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THE INTRACELLULAR LOCALIZATION OF Δ^9-TETRAHYDROCANNABINOL IN LIVER AND ITS EFFECTS ON DRUG METABOLISM *IN VITRO**†

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Abstract—In the hepatic cell Δ^9-tetrahydrocannabinol (THC) is localized in nuclei and microsomes. The intracellular binding of THC affects hepatic drug metabolism: nuclei markedly reduce the metabolism of THC by hepatic microsomes; THC inhibits the microsomal oxidation of aminopyrine and hexobarbital, the conjugation of estradiol and *p*-nitrophenol and enhances the reduction of *p*-nitrobenzoic acid. The metabolism of THC, *in vitro*, is strikingly inhibited by SKF-525A but not by desipramine, nortriptyline and iprindole which are potent inhibitors of the oxidation of other drugs.

As a consequence of its intense lipid-solubility Δ^9-tetrahydrocannabinol (THC) is capable of interesting interactions with biological material. For example, unlike most drugs which are bound in plasma mainly by albumin, THC is primarily associated with the lipoprotein components.[1,2] Although THC is extensively bound by the lipoproteins of plasma, the finding that the drug rapidly disappears from plasma after intravenous administration and accumulates in tissues[3] suggested that THC might be strongly bound by cellular components. The present studies were undertaken to investigate the binding of THC in liver and its effects on several pathways for drug metabolism.

These studies have demonstrated that THC is almost exclusively associated with the particulate material of the hepatic cell, especially nuclei and microsomes. Moreover, several effects on hepatic drug metabolism are manifested as consequences of the intracellular binding of THC. Thus, due in part to binding by nuclei, THC is metabolized more rapidly by hepatic microsomes than by liver homogenates. The binding of THC by hepatic microsomes is reflected in its ability to inhibit the metabolism of other drugs along pathways involving side chain oxidation, demethylation and conjugation with glucuronic acid.

METHODS

^{3}H-THC (8·0 mCi/m-mole) labeled in positions 2, 4, 8 and 10 and ^{14}C-THC (5·2 mCi/m-mole) labeled in positions 2 and 4 were obtained from the National Institute of Mental Health, Center for Studies of Narcotics and Drug Abuse. These preparations

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were repurified prior to use by the following procedure: ^{3}H-THC together with sufficient unlabeled THC to give an approximate specific activity of 800 μCi/m-mole was dissolved in a small volume of ethanol and quantitatively transferred to a 50-ml glass-stoppered shaking tube. After evaporation of the solvent under a stream of nitrogen, the residue was redissolved in a small volume of propylene glycol, diluted to 5 ml with water and extracted three times with 15 ml of petroleum ether.* The petroleum ether extracts were pooled, evaporated to dryness under nitrogen and the ^{3}H-THC dissolved in sufficient ethanol to give a concentration of (1·0 mg/ml, 800 μCi/m-mole). The radiopurity of this preparation determined by gas–liquid chromatography and liquid scintillation counting was in excess of 95 per cent.

Male Sprague–Dawley rats (Holtzman Co., Madison, Wis.) weighing 150–180 g were used in these experiments. The animals were allowed free access to food and water at all times.

Isolated perfused rat liver. Details of the perfusion procedure[4] and apparatus[5] have been published previously. The perfusion medium consisted of 33 ml of defibrinated rat blood and sufficient Krebs–Henseleit bicarbonate buffer, pH 7·4,[6] to make a final volume of 100 ml. After equilibration, ^{3}H-THC was added to the medium in the recycling system in a mixture of 30% propylene glycol and 70% rat serum.

Preparation of microsomes. The rats were killed by cervical dislocation. Their livers were removed and chilled immediately on crushed ice. All subsequent manipulations were carried out at 2–4°. The livers were homogenized in a motor-driven Teflon–glass homogenizer with 3 ml of isotonic KCl (1·15%) per g of tissue. The homogenate was centrifuged at 10,000 *g* (average) for 30 min in an International model HR-1 centrifuge (rotor No. 856). The supernatant fraction was then centrifuged at 105,000 *g* (average) for 1 hr in a Spinco model L preparative ultracentrifuge (rotor No. 40). The supernatant phase (soluble fraction) from the second centrifugation was removed and the microsomal pellet was then homogenized with sufficient 1·15% KCl that 1 ml of the preparation contained the microsomes obtained from 250 mg of liver. Aliquots of this preparation were used for the assay of enzymatic activity.

Preparation of cell fractions. The livers were homogenized in a motor-driven Teflon–glass homogenizer with 9 ml of isotonic sucrose (0·25 M) per gram of tissue. Cell fractions were prepared by differential centrifugation.[7] The supernatant fraction, essentially free of unbroken cells and nuclei, was prepared by centrifugation of the whole homogenate at 600 *g* for 30 min. The supernatant was then recentrifuged for 30 min at 10,000 *g* to sediment mitochondria. To isolate microsomes, the 10,000 *g* supernatant fraction was recentrifuged at 105,000 *g* for 60 min.

