the number of runs needed to attain a criterion of 3 error-free runs out of 4, to a maximum of 20 runs per problem, was also recorded.

Table 2 shows the results of the maze testing. Because of problems caused by unequal group sizes, the results were analysed in two stages. A two-way analysis of variance showed no significant main effect of treatment or sex, and no significant treatment × sex interaction. Since the sex of the animals had no significant effect, the results for males and females of each fostering group (i.e., fostered or cross-fostered) were pooled for a different two-way analysis of variance (treatment, fostering). Again there was no significant main effect of treatment, but animals that were fostered by mothers of their own treatment group per-

TABLE 2. PERFORMANCE OF RATS ON THE MAZE TEST AFTER PRENATAL TREATMENT

	Number of errors		Number of runs to criterion	
	Male	Female	Male	Female
Control				
Fostered	90.5 ± 7.5* $n = 6$	81.5 ± 6.7 $n = 4$	99.8 ± 6.4	89.3 ± 3.3
Cross-fostered	91.3 ± 10.7 $n = 3$	105.0 ± 8.8 $n = 5$	102.7 ± 8.1	90.6 ± 6.3
Cannabis treated				
Fostered	71.5 ± 7.6 $n = 6$	80.0 ± 10.6 $n = 4$	92.0 ± 5.6	90.3 ± 3.6
Cross-fostered	87.2 ± 4.6 $n = 5$	115.5 ± 13.3 $n = 4$	100.0 ± 2.6	111.0 ± 5.3

* Score ± S.E.

formed significantly better than those cross-fostered by mothers of the opposite treatment group ($F = 7.89$; d.f. = 1.33; $p < 0.01$). The interaction of treatment × fostering was not significant.

A similar analysis of the runs-to-criterion scores was carried out. Again, the only clearly significant difference was that fostered animals needed fewer runs than the cross-fostered animals ($F = 26.6$; d.f. = 1.33; $p < 0.01$).

A second experiment, using a higher dose of cannabis extract providing 20 mg of THC per kg, also failed to reveal any significant main effect of drug vs. control treatment.

DISCUSSION

The results of this experiment confirm our earlier finding[1] that prolonged treatment with high doses of cannabis or of ethanol produces significant impairment of certain learning abilities in the rat, that lasts long after the end of the drug treatment. Holding the rats at constant body weight, in better proportion to the width of the moving belt, resulted in lower variability within groups, and greater statistical significance of the between-group differences, than in the previous study.

Radouco-Thomas *et al.*[18] have also reported residual effects of THC (10 mg/kg i.p. daily, for up to 40 days) on the ability of mice to acquire a shuttle-box avoidance task. Compared to the controls, THC-treated mice showed a greater loss of performance from the last trials of one test day to the first trials of the next, suggesting impaired retention of learning. The difference in intersession loss was even more marked after an interval of 100 days between tests.

The significantly increased proportion of rats showing muricidal behavior after the end of cannabis treatment is also consistent with the findings of others. Our observation of 70% incidence agrees very closely with the 70% observed by Miczek[4] and 80% by Alves and Carlini[3]. An important difference, however, is that these authors first tested for muricidal behavior *during* the chronic drug treatment. By not testing until long after the end of drug treatment, we have avoided the possibility of conditioning of an intoxication effect, and can therefore conclude that this behavior indicates a residual change in brain function.

Prolonged isolation can produce muricidal behavior in Wistar King A female rats[2]. However, even after 7 months of isolation, only one of our 10 controls killed mice, so that the difference between groups must be attributed to the drug treatments. Alves and Carlini[3] reported that 22 hr of food deprivation daily increased the incidence of muricidal behavior in cannabis-treated rats. Since neither the weight of their rats nor the food consumption in the 2 hr feeding period are reported, it is difficult to compare their conditions with the moderate food restriction in our study. Nevertheless, it is possible that post-cannabis rats fed *ad libitum* might have shown less muricidal behavior.

Our results are consistent with the report by Novakova *et al.*[13] that mouse-killing rats were less able than non-killers to learn an avoidance response. All three animals (one cannabis and two ethanol) that failed to reach criterion on the moving belt test by 15 days were killers, and there was a clear difference in learning speed between all the killing and non-killing rats regardless of treatment. It seems likely that with more animals per group there would have been a significant learning difference between killers and non-killers

within each treatment group. The mechanism of the association between mouse-killing behavior and slow learning is unknown, but the muricide test results provide an excellent independent confirmation of the learning tests in these animals. Possibly rats that perform in a stereotyped manner, such as that involved in mouse-killing, are less able to adapt and thus to learn new behaviors.

The EEG finding of "epileptiform" spikes in the hippocampal tracings is consistent with pilot observations in our earlier study[1] and with similar findings by Heath[19] in rhesus monkeys subjected to long-term treatment with very large doses of cannabis. The combination of electrical anomalies in the hippocampus and impaired acquisition of the moving belt test performance is consistent with other evidence of learning impairment in animals with hippocampal lesions[20, 21].

The lack of treatment effects on brain weight is consistent with observations by several other groups[22–24], with the lack of any histological evidence of cortical atrophy in our animals, and with recent reports of normal tomographic scans in humans who were regular users of cannabis[25, 26]. It is rather surprising that the Golgi-stained sections suggest a loss of dendritic spines in the cortex and hippocampus of the chronic ethanol rats, but not in the cannabis group, even though both groups had equivalent impairment of learning and hippocampal EEG changes, and the cannabis group had a higher incidence of muricidal behavior. The present studies, however, employed formalin-fixed rather than fresh tissue for the Golgi stain, and the results are not properly quantitative. Therefore they should merely be considered sufficiently interesting to justify systematic study. It is not yet clear whether a change in dendritic spines demonstrable by Golgi stain can be related to electron microscopic alterations in axon terminals found in cannabis-treated monkeys[27].

Treatment of pregnant rats with the same dose of cannabis extract, but for a much shorter time, failed to produce any detectable impairment of maze learning in the offspring. Our findings are not in agreement with those of Gianutsos and Abbatiello[5]. The difference might be explained by differences in the route of drug administration, or in the type of maze used. The possible role of difference in dose cannot be assessed, since they did not state the dose used. Although THC is known to cross the placenta and to have an affinity for the fetal CNS[28], it seems likely that the fetal brain is not particularly sensitive to the drug during days 8 to 12 of pregnancy. This is the critical time for overt neurological teratogenicity in the rat[29], but learning and other relatively subtle behavioral deficits could possibly be caused at a later stage of gestation. Alternatively, it may simply be that a much longer period of drug exposure is needed to affect learning ability on our tests.

The importance of fostering and cross-fostering in studies of prenatal influences on behavior is well established[30, 10]. The offspring may be affected by residual drug effects on maternal behavior rather than by direct intrauterine actions of the drug itself. The significant finding in the present study, however, is that the offspring of both cannabis and control groups did worse when cross-fostered than when fostered by mothers in the same treatment group. This suggests that the mothers were somehow able to distinguish between treated and untreated offspring. To establish this point clearly, two further types of study are needed. One is a much larger-scale replication of this work, using the litter rather than the individual pup as the statistical unit. The other type would involve split-litter techniques, with mixed (half control, half drug-treated) litters assigned to each mother. An identifiable difference between the offspring suggests that more sensitive methods might indeed show residual behavioral effects of intra-uterine cannabis exposure. Much more extensive investigation is needed to permit a conclusion.

Résumé. Des jeunes rats mâles ont reçu par gavage quotidien des hautes doses d'extrait de cannabis (THC 20 mg/kg), d'éthanol (6 g/kg) ou de solution contrôle, pendant 6 mois. Un mois après la fin du traitement, les animaux traités à l'éthanol ou à l'extrait de cannabis étaient significativement plus lents, et faisaient plus d'erreurs, durant l'apprentissage d'un tâche psychomoteur. Ils ont aussi montré une fréquence plus élevée de muricide dans des épreuves effectuées à un et deux mois après la fin du traitement. Les mêmes groupes traités à l'éthanol et à l'extrait de marihuana manifestaient des "spikes", d'occurrence irrégulier, dans les tracés électriques dérivés de l'hippocampus dorsal. Un examen histologique exploratoire a suggéré une diminution du nombre d'épines dendritiques dans l'hippocampus et le cortex des rats traités à l'éthanol, mais pas chez ceux traités à l'extrait de cannabis.

Par contre, l'administration de doses comparables de cannabis (THC 10 ou 20 mg/kg par jour) aux femelles enceintes, pendant la phase critique du développement du système nerveux foetal (les jours 8 à 12 de gestation), n'a été suivie d'aucune altération de l'apprentissage des problèmes de labyrinthe par les jeunes. Cependant, les mères pouvaient apparemment distinguer les jeunes traités à cannabis *in utero* des jeunes contrôles, ce qui suggère l'eventuelle existence d'une subtile difference comportementale produite par le traitement.

REFERENCES

1. FEHR, K. A., KALANT, H., LEBLANC, A. E. and KNOX, G. V., Permanent learning impairment after chronic heavy exposure to cannabis or ethanol in the rat. "Marihuana: Chemistry, Biochemistry, and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 495–505, 1976.
2. UEKI, S., FUJIWARA, M., and OGAWA, N., Mouse-killing behavior (muricide) induced by Δ^9-tetrahydrocannabinol in the rat. *Physiol. Behav.* **9**,585–587 (1972).
3. ALVES, C. N. and CARLINI, E. A., Effects of acute and chronic administration of Cannabis sativa extract on mouse-killing behavior of rats. *Life Sci.* **13**,75–85 (1973).
4. MICZEK, K. A., Mouse-killing and motor activity: Effects of chronic Δ^9-tetrahydrocannabinol and pilocarpine. *Psychopharmacology* **47**,59–64 (1976).
5. GIANUTSOS, G. and ABBATIELLO, E. R., The effect of pre-natal cannabis sativa on maze learning ability in the rat. *Psychopharmacologia* **27**,117–122 (1972).
6. BORGEN, L. A., DAVIS, W. M. and PACE, H. B., Effects of prenatal Δ^9-tetrahydrocannabinol on the development of rat offspring. *Pharmacol. Biochem. Behav.* **1**,203–206 (1973).
7. UYENO, E. T., Δ^9-tetrahydrocannabinol administration during pregnancy of the rat. *Proc. West. Pharmacol. Soc.* **16**,64–67 (1973).
8. SASSENRATH, E. N. and CHAPMAN, L. F., Tetrahydrocannabinol-induced manifestations of marihuana syndrome in group-living macaques. *Fed. Proc.* **34**,1666–1670 (1975).
9. VARDARIS, R. M., WEISZ, D. J., FAZEL, A. and RAWITCH, A., Chronic administration of delta-9-tetrahydrocannabinol to pregnant rats: studies of pup behavior and placental transfer. *Pharmacol. Biochem. Behav.* **4**,249–254 (1976).
10. FRIED, P. A., Short- and long-term effects of pre-natal cannabis inhalation upon rat offspring. *Psychopharmacology* **50**,285–291 (1976).
11. LEBLANC, A. E., KALANT, H., GIBBINS, R. J. and BERMAN, N. D., Acquisition and loss of tolerance to ethanol by the rat. *J. Pharmacol. Exp. Ther.* **168**,244–250 (1969).
12. FERGUSON, G. A., "Statistical analysis in Psychology and Education" (2nd edition). McGraw Hill, New York, 1966.
13. NOVAKOVA, U., FLANDERA, V. and SANDRITTER, W., Aggressive rats—some properties of learning, memory and the limbic system. *Pharmacol. Biochem. Behav.* **2**,729–733 (1974).
14. MEHRAEIN, P., YAMADA, M. and TARNOWSKA-DZIDUSZKO, E., Quantitative study on dendrites and dendritic spines in Alzheimer's disease and senile dementia. *Adv. Neurol.* **12**,453–458 (1975).
15. GROTA, L. J., Effects of litter size, age of young, and parity on foster mother behavior in Rattus Norvegicus. *Anim. Behav.* **21**,78–82 (1973).
16. HEBB, D. O. and WILLIAMS, H., A method of rating animal intelligence. *J. Genet. Psychol.* **34**,59–65 (1946).
17. RABINOVITCH, M. S. and ROSVOLD, H. E., A closed field intelligence test for rats. *Can. J. Psychol.* **5**,122–128 (1951).
18. RADOUCO-THOMAS, S., MAGNAN, F. and RADOUCO-THOMAS, C., Pharmacogenetic studies on cannabis and narcotics: Effects of Δ^1-tetrahydrocannabinol and morphine in developing mice. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 481–494, 1976.
19. HEATH, R. G., Cannabis sativa derivatives: effects on brain function of monkeys. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas *et al.*, eds.). Springer-Verlag, New York, pp. 507–519, 1976.
20. SCHMALZ, L. W., Deficit in active avoidance learning following penicillin injection into hippocampus. *Physiol. Behav.* **6**,667–674 (1971).

21. ISAACSON, R. L., The hippocampus. "The Limbic System." Plenum Press, New York, 1974.
22. MAZURKIEWICZ-KWILECKI, I. M. and FILCZEWSKI, M., The effects of chronic treatment with Δ^9-tetrahydrocannabinol on catecholamine synthesis in the rat. *Psychopharmacologia* **33**,71–79 (1973).
23. LEITE, J. R. and CARLINI, E. A., Failure to obtain "cannabis-directed behavior" and abstinence syndrome in rats chronically treated with cannabis sativa extracts. *Psychopharmacologia* **36**,133–145 (1974).
24. ROSENKRANTZ, H., SPRAGUE, R. A., FLEISCHMAN, R. W. and BRAUDE, M. C., Oral Δ^9-tetrahydrocannabinol toxicity in rats treated for periods up to six months. *Toxicol. Appl. Pharmacol.* **32**,399–417 (1975).
25. CO, B. T., GOODWIN, D. W., GADO, M., MIKHAEL, M. and HILL, S. Y., Absence of cerebral atrophy in chronic cannabis users—Evaluation by computerized transaxial tomography. *J.A.M.A.* **237**,1229–1230 (1977).
26. KUEHNLE, J., MENDELSON, J. H., DAVIS, K. R. and NEW, P. F. J., Computed tomographic examination of heavy marijuana smokers. *J.A.M.A.* **237**,1231–1232 (1977).
27. HARPER, J. W., HEATH, R. G. and MYERS, W. A., Effects of cannabis sativa on ultrastructure of the synapse in monkey brain. *J. Neurosci. Res.* **3**,87–93 (1977).
28. KENNEDY, J. S. and WADDELL, W. J., Whole body autoradiography of the pregnant mouse after administration of C^{14}-Δ^9-THC. *Toxicol. Appl. Pharmacol.* **22**,252–258 (1972).
29. WARKANY, J., The role of sensitive or critical periods in teratogenesis. *Proceedings of the Conference on Drugs and Poisons as Etiological Agents in Mental Retardation* (G. M. McKhann and S. J. Yaffe, eds.). U.S. Department of Health, Education and Welfare, Public Health Service, No. 1791 (1967).
30. JOFFE, J. M., Environmental agents. II. Drugs. "Prenatal Determinants of Behavior." Pergamon Press, Oxford, New York: 1969.

SOCIAL BEHAVIOR OF RHESUS MONKEYS CHRONICALLY EXPOSED TO MODERATE AMOUNTS OF DELTA-9-TETRAHYDROCANNABINOL

L. F. CHAPMAN, E. N. SASSENRATH, and G. P. GOO

Departments of Behavioral Biology, Psychiatry, and Neurology, School of Medicine, University of California Davis,

and

California Primate Research Center, Davis, California 95616

Summary. The actions of delta-9-THC (2.4 mg/kg) on the social behavior of rhesus and fascicularis macaque monkeys were studied following daily oral administration for periods up to several years. With the treatment schedule and dosage employed, sedative effects (reduced social interaction, relaxed postures, reduced general activity, ptosis) predominated for the first 2–3 months; a few animals intermittently exhibited a form of behavioral activation with increased pacing and other repetitive stereotyped behavioral patterns. With the treatment schedule held constant, tolerance to these behavioral effects was apparent thereafter. As these acute effects diminished, irritability and aggressiveness in treated animals increased significantly, this behavioral change was apparently not a withdrawal effect. At this stage, previously lower-ranked animals began to rise in the dominance hierarchy; overt aggression increased in treated animals and submissive behavior increased in non-treated cagemates. Under high stress condition (adjustment to new social groupings), previously lower-ranked THC-treated animals initiated overt fighting episodes leading to elevated stress hormone levels in undrugged cagemates and injuries.

These observations indicated that repeated exposure to moderate amounts of THC over long periods resulted in a biologically serious impairment in the ability to exhibit appropriately adaptive behavior. This impairment apparently represented a shift in baseline behavioral characteristics rather than the effects of acute intoxication or withdrawal.

INTRODUCTION

CANNABIS is of intrinsic biological interest because of its potent psychoactive actions and its somewhat unusual combination of sedating and stimulating effects. There is also widespread interest in cannabis and related substances from the point of view of assessment of potential human health hazards associated with their use. A well recognized potential risk associated with potent psychoactive agents is the possibility that repeated exposure over prolonged periods may lead to tolerance and a gradual increase in amounts used, as well as to addiction or dependence and associated withdrawal symptomatology. It is now clear that tolerance and at least mild withdrawal symptoms do appear when usage is frequent

and relatively heavy[35, 58]; it is also apparent that with occasional exposure to modest amounts there is little dependence liability[60].

An additional behavioral-psychological risk common among psychoactive agents is that prolonged use may lead to adverse changes in baseline personality, mentation and behavior; a familiar extreme example would be the mental and social deterioration of chronic severe alcoholism, in which behavioral problems apparently represent cumulative and persistent personality changes extending beyond periods of acute intoxication or withdrawal. The present studies are directed towards providing information regarding potential behavioral risks of this latter type.

A number of clinical reports of persistent psychological abnormalities in at least some human subjects who have chronically been exposed to large amounts of cannabis have appeared[4, 5, 7, 13, 38, 41, 46, 53, 56, 57, 59, 60, 73, 77]. Each is difficult to assess for a number of reasons. One of the more difficult problems in assigning an etiological role to cannabis use in the subjects of these studies is recognizing and controling sampling errors. The population pool from which subjects are drawn has usually been initially based on the presence of psychological abnormalities. From this pool, subjects were then identified who had a history of chronic cannabis use. In these circumstances, it is difficult to exclude the possibility that the index cases simply represent a sample of those individuals in whom cannabis use and psychological abnormality overlap. A related problem is whether or not certain pre-existing psychological abnormalities predispose to subsequent cannabis use. Thus, while there are a number of reports suggesting that chronic cannabis use leads to persistent undesirable psychological changes, the evidence is inconclusive; most, if not all such claims have been challenged[14, 39, 40, 44, 50, 65]. It should be kept in mind, however, that the inconclusiveness of these clinical reports does not offer evidence supporting the absence of such risks.

There are also several studies[6, 10, 15, 16, 23, 48, 63, 71, 74, 76] of psychological assessment of population pools initially identified solely on the basis of chronic heavy cannabis usage. In general, these studies have failed to observe a significantly higher rate of undesirable psychological abnormalities in these heavy users as compared with a control group of non-users; a few have reported a significantly higher frequency of psychosis and psychopathic personality patterns in cannabis users[71]. Again, methodological considerations cloud the issue. Does the initial presence of psychological abnormality bias the sample towards greater probability of cannabis usage? Are the psychological measures sensitive enough to detect meaningful abnormalities? Are the results from these subject populations (largely lower socioeconomic groups in relatively underdeveloped nations) applicable to individuals in highly developed nations? This category of "negative" clinical evidence also remains inconclusive.

A relatively large number of studies, recently reviewed by Miller and Drew[54], have been concerned with behavioral effects of cannabis on laboratory animals. The species studied have largely been limited to rodents (although some studies have used pigeons or non-human primates), most studies have been concerned with acute actions or the effects of short-term repeated administration. Several generalizations can be made, although many findings remain controversial and differences in methodology often make comparisons of various studies difficult or impossible.

A reduction in spontaneous activity, sometimes accompanied by a phase of excitement or stimulation, is reported in a number of studies. Potvin and Fried[61] reported that chronic THC administration in rats leads to hyperemotionality.

Observations on acquisition (learning) of avoidance-motivated behavior have yielded inconsistent findings; there is evidence that THC can both facilitate avoidance acquisition and also impair expression of already well established avoidance responses[58]. A number of other studies have focussed on reinforcement (reward) motivated learning, examining the effects of variation in schedules of reinforcement. Again, generalizations are difficult. Some studies failed to observe decrements in learned responses (or their rate of acquisition) when cannabis was given; for example, Ferraro *et al.*,[21] found no impairment in accuracy of performance of trained chimpanzees during various reinforcement schedules. Other studies using complex discrimination tasks have found decrements in performance during cannabis intoxication[2, 22, 78].

A number of studies have been concerned with possible impairment of behavior that is critically dependent upon memory functions; although not definitive, some experiments support the conclusion that acute cannabis administration can impair certain aspects of memory.

While in humans feelings of increased hunger are commonplace in reports of recreational use of marijuana, animal studies have not revealed clear-cut increases in food or water intake after cannabis administration; in fact, a number of studies have observed a reduction.

A great deal of interest has focused on the question of possible increases in aggressive behavior. Ancient folklore has held that cannabis was used by various warriors before battle, presumably to increase aggression. Working with rats, Carlini in the 1960's[8, 9] observed an increase in aggressiveness in food-deprived animals. Several subsequent workers have failed to observe increased aggressiveness. Various factors, including the specific type of aggression (spontaneous, predatory, or "irritable"), the presence or absence of various types of aggression-influencing circumstances (social isolation, electrical shock, intraspecies territorial intrusions), differences in species, differences in dosage schedule and duration of drug administration, etc., have made conclusions uncertain and disputed. Environmental and social factors are apparently important. Ely and associates[18] emphasized the significance of the dominant-submissive status of individuals in their response to cannabis, Miczek and Barry[51] found that acute administration of THC decreased attack and threat behavior in dominant members of rat pairs. Also, THC impaired defense-submissive behavior patterns which minimized injury in spontaneous intraspecies fighting; thus, THC-treated submissive animals received more serious injuries than control submissive animals. Miczek recently[52] expanded his series to include mice, rats, and squirrel monkeys; he concluded that acute THC decreases species-specific attack behavior in amounts that induce no other grossly observable changes in behavior (up to 2.0 mg/kg). In studies with squirrel monkeys, introduction of an unfamiliar animal induced intense aggression in resident animals; THC reduced the intensity of this aggression in a dose-dependent fashion.

The effects of chronic cannabis administration on aggression has been studied only by a few investigators[8, 9, 18, 51, 52, 74]. Carlini and associates[8, 9] have reported in several studies that chronic administration, coupled with food deprivation or sleep deprivation predictably increase aggressiveness in rats.

The studies of the actions of cannabis on aggression in laboratory animals remain controversial. In general, most studies have supported the conclusion that a reduction in aggression is commonly found as an acute action under non-stressed conditions; there is also evidence for increased aggressiveness in chronically treated animals under stress.

A relatively small number of behavioral studies with laboratory primates has appeared[1, 2, 11, 12, 17, 19, 20, 21, 22, 26, 27, 30, 33, 34, 37, 42, 43, 45, 55, 66, 67, 69, 70, 72, 78]. Most of these have been limited to observations with a small number of animals over a reasonably brief period (months). For example, in 1971, Cole, Pieper and Rumbaugh[11] studied two chimpanzees and one orangutan exposed to marihuana smoke. Using an operant conditioning model as a method of behavioral assessment, they found that one animal consistently responded at a higher rate during the period of drug administration; the second showed a similar but smaller change and the third showed no change.

Harris, Waters and McLendon[30] in 1972 observed three rhesus monkeys exposed to repeated intravenous administration of delta-9-THC over a 30-day period. On this schedule they found rapid tolerance to the actions of the drug on shock avoidance behavior and a minor degree of tolerance as assessed by gross behavioral changes.

In 1974, Stadnicki, Schaeppi, Rosenkrantz and Braude[70] over a 50 day period observed the actions of a crude marihuana extract (administered by oral gavage) on 3 rhesus monkeys. The amounts of marihuana extract used were relatively large (i.e., delta-9-THC equivalents from 12.5 mg/kg to 300 mg/kg); two monkeys received the equivalent of 37.5 mg/kg delta-9-THC for 50 days. At this time behavioral tolerance to the drug had developed; abrupt withdrawal on this schedule also led to aggressive behavior and evidence of " hallucinations ".

Giono-Barber, Paris, Bertuletti and Giono-Barber[26, 27], also in 1974, reported findings on two baboons (*Papio papio*) studied in a setting which allowed observation of competitive behavior for a favored food (peanuts). Hashish was administered orally to both animals in amounts equivalent to 1.75 mg/kg of THC for 5 weeks. In the dominant animal of the pair, they observed less competitive behavior for food during the initial period of exposure to the drug, followed by increased aggression and dominance (in competition for food). During the first week, the less dominant animal appeared less inhibited in competing for food; during the subsequent weeks it exhibited increased aggression and continued to exhibit less submissive behavior. They noted that a rapid tolerance to the depressive effects of cannabis occurred in this dosage range, while increased aggression and excitability persisted. They also observed a rapid return to the pre-drug pattern when the drug was stopped after the 5 week period. Levett and associates[42] studied two established baboon pairs; they observed contrasting effects of crude marihuana, related to dominance relationships, on locomotor activity and social interaction.

The goal of the present studies has been to examine the long-term effects on social behavior of chronic administration of THC to laboratory primates (macaque monkeys) under conditions which duplicated as closely as possible a pattern of long continued moderately heavy human use. This report summarizes some of the generalizations apparent from these behavioral studies with rhesus monkeys; a more complete quantitative treatment is in preparation.

METHODS

ACUTE ADMINISTRATION

Nine subadult (aged 2 to 3 years) rhesus macaques (*M. mulatta*) and 6 sub-adult (aged $2\frac{1}{2}$ to $3\frac{1}{2}$ years) crab-eating macaques (*M. fascicularis*) were housed in mixed sex cage

groups of 3 animals each. Groups were maintained in standard Labcare cages measuring $2\frac{1}{2} \times 2\frac{1}{2} \times 3$ feet on a 12-hour light-dark cycle with feedings of monkey chow twice daily and free access to water. All cage groups were formed 2 to 3 months prior to start of behavioral observations.

In 2 of the 3 rhesus cage groups (i.e., A and B), Δ^9-THC was administered at levels of 0.6, 1.2, and 2.4 mg per kg per day to permit assessment of dose-response relationships for social interaction. This dose range can be calculated to be equivalent to smoking 1 to 3 marihuana cigarettes per day in man, correcting for differences in route of administration and body surface area[24]; recent studies of blood levels suggest it may represent an even smaller marihuana cigarette equivalent[3].

THC was administered to each individual member of the groups at weekly intervals in a randomized sequence of recipients and dosages; this permitted repeated measurement of baseline (undrugged) behavior, and definition of responses to the various dosage levels. THC was given orally on preferred food in single daily administrations at 0800 or 0830 hours each day, followed by behavioral observations at intervals of 1, 3, and 5 and 25 hours after drugging, to permit assessment of time-response relationships.

A third rhesus cage group (C) and two crab macaque groups (D and E) received THC under similar conditions at a single dose level of 2.4 mg per kg. Pre- and post-drug behavioral baseline data were collected for each group during the weeks preceding and following THC administration.

At the conclusion of the 6 weeks' period of single individual group member drugging, all members of groups A and B were drugged simultaneously at 2.4 mg per kg on 2 alternate days to permit contrast of individual response to drug in the presence of drugged *vs.* non-drugged social environment.

The 2.4 mg/kg level was chosen for further studies since it was a near-threshold dose that predictably resulted in a pattern of readily observable behavioral changes (relaxed posture, reduced activity, ptosis, etc.) which resembled the pattern observed in humans after definite but moderate intoxication.

SHORT-TERM CHRONIC STUDY

At the conclusion of the above acute study, the # 1 ranking member in Group A and the #2 ranking member in group B were given 2.4 kg/kg Δ^9-THC for 10 consecutive days. Behavioral observations were made for 5 days pre- and post-drugging and on days 1 through 4, and 7 through 10 of THC administration.

LONG-TERM CHRONIC STUDY

Thirteen rhesus macaques (*M. mulatta*) and 7 crab macaques (*M. fascicularis*) were housed in indoor cages measuring $2\frac{1}{2} \times 4 \times 3$ feet in mixed sex groups of 3 or 4 members each as described previously[8]. All animals were between 2 and $3\frac{1}{2}$ years of age at the start of the study. In each of these groups, one group member was drugged daily on a continuing basis.

An additional 12 rhesus macaques aged 3 to $3\frac{1}{6}$ years were housed in outdoor cages of

10 × 10 × 8 feet in 2 groups of 3 males and 3 females each. In each of these groups, one male and one female were drugged daily.

In both indoor and outdoor cage groups, the drug-treated group members were selected to represent both males and females, as well as both dominant and subordinate social ranks: the overall distribution of drugged subjects was 6 males and 4 females, with 3 of #1 rank, 4 of #2 rank, and 1 each of ranks #3, #4, and #5.

During the first year of the long-term study, THC was administered orally at 2.4 mg/kg once daily at 1500 hours. Each day, behavioral observations were made at 0900 and 1000 hours or at 18 and 19 hours *after* drug administration on the previous day. This interval was interposed to distinguish "residual" drug effects of long-term drug exposure from the immediate effects of each day's drugging.

During the second year of observation of the behavioral effects of daily drugging, the time of drugging was changed for 3-month intervals to 0800 and 1200 hours (as shown in Figure 3) with behavioral observations maintained at 0900 and 1000 hours. This allowed observation of intervals between drug intake and behavioral assessments of 1 and 2 hours and 21 and 22 hours.

NEW CAGE-GROUP FORMATION

After 3 to 4 years of daily THC administration, 4 adult THC-treated females and 4 control females from the previous cage groups were caged individually for 6 months and then regrouped for breeding purposes into 2 outdoor cage groups of 5 females and 1 male each, with 2 THC-treated and 2 or 3 control females in each group. Social interaction behavior and plasma cortisol levels of all females were monitored before and during the 2 months following group formation.

BEHAVIORAL ASSESSMENT

Characterization of behavioral profiles of individuals within a group-living environment was done by standardized observational procedures which used a repertoire of over 50 behaviors to define dyadic interactions as they occurred sequentially during the observation period (d). All behavior was observed and recorded under rigidly standardized procedures in the home cage setting under 2 conditions: during intervals of spontaneous (non-stimulated) interaction, and during competitive interaction elicited by timed interval presentation of small pieces of preferred food.

The observation sessions were standardized with respect to time of day and interval after drug administration. In the acute study, each cage was observed a total of 2 hours each day for 5 days per week. In the long-term chronic study, each cage was observed during alternating AM and PM sessions on a continuing basis, so that for each group, comparable 20- or 30-hour blocks of observational data were collected during each sequential 3-month interval throughout the treatment period.

Significant changes in behavior from pre-drug baseline levels were determined by comparing ranges and consistencies of daily frequencies of each behavioral index. More detailed analysis of repeated samples over time and multiple regression statistics will be reported elsewhere.

RESULTS

Although detailed analysis of the complex behavioral data is not possible here, certain aspects can be summarized as follows:

ACUTE OR SHORT-TERM EXPOSURE TO THC

Under conditions in which THC was given at weekly intervals (in the acute study) or on ten consecutive days (in the short-term chronic study), the predominant drug effect was sedative with reduction of activity and state of arousal. The degree and duration of action varied with the dosage; at 2.4 mg per day, peak effects were observed 2 to 3 hours after drug intake. Lower dose levels showed lesser and briefer behavioral changes in fewer indices.

Active *vs.* inactive behaviors: Table 1 shows that 60 to 100 percent (3/5 to 5/5) of all animals at all ranks showed a decrease in active behavior such as exploration of the cage environment and cage shaking "displays". At the same time, inactive behavior increased. In addition to reduced spontaneous activity, drugged animals showed partial or complete ptosis, relaxed or slumped postures, and decreased interaction with cagemates.

However, in 2 of the 15 animals observed, characteristic sedative type behavior decreased, suggesting a drug-induced stimulatory effect. Similarly, despite other evidence of sedation, active stereotypies (repetitive motor behaviors such as pacing and body flips)

TABLE 1. ACUTE ORAL THC: INDIVIDUAL DIFFERENCES IN DIRECTIONAL CHANGES IN BEHAVIORAL INDICES OF ACTIVITY AND/OR AROUSAL IN MONKEYS OF EQUIVALENT SOCIAL RANK IN FIVE 3-MEMBERED CAGE GROUPS AT 1 TO 2 HOURS AFTER ORAL INTAKE OF DRUG AT 2.4 mg PER kg.

		Number subjects showing change[a]		
Behaviors	Rank	Decrease	No Change	Increase
Active				
Exploration	#1	4/5	1/5	
	#2	4/5	1/5	
	#3	3/4	1/4	
Cage Shake	#1	5/5		
	#2	3/5	2/5	
	#3	5/5		
Active Stereotypies	#1	1/4	1/4	2/4
	#2	1/5		4/5
	#3			5/5
Inactive				
Sleep	#1		1/5	4/5
	#2	1/5		4/5
	#3	1/4	2/4	1/4
Sit/lie	#1		3/5	2/5
	#2	1/5	2/5	2/5
	#3	1/4	2/4	1/4

a. Number of animals of given rank showing indicated change relative to animals at that rank in all cage groups observed.

increased in 40, 80, and 100 percent of # 1, # 2, and # 3 ranking animals, respectively. Those individuals which showed increases in both active and inactive behavioral indices were frequently observed to alternate between stimulated (active stereotypies) and sedated states during the same half-hour observation sessions.

Affiliative behavior. Affiliative behavior between and among cagemates also changed significantly when one or all members of the group were drugged. Table 2 shows social grouping data for cage group A. These data were selected as representative examples of the findings from a larger series of observations in affiliative behavior. The drugging of a single cagemate changed the grouping patterns of all cagemates as indicated by the relative percentage of time each spent "alone", "together" with both other cagemates, or paired with one or the other cagemate.

The greatest change from baseline was seen when all group members were drugged simultaneously. When all were drugged, total group grooming or huddling (all together) disappeared entirely and partner preference in dyadic affiliations reversed for animals # 1 and # 2; animal # 3 withdrew entirely from association with cagemates. These drug-related changes appeared to represent changes in the direction of minimal social stimulation.

As shown in Table 3, at least half of the dominant # 1 and # 2 ranking group members showed a decrease in normal aggressive behaviors after drugging in both the non-stimulated home cage environment and in the food competition test situation. There were,

TABLE 2. CHANGES IN SOCIAL GROUPING (PROXIMAL PLACEMENTS) WITHIN CAGE GROUP A AFTER ACUTE ORAL ADMINISTRATION OF Δ^9-THC TO EACH GROUP MEMBER INDIVIDUALLY AND TO ALL GROUP MEMBERS SIMULTANEOUSLY.

Group member rank	Sex	THC dose (mg/kg)	Drugged member	Percent time in social grouping				
				Alone	All together	With # 1	With # 2	With # 3
# 1	M	0	# 1	31	32		6	31
		0.6	# 1	30	31		31	10
		1.2	# 1	50	0		25	24
		2.4	# 1	67	8		14	10
		2.4	All	43	0		57	0
# 2	M	0	# 2	69	25	6		<1
		0.6	# 2	37	43	10		10
		1.2	# 2	95	0	4		0
		2.4	# 2	87	3	9		0
		2.4	All	42	0	57		2
# 3	F	0	# 3	32	36	32	<1	
		0.6	# 3	91	0	6	3	
		1.2	# 3	40	23	36	1	
		2.4	# 3	30	16	53	1	
		2.4	All	98	0	0	2	

however, a few of the most dominant # 1 animals which showed an increase in the active contact aggressive behaviors (hit, bite and chase) in both the non-stimulated and competitive group situations. An interaction of drug effects with social factors is revealed by the finding that increases in aggressive interaction by the highest ranking group members were directed selectively at one (less favored) of the two subordinate group members in each group.

TABLE 3. INDIVIDUAL DIFFERENCES IN DIRECTIONAL CHANGES IN AGGRESSIVE BEHAVIORS AFTER ACUTE EXPOSURE TO THC IN THE HIGHER RANKING MONKEYS OF 3-MEMBERED CAGE GROUPS*.

Behavioral index	Rank	Subjects showing change[a]		
		Decrease	No change	Increase
Spontaneous aggression				
Threat	# 1	2/5	3/5	
	# 2	3/4	1/4	
Hit/bite/chase	# 1	2/5		3/5
	# 2	2/4	2/4	
Competition for food				
Threat	# 1	3/5	2/5	
	# 2	4/5	1/5	
Hit/bite/chase	# 1	3/5	1/5	1/5
	# 2	3/4	1/4	

* See Table 1 for definition of terms.

LONG-TERM CHRONIC EXPOSURE TO Δ^9-THC

High density indoor cage groups. Within 2 to 4 months after the start of daily drugging of selected group members, tolerance to the sedative effects of the drug became apparent; this change was most marked in males. Concurrently, there was a gradual appearance in the THC-treated subjects of an irritable responsiveness to social stimulation manifest principally as episodic contact aggression directed toward subordinate cagemates (i.e., hit, bite, chase, and attack behaviors). This change was also reflected in the behavior of undrugged cagemates as increased submissive or avoidance responses to THC-treated animals.

Tables 4 and 5 summarize this general change in group interaction for the five indoor 3-membered cage groups. Table 4 shows that eventually the # 1 or # 2 ranking THC-treated group members showed increases in contact aggressive behavior both in non-stimulated and in competitive group interactions. In contrast, during the same period in these same groups # 1 and # 2 ranking cagemates not receiving THC showed decreased or unchanged levels of aggressive behavior.

Table 5 shows the time course and degree of change in aggressive behavior over a one-year period as assessed in both non-stimulated and competitive social environments for the 4 THC-treated individuals ranking # 1 and # 2 in their groups. Although each animal showed increased aggressiveness in some form, the onset, intensity, and behavioral expression of irritable aggression varies among individuals and is closely related to the

TABLE 4. LONG-TERM THC: DIFFERENCES IN DIRECTIONAL CHANGES IN AGGRESSIVE BEHAVIORS BETWEEN DRUGGED AND NON-DRUGGED GROUP MEMBERS OF EQUIVALENT RANK.

Group members			Aggression category	Directional change		
Drug	Rank	N[a]		Decr.	No change	Incr.
THC	# 1	2	Spont.-H/B/C[b]	1/2		1/2
			Compet.-H/B/C			2/2
			Attack		(1/2)[c] →	
	# 2	2	Spont.-H/B/C		1/2	1/2
			Compet.-H/B/C		(1/2) →	1/2
			Attack		(1/2) →	1/2
None	# 1	4	Spont.-H/B/C	2/4	2/4	
			Elic.-H/B/C	1/4	3/4	
	# 2	3	Spont.-H/B/C	3/3		
			Elic.-H/B/C	1/3	2/3	

a. N = number of total group members at that rank and drug status. See text for description of spontaneous and competitive interaction.
b. H/B/C = summation of hit, bite, and chase behaviors.
c. ( ) → = delayed drug effect; behavioral change (increase) occurs during latter months of daily drugging.

TABLE 5. CHANGES IN FREQUENCIES OF AGGRESSIVE BEHAVIORS DURING LONG-TERM DAILY DRUGGING WITH Δ^9-THC IN FOUR DOMINANT GROUP-CAGED RHESUS MALES.

Cage and rank	Aggr. behav.	Months of daily drugging							
		1–2		4–5		7–9		11–13	
		Spont.	comp.	Spont.	comp.	Spont.	comp.	Spont.	comp.
D-1	H/B/C	+	(+)	+	(+)	(+)	+	+	+
	Attack								
H-1	H/B/C	−	(+)	−	+	−	+ +	−	+
	Attack		0		0		0		+
E-2	H/B/C	−	(+)	+	(+)	0	+	(−)	+
	Attack	0		+ +		+		0	
F-2	H/B/C	+ +	(+)	Incomplete		+ +	(+)	Incomplete	
	Attack	0	0			+	(+)		

H/B/C = summation of hit, bite, and chase behaviors.
+ or − = 100 percent increase or decrease.
+ + = > 200 percent increase.
( ) = marginal or inconsistent effect.
0 = no change.
No notation indicates absence of behavior during observation.

stress level of the psychosocial situation. This summary table shows that certain drugged subjects show a delayed onset of heightened aggressive behaviors (E-# 2); some show increased aggression only in competitive situations (H-# 1), while in others it was apparent in non-stimulated interactions as well (E-# 2 and F-# 2). Increased aggressiveness was revealed in some animals as more intense attack behaviors (E-# 2), or the more frequent hit-bite-chase behaviors (D-# 1 and H-# 1).

To assess the possible contribution of low level withdrawal symptoms to the development of the irritable aggressiveness described above, the interval between daily drug administration and daily AM behavioral assessment was changed from 18 to 2 hours, and subsequently to 22 hours. After 12 to 14 months of daily drugging at 1600 hours, THC was given at 0800 hours for 3 months and at 1200 hours for a final 3 months; behavioral assessment was maintained at 1000 (and 1400) hours throughout.

As shown in Fig. 1, in the crab macaque groups (D and E), submissive responses of subordinate cagemates to drugged dominant animals increased after the first 6 months of drugging. Although there is considerable variability in the data, there is no evidence that submissive responses increased when measured just prior to daily drugging (noon treatment) or that submission decreased markedly just after drugging (AM treatment). This observation was interpreted as indicating that the observed aggressive irritability was not a manifestation of low level "withdrawal" effects prior to each day's drugging, but represented instead a sustained cumulative effect of chronic drug exposure.

Outdoor young adult cage groups. In the two 6-membered outdoor cage groups, daily observation of group interactions were made over the initial 18 months of daily drugging. Groups of 6-month-old peers had been formed $2\frac{1}{2}$ years previously and had maintained stable dominance hierarchies during that time. As shown in Fig. 2, during the first year of

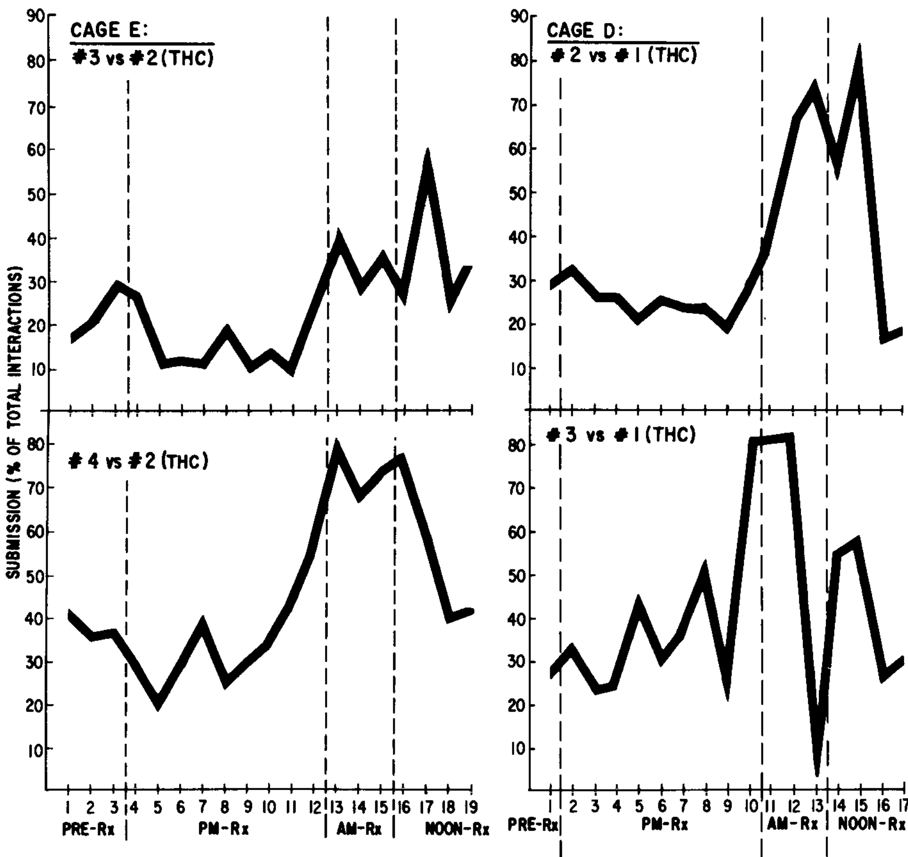


FIG. 1.

the long-term THC study, sequential changes in the hierarchial structure of both cages occurred. In each instance, these dominance shifts were initiated by changes in rank of a THC-treated female.

As shown in Fig. 2, in Cage OE, the drugged female fell from rank # 5 to # 6 during the first months of daily drugging when sedative effects predominated; she then rose in dominance position during the fifth to eighth months (as tolerance and irritability developed) to

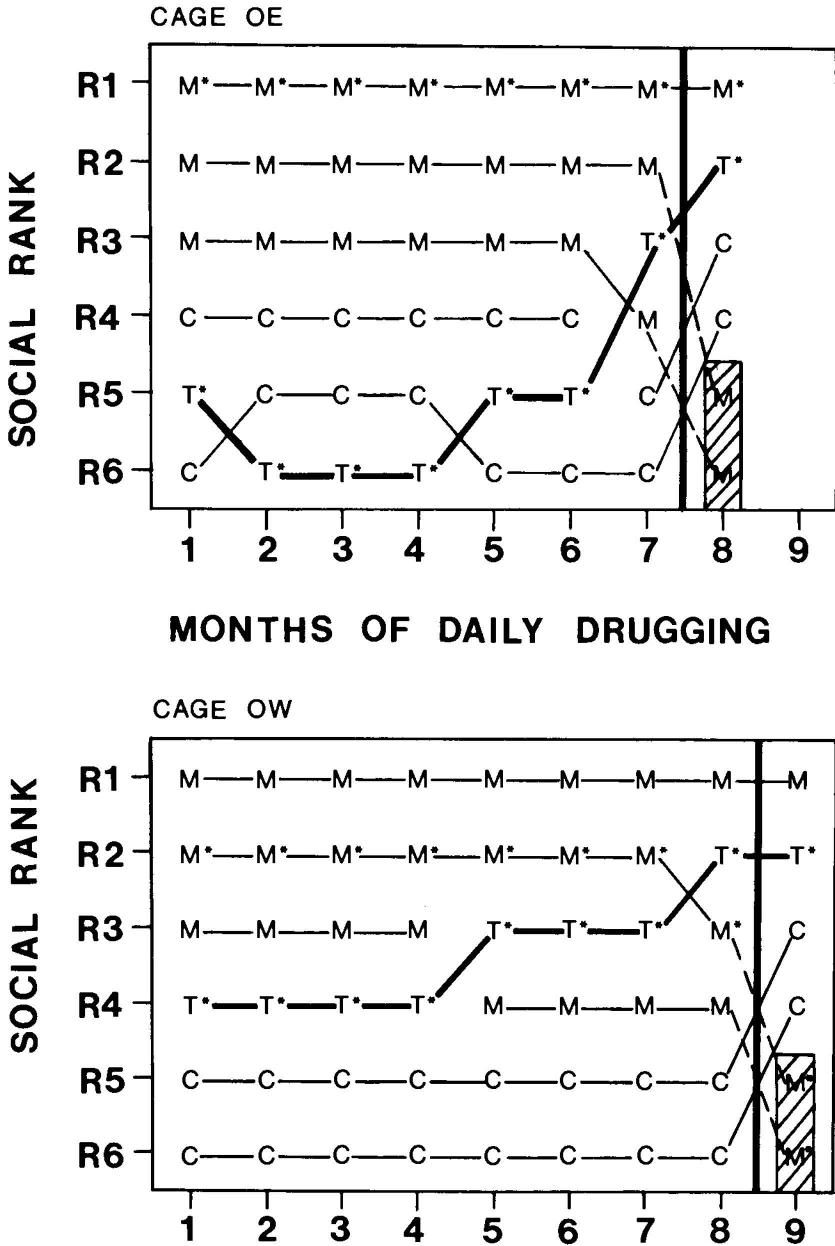


FIG. 2.

ultimately become the highest ranking female (# 2) in the group. In cage OW, although the drugged female (# 4) did not fall in rank during early months of drugging, she also showed a similar rise to the #2 rank during the fifth to eighth month interval. In both cages, the two subordinate males ultimately had to be removed from the group due to their receipt of persistent high levels of aggression and injury. At the time of group disruption, all animals were 44 to 47 months of age and, except for one # 3 male in cage OE, were sexually mature as shown by plasma testosterone levels (C).

New group formation. Prior to the third breeding season, females which had been caged individually during pregnancy and lactation with their previous infants were incorporated into groups of unfamiliar cagemates for breeding purposes.

In Group I, the smallest female (THC-treated) was killed during the first 24 hours after grouping due to the intensity of aggressive interaction and resulting injuries. In Group II, precautions were taken to avoid a similar occurrence. After the first day of intense fighting, the 2 THC-treated females were removed from the group cage for a period of 48 hours, followed by overnight removal for 4 days before being left in the group for a period of one month. At the end of this time, due to their continuing aggressive behavior, they had to be removed permanently from the group.

Figure 3 shows the 0900 circulating plasma cortisol index of stress response for both THC-treated and undrugged females in this group during these weeks after new cage

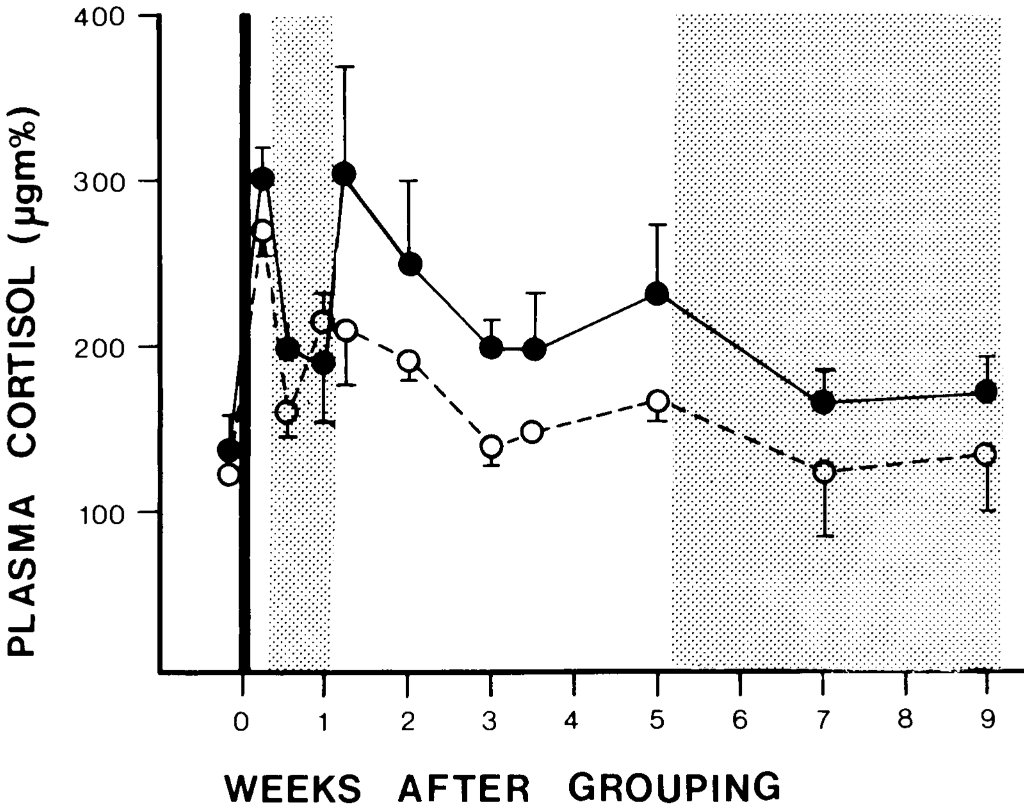


FIG. 3.

grouping, including the early days of intense fighting and intermittent cagemate removal; it also includes the month after permanent removal of the aggressive THC-treated females. The mean cortisol levels for both drugged and undrugged females indicated that adjustment to unfamiliar cagemates was stressful to all females with a gradual levelling off of the cortisol stress response after 3 weeks. However, THC-treated females showed consistently lower levels of cortisol. Further, their presence in the cage immediately prior to blood sampling appeared to be a critical determinant of stress-induced elevation of plasma cortisol for their undrugged female cagemates.

Thus, although the THC-treated females were behaviorally over-responsive to the challenge of forced proximity to unfamiliar cagemates, this stress was not accompanied by increased activation or sensitization of the hypothalamic-pituitary-adrenocortical stress response system. However, activation of this endocrine stress response pathway was greater in the undrugged females, apparently in response to the stress-enhanced aggressive behavior of the chronically THC-treated females.

DISCUSSION

ACUTE ADMINISTRATION

Sedative and Stimulant Actions. Our findings with regard to the generalized behavioral effects of acute or short-term treatment of rhesus monkeys with delta-9-THC are in substantial agreement with previous observations by others[29, 45, 70]; i.e., the agent predominantly produces sedation with reduction of active behavior, ptosis, relaxed postures, and reduced social interaction, along with "secondary" activation effects (not observed in all animals). We did not see a consistent initial phase of increased activity and behavior suggesting hallucinations such as that reported in squirrel monkeys following parenteral administration[47, 69]. Others, using parenteral routes have also failed to observe an early activated phase in rhesus monkeys[70].

In our studies with small groups of free-moving rhesus monkeys, 2 of 15 animals showed predominantly "activated" patterns of behavior. In addition, a relatively high proportion of treated animals exhibited sedation and inactivity interspersed with patterns of "active" stereotyped repetitive behavior. These observations are in keeping with the generalization that acute delta-9 THC has both stimulant and sedative effects, with the latter predominant in most instances. Similar conclusions apply for effects reported in man. There was an indication in our data that active behavior (stereotyped pacing or body flips) was more common in animals ranked lower in the dominance hierarchy, whereas changes in the inactive direction (sleeping, lying or sitting) was somewhat more common in the higher ranked animals. In those animals that exhibited conspicuous stereotyped repetitive behavior after drug treatment, the specific behavior exhibited in all instances previously had been noted to be part of that animal's characteristic behavioral repertoire before drugging.

Acute administration of THC consistently produced changes in social grouping (affiliative) behavior. There was an over-all tendency towards reduced affiliative behavior (huddling or grooming) under the influence of the drug, although there was significant individual variability and no simple relationship to amount of drug given was apparent (Table 2). Changes in dyadic preferences occurred and a relationship of the pattern of change to the total social environment, possibly including dominance positions, was also

apparent. For example, in Cage A (Table 2), when all cagemates were drugged simultaneously, the least dominant animal (#3) withdrew entirely from social interaction, reducing the time all three were together to zero, while pairings between #1 and #2 increased.

The great variability of the effects of marihuana on human affiliative behavior and social interaction is well known. In some settings, increased talking and social interaction is conspicuous; in others, a quiet, lethargic, or disinterested pattern is characteristic[39]; individual variations in characteristic styles of reaction to marihuana are also familiar. These observations with laboratory primates raise the possibility that such changes may be related to social relationships in the drug-taking environment, and indicate a general tendency towards reduction of social interaction, but with individual exceptions.

Aggression. Acute THC resulted predominantly in decreased aggression. Of 18 animals, aggressive behavior decreased in 15, and increased in 3. These observations are in accord with the majority of other animal studies on acute effects of THC. The three monkeys which showed an increase in aggressive behavior were all highly dominant (#1) ranked animals; their increased aggression was consistently directed to a single lower ranked animal. Again, these observations with monkeys are consistent with reports regarding human users in whom aggressiveness usually declines[32, 75], but occasional outbursts of aggressive activity do occur[36, 53, 59, 75]. Some authors have emphasized that this aggressiveness is related to exaggerated egotistical feelings of self-importance and superiority[59].

CHRONIC EFFECTS

Tolerance. Studies of the chronic effects of daily THC administration support the now widely accepted conclusion that tolerance to certain behavioral actions of THC develops in a relatively short time[20, 30, 43, 59, 74], whereas tolerance to other behavioral effects is greatly delayed or does not appear. In rhesus monkeys, Harris, Waters and McLendon[30] demonstrated a long-lasting behavioral tolerance as indicated both by gross behavioral changes and performance on certain operant conditioning tasks as early as the 4th or 5th day, with daily intravenous injections of delta-9 THC (2.0 mg/kg). They noted that the observed tolerance was selective for various types of operant conditioning and categories of spontaneous behavior. For example, tolerance to a shock-avoidance schedule developed rapidly, while tolerance to a food reward schedule developed slowly and incompletely. They found that animals in a shock-avoidance situation developed tolerance within a few days to such a degree that they could respond successfully (to avoid shock) at a time when they appeared grossly stuporous and could be handled by humans without eliciting defensive or aggressive behavior.

In man, it is generally accepted that tolerance is not commonly observed by marihuana users, at least in the patterns characteristic for the United States. Jones and Benowitz[35], however, demonstrated that with appropriate amounts and when the schedule of administration is arranged to yield a relatively consistent degree of intoxication over periods of days, a form of tolerance to certain behavioral effects in human subjects can be readily and rapidly demonstrated. They administered a large total dose (210 mg) of THC per 24 hours in divided doses. Under these conditions, the reactions of the subjects were reported to be pleasurable for the first few days only. Following this, an unpleasant, lethargic, sedated state developed, indicating that tolerance to the pleasurable effects apparently had oc-

curred. Dependence on the drug also developed; abrupt cessation of the drug resulted in symptoms of an abstinence syndrome with restlessness, irritability and insomnia.

In our observations with rhesus monkeys receiving a fixed amount (2.4 mg/kg) of THC daily, behavioral tolerance developed over a period of two to four months. After this time, the sedated and the stimulated types of behavior characteristic of acute administration were markedly reduced or no longer observed. At this point, the behavior of the animals did not appear grossly abnormal, and there were no differences in behavior apparent in the pre-drugging interval of the 24-hour cycle when contrasted with post-drugging interval when the direct actions of the drug would be expected to be maximal. Under this schedule of drug administration, therefore, a high degree of tolerance to the behavioral changes previously observed with acute administration occurred within a few months.

Aggression. At about the same time that tolerance to the sedative and stimulant behavioral effects became complete, a new category of behavioral effects also emerged. Two features could be identified. The first was an increase in species-specific irritable aggressive behavior of the drugged animals, evidenced by the frequencies of hitting, biting, chasing and attack incidents. The other aspect was an increase of avoidance or submissive responses to the THC-treated animals by undrugged cagemates. In outdoor 6-member cage groups, the increased aggression by treated animals, and increased submissiveness or avoidance responses to them, resulted in a rise of the drugged animals in the dominance hierarchy. In one instance, a number 5-ranked THC-treated female rose to the # 2 position just below # 1-ranked drugged male; in another instance a # 4-ranked female also rose to # 2 status. These displacements resulted in high level intragroup aggression and trauma, making it necessary eventually to remove displaced animals from the group cage to prevent serious or lethal injuries.

In an attempt to examine the possibility that the observed increase in irritable aggressiveness might be related to withdrawal effects, the time relationships between administration of the drug and the period of behavioral assessment (submissive responses by undrugged cagemates) were varied. There was no evidence of a consistent relationship between the observation period and the time since last drug ingestion; this observation supports the conclusion that the observed behavioral change persists through the 24-hour cycle on a schedule of continued daily drugging and therefore is unlikely to be associated with periods when the circulation drug level is minimal (i.e., a withdrawal effect).

The most dramatic behavioral changes observed to be associated with chronic exposure to THC occurred when chronically drugged animals were placed into the highly stressful environment of adapting to new social groups. Under these conditions, severe aggression and fighting occurred. One THC-treated female was fatally injured during the first 24 hours after being placed in a new group setting.

Increased aggression associated with chronic THC has been reported by investigators in rodents[8, 9, 18, 51], and has been seen in baboons[20, 27, 42]. Among human chronic cannabis users, irritable behavior and occasional violent acts occur, but definitive evidence of increased physical aggression is lacking[59, 75].

Plasma Cortisol. Cagemates of chronically THC-treated animals in newly formed social groups showed higher levels of plasma cortisol during the higher stressful weeks after group formation. Further, this elevated endocrine stress responses were reduced by removal of the THC-treated cagemates and rose again with their reintroduction to the group.

Elevated cortisol levels have been shown to correlate closely with submissive behaviors given and aggression received in rhesus monkey social groups. This endocrine stress

response is presumed to be correlated with perceived loss of control or inability to cope[31, 66]. Thus, these endocrine measures are in keeping with the aggression-submission behavioral interaction observed between THC-treated and undrugged cagemates, and suggest a relationship to the type of stress pattern evoked in response to the behavior of THC-treated animals.

Summary of Behavioral Changes Associated with Chronic THC. In general, observations of changes in dominance hierarchies, and observations of hyperaggressive activity in new social group formations both support the conclusion that rhesus monkeys chronically treated with THC demonstrate two categories of altered behavior; the first is an increase in irritable aggression. The second is impairment of the ability to moderate, modulate, or otherwise control such irritable aggressive behavior patterns when they are biologically inappropriate, i.e., the treated animals reacted maladaptively since their presence in the groups was associated with increased severe antagonistic interactions, fighting, and serious injuries or death.

An additional pertinent feature is the response of the undrugged cagemates to drugged animals. The over-all tendency of undrugged cagemates was to increase avoidance and submissive behavior in confrontations with drugged animals. Rhesus monkeys in group situations engage in continuous social exchanges organized according to a strict hierarchy of dominance relationships; this pattern presumably is of adaptive value for this species. The behavior of individuals in dyadic confrontations (over food, space, etc.) is clearly dependent upon a two-way communication process in which the behavior of one triggers behavioral responses in the other, back and forth, until a critical point at which one or the other individual either dominates or submits[55]. Biologically, the ability to perceive and to emit appropriate behavioral cues during these exchanges are important elements of an individual's adaptive capacity.

The possibility seems to deserve further study that animals receiving chronic THC may become impaired in their ability to generate and perceive the behavioral cues which control social interaction processes. In our observations, under conditions of high stress the presence of treated animals in the group cages led to severe fighting in the group as a whole (and serious injuries) which could be immediately reduced by removal of the drug-treated animals from the group. If the cage group is considered as a unit, under high stress conditions the presence of a drug-treated animal was maladaptive for the group, since it led to severe fighting and injuries among non-drugged as well as drugged animals. The possibility that THC might impair ability to generate and respond to social cues in rhesus monkeys was studied by Miller and Deets[55]. Using dose of 1 mg/kg of delta-9-THC, they observed a deficit in the ability of acutely drugged animals to display appropriate facial expressions (although with this degree of exposure to the drug, the ability to perceive facial expression cues emitted by partners was not impaired); communication between treated and untreated monkeys was maximally disrupted when a drugged monkey sent behavioral cues to an untreated responder.

The present studies with laboratory primates indicate that prolonged daily exposure to THC results in a biologically serious impairment of the capacity to exhibit adaptive social behavior related to dominance-submission relationships and to modulate and control aggression towards other group members. This impairment is most apparent in a stressful environment.

The question of relevance of these observations with rhesus monkeys to assessment of potential risks of adverse behavioral changes associated with long-term exposure to mari-

huana in man presents all of the familiar problems of interpreting findings from animal models. Obviously, when the topic is social behavior, these problems are especially challenging. Nevertheless, the observations reported here encourage consideration of the possibility that chronic cannabis exposure in man at comparable levels may result in adverse changes in the ability to exhibit adaptively appropriate social behavior, particularly under stressful circumstances.

ACKNOWLEDGEMENT

This investigation was supported by USPHS grant DA-00135. Additional partial support has been provided by USPHS grants RR-00169 to the California Primate Research Center and RR-05684 to the School of Medicine, University of California, Davis.

REFERENCES

1. Adams, P. M. and Barratt, E. S., The effects of a marijuana extract on the general motor activity of the squirrel monkey. *Psychonomic Science* **25**,279–280 (1971).
2. Adams, P. M. and Barratt, E. S., Effects of a marihuana extract on performance and EEG's in squirrel monkeys. "Symposium on Drug Tolerance, Addiction, Abuse, and Methadone Treatment" Futura Press, Mount Kisco, New York, 1972.
3. Agurell, S. This volume. pp. 3–13.
4. Beaubrun, M. and Knight, F., Psychiatric assessment of 30 chronic users of cannabis and 30 matched controls. *Am. J. Psychiat.* **130**,309–311 (1973).
5. Bowman, M. and Pihl, R. O., Cannabis: psychological effects of heavy use. *Psychopharmacol. (Berlin)* **29**,159 (1973).
6. Boulougouris, J. C., Social traits of heavy hashish users and matched controls. *Ann. N.Y. Acad. Sci.* **282**,17–23 (1976).
7. Campbell, A. M. G., Evans, M., Thomson, J. L. G. and Williams, M. J., Cerebral atrophy in young cannabis smokers. *Lancet* **2**,1219–1224 (1971).
8. Carlini, E. A. and Masur, J., Development of aggressive behavior in rats by chronic administration of cannabis sativa (marihuana). *Life Sciences* **8**,607–620 (1969).
9. Carlini, E. A. and Masur, J., Development of fighting behavior in starved rats by chronic administration of delta-9-THC and cannabis extracts. *Commun. Behav. Biol.* **5**,57–61 (1970).
10. Carter, W. E., Social and cultural aspects of cannabis use in Costa Rica. *Ann. N.Y. Acad. Sci.* **282**,2–16 (1976).
11. Cole, J. M., Pieper, W. A. and Rumbaugh, D. M., Effects of delta-9-tetrahydrocannabinol on spaced responding in great apes. *Communications in Behav. Biol.* **A6**,286–293 (1971).
12. Conrad, D. G., Elsmore, T. F. and Sodetz, F. J., Delta-9 THC: dose-related effects on timing behavior in chimpanzees. *Science* **175**,547–550 (1972).
13. Chopra, S. G., Marijuana and adverse psychotic reactions. *Bull Narac.* **23**,15–22 (1971).
14. Co, B. T., Absence of cerebral atrophy in chronic cannabis users. Evaluation by computerized transaxial tomography. *J.A.M.A.* **237**,1229–1230 (1977).
15. Coggins, W. J., The Costa Rica Cannabis Project: an interim report on the medical aspects. "The Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, 1976.
16. Comitas, L., Cannabis and work in Jamaica: a refutation of the amotivational syndrome. *Ann. N.Y. Acad. Sci.* **288**,24–32 (1976).
17. Deneau, G. A. and Kaymakclan, S., Physiological and psychological dependence to synthetic delta-9-THC in rhesus monkeys. *Pharmacologist* **13**,246 (1971).
18. Ely, D. L., Henry, J. P. and Jarosz, C. J., Effects of marihuana (delta-9-THC) on behavior patterns and social roles in colonies of CBA mice. *Behav. Biol.* **13**,263–276 (1975).
19. Ferraro, D. P., Grilly, D. M. and Lynch, W. C., Effects of marihuana extract on the operant behavior of chimpanzees. *Psychopharmacologia* **22**,333–351 (1971).
20. Ferraro, D. P. and Grisham, M. G., Tolerance to the behavioral effects of marihuana in chimpanzees. *Physiol. Behav.* **9**,49–54 (1972).

21. FERRARO, D. P., LYNCH, W. C. and GRILLY, D. M., Behavioral effects of small oral doses of marihuana extract in chimpanzees. *Pharmacology* (*Basel*) **7**,273–282 (1971).
22. FERRARO, D. P. and GRILLY, D. M., Lack of tolerance to delta-9-tetrahydrocannabinol in chimpanzees. *Science* **179**,490–492 (1973).
23. FINK, M. and STEFANIS, C., Interim report: study of marihuana users in Greece, prepared for the National Institute of Mental Health, Contract H.S.M. 42–70–8, pp. 53, 54. U.S. Government Printing Office, Washington, D.C. (1971).
24. FREIRICH, E. J., GEHAN, E. A., RULL, D. P., SCHMIDT, L. H. and SKIPPER, H. E., Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. *Cancer Chem. Rep.* **50**,219 (1906).
25. FRIED, P. A., Behavioral and electroencephalographic correlates of the chronic use of marihuana. *Behav. Biol.* **21**,163–196 (1977).
26. GIONO-BARBER, P., PARIS, M., BERTULETTI, G. and GIONO-BARBER, H., L'action du cannabis sur le comportement de domination du singe cynocephale. *J. Pharmacologie* **5**,591–602 (1975).
27. GIONO-BARBER, P., BERTULETTI, G. and GIONO-BARBER, H., Action due cannabis sur l' apprentissage chez le singe cynocephale. *C. R. Seances Soc. Biol. Filiales* **169**,264–270 (1975).
28. GREENBERG, F., Psychiatric and behavioral observations of casual and heavy marihuana users in a controlled research setting. *Ann. N.Y. Acad. Sci.* **282**,72–74 (1976).
29. GRUNFELD, Y. and EDERY, H., Psychopharmacological activity of the active constituents of hashish and some related cannabinoids. *Psychopharmacologia* **14**,200–210 (1969).
30. HARRIS, R. T., WATERS, W. and MCLENDON, D., Behavioral effects in rhesus monkeys of repeated intravenous doses of delta-9-THC. *Psychopharmacologia* **26**,297–306 (1972).
31. HENRY, J. P., Mechanisms of psychosomatic disease in animals. *Adv. Vet. Sci. Comp. Med.* **20**,115–145 (1976).
32. HOLLISTER, L. E., RICHARDS, R. K. and GILLESPIE, H. K., Comparison of tetrahydrocannabinol and synhexyl in man. *Clin. Pharmacol. Ther.* **9**,783–791 (1968).
33. HOUSER, V. P., The effects of delta-9-THC upon fear motivated behavior in squirrel monkeys. *Physiol. Psychol.* **3**,157–161 (1975).
34. JONES, B. C., CLARK, D. L., CONSROE, P. F. and SMITH, H. J., Effects of delta-9-THC on social behavior of squirrel monkey dyads in a water competitive situation. *Psychopharmacologia* **37**,37–43 (1974).
35. JONES, R. T. and BENOWITZ, N., The 30-day trip: clinical studies of cannabis tolerance and dependence. "Pharmacology of Marihuana" (Braude, M. C. and Szara, S., eds.). Raven Press, New York, 1976.
36. KAPLAN, H. S., Psychosis associated with marihuana. *N.Y. State J. Med.* **71**,433–435 (1971).
37. KAYMAKCALAN, S., Physiological and psychological dependence on THC in rhesus monkeys. "Cannabis and its Derivatives" (W. D. M. Paton and J. Crown, eds.). Oxford University Press, London, 1972.
38. KOLANSKY, H. and MOORE, W. T., Effects of marihuana on adolescents and young adults. *J.A.M.A.* **216**,486–492 (1971).
39. KUEHNLE, J., Computed tomographic examination of heavy marijuana smokers. *J.A.M.A.* **237**,1231–1232 (1977).
40. KUPFER, D. J., DETRE, D., KORAL, J. and FAJANS, P., A comment on the "motivational syndrome" in marihuana smokers. *Am. J. Psychiat.* **130**,1319–1322 (1973).
41. LeDain Commission, *A Report of the Commission of Inquiry into the Non-Medical Use of Drugs*. Information Canada, Ottawa (1972).
42. LEVETT, G. A., SAAYMAN, G. S. and AMES, F., The effects of cannabis sativa on the behavior of adult female chacma baboons (Papio ursinus) in captivity. *Psychopharmacology* **53**,79–85 (1977).
43. MANNING, F. J., Acute tolerance to the effects of delta-9-THC on spaced responding by monkeys. *Pharmacol. Biochem. Behav.* **1**,665–671 (1975).
44. MARCUS, A. M., KLONOFF, H. and LOW, M., Psychiatric status of the marijuana user. *Canad. Psychiat. Assoc.* **19**,31–39 (1974).
45. MARTINEZ, J. L., STADNICKI, S. W. and SCHAEPPI, U. H., Delta-9-THC: effects on EEG and behavior of rhesus monkeys. *Life Sciences* **11**,643–651 (1972).
46. MCGLOTHLIN, W. H. and WEST, L. J., The marihuana problem, an overview. *Am. J. Psychiat.* **125**,370–378 (1968).
47. MCISAAC, W. M., FRITCHIE, G. E., IDÄNPÄÄN-HEIKKILÄ, J. E., HO, B. T. and ENGLERT, L. F., Distribution of marihuana in monkey brain and concomitant behavioral effects. *Nature* **230**,593–594 (1971).
48. MEHNDIVATTA, S. S., Psychosocial effects of long-term cannabis use in India. A study of fifty heavy users and controls. *Drug. Alcohol Depend.* **1**,71–81 (1975).
49. MELGES, F. T., TINKLENBERG, J. R., HOLLISTER, L. E. and GILLESPIE, H. K., Marihuana and the temporal span of awareness. *Arch. Gen. Psychiat.* **24**,564–567 (1971).
50. MENDELSON, J. H., KUEHNLE, J. C., GREENBERG, I. and MELLO, N. K., The effects of marihuana use on operant behavior; individual data. "The Pharmacology of Marihuana" (M. C. Braude and S. Szara, eds.). Raven Press, New York, 1976.
51. MICZEK, K. A. and BARRY, H., Delta-9-tetrahydrocannabinol and aggressive behavior in rats. *Behav. Biol.* **11**,261–267 (1974).

52. MICZEK, K. A., Delta-9-tetrahydrocannabinol: antiaggressive effects in mice, rats, and squirrel monkeys. *Science* **199**,1459–1461 (1978).
53. MILES, C. G., A selective review of studies of long-term use of cannabis on behavior, personality, and cognitive functioning. Cannabis and Man, Psychological and Clinical Aspects and Patterns of Use. (*Proceedings of the 3rd International Cannabis Conference, Institute for Study of Drug Dependence, C.I.B.A. Foundation*). (P. H. Connell and N. Dorn, eds.). Churchill Livingstone, Edinburgh (1975).
54. MILLER, L. L. and DREW, W. G., Cannabis: review of behavioral effects in animals. *Psychol. Bull.* **31**,401–417 (1974).
55. MILLER, R. E. and DEETS, A. C., Delta-9-THC and non-verbal communications in monkeys. *Psychopharmacology* **48**,33–58 (1976).
56. National Institute of Mental Health, *Report on Marihuana and Health.* U.S. Government Printing Office, Washington D.C. (1971).
57. National Institute of Mental Health, *Report on Marihuana and Health.* U.S. Government Printing Office, Washington, D.C. (1972).
58. PATON, W. D. M. and PERTWEE, R. G., The pharmacology of cannabis in animals. "Marijuana" (Mechoulam, R., ed.), Academic Press, New York, 1973.
59. PATON, W. D. M. and PERTWEE, R. G., The actions of cannabis in man. In "Marijuana" (Mechoulam, R. ed.). Academic Press, New York, 1973.
60. PATON, W. D. M., PERTWEE, R. G. and TYLDEN, E., Clinical aspects of cannabis action. "Marijuana" (Mechoulam, R., ed). Academic Press, New York, 1973.
61. POTVIN, R. J. and FRIED, P. A., Acute and chronic effects on rats of delta-1-trans-tetrahydrocannabinol on unlearned motor tasks. *Psychopharmacologia* **26**,369–378 (1972).
62. ROSENKRANTZ, H., The immune response and marihuana. "Marihuana: Chemistry, Biochemistry and Cellular Effects" (G. G. Nahas, ed.). Springer-Verlag, New York, 1976.
63. RUBIN, V. and COMITAS, L., A study of the effects of chronic ganja smoking in Jamaica. Prepared for the National Institute of Mental Health, contrast H.S.M. 42–70–77. *Marihuana: A Signal of Misunderstanding*, pp. 55–56. U.S. Government Printing Office, Washington, D.C. (1972).
64. RUBIN, V. and COMITAS, L., *Ganja in Jamaica*. Mouton and Co., The Hague, 1975.
65. ROBBINS, P. R. and TANCK, R. H., Psychological correlates of marijuana use: an exploratory study. *Psychol. Rep.* **33**,703–704 (1973).
66. SASSENRATH, E. N., Increased adrenal responsiveness related to social stress in rhesus monkeys. *Hormon. Behav.* **1**,283–298 (1970).
67. SASSENRATH, E. N. and CHAPMAN, L. F., THC-induced manifestations of the marihuana syndrome in group living macaques. *Fed. Proc.* **34**,1666–1670 (1975).
68. SASSENRATH, E. N. and CHAPMAN, L. F., Primate social behavior as a method of analysis of drug action studies with THC in monkeys. *Fed. Proc.* **35**,2238–2244 (1976).
69. SCHECKEL, C. L., BOFF, E., DAHLEN, P. and SMART, T., Behavioral effects in monkeys of racemates of two biologically active marijuana constituents. *Science* **160**,1467–1469 (1968).
70. STADNICKI, S. W., SCHAEPPI, U., ROSENKRANTZ, H. and BRAUDE, M. C., Crude marihuana extract: EEG and behavioral effects of chronic oral administration in rhesus monkeys. *Psychopharmacologia* **37**,225–233 (1974).
71. STEFANIS, C., BOULOUGOURIS, J. and LIAKOS, A., Clinical and psychophysiological effects of cannabis in long term users. "The Pharmacology of Marihuana" (M. C. Braude and S. Szura, eds.). Raven Press, New York, 659–665 (1976).
72. SYNDER, E. W., LEWIS, E. G., DUSTMAN, R. E. and BECK, E. C., Sustained ingestion of delta-9-THC and the operant behavior of stump-tailed macaques. *Newsletter of Res. Ment. Health and Behav. Sci.* **15**,40–43 (1975).
73. TENNANT, F., PREBLE, M., PRENDERGAST, T. and VENTRY, P., Medical manifestations associated with hashish. *J.A.M.A.* **216**,1965–1969 (1971).
74. THOMPSON, G. R., ROSENKRANTZ, H. and BRAUDE, M. C., Neurotoxicity of cannabinoids in chronically treated rats and monkeys. *Pharmacologist* **13**,296 (1971).
75. TINKLENBERG, J. R., Marijuana and human aggression. "Marijuana, Effects on Human Behavior." Academic Press, New York, 1974.
76. WEISS, S., A general picture of charas habit in Afghanistan. Report from Kabul University Medical School. *Marihuana: A Signal of Misunderstanding* pp. 57–58. U.S. Government Printing Office, Washington, D.C. (1971).
77. World Health Organization, *World Health Organization Technical Reports Series 478* (1971).
78. ZIMMERBERG, B., GLICK, S. D. and JARVIK, M. E., Impairment of recent memory by marihuana and THC in rhesus monkeys. *Nature* **233**,343–345 (1971).

CHRONIC MARIHUANA SMOKING: ITS EFFECT ON FUNCTION AND STRUCTURE OF THE PRIMATE BRAIN*

ROBERT G. HEATH, A. T. FITZJARRELL, R. E. GAREY, and W. A. MYERS

Department of Psychiatry and Neurology, Tulane University School of Medicine, New Orleans, LA. U.S.A.

THERE is a pressing need to continue gathering objective data on the effects of use of *cannabis sativa* derivatives. Clinical reports have been made of the harmful effects induced by psychoactive components of the drug. Each time sound data have been presented which indicate the drug might be injurious to health, there have been rebuttals, the critics usually claiming the study lacked adequate controls. Typical arguments have been that subjects participating in the study used drugs in addition to *cannabis sativa* or that pathology would have developed in the absence of cannabis use. The type of rigid controls required to firmly establish the effects of cannabis derivatives, and thus refute these criticisms, can only be imposed in animal experiments.

Nearly all human consumption of cannabis is in the form of smoking marihuana or hashish. Over the past several years, we have conducted studies in rhesus monkeys to determine the effects of cannabis sativa derivatives on brain function, behavior, and brain structure. Findings previously reported from our laboratories show that monkeys exposed to significant amounts of active marihuana smoke for a period of three to six months,† or that received delta-9-THC intravenously on a regular basis over a similar period,‡ developed lasting and possibly permanent changes in brain recordings, the changes most pronounced at deep brain sites where activity has been correlated with emotion (Heath, 1972; Heath, 1975; Heath, 1976). In contrast, control monkeys, including monkeys exposed to smoke of large amounts of marihuana *without* active cannabis derivatives and those smoking significantly lower doses of active marihuana for the same period, showed no changes in recordings.

We have also reported ultrastructural changes at the septal region, the first deep brain site we chose to exhaustively study in the monkeys in this series (Heath, 1954; Harper, Heath, and Myers, 1977; Myers and Heath, 1978). Changes at the septal region consisted

* Supported in part by National Institute of Drug Abuse Grant No. DA-00311.

† Changes occurred at heavy doses (one cigarette t.i.d., five days per week) and at moderate doses (one cigarette t.i.d., two days per week), whereas monkeys receiving one-half cigarette on each of five days per week showed no changes.

‡ Dose of 0.69 mg/kg per day, five days per week, administered intravenously in fatty serum.

of alterations at the synapse and in the rough endoplasmic reticulum of monkeys that had been rested for six months following chronic smoking (six months) of active marihuana, as well as of monkeys that had received intravenous delta-9-THC. In contrast, septal region tissues of the control monkeys exposed to heavy smoke of *inactive* marihuana showed no changes, their tissues being essentially the same as those of unexposed control monkeys. A report of ultrastructural studies of a second deep brain region, the hippocampus, a site that also showed significant recording changes in those monkeys chronically exposed to psychoactive cannabis derivatives, is currently in preparation. The ultrastructural changes are similar to those we have described for the septal region.

Our reports have provoked criticism, principally on two issues: (1) that the effects we reported were the result of excessive exposure to smoke that resulted in anoxia and were not attributable to the psychoactive cannabis derivatives; and (2) that dosages of cannabis were far in excess of those used by human subjects.

In the first part of this report, I will describe the studies we have conducted recently to clarify these two methodologic issues. I will then describe acute effects on brain function that were obtained in monkeys with chronically implanted deep electrodes when we tested cannabis derivatives other than delta-9-THC. And finally, I will present additional data from our ongoing electron microscopic studies of the brains of monkeys exposed to cannabis derivatives.

NONSPECIFIC EFFECTS OF SMOKE

The statements that our findings were the result of nonspecific effects of smoke are irrelevant since we controlled for that factor in all studies. Blood gases were measured and no anoxia was indicated. Further, among the control monkeys in the studies were monkeys exposed to smoke devoid of psychoactive cannabis, the degree of smoke exposure being the same as for those monkeys maximally exposed to smoke of active marihuana. In those control monkeys, there were no electroencephalographic (EEG) changes and their brains showed none of the ultrastructural changes seen in the brains of monkeys exposed to smoke of active marihuana. Moreover, some of the monkeys were given delta-9-THC intravenously, rather than being exposed to smoke, and they also showed permanent EEG changes and ultrastructural pathology. These controls indicate that altered brain function and pathology were caused by the psychoactive components and not by the smoke.

DOSE LEVELS

The dosage factor is more complicated. In designing animal experiments, it has been difficult to simulate man's pattern of smoking. A significant contribution toward lessening this problem was made by Renault and associates (1971), who used a respirometer to trap smoke from accurately measured quantities of marihuana for delivery to an animal. But they did not determine whether psychoactive ingredients were lost or altered by their apparatus. Further, an animal's efficiency in inhaling smoke and absorbing active ingredients posed a variable when compared to a human subject's smoking pattern.

When we initiated our studies, these variables were not resolved. Also, at that time, dependable measures for blood levels of delta-9-THC were not available. Our first smoking

method was a modification of the respirometer technique of Renault and colleagues (1971). Heavy, moderate, and light dosages that we used in the monkeys were based on published reports of quantities consumed by human smokers (Tennant and Groesbeck, 1972). Our dosages were calculated on a weight basis plus Freireich's (1966) factor for relative surface area. In addition, we estimated that 50% of the smoke was lost in the respirometer and tubing of our smoking apparatus and we therefore doubled that dose. In the monkey, Freireich's factor called for a 300% increase in dosage. With the doubling for estimated smoke loss, the amount of starting material we used was therefore about six times that used by the efficient human smoker. Looked at only from the standpoint of the starting quantity of marihuana, these dosages indeed seem high, particularly if Freireich's factor is invalid as some contend. Each smoke contained 0.82 gram of marihuana assayed at 3.0% delta-9-THC. This amounted to 24.6 mg of delta-9-THC per smoke or about 5 mg/kg delta-9-THC for a monkey weighing 5 kg. The monkeys exposed to this quantity of smoke showed the same or lesser changes than those that received 0.69 mg/kg delta-9-THC intravenously. We therefore questioned the efficiency of our smoking procedure and investigated the issue of dosage in several ways.

We first compared the total quantity of delta-9-THC in our starting dose delivered to the monkeys by our smoking device with the quantity a man would receive from the same amount of starting material by drawing directly on the pipe holding the marihuana. The results shown in table 1 indicate that the human subject puffing on the pipe received eight times the amount of active ingredient that our monkeys received through delivery of smoke by the special apparatus. A significant proportion of the active ingredient apparently remained attached to the monkey smoking device.

TABLE 1. Δ^9-THC IN MARIHUANA SMOKE. HUMAN VERSUS MACHINE

	Smoke (mg Δ^9)	Ashes (mg Δ^9)
Human (N = 4)	6.45 (25.10%)	3.78 (14.70%)
Machine (N = 12)	0.86 (3.42%)	Trace (0.10%)

1 gm of marihuana (2.45% Δ^9-THC = 24.5 mg Δ^9-THC)

The factor of smoking efficiency was more difficult to compare. A human subject inhales deeply and holds the smoke in his lungs so that much is absorbed into the blood stream. The monkey, on the other hand, breathes as shallowly as possible. Blood levels of delta-9-THC were obtained in monkeys exposed to our original smoking procedure for comparison with those of human subjects smoking known quantities of marihuana (Table 2). Despite weight differences between monkey and man, these data show that man's blood levels of delta-9-THC were higher after smoking a standard size cigarette than those of our monkeys smoking marihuana at the heavy dose level. Summarizing, these studies indicate that the quantity of marihuana smoked is not a meaningful indicator of the amount of active ingredient absorbed (true dose) in monkey experiments. In light of later experiences, we estimated, when all the above factors are considered, that our monkeys to which smoke was delivered with the first apparatus were probably exposed to less than one mg/kg of delta-9-THC despite the relatively large quantity of starting material.

TABLE 2. BLOOD LEVEL Δ^9-THC AFTER ACUTE SMOKING OF MARIHUANA. GC—MS ANALYSIS. MONKEY VERSUS HUMAN

	Number of cigarettes	Δ^9-THC content	Blood level 10 minutes post
Monkey	1	19.6 mg	3.2 ng/ml
Human subject	1	10.0 mg	19–26 ng/ml

MODIFIED SMOKING APPARATUS

Following the first reports of our studies and the subsequent criticism of our dose schedule, we designed a new smoking apparatus to improve the efficiency of the monkey's smoking so that the pattern more closely simulated man's smoking pattern. The new apparatus incorporates an Isolette infant respirator and a small mask which holds the marihuana cigarette (Fig. 1). A series of valves permit the mask to be alternately filled and emptied of smoke in a manner simulating man's inhalation and exhalation of smoke. After observing the smoking pattern of a number of chronic marihuana users, we programmed the apparatus so that the respirator is activated every 30 seconds for intake of smoke, the monkey holding the smoke in its lungs for six seconds. Between puffs, the smoke is exhausted and the mask is filled with oxygen. Oxygen was added to eliminate any possi-

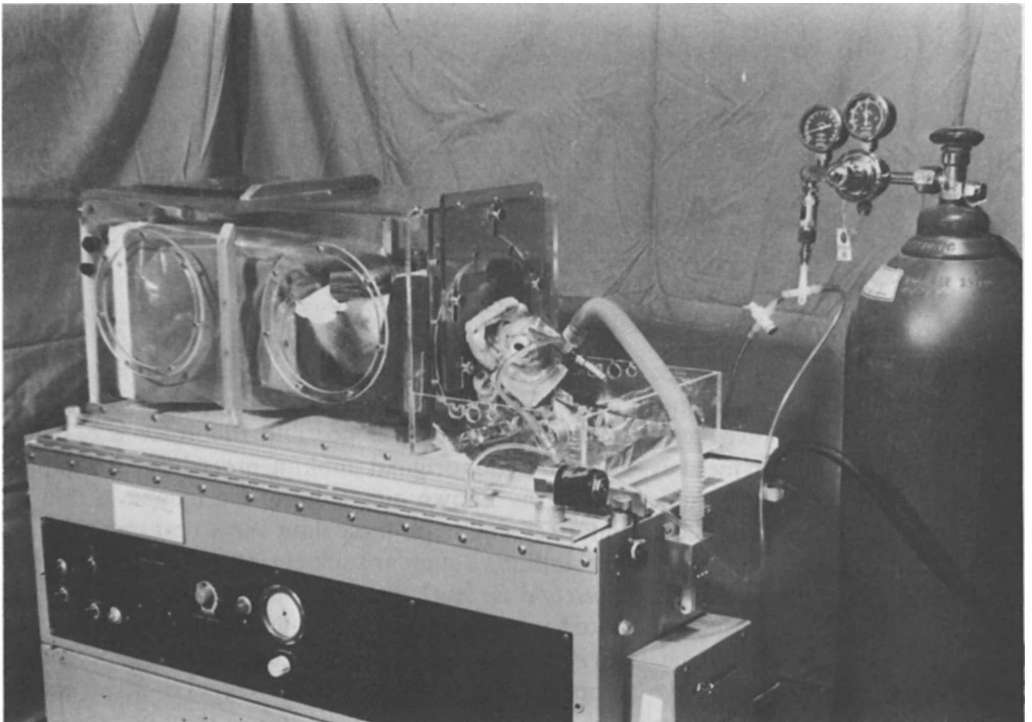


FIG. 1. Photograph of new smoking apparatus.

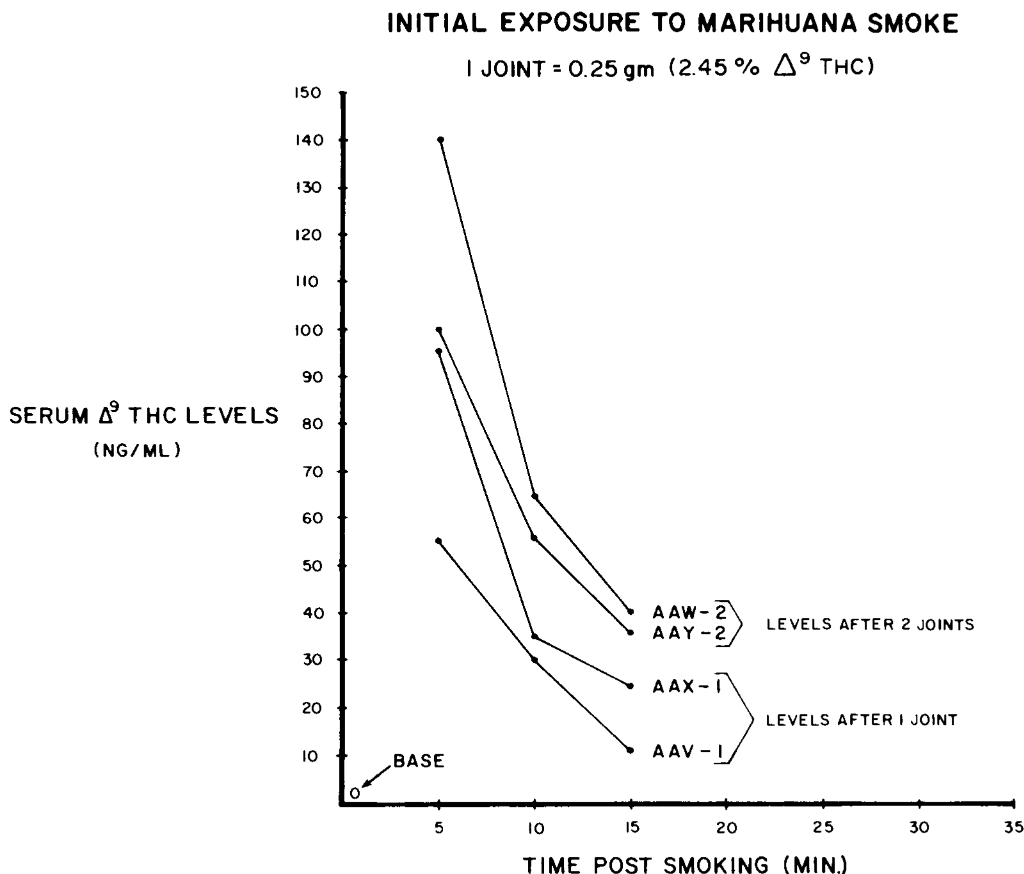


FIG. 2. Blood levels of delta 9-THC following initial exposure to marihuana smoke.

bility of carbon monoxide accumulation in the tubing. With this smoking program, blood gases remained within normal range.

By measuring blood levels of delta-9-THC, we determined that a 0.25 gram joint smoked by the monkey in the manner described induced a blood level that fell within the range of a man smoking a standard joint of the same delta-9-THC content (1.0 gram) (Figs. 2 and 3). With this procedure, the monkey is exposed to 1 to 2 mg/kg of delta-9-THC per joint. As these data obviously indicate, there is still some loss of active material due to the factors detailed heretofore, but it is considerably less than with our original smoking apparatus. Even after chronic smoking, the blood level consistently increased with exposure to smoke of a 0.25 gram cigarette although to a slightly lesser degree than with the original exposure. Because the 0.25 g cigarette produced blood levels similar to those of a man smoking a standard joint, we have standardized on the 0.25 g cigarette for our monkey studies.

OTHER CANNABIS DERIVATIVES

In our studies in monkeys, we assumed, because delta-9-THC is the known and principal psychoactive ingredient of marihuana, that it was probably the cannabis ingredient re-

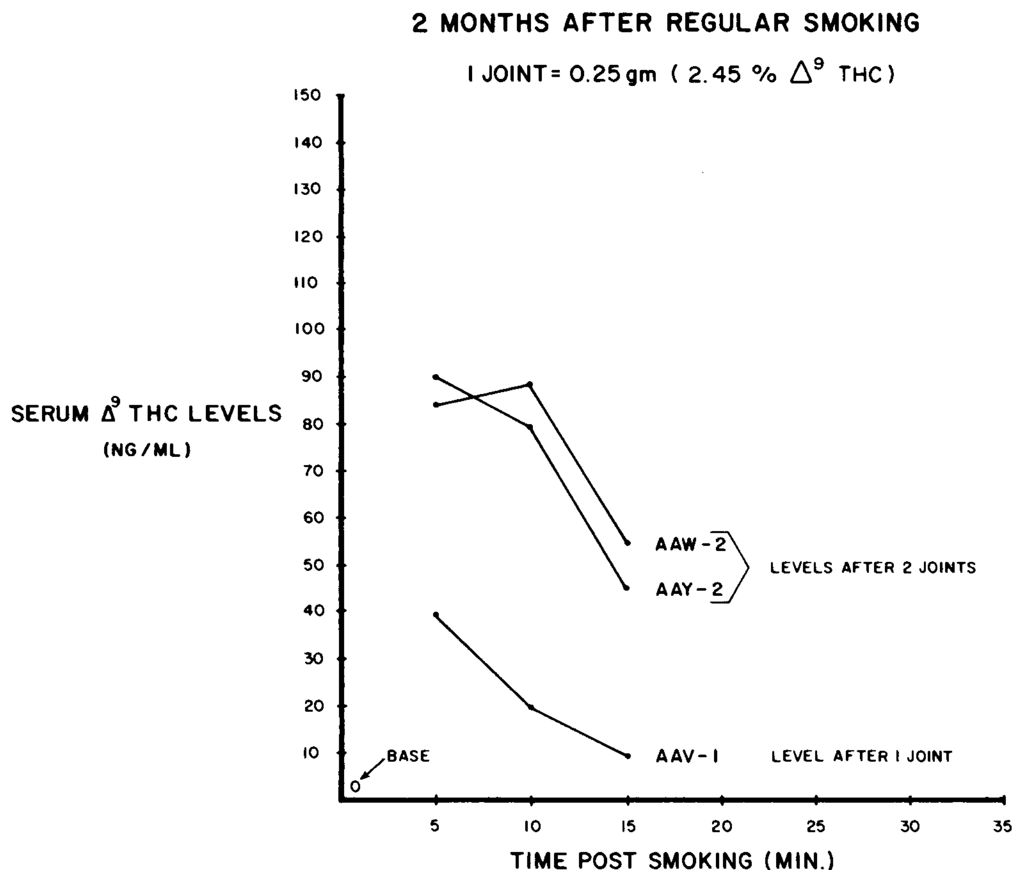


FIG. 3. Blood levels of delta-9-THC after monkeys had been smoking on a regular schedule of five days per week for two months.

sponsible for the brain changes we observed. Further, intravenous administration of pure delta-9-THC induced similar alterations in brain recordings. Assays of marihuana, however, had shown the presence of small quantities of several other ingredients. We therefore tested cannabinol, cannabidiol, and cannabichromene to determine whether they were responsible, at least in part, for the observed changes. Each compound was tested intravenously at several dose levels, the maximal dose in each instance being at least 20 times greater than the maximal amount present by assay in one standard joint of our marihuana.

The compounds proved to be inert with the exception of cannabinol which produced a questionable effect when given at a very high dosage (12.5 mg/kg versus 0.69 mg/kg delta-9-THC). The changes consisted of some sharp wave activity in the septal region followed by some slowing in the septal region, deep cerebellar nuclei, and ventroposterior lateral thalamus. They only roughly approximated alterations seen in these same brain structures of psychotic patients, being more characteristic of those seen during nonspecific toxic states. In contrast, delta-9-THC, at doses of 0.69 mg/kg, consistently induced striking changes at brain sites where activity correlates with emotional expression, the changes resembling those seen at the same brain sites of psychotic patients.

Six months after the original testing of cannabinol, the material was retested in two other monkeys. When it failed to induce changes, it was necessary to question the original results or to assume that the material had somehow deteriorated while it was stored in the cold room.

These results indicate that the acute and chronic brain changes that occurred in the monkeys with marihuana smoking were a consequence of the delta-9-THC. They are substantiated by the finding that similar brain changes were induced in other monkeys with intravenous administration of delta-9-THC.

ELECTRON MICROSCOPIC STUDIES

All light microscopic histopathologic studies that we have done on the monkey brains have yielded normal results.

Electron microscopic studies are under way on the brains of the monkeys of our first study in which we reported EEG changes. Included in the group are tissues of those monkeys that were chronically exposed to heavy, moderate, and light smoking of active marihuana (3.0% delta-9-THC), the monkey exposed to heavy smoking of *inactive* marihuana, and those that received delta-9-THC intravenously. We have also included monkeys without implanted brain electrodes to exclude the possibility that the changes obtained were the result of chronic implantation of electrodes. In addition, we are studying the brain of the one monkey that was chronically smoked two times per day for six months with the new Isolette smoking device. That monkey was then rested for six months, after which it was given a single dose of delta-9-THC intravenously and sacrificed 15 minutes later. As controls, tissues of two naive (unexposed) monkeys are also being studied. Up to now, we have completed examination of tissues of the septal region, hippocampus, amygdala, and motor cortex and we have performed some studies on tissues from other brain sites.

For these studies, the monkeys were anesthetized with sodium pentobarbital (30 mg/kg) and perfused at ambient temperature under a pressure of 125 mm Hg. A short flush of heparinized normal saline preceded the fixation solutions. The initial 30 minutes of the perfusion was done with a 1% paraformaldehyde/1.5% gluteraldehyde mixture made up in 0.1 M cacodylate buffer. This was followed for 60 minutes by a 4% paraformaldehyde/4% gluteraldehyde mixture and the same buffer. The brain was then removed from the skull and soaked in the more concentrated fix for an additional three and one-half hours. It was then washed in 0.1 M cacodylate buffer with 10% sucrose for 60 minutes. During this time, pledgets were removed from the designated loci. The cut tissue was post-fixed in 1% osmium tetroxide in buffer before dehydration in graded ethanol and embedding in Epon 812. The first step of dehydration also served to stain the tissue *en bloc* with 1% uranyl acetate in 50% ethanol for 60 minutes. Sections were cut on a Porter-Blum MT-2B at one micron and stained with toluidine blue to determine the sample site for thin sections. Light gold to silver sections were stained with uranyl acetate and lead citrate before viewing on a Philips EM-300 electron microscope.

Ultrastructural changes were consistent in the monkeys that showed lasting EEG changes consequent to heavy or moderate smoking or to intravenous delta-9-THC. Tissues of the monkey that was the heavy smoker on the new machine, the animal that failed to develop lasting EEG changes, showed similar but less pronounced ultrastructural changes. In contrast, tissues of the monkey smoked heavily with *inactive* marihuana and those of the

naive (unexposed) controls were normal. Pathologic changes were: (1) in the morphology of the synapse; (2) in the volume density of the rough endoplasmic reticulum; (3) in the nuclei, characterized by the presence of a large number of intranuclear inclusions.

Changes were essentially the same at all sites that were studied, but there was some variability in the degree of change. Pathology was greatest in the septal region, next notable in the hippocampus and amygdala, and least profound in the cortex.

At the synapse, the cleft was widened. An electron opaque material was present in the cleft and in pre- and postsynaptic regions and there was some clumping of the synaptic vessicles. Table 3 shows a comparison of axodendritic synaptic width measurements in pyramidal cells of layer five of the hippocampus. Tissues of monkeys exposed to active cannabis had a greater mean synaptic width (about 75 Å wider in the septal region and 70 Å wider in the hippocampus) than tissues of unexposed control monkeys or of the heavy-smoking monkey of *inactive* marihuana. The analysis of variance indicates the monkeys receiving the active materials did not differ significantly from each other, but had a significantly larger ($p = 0.05$) mean synaptic cleft width (277 Å) than those not exposed to psychoactive material. On the other hand, no significant differences were observed between the two unexposed control monkeys or among unexposed controls and the heavy smoking monkey of *inactive* marihuana. Measurements were obtained with Mitutoyo calipers. To avoid observer bias, all tissues were coded.

TABLE 3. HIPPOCAMPUS. COMPARISON OF SYNAPTIC WIDTH MEASUREMENTS

Monkey brain	N	Mean synaptic cleft (Å)	Standard deviation	Standard error
Heavy-smoking active marihuana	100	271.67	26.54	2.86
Intravenous delta-9-THC	100	276.84	24.22	2.07
Unexposed control 1	100	203.46	13.66	1.32
Unexposed control 2	100	205.18	10.93	1.50
Heavy-smoking inactive marihuana	100	204.18	17.10	1.44

Figure 4 illustrates the neuropil of layer 5 from a subcallosal gyrus (septal region) sample (30,000 ×) of the brain of an unexposed control monkey. Dendrites are evident in various planes of section and an electron-lucent intercellular space (100 Å–150 Å) separates all tissue components. Axodendritic synapses can also be seen, each characterized by a presynaptic bouton (B), with a complement of spherical synaptic vesicles, a distinct synaptic cleft, and a postsynaptic dendrite (D). The inset of the figure shows the synaptic cleft region of such a synapse at higher magnification (80,000 ×). The presynaptic membrane (pr), the cleft region (sc) (about 200 Å in width), and the postsynaptic membrane (po) with its subsynaptic opacity are evident.

In Fig. 5, an axodendritic synapse and astrocytic process (As) containing numerous glycogen granules (g) are seen in subcallosal layer 5 neuropil from the monkey that was exposed to large quantities of active marihuana smoke (heavy smoker). When compared to control tissue, certain fine structural differences are disclosed. The synaptic cleft region and

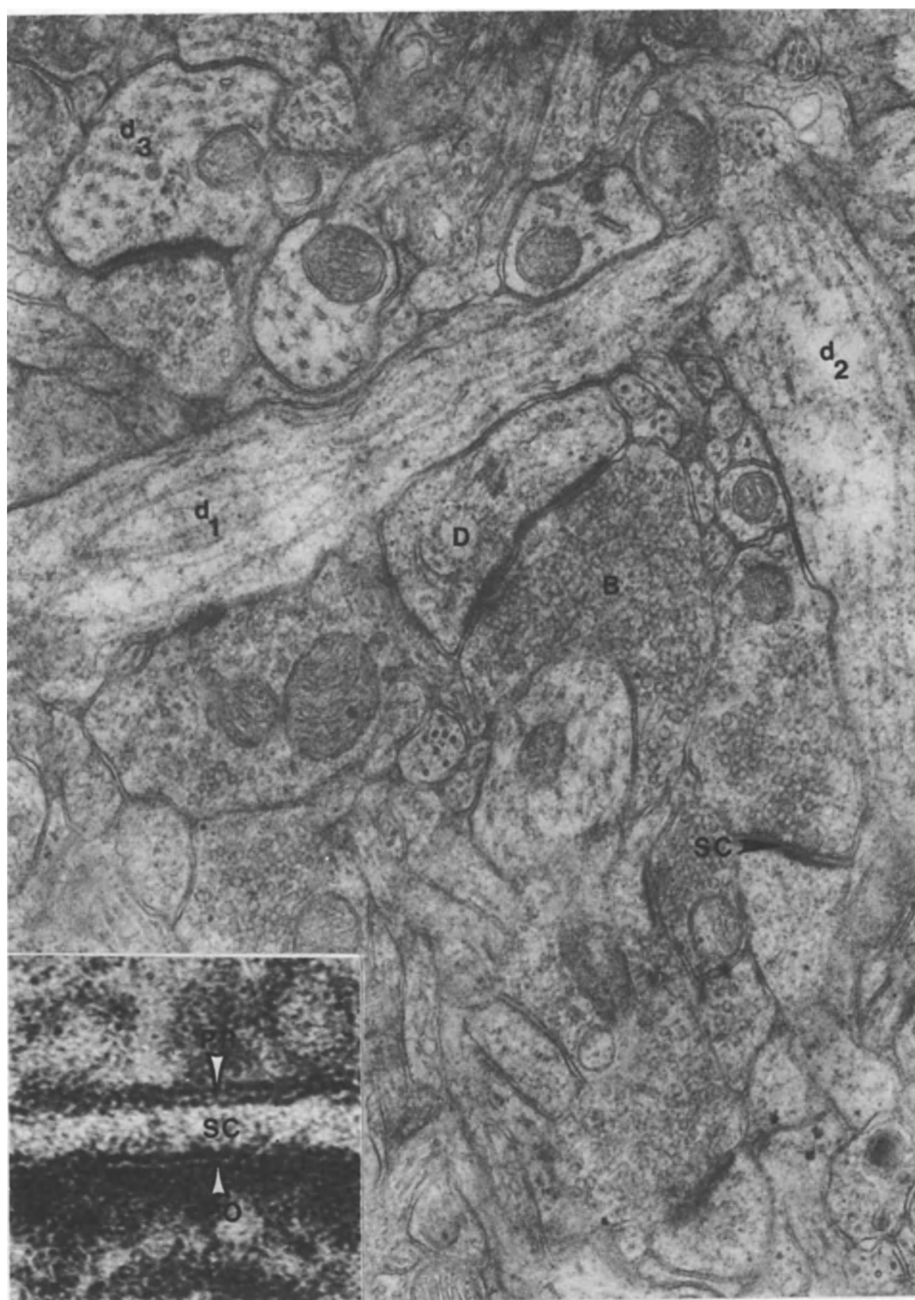


FIG. 4. Photomicrograph illustrating the neuropil of layer 5 from a subcallosal gyrus sample of the brain of a nonexposed control monkey (30,000 ×). Dendrites are evident in various planes of section (d_1, d_2, d_3). Axodendritic synapses can also be seen, each characterized by a presynaptic bouton (B), with a complement of spherical synaptic vesicles, a distinct synaptic cleft (SC), and a postsynaptic dendrite (D). The inset shows the synaptic cleft region of such a synapse at higher magnification (80,000 ×). The presynaptic membrane (PR), the cleft region (SC) (about 200 Å in width), and the postsynaptic membrane (PO) with its subsynaptic opacity are evident.

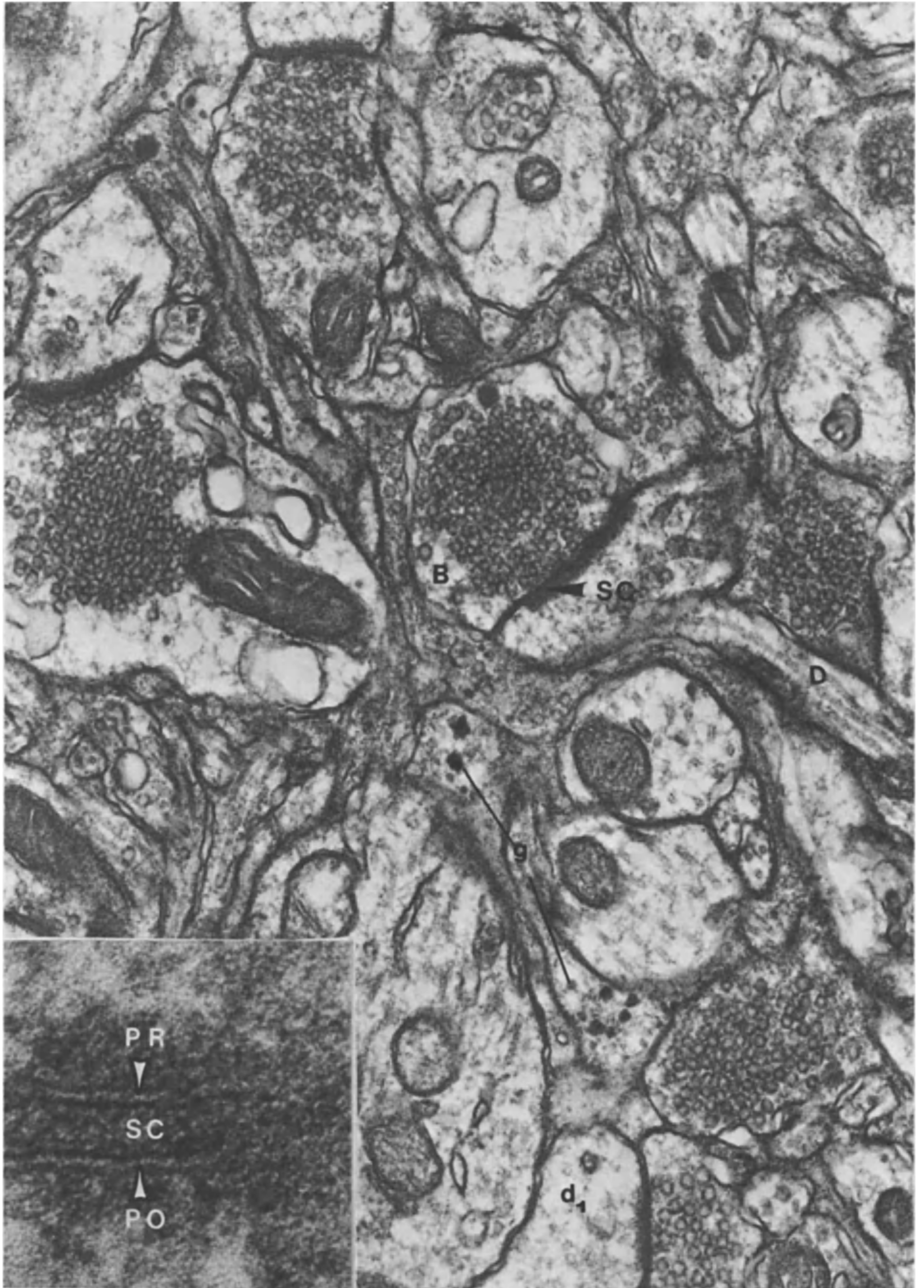


FIG. 5. An axodendritic synapse and astrocytic process containing numerous glycogen granules (g) are seen in subcallosal layer 5 neuropil from the monkey that was exposed to large quantities of active marihuana smoke (heavy smoker) (30,000 ×). When compared to control tissue (Fig. 4), certain fine structural differences are disclosed. The synaptic cleft region and some of the extracellular space (at arrow) appear to be electron-opaque. The inset shows the synaptic cleft region from a similar synapse at higher magnification (80,000 ×). Electron-opaque, granular material is seen to be accumulated in the cleft and in the pre- and postsynaptic regions.

some of the extracellular space (at arrows) appear to be electron-opaque. The inset of this figure shows the synaptic cleft region from a similar synapse at higher magnification (80,000 ×). Electron-opaque, granular material is seen to be accumulated in the cleft and in the pre- and postsynaptic regions. The distance between opposing synaptic membranes seems appreciably widened.

The next figure (6) illustrates another common observation in monkeys receiving psychoactive cannabis. The presynaptic bouton (B) appears swollen, containing aggregations of tightly packed synaptic vesicles (sv). These clumped vesicles usually occupy central regions of the bouton. Vesicles of this nature were not observed in brains of the three control monkeys that were studied.

In exposed monkeys, there were distinct ultrastructural changes in organelles of neurons, specifically of pyramidal cells ranging from 20 to 24 microns in diameter, of all brain sites studied. Alterations were in the form of reduced volume density along with various degrees of fragmentation and disorganization of the rough endoplasmic reticulum. The volume density was measured by the technique of stereologic morphometry, using a mylar screen with a point lattice (Myers and Heath, 1978; Loud, 1968; Wiebel, Kistler, and Scherle, 1966). The volume density of the organized rough endoplasmic reticulum in the brains of the monkeys that were heavily smoked with active marihuana and those that received delta-9-THC intravenously was substantially lower than that in the brains of the heavy *inactive* marihuana smoker and the two unexposed control monkeys. Values for the septal region are shown in Table 4. Similar values were found in the hippocampus and amygdala. The golgi apparatus and the mitochondria varied little in volume densities when brains of the two groups of monkeys were compared (Table 5).

TABLE 4. SEPTAL REGION. PERCENT VOLUME DENSITY CYTOPLASM. ORGANIZED ROUGH ENDOPLASMIC RETICULUM

Monkey sample	Mean	Sum of means	Standard deviation
Group I:			
Heavy active smoker	18.17	20.12	± 2.74
delta-9-THC I.V.	22.05		
Group II:			
Control 1	41.08		
Control 2	46.00	39.35	± 2.13
Heavy inactive smoker	36.97		

F 1, 3 $\quad p < 0.01$

In addition to these measured differences, we also observed various degrees of fragmentation and disorganization of the rough endoplasmic reticulum patterns with free ribosomal clusters throughout the cytoplasm, as well as swelling of the cisternal membranes (Fig. 7).

Nuclear inclusions were observed in the nuclei of the exposed monkeys in significantly greater quantity than in the nuclei of the control monkeys, both the monkey exposed to *inactive* marihuana and the unexposed controls. The inclusion bodies were of two principal types: longitudinal rodlike filaments and fibrillar or sheet-like bodies, both about 70 Å thick (Figs. 8 and 9). At each site, 130 to 500 cells were viewed. Tissue of the monkey that

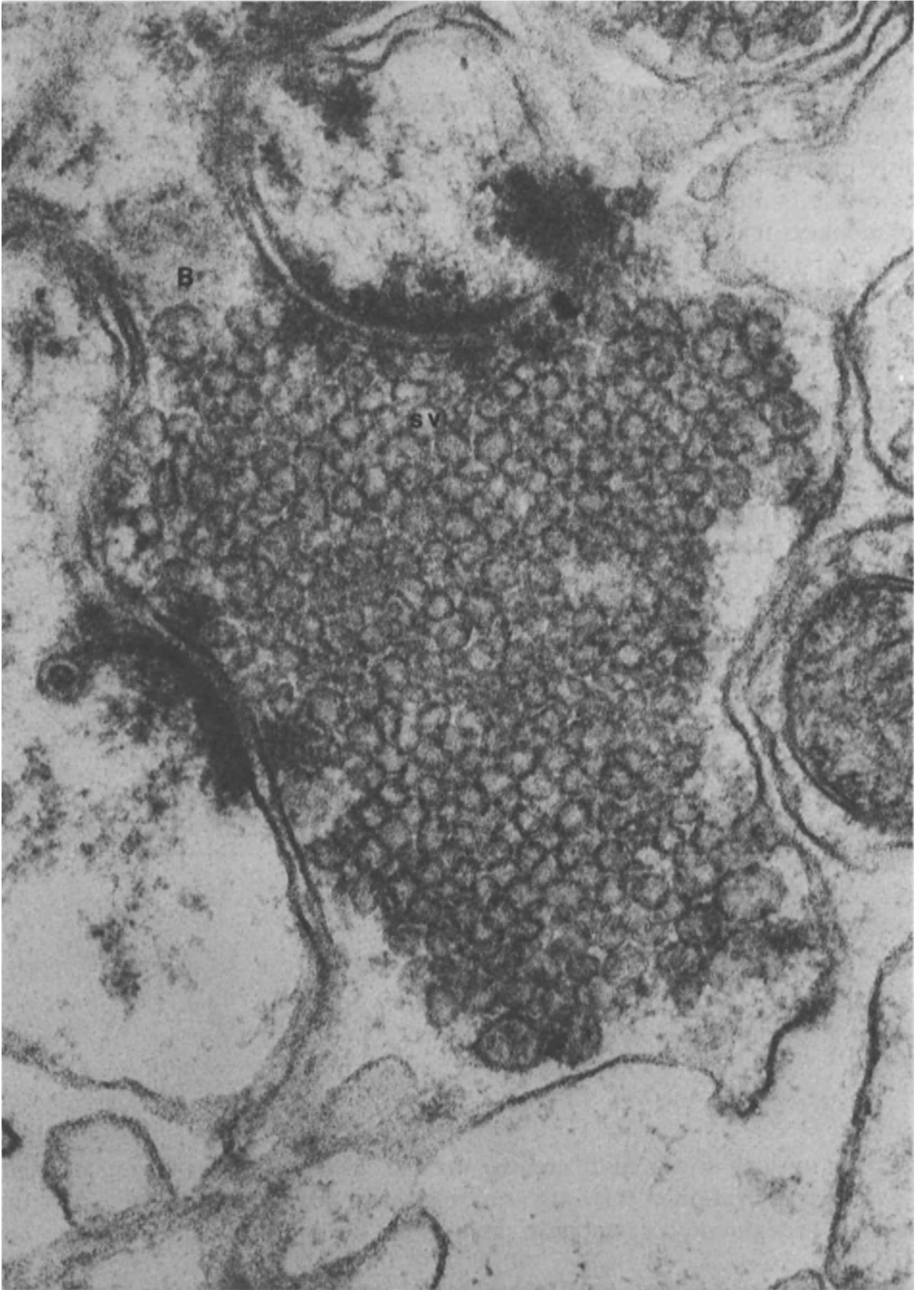


FIG. 6. This photomicrograph illustrates another common observation in monkeys receiving psychoactive cannabis. The presynaptic bouton (B) appears swollen, containing aggregations of tightly packed synaptic vesicles (sv). These clumped vesicles usually occupy central regions of the bouton.

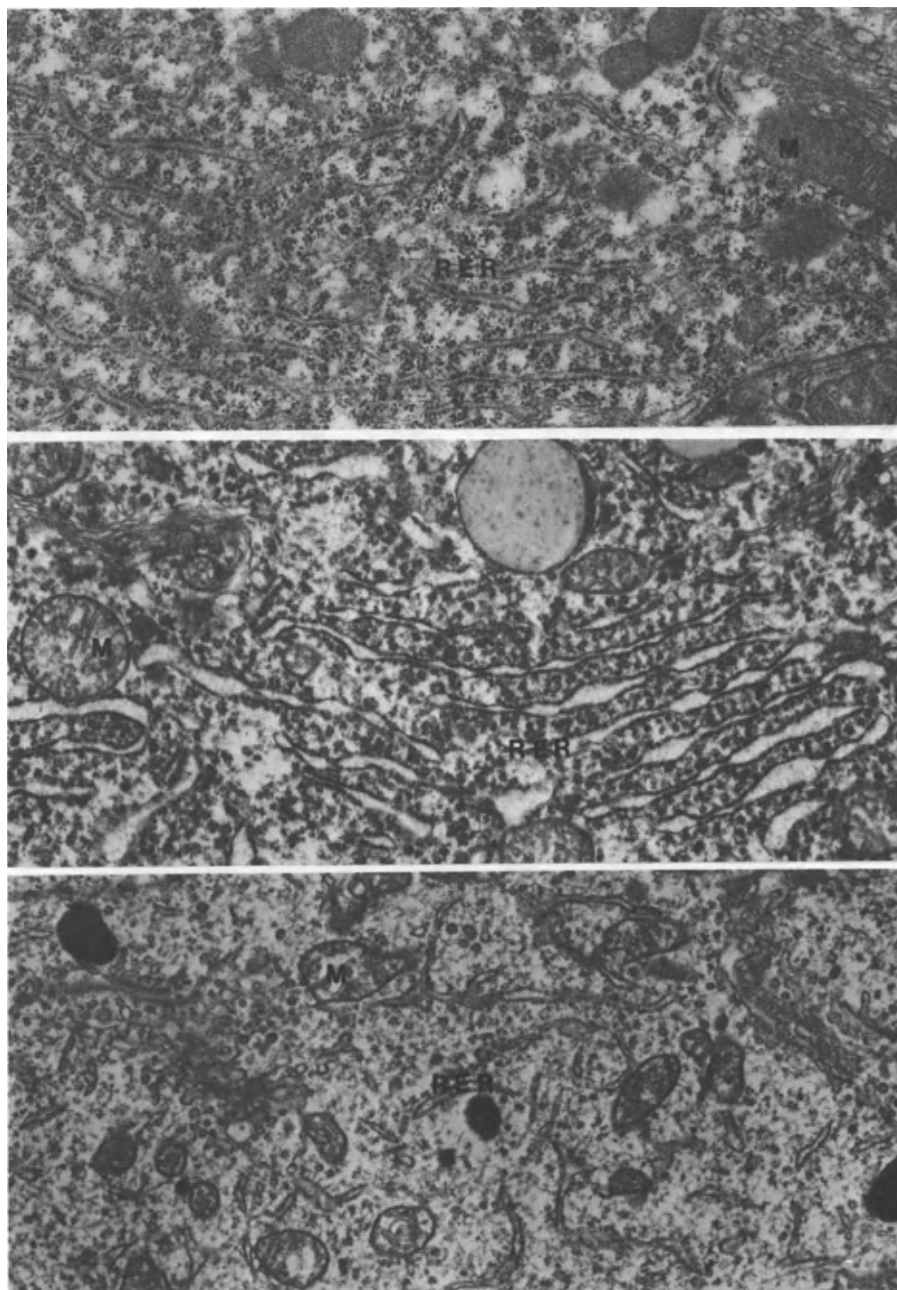


FIG. 7. Sections of hippocampal tissue. Top: Unexposed control monkey. Center: Monkey exposed to active marihuana smoke. Bottom: Monkey administered delta-9-THC intravenously for six months. This illustrates fragmentation of the rough endoplasmic reticulum (RER) with free ribosomal clusters and swelling over cisternal membranes and occasionally a breakdown in mitochondria (M) in monkeys exposed to psychoactive cannabis derivatives. 28,000 ×.

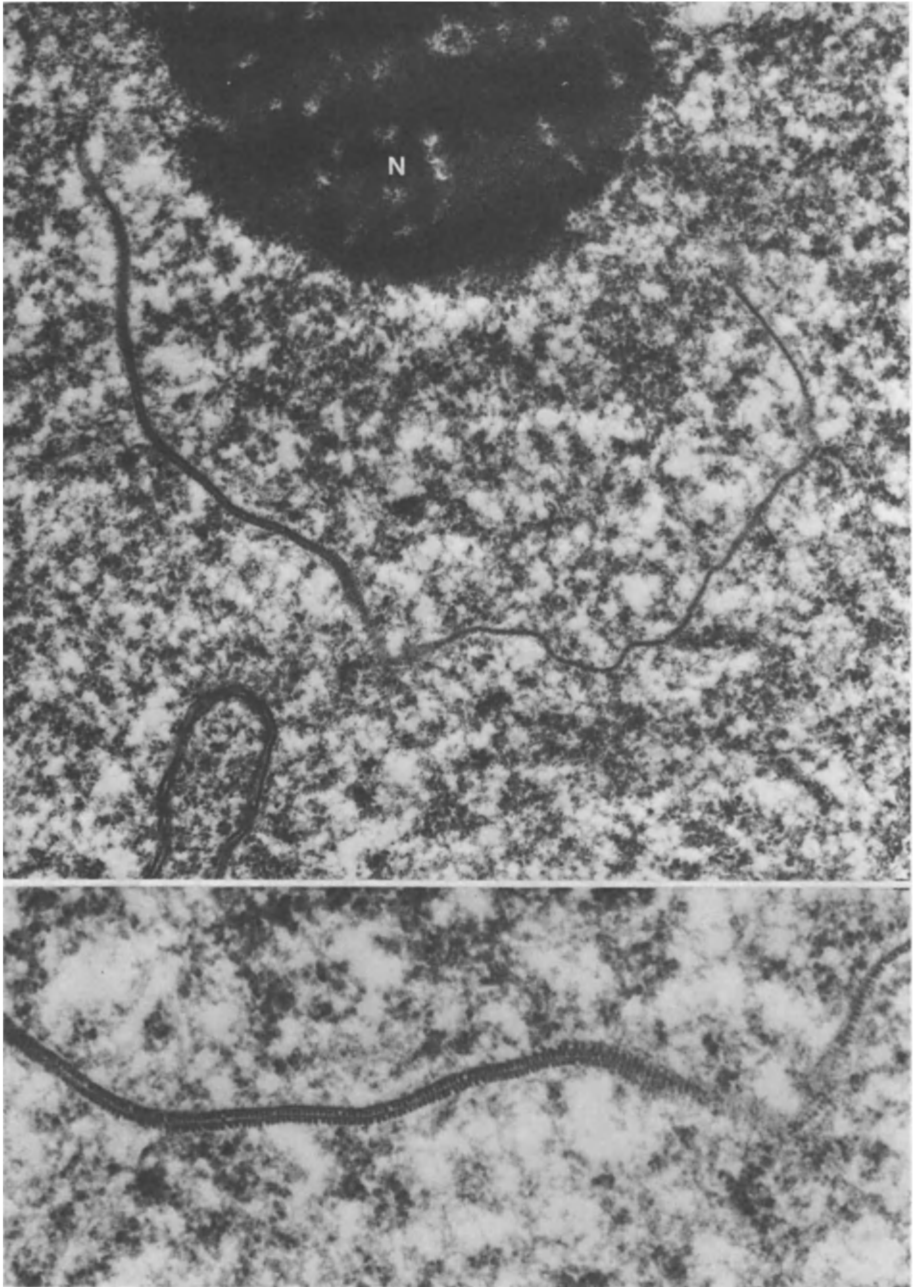


FIG. 8. Nuclear inclusion bodies, rod-like type. N = nucleolus. 21,000 × and 66,000 ×.

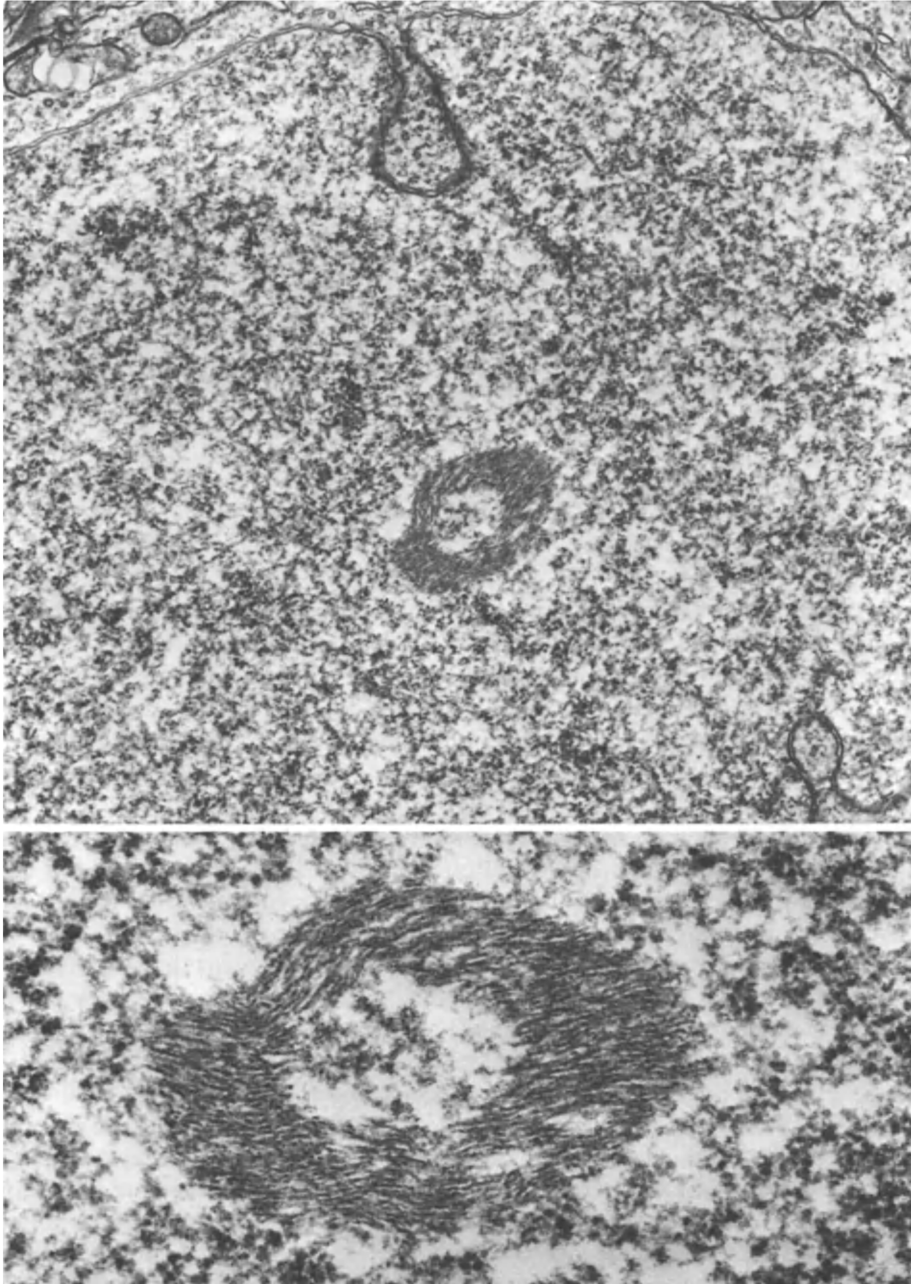


FIG. 9. Nuclear inclusion bodies, fibrilla or sheet-like type. 21,000 × and 66,000 ×.

TABLE 5. SEPTAL REGION. PERCENT VOLUME DENSITY CYTOPLASM

Monkey sample	Mean	Sum of means	Standard deviation
	Golgi		
Group I:			
Heavy active smoker	13.16	11.29	± 2.64
Δ^9-THC I.V.	9.41		
Group II:			
Control 1	12.17		
Control 2	10.08	11.70	± 1.45
Heavy inactive smoker	12.86		
	Mitochondria		
Group I:			
Heavy active smoker	13.96	13.39	± 0.80
Δ^9-THC I.V.	12.82		
Group II:			
Control 1	10.67		
Control 2	11.92	11.60	± 0.82
Heavy inactive smoker	12.23		

F 1, 3 $\quad p < 0.01$

was heavily smoked with the new smoking apparatus and later given delta-9-THC intravenously 15 minutes before sacrifice showed a uniquely higher number (24%) of inclusion bodies than tissues of the other exposed monkeys (Table 6). The inclusions were seen in 0.8% to 2.6% of the nuclei of monkeys exposed for long periods and rested, and in 0.05% of nuclei of the monkey exposed to smoke of *inactive* marihuana and rested. None were present in tissues of the two unexposed control monkeys. Rod-like inclusion bodies were seen more frequently than the sheet-like type (Table 7).

Nuclear inclusion bodies of the type seen here have been described in numerous publications (Roncoroni, 1895; Cajal, 1909–1911 and reprinted 1952). They have been noted in both pyramidal and glial cells of the central nervous system and in peripheral ganglia. They are present in many species, including the squirrel monkey (Seigesmund, Dutta, and Fox, 1964), but none have been described previously for the rhesus monkey. Their occurrence is rare in brain tissue and their significance remains obscure. They have been reported to increase in number with aging (Field and Peat, 1971) and some investigators have speculated an association with viral disease. A marked increase in numbers in the stellate ganglion has been reported following electrical stimulation (Seite, Mei, and Vuillet-Luciani, 1973), but comparable studies have not been conducted on alteration in numbers within cells of the central nervous system. Our data suggest a relationship between quantity of inclusion bodies and alteration in cell function and ultrastructure. Function was altered in the brain regions of the exposed monkeys, brain regions which showed numbers of inclusion bodies. The greatest increase in numbers appeared in the monkey that showed EEG alternations from the intravenous delta-9-THC at the time it was sacrificed.

In summary, these studies indicate that the dosages we have used in our investigations, if considered in the context of the blood levels of the psychoactive component delta-9-THC, have been in the range of levels reported in human marihuana smokers. The earlier reported physiologic changes correlate with the ultrastructural changes.

TABLE 6. INTRANUCLEAR INCLUSIONS IN NERVE CELLS

	Heavy smoker active*			Delta-9 injection			Heavy smoker active			Heavy smoker inactive			Control			Control		
	N	I	%	N	I	%	N	I	%	N	I	%	N	I	%	N	I	%
Septal	480	158	32.9%	380	15	4.0%	342	5	1.5%	385	0	0%	360	0	0%	306	0	0%
PVL Thal	282	74	26.3%	210	5	2.4%	280	1	0.4%	310	0	0%	380	0	0%	340	0	0%
Hippo	348	107	30.8%	342	12	3.5%	310	5	1.6%	314	1	0.3%	300	0	0%	310	0	0%
Amygdala	376	108	28.7%	340	9	2.7%	314	3	1.0%	309	0	0%	280	0	0%	312	0	0%
Motor Cx	340	88	25.9%	240	6	2.5%	270	2	0.7%	292	0	0%	300	0	0%	316	0	0%
Fastig	210	1	0.5%	193	1	0.5%	204	0	0%	160	0	0%	200	0	0%	182	0	0%
Caudate	205	0	0%	130	0	0%	180	0	0%	175	0	0%	140	0	0%	180	0	0%
Totals	2241	536	24.0%	1835	48	2.6%	1896	16	0.8%	1945	1	0.05%	1960	0	0%	1946	0	0%

N – number of nuclei
I – number of inclusions
% – percentage of nuclei with inclusions
* This animal was injected with radioactive delta-9-THC fifteen minutes prior to sacrifice.

TABLE 7. INCLUSION TYPES

	Heavy smoker active[a]			Delta-9 injection			Heavy smoker active			Heavy smoker inactive		
	I	R	S	I	R	S	I	R	S	I	R	S
Septal	158	99	59	15	9	6	5	4	1	0	0	0
PVL thal	74	45	29	5	3	2	1	1	0	0	0	0
Hippo	107	66	41	12	9	3	5	3	2	1	1	0
Amygdala	108	69	39	9	5	4	3	3	0	0	0	0
Motor Cx	88	63	25	6	5	1	2	2	0	0	0	0
Fastig	1	1	0	1	1	0	0	0	0	0	0	0
Totals	536	343	193	48	32	16	16	13	3	1	1	0

I – number of inclusions
R – number of rod inclusions
S – number of sheet inclusions
a. This animal was injected with radioactive delta-9-THC fifteen minutes prior to sacrifice.

REFERENCES

CAJAL, S. R., *Histologie du Systeme Nerveaux de l'Homme et des Vertebres*. Vols. 1 and 2, Maloine, Paris (1909, 1911). Reprinted Madrid Consejo Superior de Investigaciones Cienteficas, 1952.

FIELD, E. J. and PEAT, A., Intranuclear inclusions in neurones and glia: a study in the ageing mouse. *Gerontologia*. **17**,129–138 (1971).

FREIREICH, E. J., GEHAN, E. A., RALL, D. P., SCHMIDT, L. H. and SKIPPER, H. E., Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey, and man. *Cancer Chemother. Rep.* **50**,219–224 (1966).

HARPER, J. W., HEATH, R. G. and MYERS, W. A., Effects of cannabis sativa on ultrastructure of the synapse in monkey brain. *J. Neurosci. Res.* **3**,87–93 (1977).

HEATH, R. G., Definition of the septal region, in "Studies in Schizophrenia," R. G. Heath and the Tulane University Department of Psychiatry and Neurology. Harvard University Press, Cambridge, pp. 3–5, 1954.

HEATH, R. G., Physiologic basis of emotional expression: evoked potential and mirror focus studies in rhesus monkeys. *Biol. Psychiat.* **5**,15–31 (1972).

HEATH, R. G., Brain function and behavior. I. Emotion and sensory phenomena in psychotic patients and in experimental animals. *J. Nerv. Ment. Dis.* **160**,159–175 (1975).

HEATH, R. G., Cannabis sativa derivatives: Effects on brain function of monkeys, in "Marihuana: Chemistry, Biochemistry, and Cellular Effects," ed. by G. G. Nahas, Springer-Verlag, New York, pp. 507–519, 1976.

LOUD, A. V., A quantitative stereological description of the ultrastructure of normal rat liver parenchymal cells. *J. Cell Biolog.* **37**,27–46 (1968).

MYERS, W. A. and HEATH, R. G., Cannabis sativa: Ultrastructural changes in organelles of neurons in brain septal region of monkeys. *J. Neurosci. Res.*, in press.

RENAULT, P. F., SCHUSTER, C. R., HEINRICH, R. and FREEDMAN, D. X., Marihuana: Standardized smoke administration and dose effect curves on heart rate in humans. *Science*. **174**,589–591 (1971).

RONCORONI, L., Su un nuovo reperto nel nucleodelle cellule nervose. *Arch. Psichiati.* **16**,447–451 (1895).

SIEGESMUND, K. A., DUTTA, C. R. and FOX, C. A., The ultrastructure of the intranuclear rodlet in certain nerve cells. *J. Anat., Lond.* **98**,93–97 (1964).

SIETE, R., MEI, N. and COUINEAU, S., Modification quantitative des batonnets intranucleaires des neurones sympathiques sous l'influence de la stimulation electrique. *Brain Research*. **34**,277–290 (1971).

TENNANT, F. S. and GROESBECK, C. J., Psychiatric effects of hashish. *Arch. Gen. Psychiat.* **27**,133–136 (1972).

WIEBEL, E. R., KRISTLER, G. S. and SCHERLE, W. F., Practical stereologic method for morphometric cytology. *J. Cell Biolog.* **30**,23–38 (1966).

CANNABIS AND THE BRAIN: A SUMMARY

P. ETEVENON

IN THIS session, fourteen reports were presented on the effects of cannabis on the central nervous system.

Experimental results have shown catecholamine changes in the brains of self-stimulated rats, EEG changes (from deep electrodes in the lymbic area) in chronically treated rats and monkeys as well as behavioral changes. Following repeated administration, tolerance and withdrawal can be observed in animals as well as in man.

Clinical results have shown that cannabinoids affect epilepsy. Electroencephalographic studies have placed cannabis in the psychotropic group of "psychodysleptics" according to the classification of Delay and Deniker.

CATECHOLAMINES

Rats with implanted electrodes and stimulated in the reward system described by Olds, showed one hour after THC administration a decrease in brain norepinephrine, an increase in brain serotonin, together with a decrease of the rate of self-stimulation. This was followed by a rebound with reversal of the effects and later on by a persistent depression. Mescaline produced the same effects.

ELECTROPHYSIOLOGICAL AND NEUROANATOMICAL EFFECTS

In rats treated for 9 months with THC, or monkeys after 3 months exposure to marihuana via a "smoking machine", permanent subcortical EEG changes can be observed in limbic structures and sensory thalamic nuclei. "Irritative" tracings, with sharp high amplitude waves, appear. After 5 months of marihuana smoking, limbic brain tissues of a monkey, examined by electron microscopy, presented ultrastructural abnormalities.

BEHAVIORAL EFFECTS

Rats, following 6 months of chronic treatment by THC or ethanol, have presented impairment of learning curves. Following 2–3 months of THC treatment, monkeys living

in a colony became withdrawn, showed immobility and a tendency to isolation. After longer treatment they became very aggressive and they were apparently unable to cope with the demand of a new stressful situation.

TOLERANCE AND WITHDRAWAL

Tolerance following cannabis has been observed in mice, rats, pigeons, dogs and monkeys as well as in humans. A crossed tolerance exists between THC and cannabidiol, THC and diphenylhydantoin or phenobarbital, THC and ethanol, THC and morphine. A withdrawal syndrome can be obtained after 3–8 weeks in THC self-injected monkeys and in man after 10–21 days of chronic administration. THC diminishes an experimental withdrawal syndrome induced in rats but this effect is not reversed by naloxone. There is no cross-tolerance between THC and LSD or mescaline. In man, cutaneous sensitivity to pain is enhanced after THC and the reverse is observed for visceral pain.

EFFECTS IN EPILEPSY

THC can precipitate epileptic fits. In clinical trials cannabidiol showed anti-epileptic properties similar to diphenylhydantoin.

EFFECTS ON HUMAN EEG AND MEMORY

Electroencephalographic changes following small doses of THC have revealed quick shifts of vigilance between states of arousal and drowsiness. Subjects have presented body image changes together with visual hallucinations or states of "reverie" associated with intensely vivid imagery. At increasing doses, the psychodysleptic properties of cannabis are enhanced. The quantitative EEG profile of cannabis places it in a subgroup of "euphoriants" within the group of psychodysleptics such as morphinomimetics and hallucinogenic psychotropic drugs. Recall memory was also impaired. Acute and chronic cannabis intoxication is accompanied by abnormal brain function and behavior.

RESUMÉ

Quatorze rapports ont été présentés sur l'influence du cannabis sur le système nerveux central.

Les résultats expérimentaux font état de changements du métabolisme des catécholamines chex des rats autostimulés munis d'électrodes profondes dans la région limbique, de changements electrophysiologiques chez des rats et des singes soumis à des traitements chroniques et de changements comportementaux. Après administration répétée, tolérance et syndrime d'abstinence peuvent être observés chez l'animal comme chez l'homme.

Les résultats cliniques portent sur l'action des cannabinoides dans l'épilepsie. Les effets du cannabis sur l'électroencéphalogramme s'apparentent aux psychotropes psychodysleptiques de la classification de Delay et Deniker.

CATECHOLAMINES

Des rats autostimulés positivement présentent une heure après administration de THC, une diminution de la fréquence d'autostimulation concomittante d'une baisse de noradrénaline et d'une augmentation de sérotonine. Deux heures après l'administration de THC, un rebond intervient avec inversion des effets qui est suivi d'une dépression persistant plusieurs heures. La mescaline psychodysleptique produit les mêmes effets.

ELECTROPHYSIOLOGIE ET NEUROANATOMIE

Chez des rats soumis à 9 mois de traitement chronique ou des singes soumis à 3 mois d'exposition à une "machine à fumer", des altérations permanentes des tracés s'observent. Des tracés irratitifs du type à ondes pointues de grande amplitude apparaissent dans les structures limbiques et les noyax thalamiques sensoriels. Après 5 mois d'exposition à la fumée de marihuana, un singe a présenté des changements ultrastructuraux neuroanatomiques anormaux.

COMPORTEMENT

Après 6 mois de traitement chronique par le THC ou l'alcool, des rats ont présentés une diminution des courbes d'apprentissage. Après 2–3 mois d'administration de THC à des singes élevés en colonie, apparait un comportement d'immobilité et d'isolement qui fait suite, après une plus longue administration de THC, à un comportement d'agressivité active persistante. Cela pourrait être dû à une incapacité de faire face à de nouvells situations de stress.

TOLERANCE ET SYNDROME D'ABSTINENCE

La tolérance aux effets du cannabis a été observé chez les souris, rats, pigeons, chiens, singes comme chez l'homme. Il exist une tolérance croisée entre THC et cannabidiol, THC et phénylhydantoin ou phenobarbital, THC et alcool, THC et morphine. Un syndrome d'abstinence peut être observé chez des singes auto-injectés en THC pendant 3–8 semaines et chez l'homme après 10 à 21 jours d'administration. Chez le rat, un syndrome d'abstinence expérimentale peut être diminué par le THC mais n'est pas alors antagonisé par la naloxone. Chez l'homme, la sensibilité douloureuse cutanée semble augmentée après THC, à l'inverse de la douleur viscérale.

ACTION DANS L'EPILEPSIE

Le THC peut précipiter des attaques épileptiques tandis que le cannabidiol essayé en clinique présenterait des propriétés antiépileptiques proches de la phénylhydantoïne.

ACTION SUR L'EEG ET LA MEMOIRE CHEZ L'HOMME

Les altérations électroencéphalographiques après de faibles doses de THC révèlent un télescopage d'états de vigilance entre la veille et l'endormissement. Les sujets présentent alors des troubles du schéma corporel et des états de rêverie avec production visuelle intense qui peuvent aller jusqu'à des hallucinations. Aux plus fortes doses, les propriétés psychodysleptiques du cannabis se révèlent encore plus. Le profil d'électroencéphalographie quantitative des effets du cannabis permet d'en faire un sous-groupe à part, qualifié d'euphorisant, à l'intérieur du groupe des psychodysleptiques tels que les morphiniques et les hallucinogènes. La mémoire de rappel est également altérée par le cannabis. La consommation aigue et chronique du cannabis produit des anomalies de la fonction cérébrale et du comportement.

CONCLUDING SUMMARY

W. D. M. PATON

IN THE three years since the Helsinki meeting, how has knowledge advanced?

(1) One can recognize some simplifications. (a) At that meeting, much work was reported on cannabinoid metabolites, and the number of these has further increased. Yet it appears now that, for psychic action in man at least, it is only Δ^1-THC itself, and perhaps the first metabolite, 7-OH-THC, that contribute significantly; side-chain hydroxylation does not appear to occur in man. In animals, however, a contribution particularly by the 3″-hydroxyl metabolite must be allowed for. (b) The kinetics of THC *in vivo* are clearer too. The slow elimination of its metabolites is the result, not of slow metabolism, but because Δ^1-THC (and perhaps 7-OH-THC) is early sequestered and then only gradually released from the deep compartments of the body. Since metabolism is rapid and not rate-limiting, changes in metabolism are unlikely to influence duration of action, although obesity might do so. Daily dosage will lead to body levels 5–10 times higher than a single dose, a "cumulation factor" similar to that estimated from toxicity studies. (c) The clinical assay of urinary or blood cannabinoids is also at last proving accessible; and a relatively simple radioimmunoassay has been proved able to show an association of cannabis with traffic accidents, to distinguish cannabis intoxication from other conditions, and to reveal cannabis use up to 30–50% in addiction treatment clinics. (d) Finally, the "surface-area" rule for calculating equivalent doses in various species, a rule that appeared probable both in principle and from the pharmacological data, has been directly verified by the measurement of cannabinoid blood levels. Rats and mice need respectively a dosage by weight about 7 and 12 times higher than man for equivalent effect, and oral dosage appears about 5 times less effective than inhalation. These important results confirm the relevance, questioned from time to time on grounds of excessive dosage, of a great deal of experimental work.

(2) The question of the "specificity" of the various actions of the cannabinoids remains complicated. Several aspects are involved. Thus any effect which could be strongly correlated with the characteristic psychic activity would be a valuable clue to the mechanism of that action and to a possible "receptor"; on the other hand, effects which correlate simply with partition coefficient into lipid (or some equivalent parameter) could be regarded as strictly nonspecific and comparable to anaesthesia. An elegant example of the former is the manner in which Δ^1-THC is 1000 times, and 7-OH-$\Delta^{1(6)}$-THC about 10 times more active in inhibiting lysophosphatidylcholine

acyltransferase in mouse brain synaptosomes than predicted from their antihaemolytic potency (a measure of "nonspecific" action), whereas cannabinol and cannabigerol fell in line with the series of alkanols. In contrast, the ability of the cannabinoids, in higher concentrations, to inhibit the uptake of thymidine in cultured lymphocytes appears to be directly related to fat solubility. Depression of the electrically evoked twitch of the guinea-pig ileum proves to correlate as well with the psychic action of cannabinoids as it does with opiate analgesic action. But with other responses anomalies appear, such as cannabidiol or cannabigerol being unexpectedly active, on dopamine uptake into mouse cortex synaptosomes and on nuclear membrane-attached ribosomes in infant rat brain respectively. The possibility exists that particular cannabinoids may have their own specific actions, as exemplified by the clear superiority of cannabidiol over other cannabinoids in controlling epilepsy. A difficulty results, therefore, as to what substances to employ as controls for "specificity" of action: CBN, CBD, (+)-Δ^1-THC, (+)-Δ^6-THC and Δ^7-THC have been used for this purpose. Δ^7-THC, indeed, may be particularly suitable, since it is especially close to Δ^1-THC in structure, yet it is psychically inactive (an important fact about the THC "receptor") and does not form 7-OH-Δ^1-THC. But since other cannabinoids are beginning to reveal characteristic actions, non-cannabinoid controls may sometimes be advisable.

(3) At the neuronal level, the available evidence about THC action remains tantalizing. It is true that when effects on higher function are studied, as in the discussion of memory and the interaction with the limbic system in this volume, reasonably coherent pictures emerge. The introduction of sensory decision theory into study of the effects of cannabis on pain sense is a useful development, showing (for Hardy-Wolff type pain) increase both in discriminability and response bias. Tolerance to this pain enhancement develops in parallel with tolerance to the "high". Extension of studies of social behaviour from rodents to primates is also welcome; irritability seen in rodents with long-term treatment reappears, alterations in dominance hierarchy develop, and socially adaptive behaviour may be impaired. At a simpler level, it appears reasonable to attribute the central stimulant effects of cannabis to generation of hypersynchronous neural discharge, but how this is brought about is still unsettled. Δ^1-THC depresses rewarding self-stimulation behaviour, and tolerance develops to this. But we cannot yet reliably link these results to definite neurochemical changes. The curious relation of Δ^1-THC to opiates remains: totally different chemical structures; a number of shared effects; a number of interactions with naloxone; and curious patterns of cross-tolerance, never fully reciprocal, and varying with the test used. The suggestions that THC may change the affinity of opiates or of related endogenous peptides for their receptors, or that they release such peptides, deserve detailed study. But we still lack any well-defined type of neurochemical action by Δ^1-THC as a basis for all these effects. Although we must recognize radical differences between THC and general anaesthetics, perhaps it is the case that the actions of both of them are to be defined not in terms of specific transmitters, but in terms of some other characteristic of the components of synapses such as size, geometry, or membrane composition.

(4) A notable feature has been the development of work in three interlocking fields: the effects of cannabinoids on spermatogenesis, on endocrine activity and on foetal development. Three mechanisms for an effect on sperm formation are suggested: in mice the development of abnormal sperm heads, and increased ring and chain chromosomal translocation in spermatocytes could imply a direct action on germ cells; impairment

of testosterone production by isolated Leydig cells stimulated by human chorionic gonadotrophin may be connected with reduced protein synthesis; and there is a reduction of the cytochrome P450 of rat testis microsomes (required for testosterone synthesis), possibly due to a fall in gonadotrophin level. Chronic high doses of cannabis by inhalation can lead to a fall in sperm count in both rats and man. These effects do not appear to correlate with psychic potency. More work is needed to disentangle the roles of reduced gonadotrophins, directly impaired testosterone production or direct cellular effects.

An important finding is that reduction by THC of gonadotrophin blood levels, suggested also by the fall in uterine and ovarian weights in Fischer rats after THC treatment, has been demonstrated by radio-immunoassay in the rhesus monkey. This inhibition appears to be at hypothalamic level since it is overcome by LH releasing factor. No evidence was found of direct oestrogenic or anti-oestrogenic activity by THC.

The embryotoxicity of THC and cannabis is now established in the rodent and rhesus monkey, at dose levels corresponding to heavy or moderately heavy chronic marihuana use in the human. Frank teratology was not found in the studies reported here. Mid-gestational vaginal bleeding in the rodent suggested a direct endometrial effect, with impairment of foetoplacental circulatory development, and gross placental infarction was noted in one instance when a rhesus placenta was obtained; but a failure of endocrinological support also remains a possible cause.

In each field, considerably more endocrinological work is needed, particularly to distinguish actions due to cannabinoids directly on a target organ from actions of neuro-endocrine origin.

(5) Two important pulmonary studies were reported in rodents exposed to marihuana smoke. Sustained exposure to doses giving plasma Δ^1-THC levels similar to those found in human users induced a focal pneumonitis progressing to rather serious pathological changes, including focal granulomata and cholesterol clefts; these effects were distinct from those of placebo and tobacco smoke. In the other study, both marihuana and tobacco smoke were shown to impair the lung's antibacterial defences; the half-time for inactivation of staphylococci in the lung was increased from 3.5 to 4.3 hr by tobacco smoke and to 7.5 hr by marihuana. The latter observation may be related to the immobilization of alveolar macrophages by THC reported at Helsinki. Since deep inhalation and retention of marihuana smoke is necessary for efficient extraction of THC, further pulmonary study is important.

(6) Finally, two studies may be mentioned which, like the work on nuclear membrane-attached ribosomes, carry the analysis down to the ultrastructural level. First, at Helsinki, a number of electrophysiological abnormalities were found in recordings from the brains of monkeys chronically exposed to cannabis smoke, and these persisted up to 6 months after exposure ceased. Ultrastructural abnormalities have now been found to develop under these conditions; these included widening of synaptic gaps, clumping of synaptic vesicles, disorganization of rough endoplasmic reticulum, and nuclear inclusion bodies. Changes were most pronounced in the septal region, next in hippocampus and amygdala, least in cortex. Secondly, at Helsinki, reduction of arginine-rich and increase of lysine-rich histones in leucocytes and spermatozoa of chronic hashish users was reported, with accompanying structural changes. In polymorphs and lymphocytes, abnormal condensation of chromatin and reduction in number of nuclear pores has

now been found. Lysine-rich Hl histone acts as a condensing factor in somatic cells repressing genomic expression, so that impaired lymphocyte function would be expected. In the spermatozoon, where arginine-rich protamine replaces the lysine-rich histones and achieves total condensation of the protamine, acrosomes were abnormal or even absent, and chromatin condensation was incomplete. The suggestion is made, therefore, that a single biochemical lesion, arginine-depletion, may be responsible, by cell-specific mechanisms, both for lymphocyte and spermatozoal abnormalities. More generally, interference with cell histones may well underly the varied chromosomal abnormalities that have now been reported under diverse experimental conditions. The correlation of ultrastructural and functional studies in these ways may be of considerable value.

In conclusion, one may ask what answers may be given to three questions:

How does Δ^1-THC produce its effects? The idea that in low doses it acts on a "receptor" and in higher doses acts less specifically is becoming probable. High potency and loss of activity with optical isomers or small changes in chemical structure point strongly to some special recognition site, whether it is a conventional receptor or some especially well-fitting hydrophobic site. The less specific actions, now extending over a wide range, are of particular significance for the chronic user.

Does cannabis cause damage to the body? The phrase used in the summary of the session on reproduction, "incontestablement nocif", is justified. Studies with chronic administration reveal long-term damage to lungs, reproductive function, and the immune system. The fact that cannabinoids are cumulative accentuates the risk. Now that structural changes in the central nervous system have been described that outlast exposure to the drug, the reversibility of the effects of long-term use, including that on memory, comes into question. The development of a simple clinical test for cannabinoids in blood and urine should help in quantifying the risks.

Is cannabis addictive? Tolerance occurs to the psychic action, as well as to many other effects. Characteristic withdrawal symptoms develop; these are the more striking in that, because of the slow elimination of cannabis from the body, withdrawal from the tissues must be slow. Psychic dependence is shown by the fact that consumption is not merely to avoid withdrawal symptoms but to maintain the psychic effect: to quote from the study on pain and memory, as tolerance developed, "the subjects were complaining that the cigarettes were no longer producing a 'high'". Cannabis satisfies the usual criteria for an addictive drug.

APPENDIX
CANNABIS AND ALCOHOL: THE GREEN AND THE RED

THE historian conversing with scientists is at a disadvantage. His presumed facts and the conclusions based upon them cannot be either proved or disproved by constantly renewable experiments. They depend upon fossilized sources that are often limited, more often than not the result of fallible observations and hazardous transmission. And then, they are subjected to the vagaries of the combinatory powers of the human mind, which are even more fallible. On the other hand, historians can give free play to their creative imagination because of the limitations inherent in the character of their information. This makes possible an arrangement of the available data in such a way as to allow for the emergence of a clear and instructive picture of the historical impact on human society of the subject under study.

In the past, the means of written communication were much more expensive and difficult of access than they are today. Scholars and writers had to be vastly more selective with respect to what they would choose to write down. The ordinary human experiences of daily life were often taken for granted and little commented upon. This was true of many matters that appear to us to be fundamental for an understanding of man and society. One such example is the use of drugs for sensory stimulation. At certain periods in history—and our own is one of them—such use causes noticeable and unusual societal problems.

Such a period occurred in the Muslim world in the late twelfth or early thirteenth century when the use of hashish became a social problem in the far-flung regions dominated by Islam. It has remained one ever since. From Central Asia, the use of hashish worked its way rapidly and relentlessly westward until it reached Muslim Spain. "The Herb", "The Green One". "The Morsel of Thought", could not be stopped. Its many other nicknames were used as terms of endearment and also of mystification, but with increasing frequency it came to be called "The Evil Herb" or "Satan's Own Food". For the benefit of latter-day scholars, the calamity became so marked that the literature of the time could no longer overlook the existence of hashish.

With respect to its effect upon society, the use of hashish should be considered under three aspects, each very different in nature and significance: first, its use for purely medical applications; second, its sporadic and haphazard consumption for non-medical purposes; and third, its spread from casual use by individuals to wide-spread consistent use by society at large.

The knowledge of a possible medicinal use for cannabis passed from Classical Antiquity to the physicians and pharmacologists of Muslim civilization. Although the historic references are few, a small number of allegedly beneficial applications are mentioned. Problems of language and terminology, however, constantly interfere with our understanding; less

so, perhaps, in the strictly medical and pharmaceutical literature than in general reports on hashish. Sometimes drugs other than cannabis, or in addition to it, may have been involved in a so-called medicinal use. In some instances, there is no way of knowing what the confection referred to as "hashish" really was; in others there is no doubt. Furthermore, there is no clear indication from medieval times that cannabis was ever smoked. Apparently the smoking of it began about the same time that tobacco cigarettes were introduced from the New World. Before that, hashish was eaten as a confection, usually made up of a variety of ingredients.

No matter how much or how little the literature has to say about cannabis, it was always present in the Muslim pharmacopoeia, just as it had been known and used by Galen and Dioscurides long before the appearance of Islam.

The use of hemp leaves was recommended in a large number of ailments: to stimulate the appetite, to dissolve flatulence, as a diuretic, to clean up dandruff, to clear the brain, for soothing pain of the ears. It was also "good for digestion", and one report claims its usefulness in epilepsy.

That hashish was also used as a stimulant—or, to translate the Arabic term literally, as an "intoxicant"—by some individuals in the Muslim world between the seventh and twelfth centuries remains a conjecture. There is no information in the historical sources. In Islamic society, it was of no concern to public authorities what an individual did in the privacy of his home, especially if, as in the case of hashish, no explicit statement against its use existed in the authoritative religious texts. As long as individual action did not come to public attention and cause a public nuisance, it was likely to be disregarded.

There are many who feel that what an individual does in private is indeed no matter of public concern. But when sufficiently large numbers of individuals all do the same thing, it will inevitably provoke public scrutiny and, if necessary, some kind of public action.

In the case of hashish use in Muslim society, from the 12th century on it became obvious that it was a problem for society and that action was called for. But what kind of action, and how to justify it in a society held together by one thing only—the religious law of Islam? The Prophet Muhammad and the early Muslims could not be credited with an express statement declaring hashish unlawful. Because hashish use had not been a problem in the early years of Islam, there had been no reason to take note of it. In contrast, the consumption of alcohol was a different matter. Well known for its effects, it was forbidden in Muslim law on the basis of the Holy Qur'ân. Thus, legal scholars used as their principal argument for control of hashish the assumed similarity of its effects with those of wine and other alcoholic beverages. Unfortunately, just as it is true today, the factual situation was ambiguous, and the necessary legal reasoning was therefore compromised. Already the jurists had to contend with problems concerning the prohibition of wine, and any comparison of hashish with alcohol was troublesome because their effects were not identical.

Not only did the jurists compare hashish with wine, but also poets who used the terms the "green one" and the "red one". Even though wine was forbidden and illegal, it was enjoyed by many, in particular by the upper classes who could afford it. Since poetry about wine was extremely popular, with the advent of hashish, its rich repertory of poetic images and rhetorical figures could be transferred easily. In Islam, every educated individual (and many of those with little formal education) was a poet, so verses on hashish provided new sensations for the jaded tastes of the connoisseurs.

Another literary convention of the writers from Near Eastern times as well as Classical

Antiquity was the highly esteemed form of the playful exposition of the merits or faults of two comparable objects. For example, different kinds of animals, flowers, human occupations, and cities were often compared. In Muslim civilization this form of poetry reached new pinnacles of artistry. Quite naturally, as soon as the hashish habit had insinuated itself into wider social groups, writers of the day applied the literary form to an alleged rivalry between hashish and wine.

Such was the case with a poet who lived in Syria from 1222 to 1258, and who exercised his considerable wit and poetic skill by composing a long rhymed debate between imaginary pro-hashish and pro-wine parties. Characteristically, he does not reveal his own preference nor does he make any moral judgements about either hashish or wine. Whether poets approved or disapproved of the moral practices they used for themes in their poetry remains a mystery. Personal experience or opinion counted for little, linguistic and literary virtuosity was their goal. And yet, this medieval Syrian poet presents in his poem the main arguments that have been repeated over and over again in popular discussions for and against the use of hashish.

First, the word is given to the pro-hashish party:

1. Greetings, my friend! Don't listen to the critics
   And don't condemn without a legal basis!
2. You wish to know about wine and the green stuff.
   Thus listen to the words of a true expert!
3. Hashish has qualities that wine cannot boast of.
   Can wine be drunk in mosque and Sufî Convent?
4. You can obtain the green stuff without haggling.
   You do not need much gold and silver for it.
5. No! Unlike wine, it is a gift. It's gratis,
   Yes truly indispensable though cheap it may be.
6. It grows in meadows green like heavenly gardens,
   Whereas their wine is like a Hellish firebrand.
7. Their wine makes you forget all meanings. Our herb
   Recalls the mysteries of godly beauty.
8. The secret of hashish lifts up the spirit
   In an ascent of disembodied thinking.
9. It is the spirit pure. Free are its confines
   From worries. Only the elect may taste it.
10. No feet have trampled on it, nor has black pitch
    Been used for sealing casks and made them dirty.
11. The body is not tired eliminating
    And vomiting like an inflated wine-skin.
12. No one will think of you as void of reason,
    Nor call you a corrupter of religion.
13. Tucked in a handkerchief it can be carried.
    No cup is needed if you wish to use it.
14. Hashish involves no sin. You are not punished.
    There's no disgrace, no quarrelsome companions.
15. In times both good and bad you can enjoy it.
    It is no hindrance to nights of devotions.

16. There is no danger of raids by policemen.
    The government will not unjustly fine you.
17. You find yourself clean, virtuous, and witty,
    Bright, too, and free from all annoying dullness.
18. You find you have no enemies to hate you.
    You always have a lot of friends around you.
19. The beloved, tasting it, will sneak away from
    Invidious chaperones and come unbidden.
20. Another thing: it's good for your digestion.
    But all its many virtues cannot be mentioned.
21. They're all there when my company at eating
    Hashish is a gazelle like a willow slender!
22. She treats me to a pretty poem, singing,
    Her voice putting to shame the cooing pigeon.
23. She flirts with me demurely with her big eyes,
    And when she smiles shows rows of brilliant white teeth.
24. When critics talk about hashish, don't listen!
    Their aim is to deter you. Don't obey them!

Then it is the turn of the champions of wine:

25. Now, dear companion whom I love so dearly,
    Accept right guidance to preserve your pleasure!
26. Would you by eating grass that is not juicy
    Want to be like a dumb beast without reason?
27. Please disregard the views of bestial people!
    Just pass around the starry, shining jewel!
28. 'Tis wine I mean. A traveler lost is guided
    At night back to the right path by its brilliance.
29. Their herb brings shame upon a decent person
    So that he slinks about just like a killer.
30. It reproduces on his cheeks a green sheen.
    His face seems darkish, like pale dust its color.
31. When his friend thinks of him at night, he shudders.
    It is like morning turning into dark night.
32. Our wine brings honor to the lowly person
    And dignity so that none is his master.
33. When wine appears, it banishes all worry.
    The drinker's thirsty heart is fully sated.
34. When wine appears, the drinker's secret gets out
    And gladdens him, his rosy cheek wine-colored.
35. Unlike hashish, its qualities are useful.
    Speak out! Count and describe wine's many meanings!
36. That other substance is harmful to mankind.
    Thus tell us all about the evil in it!
37. No caliph surely ever tasted hashish,
    Nor did a king in full command and power.

38. No poet ever seriously praised it
    In words as artful as the singer's, Ma'bad.
39. No strings were ever plucked in praise of hashish.
    The roseate drink alone can make this happen.
40. Whatever else but wine can tinge the hands that
    Holding a cup of wine reflect its color?
41. Drunk the beloved turns and bends down, swaying
    Gracefully like the bent branch of a willow,
42. Giving you wine like wine in her saliva,
    The white teeth in her mouth agleam and brilliant.
43. She hesitates no more to join her lover.
    Then she forgets all on the day that follows.
44. Who would refrain from something of this nature?
    I was not well advised to give up drinking.
45. But for those meddlers I would ne'er be sober,
    Nor would I listen to the critics' comments.
46. Drink wine! Don't listen to censorious people,
    Though wine may be outlawed in our religion!

Note that the poet was writing at a time when the hashish wave was still in its early stages and probably had not yet engulfed urban life. Perhaps this is why he can afford to give the impression of a certain objectivity. Nonetheless, his arguments are very familiar. He begins triumphantly with a fanfare: hashish has not been proved illegal. He continues with the trump card of addicts: it is used by the representatives of true religious fervor—the mystics. It is even eaten in mosques. It involves no sin, no legal danger, no penalties. It is easily carried and consumed. Above all, it is cheap, much cheaper than wine, and everyone can afford it. But the poet describes only briefly the physical and mental deterioration caused by hashish, because the wine lovers are only too glad to make their most important point, namely, that in contrast to wine, hashish means low social status and reduces its users to the dregs of society and culture. But the hashish addicts think of themselves as an elite group. They claim beautiful experiences in the realm of pure spirit, experiences that set them apart from the rest of the common herd of the uninitiated. They are alone and withdrawn, but they have many friends. They believe that the drug makes them peaceful, and they feel that by using it, they become especially lovable individuals.

The poet permits each side to claim that its favorite intoxicant is a powerful aid to seduction. His poem, like so many others, was meant to appeal to erotic fancy. In fact, hashish use was often declared detrimental to sexual activity, but it was also described, in the same way as in the above poem, as a means to obtain sexual satisfaction from a lover unaware of being under the influence of the drug and whose inhibitions were weakened by its use.

Poets in later times continued to compose short "jeux d'ésprit" exalting the alleged virtues of hashish. Whether or not they were serious is difficult to determine. Quite often, it seems, they reflected the positive attitude toward the drug that was characteristic of certain members of the upper class intellectuals. For them, hashish is a thing of true beauty; it gives them irrepressible joy and repose and provides them with relief from worries and anxiety. It reveals to them secrets and opens to them new meanings. It increases their understanding and enlarges their imaginative perceptions. An affinity of the hashish eater

to music was occasionally reported. No truly violent actions directed against other perons under the influence of hashish are mentioned in these stories; but the pro-hashish faction never comes to grips with the points raised by the attackers.

These scholars compiled a long list of the mental and physical ill effects caused by the drug: reddening of the eye, dryness of mouth, excessive sleeping and heaviness in the head when the drug takes possession of the brain, as well as numbness of the extremities. Prolonged use dries up the semen (already noted by Galen) and cuts off the desire for sexual intercourse, cuts short the reproductive capacity, brings forth hidden disease, harms the intestine, makes the limb inactive, causes a shortage of breath, diminishes vision in the eye and increases pensiveness in the imagination after initially causing joy; hashish produces narcosis, laziness, stupor, weakening of sense perception, foul breath, ruination of color and complexion.

Hashish is mind changing and personality changing, causing "insanity in the habitual user", "changes the mind making it absent from reality".

Habituation to hashish is also stated. "Among the greatest physical harm caused by it is the fact that habitual users of it are hardly ever able to repent of it because of the effect it has upon their temper" says al-Zakarshi, and al-Badri concurs: "The user cannot separate from it and leave it alone".

Hashish is stated consistently by its adversaries to be something that saps the user's energy and ability and willingness to work. Implicitly this was considered its greatest danger to the social fabric.

Finally, a holyman, Sheikh al-Hariri described what may be the lingering effect of chronic hashish usage. He claimed that abstinence for a long period was necessary to overcome the long-term action of the drug in the organism. "One has to give it up for forty days, until the body is free from it, and forty more days until he is rested from it after becoming free". The jurists also used on occasion the persuasive form of rhyme to speak about hashish. Their attitude was extremely negative, as one from their ranks put it:

> All the destructive effects of wine
> Are found in hashish many times over.

They tried to fight hashish or assumed the official posture against the drug, but they were waging an uphill struggle. Even though they were successful in arguing for the illegality of hashish, they proved decidedly unsuccessful in devising effective means for curtailing its use. On the contrary, it appears that eventually they became resigned to letting matters take their course.

The similarity of views and arguments, then and now, is the most striking feature of the medieval debate. The dilemma still exists between the rights of individuals (which in our view, if not the Muslim, may extend to self-debasement and self-destruction) and the needs of society. It is no closer to being solved. The same romantic claims to beauty and spiritual release and other benefits derived from hashish use, and on the other hand, the same strong statements about its generally harmful effects are heard today. The most appropriate course for society to take is still mired in the same kind of helplessness and confusion. Many things have changed, of course, yet the only really new element to appear in the picture is the scientific ability modern society now possesses to understand and measure objectively the properties and effects of the drug. This is a very recent development. Until a decade ago, it was not possible to discover any satisfactory scientific literature on cannabis.

Perhaps the current efforts of scientists can be translated soon into beneficial social action. Even though the historian, familiar with the character of man and society through the ages, is not inclined to believe in the coming of the millennium in our own day, still that unscientific component of man's mind called "hope" constantly raises its small voice to say: "Maybe it will".

FRANZ ROSENTHAL
Professor, Near Eastern Literature
Yale University

Editorial note: This paper was to be read by the author at the dinner attended by the participants of the Symposium. The references to the Muslim Scholars quoted may be found in the following text: Franz Rosenthal, *The Herb, Hashish versus Muslim Medieval Society*, E. J. Brill, Leiden, 1971.

AUTHOR INDEX

Entries in ordinary type are page numbers containing citations.
Entries in *italics* are page numbers containing the references.
Entries in **bold type** refer to authors and papers in this book.

Abbatiello, E. R. 536, *536*, 682, 689, *691*
Abel, E. L. 543, 552, 559, *560*, 595, *603*, 665, 669, *679*
Abood, L. G. 243, *248*
Abood, M. E. 243, *248*
Ackerman, N. R. 280, *295*
Adams, J. E. 547, *563*
Adams, M. D. 586, 587, *588*
Adams, P. M. 548, *560*, 695, 696, *710*
Adams, R. D. 555, *562*
Adawadkar, P. D. 185, *207*
Adey, W. R. 549, 552, *560*, *561*, *562*
Adler, A. 429, *438*
Adler, I. D. 411, *417*
Affara, N. 184, *207*
Agneray, J. 138, *144*
Agnew, W. F. 494, *495*
Agrawal, M. 450, *466*
Agren, G. K. 231, 240, *242*
Agurell, S. **3**, 3, 4, 5, 7, 10, *13*, 16, *43*, 45, 53, *61*, *62*, **63**, 63, 64, 65, 67, *69*, *70*, 72, 78, *80*, 101, 102, *103*, 105, 106, 108, 118, *121*, 279, 280, 287, 289, *295*, 493, 494, *495*, *498*, 697, *710*
Aherne, G. W. 81, 82, *88*
Ahmed, K. 231, 239, *242*
Aiken, J. W. 602, *603*
Ajmone Marsan, C. 622, *639*
Åkermark, B. 72, *80*
Akiyama, A. 294, *297*
Albers, R. W. 231, *242*, 246, *249*
Aliapoulios, M. A. 359, *369*, 429, 437, *438*, 456, *466*, 520, *530*
Allen, M. A. 371, 375, *376*
Allentuck, S. 605, *617*
Allfrey, V. G. 199, *207*, 377, *387*
Allinger, N. L. 72, 74, 76, *80*, 248, *249*
Alpin, R. T. 74, *80*
Alves, C. N. 682, 688, *690*
Ambrosetto, G. 645, 654, *657*
Amelar, R. D. 431, 438, *439*
Amerson, E. W. 81, *88*
Ames, F. 696, 708, *711*
Ames, F. A. 540, *561*
Amit, Z. 252, *263*
Andén, N.-E. 616, *617*
Anderson, M. *341*
Anderson, P. 654, *655*
Anderson, P. F. 246, *249*, 586, *588*
Anderson, S. 677, *680*
Anderson, S. A. 654, *655*
Anderson, W. A. 383, 386, *388*
Anderson, W. W. 543, *566*
Andersson, S. 654, *655*
Annau, Z. 495, *496*
Ansell, G. B. 570, *582*
Anselmi, B. 676, *680*
Ansley, H. R. 377, *387*
Anton, A. H. 244, *249*
Aprison, M. H. 570, *582*
Aqleh, K. 280, *295*, 586, *588*
Archer, R. 623, 636, *640*
Archer, R. A. 72, 74, 76, *80*, 248, *249*
Arienti, G. 248, *249*
Armand, J. P. 129, 131, 132, 140, *143*, *144*, 145, *159*, 188, *207*, 222, *228*, 326, *328*, 329, 335, *342*, 377, *387*, 670, *679*
Armstrong, T. A. 89, 91, 92, *99*
Arnold, M. B. *561*
Arya, M. 495, *496*
Åsberg, M. 3, 7, 10, *13*, 16, *43*
Asch, R. H. **449**, 450, *466*
Askew, W. E. 558, *561*
Astrup, P. 495, *495*
Atkinson, A. 244, *249*
Atkinson, K. S. 325, *328*
Atkinson, P. N. 339, *342*
Atkinson, R. C. 542, *565*
Atweh, S. 352, *358*
Aulakh, C. S. **567**
Avelino, E. 379, *387*
Avery, D. H. 592, *604*
Avis, H. F. *561*
Axelrod, J. 92, *98*, 105, 106, 109, 111, 113, 115, 117, *121*, 252, *263*, 520, *530*, 587, *589*
Ayalon, D. 450, *467*, 495, *498*
Ayhan, I. H. 593, 596, 600, 601, *604*
Azen, S. P. 279, *295*

Babor, T. 280, *297*, 395, *404*
Babor, T. F. 408, *418*
Bach, D. 244, *249*
Bach, F. M. 132, *144*, 146, *159*
Bach, M. L. 132, *144*, 146, *159*
Bachelard, H. S. 535, 536, *537*
Bachman, J. *563*, 595, 599, *603*
Baglioni, C. 186, *207*
Bailey, P. T. 567, 567, 578, 580, *582*
Baker, H. W. G. 403, *405*
Baker, J. R. 280, 284, 289, *295*, *298*, 480, 481, 482, 487, *498*, 510, *512*
Banchereau, J. 129, 158, *159*, 437, *438*
Banerjee, B. M. 481, 482, *496*
Bannerjee, S. P. 71, *80*, 351, 356, *358*
Barak, A. 252, *263*
Barak, Y. 648, *656*
Baram, D. A. 592, *604*, 668, 677, *679*
Barbor, T. F. 252, *263*
Bardin, C. W. 430, *439*
Barnabel, O. 248, *250*
Barnes, P. R. 548, *565*, 646, 653, *656*
Barondes, S. H. 536, *536*
Barratt, E. S. 548, *560*, *565*, 596, *604*, 646, 653, *656*, 695, 696, *710*
Barret, K. 184, *206*
Barry, H. III 118, *121*, 229, *242*, 351, *358*, 620, *641*, 651, *656*, 695, 708, *711*
Bartke, A. 252, 261, *263*, 396, *405*, 408, *417*
Bartko, J. J. 533, *537*
Bartle, K. D. 279, 280, 294, *297*
Bartova, A. 145, *159*, 330, *341*
Bartram, S. F. 245, *250*
Bauminger, S. 450, *466*
Baxter, J. D. 220, 222, *228*
Beaconsfield, P. 326, *326*
Beals, R. K. 501, *511*
Beaubrun, M. 694, *710*
Beauge, L. A. 231, *242*
Bechner, J. S. 494, *497*
Beck, E. C. 548, *563*, 696, *712*
Becker, B. A. 480, 486, *496*
Beckner, J. S *121*, 162, *169*, 177, *206*, 528, 529, *529*
Bedford, J. M. 377, 381, 383, 386, *388*
Beeson, M. F. 570, *582*
Bell, F. R. 552, *560*
Bellet, S. 294, *297*
Bellve, A. R. 422, *427*
Bellville, J. W. 280, *295*, 586, *588*
Bemporad, B. 665
Ben-David, M. 495, *497*
Benner, J. F. 315, 319, 324, *326*
Bennett, B. M. 504, *511*
Bennett, I. F. 279, 280, *297*, 543, 544, 552, *564*
Benowitz, N. 129, 131, *144*, 541, *563*, 586, 588, *589*, 595, 599, *603*, 676, *679*, 694, 707, *711*
Bensusan, A. D. 598, *603*, *604*
Bent, M. J. 381, 383, *387*
Ben-Zvi, Z. 64, 65, 67, *69*, 71, 72, *80*, 520, 528, 529, *529*
Berardi, A. C. 549, *563*, 651, *655*
Berg, D. 184, *206*
Berman, L. 311, *327*
Berman, N. D. 682, *690*
Bernstein, J. 280, *297*
Berthiaume, A. 645, *656*
Bertuletti, G. 696, 708, *711*
Besch, N. 360, *369*, 396, *405*
Besch, N. F. 449, 450, 453, *466*, *467*, 494, *498*
Besch, P. K. 360, *369*, 396, *405*, 450, 453, *466*, *467*, 494, *498*
Beven, J. L. 282, *295*, 325, *326*, 493, *496*
Beyer, R. D. 244, *249*
Bhacca, H. S. 77, *80*
Bhargava, H. N. 602, *603*, 605, *617*
Bhatnagar, Y. M. 422, *427*
Bhattacharya, I. C. 325, *328*,
Bhattacharyya A. K. 567
Bicher, H. I. 613, *617*
Biddle, F. C. 480, *496*
Bigler, E. D. 654, *655*
Binder, M. 53, 60, *61*, *62*, 64, 69, *69*, **71**, 72, 78, *80*, 101, 102, *103*
Binns, R. 282, *295*, 325, *326*, 493, *496*
Bino, T. 243, *249*
Birch, E. A. 605, *617*
Birmingham, M. K. 145, *159*, 330, *341*
Birnie, G. 184, *207*
Björkroth, U. 63
Black, M. M. 377, *387*
Blake, I. M. 53, *61*
Blakemore, J. 89, *99*
Blevins, R. D. 132, 142, *143*, 145, 157, *159*, 188, *206*, 222, *228*
Bloch, D. P. 386, *388*
Bloch, E. 441, 441, 442, 445, 447, *447*
Bloj, B. 243, 244, 245, 247, 248, *249*
Blomstrand, C. 248, *249*
Bloom, A. S. 599, 600, 601, *603*
Bloom, F. E. 568, *582*
Bloom, L. 243, *248*
Boegli, G. 106, *121*, 549, *561*
Boehme, R. F. 311, *327*
Boff, E. 552, *565*, 696, 706, *712*
Bogden, A. E. 408, *417*
Boissier, J. R. 659, 660, 661, 662, *663*
Bondy, G. P. 225, 227, *228*, 360, *369*, 450, 458, *467*, 495, *498*
Boorman, G. A. 450, *466*
Borg, H. 105, 106, *121*
Borgen, L. A. 360, *369*, 408, *418*, 476, *477*, 480, 481, 482, *496*, *498*, 502, *511*, 587, *589*, 682, *690*
Borison, H. L. 677, *679*
Bornstein, W. 551, *561*
Borun, T. W. 176, *207*
Borys, H. K. 638, *641*, 654, *657*
Boschetti, N. V. 360, *369*
Bose, S. 568, *582*
Boulougouris, J. 694, *712*
Boulougouris, J. C. 694, *712*
Bourdon, R. 6, *13*, 280, 289, *299*
Bourne, G. H. 506, *511*
Bousfield, W. A. 672, *679*
Boutet, M. 279, 294, *298*

Bowins, B. 287, *298*, 493, *498*
Bowling, C. 568, *582*
Bowman, K. M. 605, *617*
Bowman, M. 694, *710*
Boyd, D. B. 72, 74, 76, *80*, 248, *249*
Boyd, E. H. 550, *561*
Boyd, E. S. 550, 558, *561*
Boyle, W. 330, *341*
Boyum, A. 132, *143*, 146, *159*
Brachet, P. 132, 142, *143*, 145, *159*
Braden, W. 548, *561*
Bradley, W. 638, *640*
Braithwaite, R. A. 85, *88*
Bram, S. 132, 142, *143*, 145, *159*
Brasch, K. 378, *378*
Braude, M. C. 4, *13*, 71, *80*, 252, *264*, 279, 282, 283, 285, 286, 287, 289, 290, 291, 294, *296*, *297*, *298*, 301, 326, *327*, 360, 361, 367, *369*, 407, 408, *417*, 441, *447*, 469, 471, 472, 474, *476*, 480, 481, 482, 484, 487, 491, 492, 494, 495, *496*, *497*, *498*, *499*, 501, 502, *511*, 531, 532, 533, 535, 536, *536*, *537*, 597, *604*, 645, 646, 653, *657*, 689, *691*, 694, 695, 696, 706, *712*
Breckenridge, W. C. 248, *249*
Breitman, T. 163, *169*, 183, *206*
Bremner, W. J. 403, *405*
Briant, R. H. 78, *80*
Bright, H. J. 238, *242*
Bright, T. P. *564*
Brightwell, D. 436, *439*, 542, 543, 552, 555, *564*
Brin, S. C. 129, 131, *144*
Brine, D. R. 6, 7, 10, *13*, **15**, 15, 16, 17, 22, 33, 35, 36, *42*, *43*, 78, *80*, 101, *103*, 105, 106, 107, 108, *121*, 549, *561*
Brine, G. A. 15, 17, *43*, 78, *80*
Britton, R. S. 329, *341*
Brodsky, M. 521, *529*
Bromberg, W. 540, *561*
Bromley, B. 495, *496*
Brown, D. J. 252, *263*, 495, *497*, *564*
Brown, H. 544, 558, *561*
Brown, J. H. V. 294, *296*
Brown, J. W. 554, *561*
Brown, L. E. 550, *561*
Bruce, W. R. 409, 410, 411, 412, 413, 416, *417*, *418*
Brunk, S. F. 592, *604*, 668, 677, *679*
Brunnemann, K. D. 279, *296*
Bryan, J. H. D. 386, *388*
Bryant, P. J. 8, *13*, 287, 289, *298*
Bryant, S. A. 371, 375, *375*
Buchsbaum, H. 638, *639*, 644, *655*
Buchthal, F. 637, *639*
Budzikiewicz, H. 74, *80*
Bulman, C. A. 294, *297*
Burdick, C. 377, *387*
Burger, H. 429, *438*
Burger, H. G. 403, *405*, *427*
Burkinshaw, P. M. 48, *61*
Burrows, B. 279, 295
Burrows, E. P. 459, *467*
Bursey, J. T. 6, 7, 10, *13*, 16, 22, *43*, 105, 107, 108, *121*
Burstein, S. 4, *13*, 45, *62*, 63, 64, 67, 69, *70*, 72, *80*, 101, *103*, 252, 261, *263*, 396, *405*, 408, *417*
Bursuker, I. 244, *249*
Busch, H. 189, *206*
Buschke, H. 555, *561*
Butcher, R. W. 229, *242*
Butcher, S. G. 616, *617*
Buthram, V. C. 459, *467*
Butler, R. C. 246, *250*, 586, 587, *589*
Buxbaum, D. 600, *603*
Buxbaum, D. M. 613, *617*

Cajal, S. R. 728, *730*
Calandra, J. C. 360, 361, 367, *369*, 408, *417*, 469, 471, 472, 474, *476*, 481, 482, *497*, *499*, 502, *511*
Calvin, H. 383, *388*
Calvin, H. C. 381, 383, *387*
Calvin, H. I. 377, 386, *387*
Campbell, A. M. G. 694, *710*
Canick, J. 403, *405*
Cannon, W. D. 183, *206*
Canter, A. 668, 677, *679*
Cantley, L. C. 231, *242*
Capelle, N. 142, *143*
Cappell, H. D. 543, *561*
Caputto, B. L. 238, *242*
Caputto, R. 238, *242*
Carakushansky, G. 501, *511*
Carchman, R. A. 132, 142, *143*, 145, *159*, 161, 168, 169, *169*, 188, *206*, *207*, *208*, **219**, 219, 220, 221, 222, 223, 226, *227*, 229, 239, *242*, 368, 370, 381, *387*
Carlini, E. A. 357, *358*, 559, *564*, 581, *582*, 586, 588, *589*, 595, *603*, 620, 636, *639*, 644, 651, 652, *655*, *656*, 682, 688, 689, *690*, *691*, 695, 697, 708, *710*
Carlton, P. L. 558, *561*
Carolyn, D. 326, *327*
Carson, I. M. 543, *565*
Carter, A. 592, *604*
Carter, W. E. 694, *710*
Cartier, P. *277*
Carvalho, F. V. 559, *564*
Cass, C. E. 166, 168, *169*
Casswell, S. 544, 552, *561*
Caton, J. E. 279, 284, *296*, *297*, 493, *497*
Catt, H. K. 261, 262, *263*
Cavasino, A. 548, *563*
Cavicchia, J. C. 422, *427*, 437, *438*
Cely, W. *358*, 587, *589*, 620, 621, 624, 625, 636, 637, 638, *640*, *641*, 650, 651, 652, 654, *655*, *656*
Chait, L. D. 586, 587, *588*
Chakravarty, I. 360, 367, 368, *369*, 495, *496*
Chalkley, R. 190, *207*
Chalkley, W. H. 361, *369*
Chamberlin, J. 184, *206*
Chan, P. C. 294, *297*
Chang, C. C. 570, *582*
Chang, J. J. 89, *98*
Chao, F.-C. 495, *496*

Chapman, L. F. 441, *447*, 481, 482, *498*, **501**, 502, *512*, 682, *690*, **693**, 695, 696, *712*
Chapuis, L. **209**
Chari, S. *427*
Chari-Bitron, A. 142, *143*, 158, *159*, 168, 169, *169*, 219, *227*, 243, *249*, 280, *297*, 329, 330, *341*
Chase, A. R. 81, *88*, 431, *438*
Chau-Wong, M. 219, *228*, 243, 246, *250*
Chavez-Chase, M. 129, 131, *144*
Cheng, J. T. 494, *495*
Chesher, G. B. **243**, 246, *249*, *250*, 586, *588*, **605**, 613, *617*, 650, 651, *655*
Chichibu, S. 623, *640*
Chin, L. 548, *561*, 646, *655*
Chin, S. 543, *566*, 677, *680*
Chiu, J.-F. 184, 205, *206*, *208*
Chiu, P. 244, *249*, 638, *641*, 654, *657*
Choisy, G. **265**
Choisy, H. **265**
Chopra, G. S. 280, *295*, 694, *710*
Christensen, H. D. 15, 22, *63*, 63, *70*, 78, *80*, 106, *121*, 520, 529, *530*, 549, *561*
Chronister, R. B. 638, *639*
Clare, G. 548, *566*, 585, *589*
Clark, D. L. 696, *711*
Clark, L. D. 542, *561*
Clark, R. **469**
Clark, S. 668, 677, *679*
Clark, S. C. 557, *561*
Clark, S. G. 459, *466*, *467*
Clark, W. C. 543, *566*, **665**, 665, 666, 667, 668, 669, 670, 677, 678, *679*, *680*
Clark, W. G. 558, *561*
Clarke, D. E. 244, *249*
Clarren, S. K. 502, *511*
Claussen, U. 588, *589*
Cleve, G. L. 367, *369*
Clewe, G. L. 408, *418*, 481, *497*
Cline, M. C. 279, *295*
Clotscher, W. F. 261, 262, *263*
Clough, J. M. 7, *13*, 84, 85, *88*
Co, B. T. 689, *691*, 694, *710*
Cocchia, M. A. 221, 223, *228*, 360, *369*, 437, *439*, 441, *447*, 450, 458, *467*
Cochin, J. 593, *603*
Coelingh, J. P. 377, 386, *387*
Coggins, W. J. 694, *710*
Cohen, A. B. 129, 131, *144*, 294, *297*
Cohen, S. 223, *228*, 396, *405*, 408, *417*, 586, *589*
Cohn, R. A. 548, *565*, 596, *604*, 646, 653, *656*
Colasanti, B. K. 622, *639*
Colburn, R. W. 243, *249*
Cole, J. M. 696, *710*
Collier, H. O. J. 605, 613, *617*
Collombel, Ch. 268, *277*
Collu, R. 360, *369*, 396, 403, *405*, 408, *417*, 494, *496*
Comings, D. E. 379, *387*
Comitas, L. 694, *710*, *712*
Connor, T. 416, *418*
Conrad, D. G. 696, *710*
Consroe, P. *358*, 550, *561*, 620, 638, *639*, *640*, 644, 648, 651, *655*
Consroe, P. F. 548, *561*, 620, 638, *639*, 644, 646, 648, 650, 651, 652, *655*, 696, *711*
Cook, C. E. 81, *88*
Cooke, B. A. 252, 261, 262, *263*
Cools, A. R. 358, *358*
Cooper, G. W. 383, *388*
Coper, H. 243, *250*, 587, *589*
Corcoran, M. E. 252, *263*, 620, *639*, *641*, 646, 650, *655*, *656*, *657*
Cordova, T. 450, *467*, 495, *498*
Cornett, T. L. 542, 543, 552, 555, *564*
Corrodi, H. 616, *617*
Costa, E. 280, *297*
Cote, I. L. 654, *657*
Cote, M. G. 279, 294, *297*
Cotman, C. W. 248, *249*, 331, *341*
Couineau, S. 728, *730*
Cowden, M. 248, *250*
Cox, R. H., Jr. 570, *582*
Coyle, J. T. 344, *348*, 352, *358*
Cozens, D. D. **469**, 472, *476*
Crabtree, R. E. 114, *121*
Craig, C. R. 622, *639*
Crary, D. 472, *476*
Craven, C. 244, *249*
Creasia, D. 284, *297*, 493, *497*
Crespi, H. L. 53, *61*
Criswell, B. S. 294, *299*
Crowl, C. P. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*, *99*
Crown, J. 15, *42*
Crown, P. 585, *589*, 663, *663*
Cumming, H. J. 495, *497*
Cunningham, D. D. 166, *169*
Currain, J. E. 221, *228*
Cushman, P. 129, 131, *143*, 396, *404*
Cutler, G. B. 437, *439*
Cutting, M. B. 294, *295*, *296*, 325, *327*
Cutting, M. E. 324, 325, 326, *327*

Dagirmanjian, R. 550, 558, *561*, *562*
Dahl, C. 613, *617*
Dahl, J. L. 237, *242*
Dahlberg, B. 429, 436, *438*
Dahlen, P. 552, *565*, 696, 706, *712*
Dahlström, A. 568, *582*
Dahlstrom, G. 494, *498*
Dahmén, J. 4, *13*, 45, *61*, 63, 64, 65, 67, *70*, 101, 102, *103*
Dalbey, W. 279, *296*
Dalmonis, A. 429, *438*
Dalterio, S. 252, 261, *263*, 396, *405*, 408, *417*
Dalzel, B. C. 253, *264*
Dalzell, H. C. 46, *62*, 253, *264*
Dardick, I. 381, *387*
Darley, C. F. 542, 543, *561*, *565*
Daul, C. B. 129, 131, *143*
Davies, P. 311, *327*
Davis, J. 185, *207*, 429, *438*
Davis, J. M. 558, *562*
Davis, J. P. 619, *639*, 652, *655*

Davis, K. H. 17, *43*, 63, *70*, 520, 529, *530*, 620, 623, 636, *640*
Davis, K. R. 689, *691*
Davis, R. V. 426, *427*
Davis, W. 347, *349*
Davis, W. M. 360, *369*, 408, *418*, 476, *477*, 480, 481, 482, *496*, *498*, 502, *511*, 587, *589*, 596, *603*, 682, *690*
Dawson, A. B. 470, *476*
Dawson, R. M. C. 247, *249*
Daykin, S. 252, *263*
Deets, A. C. 696, 709, *712*
De Haan, R. L. 265, *277*
Dejong, R. N. 546, *561*
Dejong, Y. 586, *589*
De Kretser, D. M. 403, *405*, *427*
Del Bianco, P. L. 676, *680*
Del Castillo, J. *341*
Delgado, C. J. 158, *159*
Delgado, J. M. R. 546, 551, *561*, *564*
Del Giudice, J. 558, *561*
DeLong, F. L. 543, *561*
Demarco, P. V. 72, 74, 76, *80*, 248, *249*
Dement, W. C. 659, *663*
Demoulin, A. *427*
Deneau, G. A. 591, 594, 596, 600, *603*, 696, *710*
Deniker, P. 659, 660, 661, 662, *663*
Dennis, B. J. 552, *560*
DeRobertis, E. 177, *206*
Derouaux, M. 646, *656*
Derrick, F. C. 429, 436, *438*
Derrick, J. C. 317, *327*
Desoize, B. **129**, 131, 140, *144*, **145**, 145, 153, 158, *159*, 161, 162, 166, 167, 168, 169, *169*, 188, *207*, 222, *228*, 229, *242*, 261, *264*, 437, *438*, 520, 528, *530*, 670, *679*
Detke, S. 186, *206*
Detre, D. 694, *711*
Detrick, R. 289, *296*
DeVita, V. T. Jr. 429, *439*
Dewey, W. L. 64, 67, 69, *70*, *121*, 129, 131, 132, *144*, 162, 168, *169*, 177, 188, *206*, *207*, 219, 220, 223, 226, *227*, **343**, 343, 345, 346, 348, *348*, *349*, 351, *358*, 368, *370*, 494, *497*, 528, 529, *529*, 581, *582*, 599, 600, 601, *603*, *604*
Dewhurst, S. A. 248, *249*
DiMartino, R. 450, 458, *467*
Diminno, R. L. 409, *418*
Dixit, B. N. 229, *242*, 351, *358*
Dixit, V. P. 252, *263*, 359, *369*, 396, *405*, 408, *417*, 419, 426, *427*, 441, *447*, 450, *466*, 495, *496*
Djerassi, C. 74, *80*
Doggett, S. 188, *207*
Dolby, T. W. 248, *249*, 347, *349*, 358, *358*
Dollery, C. T. 78, *80*
Domino, E. F. 71, *80*, 129, 131, *144*, 520, 528, *529*, 548, 553, *561*, *563*, *565*, 588, *589*, 659, *663*
Dontenwill, W. 493, 495, *498*
Doorenbos, N. J. 280, *296*
Dornbush, R. 377, *387*, 548, *566*, 659, 663, *663*
Dornbush, R. L. 542, 543, *561*, *562*, 585, *589*, 665, 670, *679*
Douglas, J. F. 158, *159*
Douglas, R. J. 546, 558, *562*
Dow, R. C. 622, *639*
Drachman, D. A. 555, *562*
Drath, D. B. 311, *327*
Drew, W. G. 542, 543, 544, 549, 550, 552, 557, 558, *562*, *564*, 694, *712*
Dubin, L. 431, 438, *439*
Dubowski, K. M. 282, *296*, 408, *418*, 483, *496*
Ducharme, J. R. 360, *369*, 396, 403, *405*, 408, *417*, 494, *496*
Dufau, M. 261, 262, *263*
Dulmanis, A. 403, *405*
Duncan, D. B. 452, *466*
Dunlop, C. W. 552, *561*
Dunn Thalma, B. 365, *369*
Dustman, R. E. 548, *563*, 696, *712*
Dutta, C. R. 728, *730*
Dwivedi, C. 650, 651, *655*
Dykstra, L. A. 357, *358*
Dym, M. 422, *427*, 437, *438*
Dyrenfurth, I. 419, 426, *427*, 432, *439*, 670, *679*

Earnhardt, J. T. 586, 587, *588*
Ebner, K. E. 225, *228*, 360, *369*
Eddie, L. W. 403, *405*
Edelstein, E. L. 645, *656*
Edery, H. 4, *13*, 63, 64, 65, 67, *69*, *70*, **71**, 71, 72, 74, 78, 79, *80*, 101, *103*, 352, *358*, 520, 528, 529, *529*, 706, *711*
Efron, D. H. 600, *603*
Ehling, U. H. 414, *417*
Ehrnebo, M. 105, 106, *121*
Eich, J. R. 542, *562*
Eichberg, J. 247, *249*
Eichelman, B. S. 559, *562*
Eicher, E. M. 386, *388*
Eimstad, W. M. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*, *99*
Eisenstein, D. 648, 651, *655*
Eisler, W. J. 22, *43*
Ekindjian, G. C. 138, *144*
Elazar, A. 549, *562*
Elgin, S. C. R. 189, *207*
Ellingboe, J. 252, *263*, 395, *404*, 408, *418*
Ellington, A. C. 480, 481, *498*, 502, *511*, *512*
Ellman, G. 280, *297*
Elsmore, T. F. 696, *710*
Ely, D. L. 695, 708, *710*
El-Yousef, M. K. 558, *562*
End, D. W. 168, *169*, **219**, 219, 220, 223, 226, *227*, 381, *387*
Endrenyi, L. 289, *299*, 493, 494, *499*
Engel, E. E. 311, *327*
Englert, L. F. 252, 262, *263*, 280, *296*, 347, *349*, 494, *497*, 550, 552, *564*, 706, *711*
Erbe, J. 166, *169*
Ercan, Z. S. 280, *296*
Erdmann, G. 45, *61*, 494, *496*
Ertel, R. J. 351, *358*
Esber, H. J. 129, 131, *144*, 408, *417*, 494, *496*

Esplin, D. W. 638, *639*
Etevenon, P. 659, 659, 660, 661, 662, *663*
Evans, M. 694, *710*
Everingham, M. 613, *617*

Fabrizio, M. 378, 383, *387*
Fahn, S. 231, *242*
Fähndrich, C. 243, *250*, 587, *589*
Fahrenholtz, K. E. 72, *80*
Fajans, P. 694, *711*
Fakunding, J. 404, *405*
Farber, J. 185, *207*
Farias, R. N. 243, 244, 245, 247, 248, *249*
Fariello, R. G. 646, *656*
Farr, A. L. 163, *169*, 230, *242*, 397, *405*
Fawcett, D. W. 383, 386, *388*, 426, *427*
Fazel, A. 481, 482, 494, *499*, 532, 535, 536, *537*, 682, *690*
Fechter, L. D. 495, *496*
Feeney, D. M. 544, 550, 551, 554, *562*, 638, *640*, **643**, 643, 646, 648, 651, 653, 654, 655, *657*
Fehr, K. A. 681, 682, 687, 688, 689, *690*
Fehr, K.O'B. 280, *296*, **681**
Feldstein, S. 548, *566*, 663, *663*
Feman, E. R. 186, *207*
Fennessy, M. R. 676, *680*
Fentiman, A. F. 7, *13*, 46, *62*, 107, *121*
Ferguson, G. A. 684, *690*
Fernandes, E. O. 450, *466*
Fernandes, M. 601, *603*
Fernandez-Guardiola, A. 622, *639*
Ferraro, D. P. 347, *349*, 502, *511*, 552, *562*, 587, *589*, 677, *679*, 695, 696, 707, 708, *710*, *711*
Ferry, J. D. 495, *498*
Fetterman, P. S. 280, *296*
Field, E. J. 728, *730*
Filczewski, M. 689, *691*
Fingle, E. 636, *641*
Fink, F. 542, *562*
Fink, M. 377, *387*, 548, *562*, *566*, 585, *589*, 659, 663, *663*, 694, *711*
Finley, T. N. 129, 131, *144*, 294, *297*
Finney, D. I. 621, *640*
Finney, D. J. 504, *511*
First, M. W. 301, 311, *327*
Fisher, H. 371, 375, *375*
Fishman, W. H. 397, *405*
Fitzgerald, M. 294, *298*, 324, 326, *328*
Fitzsimmons, R. C. 481, 482, *497*, 532, *537*
Flandera, V. 684, 688, *690*
Fleischman, R. W. 279, 279, 280, 287, 289, 291, 294, *296*, *298*, 408, *417*, 441, *447*, 480, 481, 482, 487, 491, 492, 494, *496*, *498*, 501, 502, 510, *511*, *512*, 531, *536*, 646, *656*, 689, *691*
Fletcher, J. 359, 369, *370*
Flexner, L. B. 536, *537*
Fogh, J. 211, *218*
Folch, H. 331, *341*
Folch, J. 246, *249*
Folk, J. 397, *405*
Foltz, F. 289, *296*
Foltz, R. B. 7, *13*
Foltz, R. L. 7, *13*, 46, *62*, 107, *121*
Fonseka, K. 64, *69*, 72, 78, *80*, 101, 102, *103*
Foote, R. S. 280, *296*
Forbes, W. B. 550, *563*
Forfar, J. C. 622, *639*
Forman, E. J. 81, 83, *88*, 289, *298*, 431, *439*, 493, *498*
Forney, R. 106, 112, *121*
Forney, R. B. 252, *263*, 279, 280, *297*, 481, 482, 495, *497*, *498*, 502, *512*, 543, 544, 552, *563*, *564*, 620, 636, *640*, 652, *655*
Forrest, I. S. 495, *496*, 503, *512*
Forsaith, J. 592, *603*
Fournier, E. 6, *13*, 408, *417*, 469, *476*, 481, *496*, 501, *511*
Fowler, M. S. 35, *43*
Fox, C. A. 728, *730*
Frachon-Mastour, P. 244, *249*
Frajese, G. 378, 383, *387*
Franchimont, P. 426, *427*
Francis, D. L. 605, 613, *617*
Frank, I. M. 324, 326, *328*, 371, 375, *375*, 585, *589*
Frank, I. R. 329, *342*
Frankenheim, J. M. 586, *589*, 595, *603*, *604*
Fraser, J. D. 598, *603*
Frazier, P. D. 294, *296*
Frederickson, R. C. A. 602, *603*
Freedman, A. M. 548, *566*, 663, *663*
Freedman, D. K. 351, *358*
Freedman, D. X. 280, 289, *298*, 548, 557, *563*, *565*, 585, *589*, 714, 715, *730*
Freeman, F. R. 558, *562*
Freidman, L. 533, *537*
Freirich, E. J. 697, *711*, 715, *730*
Frenk, H. 646, 654, *655*
Freudenthal, R. I. 15, 22, *43*, 78, *80*, 106, *121*, 280, *296*, 494, 495, *496*, 549, *561*
Freund, M. 383, *388*, 436, *438*
Fried, P. A. 481, 482, 494, *496*, *498*, 546, 549, *562*, 650, *655*, 682, 689, *691*, 694, *711*, *712*
Friedman, A. J. 542, *562*
Friedman, E. 601, *603*, 605, *617*
Friedman, M. A. 161, *169*, 188, *207*, 219, 220, 221, 223, 225, *227*, *228*, 368, *370*
Fritchie, G. E. 252, 262, *263*, 280, *296*, 347, *349*, 494, *497*, 550, 552, *563*, *564*, 706, *711*
Frizza, J. 246, *249*
Fry, D. 81, *88*
Fu, T.-K. 371, 375, *375*
Fujimoto, G. I. 419, 426, *427*, **441**, 441, 442, 445, 447, *447*
Fujimoto, J. M. 650, *655*
Fujiwara, M. 682, 688, *690*
Fukuhara, Y. 294, *297*
Fuld, P. A. 555, *562*
Fuller, J. L. 546, *562*
Furrer, R. 409, *417*
Fuxe, K. 568, *582*, 616, *617*

Gabbay, E. J. 185, *207*
Gado, M. 689, *691*
Gaensler, E. 294, *298*, 324, 326, *328*
Gaffney, T. E. 52, *62*
Gailis, L. 240, *242*
Galanter, M. 493, *496*, 659, *663*
Galanter, R. 287, 289, *296*
Galbreath, C. 481, 482, *496*
Galef, B. G. 546, *562*
Gallager, D. W. 559, *562*
Gan, O. H. M. 8, *13*, 287, 289, *298*
Gaoni, Y. 74, *80*, 301, *327*, 620, *640*
Garcia, J. F. 494, *497*
García-Castiñeiras, S. 231, *242*
Garelis, E. 378, *387*
Gardner, W. U. 367, *370*
Garner, L. I. 145, *159*, 501, *511*
Garrett, C. P. O. 85, *88*
Garrett, E. R. 3, 4, 6, 91, *98*, **105**, 105, 106, 107, 108, 110, 113, 115, 116, 117, 118, 119, *121*, 289, *296*
Garriott, J. C. 620, 636, *640*, 652, *655*
Gasser, J. C. 280, *295*, 586, *588*
Gastaut, H. 645, 654, *657*
Gatenby, A. D. 244, *249*
Gaul, C. C. 129, 131, *144*, 294, *296*
Gay, V. L. 450, *466*
Geber, W. F. 480, 481, *496*, 502, *511*
Gehan, E. A. 697, *711*, 715, *730*
Gelehreter, T. 221, *228*
Gelfand, D. 220, 222, *228*
German, A. L. 49, *61*
Gershon, S. 601, 602, *603*, 605, 616, *617*
Gharibian, J. 311, *327*
Ghosh, J. J. 243, 244, 245, 247, *250*, 360, 367, *369*, 495, *496*
Gianutsos, A. 536, *536*
Gianutsos, G. 682, 689, *691*
Gibb, W. 580, *582*
Gibbins, R. J. 682, *690*
Gidley, J. T. 106, *121*, 549, *561*
Gilbert, J. C. 244, *249*, *358*
Gill, E. W. 64, *69*, 72, 78, *80*, 106, *121*, 219, *227*, 244, 245, 248, *249*, *250*, 289, *296*, 493, 494, *496*
Gillespie, H. K. 3, 4, *13*, 520, *530*, 541, 552, 557, *563*, *564*, 707, *711*
Gillin, J. C. 543, *562*
Gilmore, W. 648, *655*
Gilmour, R. S. 184, *207*
Ginestet, D. 659, 660, 661, 662, *663*
Ginsberg, M. D. 495, *496*
Ginsburg, J. 326, *326*
Giono-Barber, H. 696, 708, *711*
Giono-Barber, P. 696, 708, *711*
Glancy, D. L. 294, *296*
Glassman, E. 536, *536*
Glazer, R. I. 521, *529*
Gledhill, B. L. 381, *387*
Gledhill, M. P. 381, *387*
Glenner, G. 397, *405*
Glenner, G. G. 361, *369*
Glick, S. D. 552, *566*, 695, 696, *712*
Glynn, I. M. 231, *242*
Goddard, G. V. 622, *640*
Godowicz, B. 378, 386, *387*
Goetz, R. R. 665
Gogolak, G. 551, *565*
Gold, R. Z. 436, *439*
Goldman, H. R. 550, *562*
Goldman, R. 243, 244, 246, *249*, 329, 330, *342*, 351, 352, 356, *358*
Goldman, S. S. 246, *249*
Goldsmith, J. R. 293, *296*
Goldstein, E. 311, 313, 325, *327*
Golstein, H. 360, *369*, 396, *405*
Gombos, G. 248, *249*
Gonzalez, S. C. 595, *603*
Goo, G. P. 501, *511*, **693**
Goodenough, S. 294, *295*
Goodman, J. S. 677, *679*
Goodman, L. S. 619, *640*, 645, 652, *656*
Goodwin, D. W. 669, 676, 677, *679*, 689, *691*
Goracci, G. 248, *249*
Gordon, R. 599, *603*
Gordon, R. J. 599, *603*
Gordon, S. 670, *679*
Gori, G. B. 279, *296*
Gori, G. E. 280, 285, 293, *296*
Gottesfeld, Z. 352, *358*
Gould, I. A. 324, 326, *328*
Gould, M. H. 588, *589*
Gouyette, A. J. 119, *121*
Gowen, J. W. 386, *388*
Grabowski, C. T. 495, *496*
Graham, J. 131, 132, *144*
Grandstand, B. 105, 106, *121*
Granner, D. 221, *228*
Gray, R. 221, 223, *228*, 360, *369*, 437, *439*, 441, *447*
Green, D. E. 4, 7, *13*, 495, *496*, 503, *512*
Green, G. M. 311, 313, 325, 326, *327*
Greenberg, D. B. 678, *679*
Greenberg, F. *711*
Greenberg, I. 280, *297*, *582*, 694, *711*
Greenberg, J. H. 72, *80*, 158, *159*, 168, 169, *169*, 229, *242*, 330, 332, 334, 339, *341*, *342*
Greene, C. 557, *561*
Greene, G. H. 310, *328*
Griesemer, R. 279, *296*
Grilly, D. M. 347, *349*, 502, *511*, 552, *562*, 587, *589*, 695, 696, *710*, *711*
Grishman, M. G. 347, *349*, 696, 707, 708, *710*
Grlic, L. 351, *358*
Groesbeck, C. J. 715, *730*
Gross, P. 294, *296*
Gross, S. J. 81, *88*, 288, 289, *296*, 493, *496*
Gross, T. 319, 324, *327*
Grota, L. J. 687, *690*
Grunfeld, Y. 64, 65, 67, *69*, 71, 72, 74, *80*, 520, 528, 529, *529*, 706, *711*
Guerin, M. R. 279, 284, *297*, 309, *327*, 493, *497*
Guerry, R. 280, *296*, 326, *327*
Guido Hi, G. 231, *242*
Gullotta, F. P. 648, 654, *655*, *657*

Gunn, C. G. 408, *418*
Gunnell, D. L. 653, *657*
Gupta, C. L. 450, *466*
Gurny, O. 78, *80*
Gurof, G. 521, *529*
Gustafsson, B. 3, 7, 10, *13*, 16, *43*, 45, *61*, 287, *295*, 493, *495*
Gutshall, P. L. 8, *13*, 287, 289, *298*
Guy, G. 220, 222, *228*

Haas, A. E. 171
Hackney, J. D. 279, *295*
Hadfield, M. G. 357, *358*
Haefliger, W. 72, *80*
Haegele, K. 53, *62*
Haertzen, C. A. 541, *562*, *563*
Hahn, P. M. 585, *589*
Haine, S. E. 279, 280, *297*, 543, 544, 552, *564*
Halaris, A. 280, 289, *298*, 585, *589*
Haley, S. L. 360, 361, 367, *369*, 408, *417*, 481, 482, *497*, 502, *511*
Hall, M. 559, *563*
Hall, R. D. 568, *582*
Halldin, M. 101
Hallé, S. 645, *656*
Hamberger, A. 247, 248, *249*
Hambrey, P. N. 333, *342*
Hamlin, H. 546, *564*
Hammar, C.-G. 16, *43*
Hanahan, D. J. 333, *342*
Handrick, G. R. 46, *62*
Harbison, R. D. 367, *369*, 408, *417*, 476, *477*, 481, 482, 484, 494, *497*, 501, 502, *511*, 650, 651, *655*
Harclerode, J. 245, 246, *250*, 360, *369*, **395**, 396, 398, 399, 400, *405*, 587, *589*
Hardman, H. F. 325, *327*
Hardy, N. 408, *417*, **469**, 469, *476*, 481, *496*, 501, *511*
Harkness, D. R. 183, *207*
Harkonen, N. 396, *405*
Harmon, J. 359, *369*, 429, 437, *438*, 456, *466*, 520, *529*
Harmon, J. W. 458, *467*
Harper, A. 311, *327*
Harper, C. E. 280, 294, *298*, *328*
Harper, J. W. 689, *691*, 713, *730*
Harris, J. R. 381, *387*
Harris, L. S. *121*, 129, 131, 132, 142, *143*, *144*, 145, *159*, 161, 162, 168, 169, *169*, 177, 188, *206*, *207*, 219, 220, 221, 222, 223, 226, *227*, 229, 239, *242*, 265, *277*, 279, *297*, 343, 346, 348, *348*, *349*, 351, 357, *358*, 368, *369*, 494, 495, *499*, 528, 529, *529*, 557, *562*, 581, *582*, 586, *589*, 595, 600, *603*, *604*, 669, 677, *679*
Harris, R. A. 243, *250*, 329, *342*
Harris, R. T. 696, 707, *711*
Harris, S. E. 184, *208*
Harrison, P. 184, *207*
Hart, B. L. 568, *582*
Hartman, E. R. 622, *639*
Hartzman, R. J. 132, *144*, 146, *159*
Harvey, D. J. 3, **45**, 45, 47, 48, 50, 51, 52, 53, 54, 58, *61*, *62*, **123**, **469**, 480, *497*
Harvey, T. M. 6, 7, 10, *13*, 16, 22, *43*, 105, 107, 108, *121*
Harwood, T. 81, *88*, 431, *438*
Haseman, J. K. 472, *476*
Hatch, T. F. 294, *296*, 319, 324, *327*
Hattori, T. 494, 495, *497*, 520, 522, 523, 526, 527, *530*, 532, *536*
Hauman, J. R. 22, *43*
Haverkate, F. 333, *342*
Hawes, M. L. 81, *88*
Hawks, R. L. 437, *439*
Hayashi, S. 221, *228*
Hayden, D. W. 279, 289, 291, 294, *296*, 408, *417*, 480, 481, 482, 487, 491, 492, 494, *496*, *498*, 501, 502, *511*, 531, *536*
Heath, R. B. 546, *562*
Heath, R. G. 129, 131, *143*, 546, 549, 551, *562*, 689, *690*, *691*, 713, 723, *730*
Hebb, D. O. 687, *690*
Hecht, F. 501, *511*
Heddle, J. A. 409, 416, *417*
Heinrich, R. 557, *565*, 714, 715, *730*
Hell, A. 184, *207*
Heller, C. G. 437, *439*
Hembree, W. C. III 252, *263*, 377, 386, *387*, 396, *405*, 408, *417*, **419**, 419, 420, 422, 426, *427*, **429**, 429, 430, 431, 432, *438*, *439*, 456, *466*, 670, *679*
Hendershot, L. S. 592, *603*
Henderson, G. 605, 613, *617*
Henderson, R. L. 280, *296*, 326, *327*
Hendrix, C. E. 552, *561*
Henrich, R. T. 222, *228*, **371**, 371, 372, 373, 374, 375, *375*, *376*
Henrikson, B. G. 596, *604*, 609, *617*
Henry, J. P. 695, 708, 709, *710*
Hepler, R. S. 329, *342*
Heppel, L. A. 183, *207*
Herbert, D. 403, *405*
Herrick, C. J. *562*
Hershkowitz, M. 243, *249*, 329, 330, *342*, **351**, 351, 352, 356, *358*
Hew, H. Y. C. 386, *388*
Heyman, I. A. 282, 290, *298*, 480, *498*, 531, 532, 533, 536, *537*
Hieger, L. 368, *370*
Higgins, S. J. 220, 222, *228*
Hilado, C. J. 495, *497*
Hill, J. G. 623, *641*
Hill, R. 243, *250*, 601, *603*
Hill, S. Y. 669, 676, 677, *679*, 689, *691*
Hilmoe, R. J. 183, *207*
Himmelsbach, C. K. 542, 548, *566*, *583*, 585, *589*
Hinds, W. C. 301, 309, 311, 316, 317, 324, 326, *327*
Hine, B. 601, 602, *603*, 605, 616, *617*
Hines, G. 552, 553, *565*
Hirschhorn, I. D. 601, *603*
Hnilica, L. 184, *206*
Hnilica, L. S. 199, 205, *207*, *208*

Ho, B. T. 71, *80*, 219, *228*, 252, 262, *263*, 280, *296*, 345, 347, *349*, 494, *497*, 550, 552, 558, *562*, *564*, 706, *711*
Hobbs, D. T. 17, 35, *43*
Hochrainer, D. 495, *498*
Hockman, C. H. 549, *562*
Hodge, H. C. 558, *561*
Hodgen, G. 397, *405*
Hodgen, G. D. 426, *427*
Hoellinger, H. 6, *13*, 142, *143*
Hoffer, A. 558, *562*
Hoffmann, D. 279, *296*
Hokin, L. E. 237, *242*
Hollander, W. F. 386, *388*
Holleman, C. L. 294, *299*
Hollister, L. E. 3, 4, 7, *13*, 265, *277*, 334, *342*, 520, *530*, 541, 542, 548, 552, 557, 559, *563*, *564*, *565*, 585, *589*, 620, 623, 636, *640*, 651, *655*, 659, *663*, 707, *711*
Holm, R. 509, *512*
Holmstedt, B. 3, 7, 10, *13*, 16, *43*, 287, *295*, 493, *495*
Hologgitas, J. 403, *405*
Holt, P. G. 294, *296*
Holtzman, D. 351, *358*, *563*
Hopkinson, J. A. V. 49, *62*
Horning, E. C. 49, *61*
Horning, M. G. 53, *62*
Horowitz, M. J. 547, *563*
Horton, R. 429, *439*
Hosko, M. J. 325, *327*
Hotchkiss, R. S. 409, *418*
Houser, V. P. 696, *711*
Howes, J. F. 253, *264*, 351, *358*
Hsu, J. T. 129, 131, 140, *143*, *144*, 153, *159*, 188, *207*, 222, *228*, 229, *242*, 261, *264*, 520, 528, *530*, 670, *679*
Hsu, P. 504, *511*
Hu, M. W. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*
Huang, H. F. S. **419**, 419, 420, 422, 426, *427*, **429**, 430, *439*, 670, *679*
Huber, G. L. 294, *295*, *296*, **301**, 311, 313, 324, 325, 326, *327*, *328*
Huckins, C. 404, *405*
Hudson, B. 403, *405*, *427*, 429, *438*
Hugenholtz, A. P. 409, *417*
Huggins, C. 397, *405*
Hughes, F. W. 620, 636, *640*, 652, *655*
Hughes, R. 542, *561*
Huisjen, J. 90, *99*
Humphries, S. 184, *207*
Hunt, C. A. 4, 6, 91, *98*, 105, 106, 107, 108, 110, 113, 115, 116, 117, 118, 119, *121*, 289, *296*
Hunter, R. B. 314, *327*
Hunter, S. A. 72, *80*
Huot, J. 132, *144*, 145, *159*, 219, *227*, 240, *242*, *263*, 329, 330, *342*, 381, *387*, 520, 528, *530*
Hurlimann, J. 210, *218*
Huszar, L. A. 330, 332, *342*
Huy, N. D. 279, 294, *298*
Idänpään-Heikkilä, J. E. 252, 262, *263*, 280, *296*, 494, 495, *497*, 550, 552, *564*, 706, *711*
Idle, M. 293, *299*
Ingelman-Sundberg, M. 243, *249*
Inui, N. 209, 210, 211, 217, *218*
Iramain, C. A. 459, *467*
Isaacson, R. L. 689, *691*
Isbell, H. 541, *563*
Iscove, N. N. 148, *159*
Ismay, J. A. 357, *358*
Israël, M. 244, *249*
Issidorides, M. R. **377**, 377, 378, 379, 381, 383, 386, *387*
Itoh, T. 521, *530*
Izquierdo, I. 549, 550, *563*, 620, 636, *640*, 651, 653, *655*
Izumi, F. 231, *242*

Jackson, D. M. **243**, 246, *249*, *250*, 586, *588*, **605**, 613, 616, *617*, 650, 651, *655*
Jaffe, J. 543, *566*, 677, *680*
Jaffe, J. H. 548, *563*, 591, *603*
Jakubovic, A. 243, 244, *250*, **251**, 251, 252, 253, 261, *263*, 481, 482, 495, *497*, **519**, 520, 521, 522, 523, 524, 528, 529, *530*, 532, 535, *536*, *537*
Jandhyala, B. 244, *249*
Janicki, B. W. 129, 131, *144*
Janowsky, D. S. 558, *562*
Jansing, R. L. 171, 184, 188, *207*
Janszen, F. H. A. 252, 261, 262, *263*
Järbe, T. U. C. 596, *604*, 609, *617*
Jardillier, J.-C. 138, *144*, **145**, **389**
Jarlitsch, A. 89, *99*
Jarosz, C. J. 695, 708, *710*
Jarvik, L. F. 371, 375, *375*
Jarvik, M. 554, *563*
Jarvik, M. E. 552, *566*, 695, 696, *712*
Jasinski, D. R. 541, *563*
Jayaraman, J. **371**
Jennings, J. R. 429, *439*
Jering, H. **161**, **229**, 242, *242*
Joffe, J. M. 689, *691*
Johanson, W. G. Jr. 313, 325, *327*
Johansson, U.-B. 45, *61*
Johnson, K. M. 71, *80*, 219, *228*, **343**, 345, 346, *349*, *358*, *563*
Johnson, S. 557, *563*
Johnstone, R. E. 280, *296*
Jolly, H. 501, *511*
Joneja, M. G. 408, *417*, 481, 482, 494, *497*, *511*
Jones, B. 620, *639*, 644, *655*
Jones, B. C. 548, 550, *561*, 696, *711*
Jones, G. 78, *80*, 105, 106, *121*, 219, *227*
Jones, L. A. 280, *296*
Jones, M. P. 279, *295*
Jones, R. 280, *297*, 541, *563*, 586, 588, *589*
Jones, R. T. 81, *88*, 129, 131, *144*, 431, *438*, 520, 523, *530*, 541, 542, 544, 548, *563*, *565*, 595, 599, *603*, 676, *679*, 694, 707, *711*
Jong, Y. 229, *242*
Josephson, L. 231, 237, *242*

Jou, S. 372, 373, 375, *376*
Judah, D. 231, 239, *242*
Just, W. W. 45, *61*, 494, *497*

Kalant, H. 280, 289, *296*, *299*, 493, 494, *499*, 549, *562*, **681**, 681, 682, 687, 688, 689, *690*
Kam, J. K. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*
Kandel, E. R. 653, *656*
Kanter, S. 559, *563*
Kanter, S. L. 4, 7, *13*
Kaplan, H. S. 707, *711*
Kaplan, J. N. 495, *496*
Karler, R. 244, *249*, *358*, 550, *563*, 587, *589*, **619**, 620, 621, 623, 624, 625, 630, 635, 636, 637, 638, *640*, *641*, 650, 651, 652, 654, *655*, *656*, *657*
Karniol, I. G. 357, *358*, 644, 651, *656*
Karnovsky, M. 311, *327*
Karr, G. 668, 677, *679*
Karr, G. W. 557, *561*
Kashket, E. R. 148, *159*
Kasinsky, N. 651, *656*
Kass, E. H. 311, 313, 325, *327*
Katsorchis, T. 378, 381, *387*
Katz, J. J. 53, *61*
Katz, M. M. 552, *566*
Kaufman, R. H. 453, *466*
Kayan, S. 599, *604*
Kaymakcalan, S. 280, *296*, 586, 588, *589*, **591**, 591, 592, 593, 594, 595, 596, 599, 600, 601, *603*, *604*, 696, *710*, *711*
Kearns, G. L. 49, *62*
Keast, K. 294, *296*
Kedia, K. 436, *439*
Keeler, M. H. 638, *640*, 644, *656*
Keith, C. H. 317, *327*
Kelleher, P. C. 294, *296*
Kelley, P. R. 81, *88*, 431, *438*
Kelly, W. J. 311, *327*
Kendrick, J. 279, 284, *296*, *297*, 493, *497*
Kennedy, J. S. 280, *296*, 476, *477*, *494*, *497*, 532, *537*, 586, *589*, 595, *604*, 689, *691*
Kenney, A. F. 549, *565*
Keogh, E. J. 403, *405*
Kephalas, T. A. 280, *296*
Keplinger, M. L. 360, 361, 367, *369*, 408, *417*, 469, 471, 472, 474, *476*, 481, 482, *497*, *499*, 502, *511*
Kershbaum, A. 294, *297*
Kesner, R. 542, *563*
Ketkar, M. B. 279, 294, *297*
Keuhnle, J. 280, *297*
Khan, M. A. 481, 482, *497*
Khazan, N. 653, *656*
Khorsandian, R. 294, *297*
Khurana, R. 129, 131, *143*
Kiburis, J. 280, *296*
Kielholz, P. 598, *604*
Kierstead, R. W. 72, 78, *80*
Kilbey, M. M. 550, 559, *563*
Kilburn, K. H. 294, *297*
Killam, E. K. 646, *656*
Killam, K. F. 646, *656*
Kimball, A. P. 558, *562*
Kimelberg, H. K. 243, 245, 246, 247, 248, *250*
Kinchi, M. 131, *144*
King, L. J. 7, *13*, 81, 83, 84, 85, *88*, 289, *297*, *298*, 431, *439*, 493, *498*
King, R. J. B. 462, *467*
King, S. 145, *159*
King, W. 347, *349*
King, W. T. 596, *603*
Kiplinger, G. 52, *62*
Kiplinger, G. F. 279, 280, *297*, 542, 543, 544, 552, *562*, *563*, *564*
Kish, V. M. 205, *207*
Kjeldsen, K. 495, *495*
Klee, W. A. 605, *618*
Kleeman, W. 243, *250*
Klein, P. D. 22, *43*
Kleinsmith, L. J. 184, 189, 202, 205, *207*, *208*, 248, *249*, 347, *349*, 358, *358*
Klippel, R. A. 568, *582*
Klonoff, H. 544, 547, 548, 549, 552, *563*, *564*, *582*, 659, *663*, 665, 670, *679*, 694, *711*
Kluver, H. 554, *563*
Knapp, D. R. 52, *62*
Knight, F. 694, *710*
Knobil, E. 450, *467*
Knox, G. V. **681**, 681, 682, 687, 688, 689, *690*
Knudson, R. J. 279, *295*
Koch, F. 135, *144*
Koch, G. 135, *144*
Koivistoinen, P. 533, *537*
Kokka, N. 494, *497*
Kokkevi, A. 665, 670, *679*
Kokko, A. 396, *405*
Kolansky, H. *530*, *582*, 694, *711*
Kolodner, R. M. 252, *263*, 359, *369*, 395, 403, *404*, *405*, 408, *417*, 419, *427*, 429, *439*, 456, *467*, 520, *530*
Kolodny, R. C. 223, *228*, 252, *263*, 359, *369*, 395, 396, 403, *404*, *405*, 408, *417*, 419, *427*, 429, *439*, 456, *467*, 520, *530*
König, J. F. R. 568, *582*
Kopecny, V. 378, *387*
Kopell, B. S. 542, 543, 548, *565*
Kopin, I. J. 92, *98*, 105, 109, 113, 115, *121*, 243, *249*, 252, *263*, 520, *530*, 559, *565*, 587, *589*
Koral, J. 694, *711*
Kornetsky, C. 593, *603*
Kostellow, A. B. **441**, 441, 442, 445, 447, *447*
Koukkou, M. 548, *563*, 659, *663*
Koval, G. J. 231, *242*
Kralik, P. M. 252, 262, *263*, 280, *296*
Kramer, J. 495, *497*
Krause, M. O. 201, 202, 204, *207*
Kreuz, D. S. 106, 111, 117, *121*
Kreuz, L. E. 429, *439*
Kristler, G. S. 723, *730*
Krueger, P. M. 426, *427*
Kruskal, W. H. 472, *476*
Kubena, R. K. 118, *121*
Kuebbing, D. 168, *169*

Kuehnle, J. 252, *263*, 395, *404*, 408, *418*, 689, *691*, 694, 707, *711*
Kuehnle, J. C. *582*, 669, 670, *679*, 694, *711*
Kuhar, M. J. 352, *358*
Kulp, R. A. 280, *296*
Kumar, R. 568, *582*
Kunysz, T. J. 371, 375, *376*
Kuo, E. H. 494, *496*
Kupfer, D. J. 694, *711*
Kurosumi, K. 403, *405*
Kutt, H. 638, *640*
Kyrkou, K. A. 372, 375, *375*

Labrecque, G. 645, *656*
Lacy, D. 429, *439*
Ledewig, G. 598, *604*
Ladman, A. J. 129, 131, *144*, 294, *297*
Laemmli, U. K. 190, *207*
LaForce, F. M. 311, 313, 325, *327*
Lagerlund, I. 72, *80*
Laguarda, R. 294, *295*, *296*, 325, *327*
Laird, H. II 550, *561*, 620, *639*, 644, *655*
Lal, H. 616, *617*
Lamprecht, F. 559, *565*
Landaw, S. A. 293, *296*
Lander, N. 71, *80*, 219, *227*, 620, 636, *639*, 651, 652, *655*
Landesman-Dwyer, S. 509, *512*
Larson, P. S. 279, 294, *297*
Latif, S. 397, 403, *405*
Latscha, R. 504, *511*
Lau, R. J. 129, 131, *144*
Laurent, B. 168, 169, *169*, 240, *242*, 243, 245, *250*, 330, *342*
Laurenzi, G. 311, *327*
Lavappa, K. S. 244, *249*
Lawrence, D. K. 72, 78, *80*, 244, 245, 248, *249*, *250*, 289, *296*, 493, 494, *496*
Lawson, A. M. 47, *62*
Layman, J. M. 494, *497*
Leander, K. 3, 4, 7, 10, *13*, 16, *43*, 45, 53, 60, *61*, *62*, **63**, 63, 64, 65, 67, *69*, *70*, 72, 78, *80*, 101, 102, *103*, 105, 108, *121*, 279, 280, 289, *295*
Leaton, R. N. 545, *563*
LeBlanc, A. F. 681, 682, 687, 688, 689, *690*
Leboeuf, G. 360, *369*, 396, 403, *405*, 408, *417*, 494, *496*
Lebowitz, M. D. 279, *295*
Lechene, C. 231, *242*
Ledbetter, M. L. S. 169, *169*
Lee, A. G. 247, *250*
Lee, C. L. 670, *679*
Lee, M. L. 279, 280, 294, *297*
Lee, T. 357, *358*
Lee, V. W. K. 403, *405*
Lee, Y. E. 280, 294, *298*, *328*
Leech, C. K. 622, *640*
Lees, M. 246, *249*
Lees, M. H. 501, *511*
Lefkowitz, S. S. 131, *144*
Legator, M. S. 416, *418*
Leger, C. **129**, **145**, 158, *159*, 437, *438*
Legris, H. **265**
Legros, J. J. 426, *427*
Lehmann, D. 548, *563*, 659, *663*
Lehninger, A. L. 230, *242*
Lehrer, G. M. 481, 482, *497*
Leighty, E. G. 46, *62*, 107, *121*, 289, *297*, 431, *439*, 493, 494, *497*
Leite, J. R. 651, *655*, 689, *691*
Lemberger, L. 4, 6, *13*, 15, *43*, 92, *98*, 105, 106, 107, 109, 112, 113, 114, 115, 117, 118, 119, *121*, 131, 132, *144*, 243, *249*, 252, *263*, 287, 289, *296*, *297*, 493, *496*, *497*, 520, *530*, 587, *589*, 623, 636, *640*
Lennox-Buchthal, M. A. 637, *639*
Lerer, B. 645, *656*
Lerner, C. B. 129, 131, *144*
Lerner, M. 280, *297*
Leroux, J. P. *277*
Lessin, P. 131, *144*, 223, *228*, 408, *417*
Lessin, P. J. 129, *144*, 585, *589*
Letarte, J. 360, *369*, 396, 403, *405*, 408, *417*, 494, *496*
Letawe, J. 426, *427*
Leuchtenberger, C. **209**, 209, 210, 211, 217, *218*, 280, *297*, 326, *328*, 372, *375*
Leuchtenberger, R. **209**, 209, 210, 211, 217, *218*, 280, *297*, 326, *328*, 372, *375*
Leuschner, F. 493, 495, *498*
Leute, R. K. 89, *99*
Levett, G. A. 696, 708, *711*
Levin, H. S. 555, *565*
Levy, B. I. 543, *561*
Levy, J. A. 129, 131, 132, *144*
Levy, S. 4, *13*, 33, 35, *43*, 63, 64, 65, 67, *70*, 71, 78, *80*, 101, *103*, 219, *227*
Lewis, C. I. 309, 310, *328*
Lewis, E. G. 548, *563*, 696, *712*
Liakos, A. 694, *712*
Libet, B. 623, *640*
Liebeskind, J. C. 646, 654, *655*
Lief, P. L. 280, *296*
Lightner, D. A. 74, *80*
Lillie, R. D. 361, *369*
Lindgren, J.-E. **3**, 3, 7, 10, *13*, 16, *43*, 53, 60, *61*, *62*, 64, *69*, *70*, 72, 78, *80*, 101, 102, *103*, 105, 108, *121*
Lindler, H. R. 450, *467*, 495, *498*
Lindsey, C. J. *561*
Linkie, D. 432, *439*
Linn, W. S. 279, *295*
Linquist, P. 89, *99*
Lipparini, F. 549, *563*, 646, 653, *656*
Lipsett, M. 450, 458, *467*
Lipton, A. 132, *144*
Lipton, M. 17, *43*, 63, *70*
Lipton, M. A. 520, 529, *530*
List, A. F. 245, *250*, 396, 398, *405*
Littau, V. C. 377, *387*
Littleton, J. M. 559, *563*
Livingston, S. 654, *656*
Livne, A. 188, *207*, 329, *342*

Lloyd, B. J. 542, 548, *566*, *583*, 585, *589*
Locke, D. 359, *369*
Loewe, S. 619, *640*, 645, 652, *656*
Logie, P. 598, *604*
Lohiya, N. K. 252, *263*, 359, *369*, 396, *405*, 408, *417*, 419, 426, *427*, 441, *447*, 450, *466*, 495, *496*
Loizzo, A. 549, *564*
Lomax, P. 586, *589*, 599, *604*
London, S. W. 677, *679*
Longo, V. G. 549, *563*, *564*, *566*, 646, 653, 654, *656*, *657*
Loregren, P. D. 8, *13*
Loriaux, D. L. 437, *439*
Loud, A. V. 723, *730*
Louis, S. 638, *640*
Lousberg, R. J. J. Ch. 280, *298*
Lovell, R. A. 351, *358*, 548, *563*
Low, C. E. 280, *297*
Low, M. 665, 670, *679*, 694, *711*
Low, M. D. 544, 547, 548, 549, 552, *563*, *564*, 659, *663*
Low, R. B. 294, *297*
Lowe, A. G. 244, *249*
Lowe, D. 395
Lowe, M. *582*
Lowry, O. H. 163, *169*, 230, *242*, 397, *405*
Lubin, D. J. 481, *497*, 501, 502, *511*
Lubin, M. 169, *169*
Lubin, S. J. 408, *417*
Lugton, W. G. D. 282, *295*, 325, *326*, 493, *496*
Luly, P. 248, *250*
Lurie, M. 72, *80*
Luthra, Y. K. 290, *297*, **531**, 531, 532, 533, 536, *537*
Lutz, M. P. 588, *589*
Lyerly, L. A. 310, *328*
Lykouras, E. 378, 381, *387*
Lyman, J. M. 494, *497*
Lynch, W. C. 347, *349*, 695, 696, *710*, *711*
Lytle, L. D. 676, *679*

MacCannell, K. 668, 677, *679*
Maccioni, A. H. R. 238, *242*
MacConnell, K. L. 557, *561*
Machleidt, H. 333, *342*
Macindol, J. H. 359, *369*
MacKay, D. M. 551, *564*
MacLean, K. I. 559, *563*
MacLean, P. D. 546, 547, *564*
MacLeod, J. 409, *418*, 429, 436, 437, *439*
MacNeil, M. 243, *248*
MacPherson, A. S. 287, *298*, 493, *498*
Maddox, W. 279, 284, *296*, *297*, 493, *497*
Magnan, F. 688, *690*
Magnan-Lapointe, F. 279, 294, *298*
Magour, S. 243, *250*, 587, *589*
Magus, R. D. 279, *297*
Mahajan, V. K. 311, 324, 325, 326, *327*, *328*
Mahl, C. F. 546, *564*
Mahoney, J. M. 243, *250*, 329, *342*
Maickel, R. P. 570, *582*
Main, S. J. 426, *427*
Mainwaring, W. I. P. 462, *467*
Maker, B. S. 481, 482, *497*
Malamud, N. 546, *564*
Malitz, S. 543, *566*, 677, *680*
Malor, R. 243, 246, *249*, *250*, 586, *588*, **605**
Malsbury, C. W. 252, *263*
Man, D. P. 620, *639*, 646, 650, 651, *655*
Manjula, A. 441, *447*
Mann, P. E. G. 129, 131, *144*, 294, *297*
Manni, E. 622, *639*
Manning, F. J. 587, *589*, 696, 707, *711*
Manno, B. R. 543, 544, *564*
Manno, J. E. 279, 280, *297*, 543, 544, 552, *563*, *564*
Mans, R. J. 184, 185, *207*, *208*
Manson, J. R. 654, *655*
Mantilla-Plata, B. 367, *369*, 408, *418*, 481, 482, 484, 494, *497*, 501, 502, *511*
Marchand, J. Ch. *277*
Marchant-Williams, H. 613, *617*
Marcotte, J. 279, 294, *297*
Marcus, A. 548, *564*, 659, *663*, 665, 670, *679*
Marcus, A. M. *582*, 694, *711*
Markidou, S. G. 372, 375, *375*
Markland, C. 436, *439*
Marks, B. H. 450, *467*
Marks, D. 544, 552, *561*
Marks, V. 3, 7, *13*, **81**, 81, 82, 83, 84, 85, *88*, 289, *297*, *298*, 360, 369, *369*, *405*, 408, *418*, 431, *439*, 493, 494, *498*
Martin, B. R. 45, 45, 47, 51, 52, 53, 54, *61*, *62*, **63**, 64, 67, 69, *69*, *70*, 72, 78, *80*, **101**, 101, 102, *103*, *121*, 162, *169*, 177, *206*, 480, 494, *497*, 528, 529, *529*, 581, *582*
Martin, J. 280, *296*, 494, 495, *496*
Martin, P. 638, *640*, 648, 651, *655*
Martin, P. A. 371, 375, *375*
Martin, R. R. 294, *299*
Martin, W. R. 591, *603*
Martinez, J. L. 646, 653, *656*, 696, 706, *711*
Martz, R. 106, 112, *121*, *564*
Marx, M. 544, *562*
Maskarinec, M. P. 252, *263*, 495, *497*
Mason, M. M. 252, *264*, 531, *537*
Masters, W. H. 223, *228*, 252, *263*, 359, *369*, 395, 403, *405*, 408, *417*, 419, *427*, 429, *439*, 456, *467*, 520, *530*
Masur, J. 653, *656*, 695, 697, 708, *710*
Mathe, A. A. 280, *298*
Mathieu, M. 268, *277*
Matsudo, V. K. R. 595, *603*
Matsumoto, K. 623, 636, *640*
Matsuyama, S. S. 371, 375, *375*
Mattison, J. B. 605, *617*
Maugh, T. H. 326, *328*
May, E. L. 64, 67, 69, *70*, 601, *604*, 669, *680*
Maynard, D. E. 78, *80*
Mazurkiewicz-Kwilecki, I. M. 689, *691*
McCallum, N. K. 4, *13*, 33, 35, *43*, 45, *62*, 63, 64, 67, 69, *70*, 71, *80*, 101, *103*, 219, *227*
McCaman, R. E. 248, *249*

McCarthy, C. R. 294, *296*, 311, 325, *327*
McCarthy, K. D. 580, *583*
McCarthy, L. E. 677, *679*
McCarthy, R. H. 665
McCaughran, J. A. Jr. 620, *639*, 650, *655*, *656*
McClean, D. K. 142, *144*, 145, *159*, 188, *208*
McClendon, D. 696, 707, *711*
McCloskey, J. A. 47, *62*
McCullagh, D. R. 426, *427*
McDonald-Gibson, W. J. 605, *617*
McDowell, F. 638, *640*
McFarland, D. 542, 543, 552, 555, *564*
McGeady, J. C. 309, 310, *328*
McGeer, E. G. 251
McGeer, P. L. 243, 244, *250*, **251**, 251, 252, 253, 261, *263*, 481, 482, 494, 495, *497*, **519**, 520, 521, 522, 523, 524, 526, 527, 528, 529, *530*, 532, 535, *536*, *537*
McGlothin, W. H. *582*, 694, *711*
McGowan, J. C. 330, 333, 339, *341*, *342*
McIlwain, H. 535, 536, *537*
McIntyre, D. C. 549, *562*, 622, *640*, 650, *655*
McIsaac, W. M. 252, 262, *263*, 280, *296*, 347, *349*, 494, *497*, 550, 552, *564*, 706, *711*
McLendon, D. M. *563*
McMahon, R. 623, 636, *640*
McMahon, R. E. 52, *62*
McMillan, D. E. 357, *358*, 581, *582*, 586, *589*, 595, 600, *603*, *604*
McMillan, P. 397, *405*
McNamara, M. C. 550, *562*, 638, *640*, 648, 653, *655*
McNicol, D. 666, *679*
McQueen, J. K. 622, *639*
Means, A. 397, 404, *405*
Mechoulam, R. 4, *13*, 15, 33, 35, *42*, *43*, 45, *62*, **63**, 63, 64, 65, 67, 69, *69*, *70*, 71, 72, 74, 78, 79, *80*, 101, *103*, 219, *227*, 301, *327*, 351, 356, *358*, 520, 528, 529, *529*, 613, *617*, 620, 636, *639*, *640*, 651, 652, *655*
Mehndivatta, S. S. 694, *711*
Mehraein, P. 685, *690*
Mei, N. 728, *730*
Meissner, W. W. 546, 553, 555, *564*
Melchers, F. 148, *159*
Meldrum, B. S. 646, *656*
Melges, F. T. 520, *530*, 541, 542, 548, 552, *564*, *565*, *711*
Mello, N. 280, *297*
Mello, N. K. *582*, 694, *711*
Mellors, A. 72, *80*, 129, 131, *144*, 145, 158, *159*, 168, 169, *169*, 219, *227*, 229, *242*, 294, *296*, **329**, 329, 330, 332, 333, 334, 339, *341*, *342*
Menard, R. 397, 403, *405*
Mendelson, C. 261, 262, *263*
Mendelson, J. 280, *297*, 586, *589*
Mendelson, J. H. 252, *263*, 395, *404*, 408, *418*, *582*, 669, 670, *679*, 689, *691*, 694, *711*
Merari, A. 252, *263*
Meresz, O. 289, *299*, 493, 494, *499*
Messiha, F. S. 559, *564*
Messing, R. B. 676, *679*
Metcalfe, J. C. 248, *250*
Meyer, A. Y. 76, *80*
Meyer, R. 586, *589*
Miale, J. B. 378, *387*
Michael, M. C. 280, *296*
Michell, R. 334, *342*
Michelle, C. F. 422, *427*
Mickey, M. R. 131, *144*
Miczek, K. A. 682, 688, *690*, 695, 708, *711*, *712*
Midgley, A. R. 450, *467*
Mikes, F. 280, *297*
Mikhael, M. 689, *691*
Mikus, P. 294, *298*, 324, 326, *328*
Miles, C. G. 694, 707, *712*
Millart, H. 265
Miller, A. J. 129, 131, *144*
Miller, F. P. 570, *582*
Miller, L. L. 539, 542, 543, 544, 549, 550, 552, 555, 557, 558, *562*, *564*, 694, 696, 709, *711*
Millet, D. 426, *427*
Millichap, J. G. 637, *640*
Mills, N. 397, *405*
Milner, B. 546, *565*
Milner, P. 547, *565*
Milstein, M. 222, *228*, 371, 373, 374, 375, *376*
Milstein, S. L. 555, *561*, 668, 677, *679*
Milton, A. S. 494, *497*
Miras, C. J. 280, *296*, 360, *369*, 372, 375, *375*, 481, *497*, 598, *604*
Mirsky, A. E. 377, *387*
Mitra, G. 244, *250*
Miyachi, Y. 450, 458, *467*
Miyake, T. 280, *295*, 586, *588*
Mizusaki, S. 294, *297*
Moehring, J. M. 294, *296*
Moffat, A. C. 7, *13*, 84, 85, 87, *88*
Mohr, U. 279, 294, *297*
Mon, M. J. 171, 188, *207*
Monfoort, C. H. 377, 386, *387*
Monroe, S. E. 450, *467*
Monti, J. 651, *656*
Monti, J. M. *561*
Moore, C. E. 395, *405*, 450, *467*, 494, *498*
Moore, F. 4, 7, *13*, 559, *563*
Moore, J. W. 559, *563*
Moore, W. T. *530*, *582*, 694, *711*
Morero, R. D. 243, 244, 245, 247, 248, *249*
Moreton, J. 347, *349*
Moreton, J. E. 596, *603*
Morgan, E. D. 48, *61*
Morgan, I. G. 248, *249*, *250*
Morgane, P. J. 549, *564*
Morin, G. 645, *656*
Morin, P. J. 645, *656*
Morishima, A. 129, 131, 132, 140, *144*, 145, 153, 158, *159*, 161, 162, 166, 167, 168, 169, *169*, 188, *207*, 222, *228*, 229, *242*, 261, *264*, 326, *328*, 329, 335, *342*, **371**, 371, 372, 373, 374, 375, *375*, *376*, 377, *387*, 520, 528, *530*, 670, *679*
Morley, J. E. 598, *604*
Morrel, F. 638, *640*

Morrill, G. A. 419, 426, *427*, **441**, 441, 442, 445, 447, *447*
Morris, B. A. 81, 82, *88*
Morris, T. G. 326, *327*
Morrissey, M. W. 324, 326, *328*
Morrissey, W. 294, *298*
Moskowitz, H. 541, *564*
Mosnaim, A. D. 559, *565*
Mott, D. E. W. 357, *358*
Mould, G. P. 81, *88*
Moyyen, S. 219, *228*, 243, 246, *250*
Muchow, D. C. 595, *604*
Mullane, J. F. 311, *327*
Mullen, R. J. 386, *388*
Munson, A. E. 129, 132, *144*, 145, *159*, 161, 168, 169, *169*, 188, *207*, *208*, 219, 220, 222, 226, *227*, 229, 239, *242*, 368, *370*
Munson, J. A. 161, 168, 169, *169*, 188, *208*, 219, 220, 226, *227*
Murison, G. L. 359
Murphy, S. 550, *562*
Musty, R. E. *561*
Myers, G. H. Jr. 429, *439*
Myers, R. E. 495, *496*
Myers, W. A. 689, *691*, 713, 723, *730*

Nagai, K. 231, *242*
Nagel, M. D. 131, *144*
Nahas, G. G. 4, *13*, 71, *80*, **129**, 129, 131, 132, 140, *143*, *144*, **145**, 145, 153, 158, *159*, 161, 162, 166, 167, 168, 169, *169*, 188, *207*, 222, *228*, 229, *242*, 252, 261, *263*, *264*, 280, 289, *299*, 326, *328*, 329, 335, *342*, **371**, 371, 372, 373, 374, 375, *376*, 377, 378, 386, *387*, 396, *405*, 407, 408, *417*, 418, **419**, 419, 426, *427*, **429**, 429, 430, 432, 437, *438*, *439*, 456, *466*, **469**, 469, *476*, 481, 495, *496*, *497*, 501, *511*, 520, 528, *530*, 548, *565*, 670, *679*
Nakashima, E. N. 542, *561*
Nakazawa, K. 280, *297*
Nallan, G. 543, *564*
Naor, Z. 450, *466*
Naquet, R. 646, *656*
Naqvi, R. H. 482, 487, 491, 492, 494, *496*
Narasimhulu, S. 243, *250*
Nasello, A. G. 550, *563*, 651, 653, *655*
Nazar, B. L. 245, 246, *250*, **395**, 396, 398, *405*, 587, *589*
Nebel de Mello, D. 280, 289, *298*, 585, *589*
Needle, M. 129, 131, *144*
Neergaard, J. R. 459, *467*
Neff, N. H. 570, *582*
Neimi, M. 396, *405*
Neischlag, E. 450, 458, *467*
Nelson, J. 542, *566*
Nelson, J. M. 586, *589*
Netter, A. 426, *427*
Nettesheim, P. 279, 284, *296*, *297*, 309, *327*, 493, *497*
Neu, R. L. 501, *511*
New, P. F. J. 689, *691*
New, R. L. 145, *159*
Newhall, A. R. 533, *537*
Newman, L. M. 588, *589*
Ng, L. K. Y. 243, *249*, 559, *565*
Ngyen-Hoang-Nam 6, *13*
Nieman, G. W. 482, *496*
Nikitovitch-Winer, M. B. 495, *498*
Nilsson, I. M. 3, 7, 10, *13*, 16, *43*, 45, *61*, 64, *69*, 72, 78, *80*, 101, *103*, 105, 106, 118, *121*, 494, *498*
Nilsson, J. L. G. 45, *61*, 72, 78, *80*, 105, 106, *121*
Nimitkipaisan, Y. 568, *582*
Nir, I. 450, *467*, 495, *498*
Nirenberg, M. 605, *618*
Nishioka, I. 280, *298*
Niswender, G. D. 450, *466*, *467*
Niu, H. C. 8, *13*, 287, 289, *298*
Noble, E. 559, *563*
Nogawa, T. 372, 373, 375, *375*
Nordqvist, M. 45, 53, *61*, *62*, 64, *69*, 72, 78, *80*, 101, 102, *103*
Novakova, U. 684, 688, *690*
Novotny, M. 252, *263*, 279, 280, 294, *297*, 495, *497*
Nowlan, R. 586, *589*
Nowlin, J. 53, *62*
Noyes, R. Jr. 592, *604*, 668, 677, *679*
Nyquist, S. E. 360, *369*, **395**, 396, 398, 399, 400, *405*

Oakberg, E. F. 409, *418*
O'Brien, D. A. 422, *427*
Ogawa, N. 682, 688, *690*
Ohlsson, A. **3**, 4, 7, 10, *13*, **63**, 63, 64, 65, 67, *69*, *70*, 72, 78, *80*, 101, *103*, 105, 108, 118, *121*
Okamoto, H. 294, *297*
Okamoto, T. 294, *297*
Okey, A. B. 225, 227, *228*, 252, *264*, 360, *369*, 408, *418*, 441, *447*, 450, 458, *467*, 495, *498*
Olds, J. 547, *565*, 567, 568, *582*
Olds, M. E. *565*
Olivetti, C. C. 550, *563*
Olsen, D. M. 620, *641*
Olsen, H. M. 495, *495*
Olsson, J. E. 552, *565*
O'Malley, B. W. 184, *208*, 459, *467*
Omura, T. 397, *405*
Opelz, G. 129, 131, 132, 142, 143, *144*
Orsingher, O. A. 549, *563*, 651, *655*
Osawa, T. 620, *641*, 646, *657*
Osgood, P. E. 229, *242*, 351, *358*
O'Shaughnessy, W. B. 619, *640*
Osmond, H. 558, *562*
Osterholm, K. C. 596, *604*
Otis, L. 503, *512*
Otken, L. B. 294, *298*
Overall, J. E. 541, *563*
Overstreet, J. 431, *439*
Ozzello, L. 210, *218*

Pace, H. B. 360, *369*, 408, *418*, 476, *477*, 480, 481, 482, *496*, *498*, 502, *511*, 596, *603*, 682, *690*
Pace, S. H. 347, *349*
Palermo-Neto, J. 559, *565*
Palmer, A. K. **469**, 472, 474, *476*, *477*
Panayiotopoulos, C. P. 585, *589*, 663, *663*
Panyim, S. 190, *207*
Papadakis, D. P. 280, *296*
Papahadjopoulos, D. 243, 245, 246, 247, 248, *250*
Papez, J. W. 545, *565*
Paris, H. G. 253, *264*
Paris, M. 696, *711*
Park, W. D. 184, *207*
Parker, M. R. **219**, 381, *387*
Pars, H. G. 229, *242*
Paton, W. D. M. 15, *42*, **45**, 45, 47, 50, 51, 52, 53, 54, *61*, *62*, 64, *70*, 78, 79, *80*, 97, *98*, 142, *144*, 145, *159*, 219, *227*, 244, 246, *250*, 329, *342*, 357, *358*, 480, 495, *497*, 540, 541, 542, *565*, 654, *656*, 694, 707, 708, *712*
Patterson, A. R. P. 166, 168, *169*
Pauerstein, C. J. 450, *466*
Paul, J. 184, *207*
Paul, M. I. 543, *565*
Pauli, L. L. 654, *656*
Pavlok, A. 378, *387*
Pearse, A. G. E. 294, *297*
Peat, A. 728, *730*
Pecknam, W. D. 450, *467*
Pedemonte, W. A. 559, *565*
Peeke, S. C. 544, *565*
Pellegrino de Iraldi, A. 177, *206*
Penfield, W. 546, *565*
Penry, J. K. 638, *640*
Pereira, W. 294, *296*, 325, 326, *327*
Perez-Reyes, M. 15, 16, 17, 22, 33, 35, *42*, *43*, 63, *70*, 101, *103*, 132, *144*, 520, 529, *530*, 620, 623, 636, *640*, 651, *656*
Perhach, J. L. Jr. 570, *582*
Perkins, J. 287, *298*, 493, *498*
Peron-Magnan, P. 659, 660, 661, 662, *663*
Perrin, R. G. 549, *562*
Persaud, T. V. N. 480, 481, *498*, 502, *511*, *512*
Pertwee, R. G. 78, *80*, 97, *98*, 142, *144*, 145, *159*, 219, *227*, 244, 246, *249*, *250*, 329, *342*, *358*, 540, 541, 542, *565*, 654, *656*, 694, 707, 708, *712*
Peterkofsky, B. 221, *228*
Peters, B. A. 548, *563*
Peters, B. H. 555, *565*
Petersen, B. H. 131, 132, *144*
Peterson, H. 438, *439*
Petersson, K. 101, 102, *103*
Petrzilka, T. 72, *80*
Petsche, H. 551, *565*
Petty, W. C. 635, *640*
Pfeferman, A. 651, *656*
Pfefferbaum, A. 543, *565*
Pfeiffer, C. A. 367, *370*
Phillips, D. M. 383, 386, *388*
Phillips, G. H. 340, *342*
Phillips, R. N. 481, 482, *498*, 502, *512*
Piall, E. M. 81, 82, 83, *88*, 431, *439*
Picchioni, A. L. 646, *655*
Pickens, R. 595, *604*
Pieper, W. A. 696, *710*
Pietras, R. J. 462, *467*
Pihl, R. O. 694, *710*
Piper, D. W. 294, *298*
Pirch, J. H. 548, *565*, 596, *604*, 646, *656*
Pitcher, R. G. 78, *80*
Pitt, C. G. 15, 17, 22, 35, 36, *43*, 78, *80*, 81, *88*, 106, *121*, 459, *561*
Pittman, J. C. 654, *656*
Pivic, R. T. 659, *663*
Plagemann, P. G. W. 166, *169*
Plank, J. B. 360, 361, 367, *369*, 408, *417*, 481, 482, *497*, 502, *511*
Plaves, M. 252, *263*
Pliner, P. L. 543, *561*
Plotnikoff, N. P. 636, *641*, 652, *656*
Pochay, V. E. **301**, 311, 326, *327*
Poddar, M. K. 243, 244, 245, *250*, **343**, 348, *349*
Poole, C. F. 48, *61*
Porath, G. 4, *13*, 63, 64, 65, 67, *69*, *70*, **71**, 78, *80*, 101, *103*
Porcellati, G. 248, *249*
Porzak, J. P. 548, 553, *565*, 659, *663*
Potvin, R. J. 694, *712*
Powell, B. J. 669, 676, 677, *679*
Powell, E. W. 550, 553, *565*
Powell, G. M. 326, *327*
Powers, M. 145, *159*
Powers, P. 49, *62*
Pradhan, S. **567**, 570, *582*
Pradhan, S. N. 325, *328*, **567**, 567, 568, 570, 578, 580, 581, *582*
Preble, M. 280, 289, *298*, 694, *712*
Prendergast, T. 694, *712*
Prendergast, T. J. 280, 289, *298*
Preston, A. M. 168, 169, *169*, **229**, 231, *242*, 243, 244, *250*
Pribram, K. H. 546, *562*
Prigge, E. 293, *297*, 495, *498*
Prockop, L. D. 494, *498*
Proctor, R. C. 605, *618*
Ptashne, M. 638, *640*
Puder, M. 450, *466*
Puil, E. A. 646, *656*
Puri, S. K. 616, *617*
Purvis, J. 397, 403, *405*

Quarles, W. 280, *297*
Quastel, J. H. 521, *530*
Quimby, M. W. 280, *296*

Rabinovitch, M. S. 687, *690*
Rachelefski, G. S. 129, 131, 132, 142, 143, *144*
Racine, R. J. 622, *641*
Radouco-Thomas, C. 688, *690*
Radouco-Thomas, S. 688, *690*

Rainsburg, R. 326, *326*
Raj, A. Y. 407
Raj, Y. 386, *388*
Rall, D. P. 715, *730*
Ramamnna, L. 280, 294, *298*
Ramsay, C. H. 45, *61*, 106, *121*, 494, *498*
Ramsey, H. H. 619, *639*, 652, *655*
Randall, R. J. 163, *169*, 230, *242*, 397, *405*
Randerath, E. 163, *169*, 183, *207*
Randerath, K. 163, *169*, 183, *207*
Rawitch, A. 682, *690*
Rawitch, A. B. 225, *228*, 360, *369*, 481, 482, 494, *499*, 532, 535, 536, *537*
Rawley, M. J. 437, *439*
Raymond, A. 280, *297*
Raz, A. 188, *207*, 243, 244, *249*, 329, 330, *342*, 351, 352, 356, *358*
Razdan, R. K. 46, *62*, 219, *227*, 253, *264*, 343, *348*
Rebbe, H. 438, *439*
Reccius, N. 371, 375, *375*
Reckzeh, G. 493, 495, *498*
Regan, J. D. 132, 142, *143*, 145, 157, *159*, 188, *206*, 222, *228*
Reichert, L. E. 450, *467*
Reifler, C. B. 638, *640*, 644, *656*
Reinking, J. 550, *561*, 620, *639*, 644, *655*
Remo, R. A. 166, *169*
Renault, P. F. 280, 289, *298*, 557, *565*, 585, *589*, 714, 715, *730*
Renne, G. C. 403, *405*
Rettura, G. 419, 426, *427*
Reuber, M. D. 162, *169*
Reznik, G. 279, 294, *297*
Rich, E. S. 559, *565*
Richards, A. B. 620, 636, *640*, 652, *655*
Richards, R. K. 557, *563*, 707, *711*
Richter, D. 535, 536, *537*
Rigler, R. Jr. 381, *387*
Rifka, S. M. 437, *439*
Ringertz, N. R. 381, *387*
Ritter, U. 209, 210, 211, 217, *218*, 326, *328*, 372, *375*
Robbins, P. R. *582*, 694, *712*
Roberts, J. 287, *298*, 493, *498*
Roberts, P. 501, *511*
Roberts, R. B. 536, *537*
Roberts, W. C. 294, *296*
Robinson, G. A. 229, *242*
Robinson, J. D. 82, *88*
Rodda, B. 106, 112, *121*
Rodda, B. E. 543, *563*, *564*
Rodgers, R. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*
Rodin, E. A. 548, 553, *565*, 659, *663*
Rodríguez, L. D. 231, *242*
Rodríguez, M. B. 168, 169, *169*, **229**, 231, 240, *242*, 243, 244, *250*
Rodriguez de Lores Arnaiz, G. 177, *206*
Rohde, C. 494, *498*
Roll, S. K. 568, *582*
Roman, A. 361, *369*
Ronald, R. C. 7, *13*, 88, *88*, 89, 91, 92, 95, 96, *98*
Roncoroni, L. 728, *730*
Ronquist, G. 231, 240, *242*
Rose, F. C. 555, *565*
Rose, R. M. 429, *439*
Roseboom, H. 119, *121*
Rosebrough, N. J. 163, *169*, 230, *242*, 397, *405*
Rosell, S. 63, 63, 64, *70*
Rosen, L. J. 670, *679*
Rosen, M. G. 495, *498*
Rosenbaum, J. 403, *405*
Rosenbaum, R. 441, 442, 445, 447, *447*
Rosenbaum, R. M. 419, 426, *427*
Rosenberg, E. 408, *417*, 469, *476*, 501, *511*
Rosenberg, M. 481, *496*
Rosenblatt, J. E. 558, *562*
Rosencrans, J. A. 601, *604*
Rosenfeld, J. 7, *13*
Rosenfeld, J. J. 287, *298*, 493, *498*
Rosenfeld, R. 106, *121*, 549, *561*
Rosenkrantz, H. 129, 131, *144*, 252, *264*, **279**, 279, 280, 282, 283, 284, 285, 286, 287, 289, 290, 291, 294, *295*, *296*, *297*, *298*, 408, *417*, 420, *427*, 441, *447*, **479**, 480, 481, 482, 483, 484, 487, 491, 492, 494, *496*, *498*, 501, 502, 510, *511*, *512*, 531, 532, 533, 535, 536, *536*, *537*, 597, *604*, 645, 646, 653, *656*, *657*, 689, *691*, 694, 695, 696, 706, 709, *712*
Rosenthal, D. 6, 7, 10, *13*, 16, 22, *43*, 105, 107, 108, *121*
Rosenthal, H. E. 459, *467*
Rossi, A. 280, *297*, 586, *589*
Rossi, A. M. 669, 670, *679*
Rosvold, H. E. 546, *562*, 687, *690*
Roth, R. I. 246, *250*, 587, *589*
Roth, S. 333, 339, *342*
Roth, W. T. 287, 289, *296*, 493, *496*, 543, 548, *565*, 659, *663*
Rotherberg, A. 546, *564*
Rouse, H. 381, 386, *388*
Rousseau, G. G. 220; 222, *228*
Rowe, H. 623, 636, *640*
Rowe, H. M. 106, 112, 114, *121*
Rowley, G. L. 7, *13*. 88, *88*, 89, 90, 91, 92, 95, 96, *98*, *99*
Roy, A. C. 605, *617*
Roy, P. E. 168, 169, *169*, 240, *242*, 243, 245, *250*, 279, 294, *298*, 330, *342*
Roy, S. N. 570, *582*
Rozijn, T. H. 377, 386, *387*
Rubenstein, K. E. 89, 89, 90, *99*
Rubin, A. 636, *640*
Rubin, I. 279, 284, *297*, 493, *497*
Rubin, V. 694, *712*
Ruble, D. C. 542, 548, *566*, *583*, 585, *589*
Rubottom, G. M. *341*
Ruddle, L. H. 339, *342*
Ruiz-Carrillo, A. 199, *207*
Rull, D. P. 697, *711*
Rumbaugh, C. L. 494, *495*
Rumbaugh, D. M. 696, *710*
Rylander, R. 289, 294, *298*
Ryrfeldt, A. 45, *61*, 106, *121*, 494, *498*

Saayman, G. S. 696, 708, *711*
Sabelli, H. C. 559, *565*
Sackett, G. 509, *512*
Saeed, S. A. 605, *617*
Saillant, J. 570, *582*
Sakuma, K. 184, *206*
Salem, N. 243, *248*
Salganicoff, O. 177, *206*
San, R. 416, *418*
Sandberg, F. 3, 7, 10, *13*, 16, *43*, 45, *61*, 78, *80*, 105, 106, 118, *121*
Sanders, J. 616, *617*
Sanders-Bush, E. 559, *562*, 600, *603*
Sandritter, W. 684, 688, *690*
Santen, R. J. 430, *439*
Santiago, J. 361, *369*
Sarfaty, G. 429, *438*
Sarkar, C. 243, 247, *250*
Sarma, M. H. 186, *207*
Sassenrath, E. N. 441, *447*, 481, 482, *498*, **501**, 502, *512*, 682, *690*, **693**, 695, 696, *712*
Sathe, S. 35, *43*
Sato, M. 650, *657*
Sato, R. 397, *405*
Sato, T. 280, *298*
Sauer, M. 437, *439*
Saunders, M. E. 72, *80*, 168, 169, *169*, 229, *242*, 330, *341*
Sawin, P. B. 472, *476*
Sayed, Y. 36, *43*
Scatchard, G. 459, *467*
Schaefer, C. F. 408, *418*
Schaeppi, U. 597, *604*, 653, *657*, 696, 706, *712*
Schaeppi, U. H. 646, 653, *656*, *657*, 696, 706, *711*
Schapero, M. 541, *564*
Scheckel, C. L. 552, *565*, 696, 706, *712*
Schellen, A. M. C. M. *427*
Scherle, W. F. 723, *730*
Schermann, J. M. 142, *143*
Scherrman, J. 6, *13*
Schleh, E. 209, 210, 211, 217, *218*, 280, *297*
Schmalz, L. W. 689, *690*
Schmid, W. 416, *418*
Schmidt, L. H. 697, *711*, 715, *730*
Schneider, A. 209, 211, 217, *218*, 326, *328*, 372, *375*
Schneider, C. 605, 613, *617*
Schneider, R. S. 89, *98*, *99*
Schoenfeld, C. 431, 438, *439*
Schoentgen, S. 279, *295*
Schou, J. 4, 494, *498*
Schramm, L. C. 480, 481, *496*, 502, *511*
Schŕan, H. 17, 35, *43*
Schultz, F. J. 306, 309, 310, 323, *328*
Schultz, G. S. 225, *228*, 360, *369*
Schurr, A. 188, *207*, 329, *342*
Schuster, C. R. 280, 289, *298*, 557, *565*, 585, *589*, 714, 715, *730*
Schuster, R. E. 288, 289, *296*, 493, *496*
Schwartz, I. W. 131, *144*
Schwartzfarb, L. 129, 131, *144*
Schwarz, S. 396, 398, 399, 400, *405*
Schwin, R. 669, 676, 677, *679*
Scotti de Carolis, A. 549, *563*, *566*, 646, 653, 654, *656*, *657*
Seamans, C. 311, *327*
Sedvall, G. 357, *358*
Seeman, P. 142, *144*, 158, *159*, 219, *228*, 243, 246, *250*, 333, 339, *342*, 357, *358*
Segal, M. 549, 550, *565*, 645, 646, 648, *656*
Segall, M. 132, *144*, 146, *159*
Seibetta, J. J. 495, *498*
Selmonosky, C. A. 294, *298*
Seltzman, H. H. 36, *43*
Serrano, A. 244, *249*
Setchell, B. P. 426, *427*
Setterfield, G. 381, *387*
Shani, A. 64, 65, 67, *69*
Shannon, M. E. 494, *498*
Shapiro, B. J. 280, 294, *298*, 324, 326, *328*
Sharma, S. 541, *564*
Sharma, S. K. 605, *618*
Sharma, V. N. 252, *263*, 359, *369* 396, *405*, 408, *417*, 419, 426, *427*, 450, *466*
Shattuck, D. 221, 223, *228*, 360, *369*, 437, *439*, 441, *447*
Shea, J. W. **301**, 311, 324, 325, 326, *327*, *328*
Shea, P. A. 570, *582*
Sheehan, J. C. 253, *264*
Sheehan, J. M. 148, *159*
Sherins, R. 397, *405*
Sherins, R. J. 426, *427*, 429, 436, *439*
Sherwood, S. L. 548, *563*
Sheth, A. R. 367, *369*, 495, *496*
Shibko, S. 533, *537*
Shipley, G. 495, *497*
Shirakawa, I. 651, *656*
Shirley, G. 252, *263*
Shoemaker, R. H. 458, *467*
Shoyama, Y. 280, *298*
Shriffin, R. M. 542, *565*
Sicuteri, F. 676, *680*
Sidman, R. L. 386, *388*
Siegel, G. J. 237, *242*
Siegesmund, K. A. 728, *730*
Siete, R. 728, *730*
Sikemeier, C. 72, *80*
Sikic, B. 280, 289, *298*, 585, *589*
Silberstein, S. D. 105, 113, *121*
Silva, M. 581, *582*
Silva, M. T. A. 588, *589*
Silvestein, M. J. 129, 131, *144*
Silvestroni, L. 378, 383, *387*
Silvette, H. 279, 294, *297*
Simmons, G. A. 294, *295*, *296*, 325, *327*
Simon, J. R. 352, *358*
Sinclair, D. 309, *328*
Sinclair, G. D. 378, *387*
Sineriz, F. 243, 245, 247, 248, *249*
Singer, P. R. 495, *498*
Singh, R. 396, *405*
Sitterson, B. W. 409, *418*
Sivil, S. 595, *604*
Sjöden, P. O. 596, *604*

Skelton, F. S. 279, 294, *297*
Skinner, J. E. 653, *656*
Skinner, R. F. 287, *298*, 493, *498*
Skipper, H. E. 697, *711*, 715, *730*
Sloane Stanley, G. H. 246, *249*
Smart, T. 552, *565*, 696, 706, *711*
Smith, C. G. 360, *369*, 396, *405*, **449**, 450, 453, *466*, *467*, 494, *498*
Smith, D. W. 502, *511*
Smith, H. E. 459, *467*
Smith, H. J. 696, *711*
Smith, M. T. 449
Smith, P. G. 293, *299*
Smith, R. G. 360, *369*, **449**, 450, 459, *466*, *467*, 494, *498*
Smith, S. H. 469, 471, 472, 474, *476*, 481, *499*
Smith, T. C. 280, *296*
Smythies, J. R. 547, *565*
Snedecor, G. W. 230, *242*
Sniffin, R. C. 294, *299*
Snyder, S. 71, *80*
Snyder, S. H. 344, *348*, 351, 352, 356, *358*
So, B. T. 294, *297*
Soates, J. R. 81, *88*, 288, 289, *296*, 493, *496*
Sodetz, F. J. 696, *710*
Sofia, R. D. 118, *121*, 158, *159*, 229, *242*, 351, *358*, 481, 482, *496*, 599, *603*, 620, *641*, 651, *656*
Soldin, S. J. 623, *641*
Solliday, N. H. 324, 326, *328*
Solomon, J. 221, 223, *228*, 360, *369*, 437, *439*, 441, *447*, 450, 458, *467*
Solomon, T. A. 620, *641*, 651, *656*
Solzman, G. 552, *566*
Sommer, K. R. 53, *62*
Sommerhoff, G. 547, 551, 554, *565*
Sornberger, G. C. 301, 311, 324, 325, 326, *327*
Soskin, R. A. 559, *564*
Spano, P. F. 570, *582*
Sparber, S. B. 536, *537*
Sparkes, R. S. 371, 375, *375*
Spears, A. W. 309, 310, *328*
Spelsberg, T. C. 189, *207*
Spencer, H. 294, *298*
Spencer, W. A. 653, *656*
Spiker, M. 550, *562*, 638, *640*, 646, 651, 653, *655*, *657*
Sprague, R. A. 287, *298*, 441, *447*, 646, *656*, 689, *691*
Spronk, H. J. W. 280, *298*
Srinivasan, P. R. 229, *242*
Srivastava, S. C. 35, *43*
Stadnicki, S. W. 597, *604*, 646, 653, *656*, *657*, 696, 706, *711*, *712*
Stafford, M. 53, *62*
Stahl, W. R. 310, 318, 319, *328*
Staples, R. E. 472, *476*
Starmer, G. A. 246, *249*, 613, 616, *617*
Steel, R. G. D. 608, *618*
Stefanis, C. 585, *589*, 659, 663, *663*, 694, *711*, *712*
Stefanis, C. N. 377, 378, 379, 381, 383, 386, *387*
Stein, G. S. 171, 176, 184, 185, 186, 188, 189, 201, 202, 204, *206*, *207*, *208*
Stein, J. L. 171, 184, 185, 186, 189, *189*, *206*, *207*, *208*
Stein, L. 568, *583*
Steinberger, A. 426, *427*
Steinberger, E. 381, *387*, 426, *427*, 429, *439*
Stenchever, M. A. 371, 375, *376*
Stern, W. C. 549, *564*
Sternthal, P. M. 436, *439*
Stich, H. 416, *418*
Stiehm, E. R. 131, *144*
Stillman, R. C. 543, 548, *561*, *562*
Stillwell, R. N. 47, *62*
Stoeckel, M. 416, *418*
Stokely, J. 284, *297*, 493, *497*
Stone, G. C. 544, *565*
Stone, G. R. 542, 548, *563*
Straight, R. C. 548, *563*
Stratton, M. 429, *438*
Strauss, R. P. 148, *159*
Stumpf, C. 551, *565*
Suciu-Foca, N. 129, 131, 132, 140, *144*, 145, *159*, 188, *207*, 222, *228*, 326, *328*, 329, 335, *342*, 377, *387*
Sulkowski, A. 559, *565*
Sulser, F. 559, *562*
Sun, G. Y. 248, *250*
Sutherland, E. W. 229, *242*
Svacha, A. J. 294, *298*
Svennerholm, L. 247, *249*
Swerdloff, R. S. 429, *439*
Swinyard, E. A. 621, *641*, 650, *657*
Symons, A. M. 360, *369*, 396, *405*, 408, *418*, 494, *498*
Symonds, C. P. 555, *565*
Synder, E. W. 696, *712*
Szara, S. 4, *13*, 71, *80*, 301, 326, *327*, 407, *417*, 495, *496*, *589*
Szego, C. M. 462, *467*
Szepsenwol, J. 359, 360, 361, 368, 369, *369*, *370*

Taillemite, C. 138, *144*
Tait, R. M. 532, 535, *537*
Talalay, P. 397, *405*
Tamarkin, N. R. 115, *121*, 252, *263*, 587, *589*
Tanck, R. H. *582*, 694, *712*
Tannhauser, M. 620, 636, *640*, 651, *655*
Taplin, G. V. 280, 294, *298*
Tarnowska-Dziduszko, E. 685, *690*
Tart, C. T. 540, 541, *565*
Tashkin, D. P. 280, 294, *298*, 324, 326, *328*
Tassinari, C. A. 645, 654, *657*, 659, *663*
Tassinari, C. T. 659, *663*
Taunton-Rigby, A. 81, *88*, 431, *438*
Tayler, T. 294, *298*
Taylor, D. A. 676, *680*
Taylor, D. V. 347, *349*
Teale, J. D. 7, *13*, **81**, 81, 82, 83, 84, 85, *88*, 289, *297*, *298*, 360, *369*, 396, *405*, 408, *418*, 431, *439*, 493, 494, *498*

Teece, J. J. 548, *565*
Teitel, B. 598, *604*
Temple, D. 145, *159*, 219, *227*, 244, 246, *250*, 329, *342*
Ten Ham, M. 229, *242*, 586, 587, *589*
Tennant, F. 694, *712*
Tennant, F. S. 280, 289, *298*, 326, *327*, 715, *730*
Teresa, A. 581, *582*
Terner, J. Y. 379, *387*
Testing, L. 106, *121*
Testino, L. 549, *561*
Teyler, T. J. 550, *565*
Thaggard, N. A. 310, *328*
Thanassi, N. M. 294, *296*
Thel, S. 45, *61*, 494, *496*
Thenot, J.-P. 53, *62*
Thoa, N. B. 559, *562*
Thomas, R. J. 408, *418*, 481, 482, *498*
Thompson, E. B. 221, *228*
Thompson, E. D. 8, *13*, 287, 289, *298*
Thompson, G. R. 252, *264*, 287, *298*, 480, *498*, 531, *537*, 646, *657*, 694, 695, *712*
Thompson, L. J. 605, *618*
Thompson, S. W. 294, *298*
Thompson, T. 595, *604*
Thompson, W. C. 310, *328*
Thomson, J. A. 189, 205, *208*
Thomson, J. L. G. 694, *710*
Thorburn, M. J. 371, 375, *375*
Thornton, N. 294, *298*
Thoursen, K. 168, *169*, 219, 220, 223, 226, *227*
Thrall, C. 184, *208*
Timmons, M. 17, *43*
Timmons, M. C. 63, *70*, 132, *144*, 520, 529, *530*, 620, 623, 636, *640*
Tindall, D. 404, *405*
Tinklenberg, J. R. 520, *530*, 541, 542, 543, 548, 552, *561*, *564*, *565*, 585, *589*, 707, 708, *711*, *712*
Toft, D. O. 459, *467*
Tomasi, V. 248, *250*
Tomer, K. 17, *43*
Tomkins, G. M. 220, 221, 222, *228*
Tong, H. S. 309, 310, *328*
Toro, G. 223, *228*, 252, *263*, 359, *369*, 395, 403, *405*, 408, *417*, 419, *427*, 429, *439*, 456, *467*, 520, *530*
Toro-Goyco, E. 161, 168, 169, *169*, **229**, 231, 240, 242, *242*, 243, 244, *250*, **359**, 369, *370*
Torrelio, M. 601, 602, *604*, 605, *617*
Torrie, J. H. 608, *618*
Tosaka, T. 622, *640*
Tratnyek, C. A. 533, *537*
Traurig, H. H. 294, *298*
Treich, I. 142, *143*
Trempe, G. 211, *218*
Trentin, J. J. 367, *370*
Trevisani, A. 248, *250*
Tria, E. 248, *250*
Trifaró, J. M. 336, *342*
Trolle, D. 495, *495*
Truant, G. S. 252, *264*, 360, *369*, 408, *418*, 441, *447*, 495, *498*
Trucco, R. E. 243, 244, 245, 247, 248, *249*
Truitt, E. B. Jr. 280, *298*
Tsafriri, A. 450, *467*, 495, *498*
Tsai, M. J. 184, *208*
Tsai, S. Y. 184, *208*
Tsai, Y. H. 184, *206*
Tsau, J. 119, *121*
Tubergen, D. G. 129, 131, *144*
Tuchmann-Duplessis, H. 513
Tulunay, F. C. 593, 596, 600, 601, *603*, *604*
Turk, R. F. 481, 482, *498*, 502, *512*
Turkanis, S. A. *358*, 550, *563*, 587, *589*, **619**, 620, 621, 623, 624, 625, 630, 636, 637, 638, *640*, *641*, 650, 651, 652, 654, *655*, *656*, *657*
Türker, M. N. 592, *604*
Türker, R. K. 280, *296*, 592, *604*
Turner, C. E. 280, *296*
Twine, C. E. 17, 35, 36, *43*
Tyminski, I. J. 72, 74, 76, *80*, 248, *249*
Tyrrell, E. D. 585, *589*

Ueki, S. 682, 688, *690*
Ullman, E. F. 7, *13*, 88, *88*, 89, 90, 91, 92, 95, 96, *98*, *99*
Ungerstedt, U. 616, *617*
Urca, G. 646, 654, *655*
Uyeno, E. T. 481, 482, *498*, 502, *512*, 682, *690*

Vachon, L. 280, 294, *298*, 302, 324, 326, *328*, 559, *565*
Vaitukaitis, J. L. 450, 458, *467*
Valentine, J. L. 8, *13*, 287, 289, *298*
Van Deenen, L. L. M. 333, *342*
Van der Molen, H. J. 252, 261, 262, *263*
Vanderwolf, C. H. 549, *565*
Van Driel, M. J. A. 252, 261, *263*
Van Noordwijk, J. 587, *589*
Van Rossum, J. M. 358, *358*
Van Thiel, D. H. 429, *439*
Vanwent, G. F. 279, 289, *298*
Vardaris, M. 225, *228*, 360, *369*
Vardaris, R. M. 481, 482, 494, *499*, 532, 535, 536, *537*, 550, *565*, 654, *657*, 682, *690*
Varsou, E. 378, 381, *387*
Vaughan, T. B. 287, 289, *296*, 493, *496*, 659, *663*
Vaughn, J. C. 377, 386, *387*
Vazquez, A. J. 559, *565*
Venable, J. W. 492, *499*
Vendesely, E. 426, *427*
Ventry, P. 280, *298*, 694, *712*
Verdeaux, G. 659, 660, 661, 662, *663*
Villee, C. A. 246, *250*
Vinson, J. A. 3, 6, 7, 10, *13*
Vitale, R. 404, *405*
Volavka, J. 548, *566*, 585, *589*, 663, *663*
Von Ledebur, M. 416, *418*
Vossmer, A. 221, 223, *228*, 360, *369*, 437, *439*, 441, *447*
Vyas, D. K. 396, *405*

Wada, J. A. 620, *639*, *641*, 646, 650, *655*, *656*, *657*
Waddell, W. J. 280, 294, *296*, *299*, 476, *477*, 494, *497*, 532, *537*, 689, *691*
Wagner, H. R. 550, *562*, 638, *640*, 648, 653, 654, *655*, *657*
Wagner, J. R. 306, 310, 323, *328*
Wahlqvist, M. 78, *80*, 105, 106, *121*
Wake, A. 650, *657*
Waksman, B. 331, *341*
Wald, N. 293, *299*
Wall, M. E. 3, 4, 6, 7, 10, *13*, **15**, 15, 16, 17, 22, 23, 35, 36, *42*, *43*, 63, *70*, 78, *80*, 101, *103*, 105, 106, 107, 108, *121*, 280, *296*, 494, 495, *496*, 520, 529, *530*, 549, *561*, 620, 623, 636, *640*
Wallington, M. J. 49, *62*
Wallis, W. A. 472, *476*
Wangh, L. J. 199, *207*
Warburton, D. M. 558, *566*
Ward, A. A. 648, *657*
Warkany, J. 689, *691*
Warkary, J. 485, *499*
Warner, R. 231, *242*
Warner, W. 132, 142, *143*, 168, *169*, 188, *206*, **219**, 219, 220, 221, 222, 223, 226, *227*, 229, *242*, 381, *387*, 495, *499*
Warr, G. A. 294, *299*
Warren, G. B. 248, *250*
Warrington, E. K. 546, *566*
Waser, P. G. 280, *297*
Waskow, I. E. 552, *566*
Waslien, C. I. 294, *298*
Watson, A. 294, *295*
Waters, W. 696, 707, *711*
Weber, E. 416, *418*
Weber, G. 521, *529*
Weil, A. T. 542, 543, *566*, 586, *589*
Weil, C. S. 472, *476*
Weiner, B. Z. 76, *80*
Weingartner, H. 287, 289, *296*, 493, *496*, 543, *562*, 659, *663*
Weinhold, P. A. 246, *250*
Weintraub, H. 189, *207*
Weiss, G. 646, 648, 653, *655*
Weiss, G. K. 550, *562*, *565*, 638, *640*, 651, *657*
Weiss, S. 694, *712*
Weissfeld, A. S. 381, 386, *388*
Weissman, B. 280, *298*
Weisz, D. J. 481, 482, 494, *499*, 532, 535, 536, *537*, 550, *565*, 653, *657*, 682, *690*
Weker, R. R. **301**, *327*
Weller, M. 248, *250*
Wells, M. A. 333, *342*
Werner, G. 45, *61*, 494, *496*
Werner, R. 168, *169*
Wesson, S. C. 294, *298*
West, L. J. 548, *566*, *582*, 694, *711*
White, A. C. 132, 142, *143*, 161, 168, 169, *169*, 188, *206*, *208*, 219, 220, 221, 223, 226, *227*, 229, *242*
White, J. I. 231, *242*
White, L. E. Jr. 638, *639*
White, S. C. 129, 131, *144*
Whittaker, V. P. 247, *249*
Whytehead, L. L. 294, *299*
Widman, M. 4, *13*, 45, *61*, 64, *69*, *70*, 72, 78, *80*, **101**, 101, 102, *103*, 105, 106, *121*, 494, *498*
Wiebel, E. R. 723, *730*
Wiechmann, M. 45, *61*, 494, *496*
Wikler, A. 542, 543, 548, 552, *564*, *566*, *583*, 585, *589*
Wilcoxon, F. 472, *476*
Wilkinson, G. N. 230, *242*
Willette, R. E. 3, 4, 6, 7, 8, 10, *13*, 57, *62*
Williams, D. 81, *88*
Williams, D. H. 77, *80*
Williams, D. L. 17, 35, 36, *43*
Williams, E. G. 542, 548, *566*, *583*, 585, *589*
Williams, H. 687, *690*
Williams, M. 547, *566*
Williams, M. J. 694, *710*
Williams, P. L. 7, *13*, 84, 85, 87, *88*
Williams, R. B. 559, *565*
Willinsky, M. D. 289, *299*, 493, 494, *499*, 549, *566*, 654, *657*
Wilson, J. C. 485, *499*
Wilson, R. S. 64, 67, 69, *70*, 601, *604*, 669, *680*
Wilton, L. V. 282, *295*, 325, *326*, 493, *496*
Windass, J. 184, *207*
Wingfield, M. 651, *656*
Winship-Ball, A. 495, *496*
Witschi, H. 279, 294, *297*
Wolkin, A. *358*, 620, *639*, 651, 652, *655*
Wolkin, A. L. 620, *639*
Wong, E. T. 89, *99*
Wong, S. L. R. 288, 289, *296*, 493, *496*
Woo, N. 289, *299*, 493, 494, *499*
Wood, G. C. 638, *639*, 644, *655*
Woodbury, D. M. 636, *641*, 650, 653, *657*
Wralley, C. 559, *565*
Wrenn, J. M. 221, 223, 225, *228*
Wright, P. L. 360, 361, 367, *369*, 408, *417*, 469, 471, 472, 474, *476*, 481, 482, *497*, *499*, 502, *511*
Wursch, M. S. 503, *512*
Wyatt, R. J. 287, 289, *296*, 493, *496*, 548, *561*, 659, *663*
Wyllie, M. G. 244, *249*, *358*
Wynder, E. L. 279, *296*
Wyrobek, A. J. 409, 410, 411, 412, 416, *418*

Yaffe, J. H. 351, *358*
Yagen, B. 33, 35, *43*
Yagiela, J. A. 580, *583*
Yamada, M. 685, *690*
Yamaguchi, A. 280, *298*
Yamauchi, T. 280, *298*
Yanagisawa, M. 231, *242*
Yang, C. 131, *144*
Yang, J. C. 665, 667, *679*, *680*
Ydrach, A. A. 360, 361, *369*
Yen, F.-S. 371, 375, *375*

Yisak, W.-A. 64, *70*
Yoshida, M. 231, *242*
Young, B. 184, *207*

Zacs, G. C. A. 663, *663*
Zagury, D. 131, *144*
Zaks, A. 548, *566*, 585, *589*
Zalta, E. 459, *467*
Zaluzny, S. G. 605
Zangwill, O. L. 547, *566*
Zarcone, V. 659, *663*
Zbinden, J. 209, 210, 211, 217, *218*, 280, *297*
Zeffert, J. T. 280, *287*
Zeidenberg, P. 252, *263*, 280, 289, *299*, 377, 386, *387*, 396, *405*, 408, *417*, 419, 426, *427*, **429**, 429, 432, *438*, *439*, 456, *466*, 543, *566*, **665**, 670, 677, *679*, *680*
Ziegler, D. 419, 426, *427*
Zieher, L. M. 177, *206*
Zimmerberg, B. 552, *566*, 695, 696, *712*
Zimmerman, A. 145, *159*
Zimmerman, A. M. 142, *144*, 188, *208*, 222, *228*, 386, *388*, **407**, 410, 411, 412, 413, 416, *418*, 520, 528, *530*
Zimmerman, E. 495, *496*
Zimmerman, S. 407, 410, 411, 412, 413, 416, *418*
Zimmerman, S. B. 188, *208*, 222, *228*, 386, *388*, 520, 528, *530*
Zinberg, N. E. 542, 543, *566*, 586, *589*
Zioudrou, C. 378, 381, *387*
Zitko, B. A. 253, *264*
Zivin, J. A. 533, *537*

SUBJECT INDEX

Abortion 505–506
Abstinence syndrome (*see also* Dependence, Withdrawal) 596–599
Abuse, of cannabis by epileptics 643–644
Accumulation, of THC in tissues 111, 117
Acetylation, effect of cannabinoids on 199, 201
Acetylcholine 558
  in self stimulation 570, 573–575
Acrosomes 383–386
Acute effects
  analgesia 592
  anticonvulsive 619–657
  biogenic amine uptake 351–358
  brain 519–530
  female reproductive function 449–467
  lung 287–295, 301–326
  single dose tolerance 586, 592, 593
  social behaviour 699–701
  spontaneous motor activity 567–583
Acyltransferases 329–342
Adenosine, effect of THC on uptake 161–169
Administration (*see also* Vehicle)
  comparison of routes 8–12, 118–119
  inhalation 8–12, 279–299, 301–326, 482–485, 493–494, 585, 586, 645, 670–671, 714–717
  intramuscular 452, 586
  intraperitoneal 47, 119, 458, 567–569, 586, 587, 601, 606, 620
  intraventricular 17, 118, 352, 522, 524, 592, 646, 719
  oral 96, 119, 441–442, 469–471, 482–485, 493–495, 503, 522, 523, 532, 533, 586, 596, 648, 651, 682, 686, 697
  self 588, 595–597
  subcutaneous 119, 359–370, 522, 592, 600
Adrenal gland 421, 441–447
  weight of 533
After-discharges 622, 631–639
Aggression 290, 559, 700–710
Albumin, metabolite binding to 105
Alcohol (*see also* Vehicle) 681–691
  cross tolerance to 587–588
Ames test 416
Amines *see* Neurotransmitters
Amino acids (*see also* individual acids)
  effect of THC on uptake into
    HeLa cells 171–208
    HTC, Y-1, NB2A, C-6 cells 219–227
    Leydig cells 251–264
    rat brain 520–522
    Reuber hepatoma cells 161–169
Aminoisobutyric acid 145–158
Anaesthetics (*see also* Sleeping time)
  comparison of cannabinoids with 336–341
Analgesia (*see also* Pain) 591, 592, 600, 601, 613–616, 665–680
Aneuploidy 409, 413–416
Animal experiments (*see also* individual species)
  analgesia 591, 592, 600
  anticonvulsant effects 619–657
  activity of Δ^7-THC 77–78
  carcinogenesis 359–370
  comparison with opioids 591–618
  dependance 595–597
  distribution of cannabinoids
    in brain 522–529
    in neonate 531–537
  effects of cannabinoids on
    adrenal gland 441–447
    ATP-ases 254–246
    biogenic amines 351–358
    brain function 713–730
    brain structure 713–730
    female reproductive function 449–467
    foetal development 479–499
    learning 681–691
    lungs 279–299
    membrane bound enzymes 329–342
    ovary 441–447
    muricidal behavior 681–691
    pulmonary defenses 301–328
    reproduction 469–477, 501–512
    self stimulation reward 567–583
    sleeping time 247–248
    social behaviour 693–712
    spermatogenesis 407–417, 419–427
    testicular enzymes 395–405
    testosterone 395–398
    uterus 441–447
  estrogenic effects 359–370
  pharmacokinetics 105–121
  tolerance 585–589, 592–595, 627–628, 707–708
Antibodies, to cannabinoids 81–84, 89–98
Anticonvulsive effects 550–551, 619–657
  tolerance to 587
Antineoplastic effect 161–169

Ataxia, tolerance to 586, 593–594
ATP-ases 229–242, 243–250

Baboon 646
Bacteria (*see also* Ames test)
effect of marihuana smoke on 310–313, 320–326
Behavior
affiliative 700–710
aggressive 290, 559, 700–710
changes after smoking 289–291, 486
correlation with brain amines 347–348, 357
in monkey neonates 508–509
muricidal 681–691
self stimulation 567–583
social 693–712
Bile, metabolites in 107
Blood, measurement of THC and metabolites in 5–12, 17–33, 81–98
THC levels after
smoking 287–290, 484, 493–494, 713–717
oral cannabis 475–476
chronic use 587
pharmacokinetics 27–36, 105–120
nuclear aberrations 377–388
Bradycardia 586, 595
Brain, anticonvulsant properties of cannabinoids 641–657
assay of 3″-hydroxy-Δ^1-THC 36–37
biochemical changes 520–522, 531–537
cannabinoids in 106, 623, 628, 629
effect of cannabinoids on
function 713–730
neurotransmitters 343–349, 351–358, 557–560
structure 522–523, 688, 713–730
limbic system 539–566
Breeding 503–506

Calcium, uptake into lymphocytes 332, 335–336
Cancer cells, ATP-ases from Ehrlich ascites cells 231
effect of cannabinoids on
HeLa cells 171–208
murine lymphoma cells 145–158
Reuber hepatoma cells 161–169
effect of marihuana smoke on breast cancer cells 231
Cannabichrome (CBC), anticonvulsant properties (lack of) 624–626
effect on brain 718
in cannabis tincture 46
Cannabidiol (CBD), anticonvulsive effects 587, 652
cross reactivity
in EMIT assay 94
in radioimmunoassay 84
effect on
ATP-ase activity 239
brain 718
Leydig cells 254–264
neurotransmitters 343–349, 351–358
ribosomes 523–525
spermatogenesis 407–417
testosterone 395–398
estrogenic activity (lack of) 460–462
metabolism 53
tolerance 587
Cannabigerol (CBG), effect on
Leydig cells 257–264
lipid metabolism 333–341
ribosomes 522–525
Cannabinoids *see* individual compounds
Cannabinol (CBN), anticonvulsant activity 623–625
cross reactivity
in EMIT assay 94
in radioimmunoassay 84
effect on
ATP-ases 239
brain 718
Leydig cells 254–264
lipid metabolism 333–339
macromolecular synthesis 171–208
neurotransmitters 342–349
ribosomes 523–525
spermatogenesis 407–417
in crude marihuana extract 469–471
in cannabis tincture 46
metabolism 54
plasma levels 19–35
teratogenesis 469–477
Cannabis *see* Cannabinoids, Cannabis tincture, Crude marihuana extract, Marihuana cigarettes, Smoke
Cannabis tincture (*see also* Crude marihuana extract)
analysis 46
metabolism 54–57
Carbon monoxide 286–287, 315, 318, 480, 483, 493–495
Carboxyhaemoglobin 283–287, 318, 480, 483, 493–495
Carcinogenesis, fibrosarcoma 359–370
mammary adenocarcinoma 359–370
lung 294
Cat, anticonvulsant effects in 648
effects on morphine response 600
hypothermia 593
Catecholamines (*see also* individual compounds)
in brain 558–560, 570, 573–575
Cells, experiments on
breast cancer 209–228
embryonic heart 265–277
Ehrlich ascites 229–242
HeLa 171–208
HTC, Y-1, NB2A, C-6 219–228
Leydig 251–264
lung 209–218
lymphocytes 129–144, 145–159, 329–342
murine lymphoma 145–159
Reuber hepatoma 161–169
Cell proliferation 171–208
Central nervous system (*see also* Brain)

changes in 519–530
depression of 567–583
Chicken 265–277
Chimpanzee 587
Cholesterol clefts 292–294
Choline, uptake 333–334, 343–344
Cholinergic centres 558
Chromatin 184–185, 378–381, 381–386
Chromatography, of cannabinoids (*see also* Gas chromatography—mass spectrometry (GC–MS))
comparison of methods 106
gas-liquid (GLC) 6, 49, 73, 282, 288, 289, 310, 484
high performance liquid (HPLC) 20, 87–88
Sephadex LH-20 7, 19, 47, 102
thin layer (TLC) 6, 73, 135, 623
Chromosomal proteins (*see also* Histones) 189–195
Chromosomes 371–376, 409, 413–416
Chronic effects (*see also* Dependence, Tolerance)
blood cells 377–388
brain 713–730
brain amines 351–358
carcinogenic 359–370
estrogenic 359–370
female reproductive system 441–447
foetal development 479–499, 501–512
learning 681–691
lung 279–299, 301–326
lymphocytes 371–376
muricidal behavior 681–691
reproduction 441–447, 501–512
social behavior 693–712
sperm 377–388
spermatogenesis 407–417, 419–427, 429–439
testicular enzymes 395–405
Clinical studies 84–88, 96–98, 540–541, 670–671
Cobalt, in epilepsy 621, 622, 630, 631
Conception 504–506
Convulsive effects 643–657
Corpus striatum 342–349, 351–358
Cross tolerance, to
alcohol 588
LSD 588
mescaline 578, 588
opioids 599–602, 605–618
Crude marihuana extract (CME) (*see also* Cannabis tincture, Marihuana cigarettes)
analysis of 469–470
anticonvulsive effects 644–645
reproduction 441–447, 449–467
teratology 469–477
Cyclic AMP 431–438
Cytidine, uptake into
HTC, Y-1, NB2A, C-6 cells 219–227
Reuber hepatoma cells 161–169
Cytochrome P-450 395–405

Daunorubicin 270–277
Decachlorobiphenyl, as smoke particle tracer 309, 310, 318
Dendrites 528, 535
Dependence (*see also* Abstinence syndrome, Withdrawal) 588, 595–602
Depression 486, 567–583
tolerance 586, 594
Derivatives
antiepileptic properties 652
for antibody production 81–84, 90–92
for GC-MS 48, 58–59
psychotropic activity 63–70
water soluble 253–264, 352–358
Deuterated cannabinoids
as internal standard for GC-MS 33–35, 57–59
metabolism of 36–37, 52–53
Dexamethasone 221–227
Diethylstilbestrol 459–462
6α,7-Dihydroxy-Δ^1-THC 50–53, 59, 60, 630
Dimethylheptylpyran (DMHP) 558
anticonvulsant activity 623–625, 636–637, 652
Diphenylhydantoin 129–143, 619–641
Disproportionation, of Δ^1-THC 120
Distribution, of cannabinoids in
brain 523–528
cells 221–223, 524–526
dog 105–118
tissues 523–528
DNA 531–537
synthesis, effect of cannabinoids on 129–143, 145–158, 161–169, 171–208, 219–227, 332–340, 420, 326
Dog, analgesis 592
anticonvulsant properties of cannabinoids 645–648
hypothermia 593
pharmacokinetics 105–120
tolerance 586, 593–594
Dominance (social) 700–710
Dopamine 343–349, 351–358, 570, 573, 575
Dose, of cannabinoids in animals related to humans 281–282, 483–485, 493–494, 715–717

Edema, lungs 291–295
Ehrlich ascites tumor cells 231, 240–242
Electroencephalogram (EEG) 547–550, 585, 595, 630–633, 645–654, 659–663, 685, 686, 689, 714, 719
Embryo 265–277, 469–477
EMIT immunoassay 7, 89–99
Endoplasmic reticulum 723
Enterohepatic circulation 107
Enzymes, acyltransferases 329–342
aldolase 265–277
ATP-ases 229–242, 243–250
creatinine phosphokinase 265–277
cytochrome P-450 395–405
glucose-6-phosphate dehydrogenase 265–277
β-glucuronidase 399, 400
hydroxybutyrate dehydrogenase 265–277
lactate dehydrogenase 265–277
malate dehydrogenase 89–98, 265–277
phosphatase 237, 238, 243–250

testicular enzymes 395–405
thymidine kinase 163, 167–169
Epilepsy 619–657
Erythrocytes 333
Estradiol 459–462
Estrogens 359–370
Euphoria 540
Eye, teratology 475

Fat, accumulation of THC in 105–120
Foetus 469–477, 479–499, 481, 504–511
Forensic studies 5, 85
Frog 623, 630
Follicle stimulating hormone (FSH) 396, 397, 400–404, 430, 437, 450–456

GABA 351–358
Ganglia 623, 630
Gas chromatography *see* Chromatography
Gas chromatography-mass spectrometry (GC-MS) (*see also* Mass fragmentography)
cannabinol 19–35
cannabis metabolites 22–36, 45–60, 102
crude marihuana extract 477–479
paraquat 37–42
Δ^1-THC 7, 12, 22–36
Geographical variation
in cannabis use 595, 598
in cannabis dose 595
Gestation 531–537
Giemsa stain 378–381
Glial cells 535
Glucose 268–277
β-Glucuronidase 399, 400
Golgi apparatus 527
Golgi stain 685–689
Gonadotropins 395–405, 450–458
Growth 367–368, 421, 487–488, 507–509
Guanosine 161–169
Guinea pig
ileum 63–70, 602
metabolites of
CBD 53
CBN 54
Δ^1-THC 51–53

Haemorrhages
lungs 291–295
skin 488
Hallucinations 548, 554
Hamster 587
Heart (*see also* Bradycardia, Tachycardia)
degeneration in foetus 507
effect of Δ^1-THC on chicken embryo 265–277
HeLa cells 171–208
Histology 211–218, 221–225, 284, 291–295, 361, 377–388, 410–416, 421–426, 445–447, 462–466, 485–486, 506, 526–528, 685, 689, 719–730
Histones 195–199, 377–388
Homologues, of cannabinoids
in tincture 46, 54–57
anticonvulsant action 652
Hormones *see* individual hormones
Hot plate test 592, 600
Human, analgesia 592
blood levels of cannabinoids 8–12, 17–31, 35
consumption 595, 598
dependence 595, 597–599
effect of cannabinoids on
brain 539–566
EEG 659–663
lymphocytes 129–143, 145–145–159
memory 665–680
nucleus 371–376, 377–388
pain 592, 665–680
sperm 377–388
epilepsy 643–657
hypoploid metaphases 371–376
limbic system 539–566
metabolites of Δ^1-THC 101–103
tolerance 585–589
Hydrocephalus 507
3″-Hydroxy-Δ^1-THC
assay by GC-MS 36, 37
psychotropic activity 63–70
6α-Hydroxy-Δ^1-THC
anticonvulsant properties 623–626
in liver 50–53, 101–102
6β-Hydroxy-Δ^1-THC
anticonvulsant properties 623–626
effect on Leydig cells 251–264
in liver 50–53, 101–102
7-Hydroxy-Δ^1-THC
analgesic effects 669
anticonvulsant effects 623–625, 630–634
effect on
cell proliferation 161–208
Leydig cells 251–264
lipid metabolism 329–342
neurotransmitters 343–349
ribosomes 522–525
identification by GC-MS 45–57
in brain 629
in human liver 102
quantitation in plasma 19–36
pharmacokinetics 35, 107–108
radioimmunoassay, cross reactivity 84
tolerance 587
7-Hydroxy-Δ^6-THC, analgesic effect 601
8α-Hydroxy-Δ^9-THC *see* 6α-Hydroxy-Δ^1-THC
8β-Hydroxy-Δ^9-THC *see* 6β-Hydroxy-Δ^1-THC
11-Hydroxy-Δ^9-THC *see* 7-Hydroxy-Δ^1-THC
5-Hydroxytryptamine 343-349, 357–358, 570, 573, 575
Hypersensitivity 486
Hypoploid metaphases 371–376
Hypothalamus (*see also* Brain, Limbic system) 343–349, 456, 545–547, 567-583
Hypothermia 573–574
tolerance to 586, 593

Ileum 63–70, 602
Immune response 129–143, 301–328
Implantation of foetus 472–473, 487, 533
Indoleacetic acid 343–349
Infection *see* Bacteria
Inguinal hernia 507
Inhalation *see* Administration, Smoke
In vitro experiments (*see also* Cells—experiments on, Ileum)
  brain—macromolecular synthesis 520–523
  synaptosomes 343–349, 351–358
  tumors 366
In vivo experiments *see* Animal experiments
3-Isobutyl-1-methylxanthine, as phosphodiesterase inhibitor 600–617

Lactate 268–277
Lactation 361–362, 523–524, 531–537
Learning 536, 569, 681–691, 686–688
  tolerance 587
Leucine, effect of Δ^1-THC on uptake into
  HeLa cells 171–208
  HTC, Y-1, NB2A, C-6 cells 219–227
  Leydig cells 251–264
  lymphocytes 145–158
  rat brain 520–522
  Reuber hepatoma cells 161–169
Leydig cells 251–264, 403–404
Limbic system 539–566
Lipids (*see also* Phospholipids)
  triglycerides 334–336
Lipoprotein, binding of THC to 105
Liver, efficiency 113–115
  hepatoma cells 161–169
  metabolites in 45–60, 101–103
LSD-25 558, 588
Lungs (*see also* Smoke) 279–299, 301–328
  cancer (metastases) 368
Leuteinizing hormone (LH) 367–369, 396, 397, 400–404, 430, 437, 450–456
Lymphocytes 129–143, 145–158
  acyltransferases from 329–342
  hypoploid metaphases in 371–376
  nuclear aberrations in 378–381

Macromolecular synthesis, effect of
  CBD 251–264
  CBG 251–264
  CBN 171–208, 251–264
  6β-hydroxy-Δ^1-THC 251–264
  7-hydroxy-Δ^1-THC 171–208, 251–264
  marihuana cigarettes 420, 426
  Δ^1-THC 129–143, 145–158, 161–169, 171–208, 219–227, 240–242, 251–264, 520–522
  Δ^6-THC 171–208, 219–227
Macrophages (alveolar) 291–295, 310–314, 325–326
Malate dehydrogenase *see* Enzymes
Mammary gland, carcinogenesis 359–370
Marihuana cigarettes (*see also* Administration, Smoke) 281–299, 306–326, 372–375, 420, 430, 483–485, 585, 586, 670–671
Mass fragmentography 7–12, 22–42, 57–59, 106, 288–289
Mass spectrometry (*see also* Gas chromatography-mass spectrometry, Mass fragmentography) 7–62, 74
Mating 504–506
Membranes, effect of cannabinoids on 157–158, 226
    bound enzymes 329–342
    bound ribosomes 522–525
    fluidity 248, 340
    neurotransmitters 343–349
  synaptosomal 243–250
Memory 542–544, 552, 554–557
Menstrual cycle 456–458
Metabolism of cannabinoids (*see also* Metabolites and individual compounds, Side-chain hydroxy metabolites)
  dog 105–120
  guinea pig 51–54
  human 101–102
  mouse 50–54, 57–60
  rat 50–54
  Rhesus monkey 101–102
  in vitro 101–102
  in vivo 45–60
Metabolites of cannabinoids (*see also* Metabolism and individual compounds)
  activity related to receptors 78–80
  analgesic effect 601, 669
  anticonvulsant properties 623–625, 630–634
  effect on
    Leydig cells 251–264
    lipid metabolism 329–342
    neurotransmitters 343–349
    ribosomes 552–555
  EMIT assay cross reactivity 94–95
  identification of 47, 49–57, 101–103
  inhibition by SKF-525A 587
  of CBD 53
    CBN 54
    Δ^1-THC 4, 16, 50–53, 101–103
    Δ^6-THC 16, 101–103
  pharmacokinetics 105–120
  quantitation in blood 17–36
  radioimmunoassay 87
  radioimmunoassay cross reactivity 83, 84
  stimulation by phenobarbitone 587
  test for psychotropic activity 63–70
  tissue levels 48, 629
  tolerance 587
Methyldeoxythymidine, uptake by Reuber hepatoma cells 161–169
Microsomes, binding of THC to 524–526
Milk, effect of THC on production 361–362
  THC in 523–524, 533
Mitochondria, binding of THC to 524–526
Mitomicin-C 411–412

Monkey
effects of THC on
brain function 713–730
brain structure 713–730
hypothermia 593
tolerance 594
withdrawal symptoms 588
crab-eating—social behavior 693–712
Rhesus
activity of Δ^7-THC 77–80
dependence 596–597
reproduction 449–467, 501–512
metabolism of Δ^6-THC 101–103
psychotropic properties in 63–70, 77–80
social behavior 693–710
tolerance 707–708
Mood 540
changes during withdrawal 599
Morphine *see* Opioids
Motor activity 567–583
tolerance 586
Mouse, analgesia 591
anticonvulsant properties of
cannabinoids 621–625. 651
ATP-ases from 231, 240–242, 244–250
carcinogenesis 359–370
chronic effects of cannabinoids 359–370
effects of cannabinoids on
brain synaptosomes 329–342
foetal development 479–499
lymphocytes 329–342
neurotransmitters 351–358
spermatogenesis 407–417
metabolites of
CBD 53
CBN 54
Δ^1-THC 50–53, 57–60
tolerance in 586–587
Muricidal behavior 681–691
Murine lymphoma cells 145–158
Mutagenesis 413–416
Myelination 535
Myocardium (*see also* Heart)
effect of Δ^1-THC on 265–277
degeneration of 507

Naloxone 599–602, 605–618
Neonates 361–362, 523–526, 531–537
Nerve cells 719–730
Neurotransmitters (*see also* individual
compounds) 343–349, 351–358, 557–560, 570,
573, 575
Neutrophils 379–382
Nissl bodies 527
Noradrenaline 343–349, 351–358, 570, 573, 575
Norepinephrine *see* Noradrenaline
7-Nor-Δ^1-THC-7-oic acid
EMIT assay for 94–98
identification in tissues 45–60
measurement in
plasma 19–36
tissues 45–60
Nuclear inclusions 723–730
Nuclear magnetic resonance (NMR) 74–77
Nucleic acids (*see also* DNA, RNA) 531–537
Nucleosides (*see also* individual compounds), effect
of Δ^1-THC on uptake into
brain 520, 522
Ehrlich ascites cells 240–242
HeLa cells 171–208
HTC, Y-1, NB2A, C-6 cells 219–227
Leydig cells 251–264
lymphocytes 129–143, 145–148, 332, 335, 336,
340
murine lymphoma cells 145–158
Reuber hepatoma cells 161–169
Nucleotide pools 166–169
Nucleus (*see also* Chromosomes, Histones)
binding of THC 524–526
chromatin 377–388
hypoploid metaphases 371–376

Octanol-water partition 146–147
Oestradiol *see* Estradiol
Olivetol
analogues 148–151
effect on thymidine uptake into
lymphocytes 145–158
liposolubility 146–147
Opioids 591–618
Ovary 441–447, 533
Ovariectomized monkeys 449–467
Ovulation 456–458

Pain (*see also* Analgesia) 600, 601, 613–616,
665–680
Pancreas, ectopic 507
Paraquat, GC-MS assay 37–42
Particle size, of smoke 309, 315–317
Pentobarbitone, sleeping time 568, 573, 576
Perceptual changes 540, 554
Pharmacokinetics, Δ^1-THC 4, 27–36, 105–120
metabolites 27–32, 35–36, 105–120
Phenobarbitone, effect on metabolism 587
anticonvulsant effects 619–641
Phenytoin *see* Diphenylhydantoin
Phospholipids 243–250, 329–342
Phosphorylation 201
Physiological effects (*see also* individual
effects) 605–617
Phytohemagglutinin 129–158
Pigeons, behavioral tolerance 586, 595
Pituitary gland
effect of THC on 365–369
hormones from 450–456
weight 533
Placenta 507
Plasma *see* Blood
Polymorphonuclear leukocytes 291–295
"Popcorn" response 289–290, 494, 645

Postnatal development 494, 507–509
Pregnancy, monkey 503–511
  mouse 474–499
  rabbit 469–477
  rat 479–499, 532–533, 686–688
Progesterone 456–458, 504
Prolactin 367, 368
Proline, uptake into Reuber hepatoma
    cells 161–169
Proteases 265–277
Protein (*see also* Histones) 531–537
  binding of cannabinoids 105
  synthesis 161–169, 171–208, 219–227, 251–264,
    520–522
Psychotropic properties (*see also* individual
    properties) 539–566, 659–663
  test for 63–70
  tolerance 585–589
Pulmonary defenses 301–328

Quasi morphine withdrawal syndrome 605–618
Questionnaires 541, 644

Rabbit, antibody production 81–84
  anticonvulsant effect of cannabinoids 646–648,
    651
  hypothermia 593
  teratology 469–477
Radioimmunoassay, of cannabinoids (*see also*
    EMIT immunoassay) 6, 7, 81–88, 282, 288,
    289, 431
Rank (social) 700–710
Rat, analgesia 591, 592
  ATP-ases from 229–242
  dependence 596
  distribution of Δ^1-THC 523–528
  effects of cannabinoids on
    adrenals 441–447
    brain biochemistry 520–523, 531–537
    convulsions 620–639, 645–654
    female sex organs 441–447
    foetal development 479–499
    learning 681–691
    Leydig cells 251–264
    lungs 279–299
    muricidal behavior 681–691
    pulmonary defenses 301–328
    self stimulation reward 567–583
    synaptosomes 343–349
    spermatogenesis 419–427
    testicular enzymes 395–405
    testosterone 395–398
  hypothermia 593
  interaction between cannabinoids and
    opioids 600–602
  metabolism of
    CBN 54
    Δ^1-THC 50–53
  tolerance 586–588, 592–593
  vitamin A deficiency 419–427
Receptors, cannabinoid 78–80, 357–358
  opioid 602–603, 613
Rectal temperature 573–574
Reproduction (*see also* Sperm, Spermatogenesis,
    Teratology) 559–570, 441–447, 449–465,
    469–477, 501–512
Resorption of foetus 472–473, 487–493, 533
Reuber hepatoma cells 161–169
Reward, self stimulation 567–583
Ribosomes 522–525
RNA 531–537
  synthesis, effect of cannabinoids on 171–208,
    219–227, 251–264, 520–522
Road traffic accidents 85

Sedation 540
Self administration 588, 595–597
Self stimulation 567–583
Semen 429–439
Sensory decision theory 665–680
Sensory stimuli 540
Serotonin *see* 5-Hydroxytryptamine
Sertoli cells 383, 395–405, 426, 437
Sesame oil *see* Vehicle
Sheep, Δ^1-THC antibody production in 81–84,
    91–92
Siamese fighting fish 595
Side-chain hydroxy metabolites
  activity 78–80
  identification 49–57, 102
  quantitation 36–37, 57–60
  test for psychotropic activity 63–70
Skeleton, abnormalities 472–475, 487–489
SKF 525A, as metabolic inhibitor 587
Skinner box 569
Sleeping time, barbiturate 248, 568, 573, 576
  tolerance 587
Smoke, marihuana,
  comparison with tobacco smoke 209–218
  effect on
    brain function 713–730
    brain structure 713–730
    breast cancer cells 209–218
    foetal development 479–499
    lungs 279–299
    memory 665–680
    pain 665–680
    pulmonary defenses 301–328
    spermatogenesis 419–427, 429–439
  non-specific effects 714
  plasma levels of cannabinoids after
    smoking 8–12, 287–289, 715–718
  urinary levels of cannabinoids after
    smoking 431, 435
Smoking machine 281–293, 302–310, 420, 483–485,
    714–715
Solvolysis of Δ^1-THC 119–120
Sorbitol dehydrogenase 399, 403
SP-111 251–264, 351–358
Species differences

metabolism 50–54, 101–103
anticonvulsant activity 623–624
Speech 540
Sperm 251–264
count 421, 423, 426, 432–438
head 381–386, 411–413
morphology 381–386, 411–413, 420–426, 432
motility 432–438
nuclear aberrations 377–378
Spermatogenesis 251–264, 407–418, 419–427, 429–439
Spermiogenesis 419–427, 429–439
Spleen, lymphocytes from 329–342
Stability, of Δ¹-THC 119–120
of crude marihuana extract 469–471, 476
Stable isotope *see* Deuterated cannabinoids
Steroids (*see also* individual compounds) 221–227, 251–264, 431, 456–462, 504, 705–706, 708–709
Subcellular fractions *see* individual fractions
Subjective effects 540–542
Surface area, dosing by 282, 483, 715
Synapse 720–730
Synaptosomal membrane 243–250
enzymes from 329–342
neurotransmitters 345–348
Synaptosomes 345–348, 351–358

Tachycardia 585, 586, 659
Teratology 408
monkey 501–512
mouse 479–499
rabbit 469–477
rat 479–499, 531–537, 686–687
Terminal boutons 526–529
Testis (*see also* Sperm, Spermatogenesis)
enzymes 251–264, 383, 395–405, 419–427
Testosterone 251–264, 395–398, 408, 430, 436–438
Δ¹-Tetrahydrocannabinol (Δ¹-THC) (*see also* Smoke)
accumulation in tissues 117
administration, comparison of methods 8–12
analgesic effects 591–592, 600
brain levels 623, 628, 629
carcinogenic effect 359–370
chronic effects 359–370
convulsant effects 643–657
dependence 595–603
distribution in
brain 523–528
cells 220–223
organs 523–528
EEG 659–663
effects on
adrenals 441–447
ATP-ases 229–242, 243–250
brain biochemistry 531–537
convulsions 619–657
embryonic heart cells 265–277
female reproductive function 449–467
female reproductive organs 441–447
limbic system 539–566
lipid metabolism 329–342
lungs 279–299
lymphocytes 129–143, 145–159
macromolecular synthesis 129–143, 145–159, 161–169, 171–208, 219–227, 251–264, 520–522
neurotransmitters 343–349, 351–358
reproduction 501–512
ribosomes 522–525
testis 251–264, 395–405
self stimulation reward 567–583
social behavior 693–712
steroids 251–264, 395–398
spermatogenesis 407–417
EMIT immunoassay 89–98
excretion 107, 118
metabolism 4, 17–37, 45–60, 101–103, 105–120
pharmacokinetics 4, 27–36, 105–120
physical properties 3, 18
quantitation in
liver 47–48, 714–719
plasma 5–12, 17–33, 81–98
radioimmunoassay 81–88
stability 119–120
teratology 479–499
tolerance 290–291, 293–294, 486, 585–589, 592–595
withdrawal 595–603
Δ⁶-Tetrahydrocannabinol (Δ⁶-THC)
distribution 524–528
effect on
ATP-ase activity 291
convulsions 623–626, 648–651
lipid metabolism 329–342
macromolecular synthesis 171–208
neurotransmitters 343–349, 351–358
ribosomes 522–525
spermatogenesis 407–417
metabolites
identification 101–103
radioimmunoassay, cross reactivity 84
test for psychotropic activity 63–70
tolerance 586–587
(+)-Δ⁶-Tetrahydrocannabinol ((+)Δ⁶-THC) 351–358
Δ⁷-Tetrahydrocannabinol (Δ⁷-THC)
synthesis 72–73
activity 77–80
Δ⁸-Tetrahydrocannabinol (Δ⁸-THC) *see* Δ⁶-Tetrahydrocannabinol
Δ⁹-Tetrahydrocannabinol (Δ⁹-THC) *see* Δ¹-Tetrahydrocannabinol
Tetrahydrocannabinol acids 624
Theophylline 431–438
Thiopentone 248
Thought 540
Thymidine, effect of Δ¹-THC on uptake into
Ehrlich ascites cells 240–242
HeLa cells 171–208
HTC, Y-1, NB2A, C-6 cells 219–227
lymphocytes 129–143, 144–158, 332, 335, 336, 340
murine lymphoma cells 145–158

Reuber hepatoma cells 161–169
testis 422, 426
Thymidine kinase 163, 167–169
Time perception 540, 542, 553–554
Tissues (*see also* individual tissues)
accumulation of THC in 105–120
cannabinoids in rabbit foetal tissues 475–476
distribution of THC in suckling rats 523–526
metabolites in 45–60, 101–103, 105–120
Tobacco, comparison with marihuana 279–299, 306–326
Tolerance, after smoking 290–291, 293–294, 486
anticonvulsant effects 627–628
behavior 707–708
depressant effects 573–581
reviews 585–587, 592–595
self stimulation reward 573–578
sleeping time 247, 248
Toxicity (*see also* Teratology) 287, 288, 366, 486
Translocations (chromosomal), sperm 413–416
Triglycerides 333–335
Tryptophan 343–349

Uridine, effect of THC on uptake into
brain 520–522
HeLa cells 171–208
Leydig cells 251–264
lymphocytes 129–143, 145–158
Reuber hepatoma cells 171–208
Urine, cannabinoids in 96–98, 107, 431, 435
Uterus (*see also* Female reproductive organs) 441–447

Vagina 441–447, 462–466
Vehicle, for cannabinoid administration
albumin 118
alcohol 146, 162, 172, 244, 255, 522
dimethylsulphoxide (DMSO) 410–416
food 697
olive oil 682, 686
propylene glycol 244, 397
propylene glycol and Tween-80 607
serum 118, 133
sesame oil 244, 361–369, 469–471, 483, 532
sesame oil and Tween-80 607
Tween-80 47, 231, 244, 352, 397, 452, 567, 569, 620
Vitamin A deficient rats 419–427
Vitamin C 209–218

Weight, body
foetal 487–489
neonatal, monkey 508–509
pregnant mouse 487
rabbit 471
rat 487, 533
tolerance 586
organ weight 533
Withdrawal symptoms (*see also* Abstinence syndrome, Dependence) 435, 436, 588, 596–599
lack of 294
quasi morphine 605–618
Writhing test 592, 616