Enzyme assays. For the measurement of microsomal oxidation and reduction, microsomes (1·0 ml, obtained from 250 mg of liver) were incubated with 2·0 ml of 0·2 M buffer, potassium phosphate or Tris (pH 7·4), $MgCl_2$ (20 μmoles), NADP (2 μmoles), sodium isocitrate (35 μmoles), isocitric dehydrogenase (2·5 units) and semicarbazide (30 μmoles). One of the following drugs was added to this mixture: aminopyrine (5 μmoles); hexobarbital (1 μmole); *p*-nitrobenzoic acid (10 μmoles); or ^{3}H-THC (0·65 μmole). THC was added to the incubation mixtures in 0·5 ml of a mixture of 20% propylene glycol and 80% rat serum. The incubation mixture was

* A.C.S. grade, obtained from Fisher Scientific Co.; purified by successive washings with 1 N NaOH, 1 N HCl and two washings with water.

adjusted to a final volume of 5 ml with distilled water. The reaction mixtures were incubated in a Dubnoff metabolic shaker for 30 min at 37° under an atmosphere of air. Those containing *p*-nitrobenzoic acid were incubated under nitrogen.

For the measurement of the activity of uridine diphosphate glucuronyl transferase, microsomes (0·5–1·0 ml, obtained from 125–250 mg of liver) were incubated with 0·5 ml of 0·2 M Tris buffer, uridine diphosphate glucuronic acid (9 μmoles) and either *p*-nitrophenol (200 mμmoles) or ^{14}C estradiol (100 mμmoles, 52 mCi/m-mole). The incubation mixture was adjusted to a final volume of 3 ml with distilled water. The reaction mixtures which contained estradiol were incubated for 10 min with Tris buffer, pH 8·4; those containing *p*-nitrophenol were incubated for 30 min with Tris buffer, pH 7·6.

The rate of metabolism of hexobarbital was measured by estimation of the disappearance of substrate.[8] The reduction of *p*-nitrobenzoic acid was determined by measurement of the *p*-aminobenzoic acid which was formed during the incubation.[9] The rate of demethylation of aminopyrine was measured by estimation of the amount of formaldehyde formed by the Nash procedure.[10] The metabolism of ^{3}H-THC was measured by estimation of the disappearance of substrate. ^{3}H-THC was recovered from incubation mixtures by extraction into 4 volumes of petroleum ether according to the procedure previously described.[3] The conjugation of *p*-nitrophenol was measured according to the method of Isselbacher.[11] The conjugation of ^{14}C-estradiol was measured by estimation of the formation of its glucuronide. Unconjugated estradiol was removed from the incubation mixtures by two extractions with 10 ml of toluene. The tubes were shaken for 45 min, centrifuged and 8-ml aliquots of the organic phase were transferred to scintillation vials containing 3 ml of ethanol, 5 ml of toluene, and 2 ml of phosphor [50 g of 2,5-diphenyloxazole (PPO) and 0·625 g of 1,4-bis[2-(5-phenyloxazolyl] benzene (POPOP) per liter of toluene]. After each extraction, the aqueous phase was frozen in an acetone–dry-ice bath and the remaining organic phase removed by aspiration. After removal of the unconjugated estradiol, the aqueous phase was acidified with 0·5 ml of 2 N HCl and the glucuronide of estradiol was extracted into 6 ml of water-saturated *n*-butanol. After being shaken for 30 min, the tubes were centrifuged and 3-ml aliquots of the organic phase were transferred to scintillation vials containing 3 ml of ethanol, 10 ml of toluene and 2 ml of phosphor. Radioactivity was measured by liquid scintillation counting and all data were corrected for counting efficiency by external standardization and for the small amount of radioactivity extracted into butanol from nonincubated controls. Using this procedure, 100 per cent of the radioactivity added to the incubation mixture was consistently recovered as estradiol or its glucuronide.

RESULTS

Intracellular localization of THC in liver. ^{3}H-THC (0·1–250 μg/g) was added to 10 per cent homogenates of liver which had been preincubated for 20 min at 37°. After equilibration for 20 min, aliquots of the homogenate were fractionated by differential centrifugation as described in Methods. Under these conditions, none of the THC added to liver homogenates was metabolized during the fractionation procedure. At all concentrations of THC, the drug was almost exclusively associated with the particulate material (Table 1). Of particular interest was the finding that about 30 per cent of

TABLE 1. DISTRIBUTION OF THC IN LIVER HOMOGENATES*

	THC added (μg/g)				
	0·1	1	10	25	250
	Drug in fraction				
	(%)	(%)	(%)	(%)	(%)
Nuclei	41	33	31	30	34
Mitochondria	26	30	29	29	25
Microsomes	25	29	27	31	34
Soluble	7	8	13	10	7

* ^{3}H-THC was added to 10 per cent homogenates of rat liver prepared in 0·25 M sucrose, and aliquots of the mixture were fractionated by differential centrifugation as described in Methods. The results were obtained by fractionation of a single mixture and are typical of at least two experiments.

the drug was associated with the microsomal fraction. Since THC is highly lipid-soluble, it was considered possible that the insoluble THC might have sedimented along with one of the cell fractions. The intracellular localization of THC was thus reinvestigated using the isolated perfused liver preparation. After equilibration of the system for 20 min, 500 μg of ^{3}H-THC was added to the recycling system and the livers were perfused for 15 min. After fractionation of the liver homogenate, most of the THC was again found associated with the particulate material, especially nuclei and microsomes (Table 2).

TABLE 2. DISTRIBUTION OF THC IN THE ISOLATED PERFUSED RAT LIVER*

	Experiment 1		Experiment 2	
	(dis/min/g/liver)	(%)	(dis/min/g/liver)	(%)
Homogenate	7920	100	7395	100
Nuclei	3188	40	2175	29
Mitochondria	653	8	702	10
Microsomes	2730	35	2070	28
Soluble	540	7	698	9
Total recovered	7111	90	5645	76

* ^{3}H-THC (500 μg) was added to the recirculating system and perfused for 15 min at 37°. Liver weights: exp. 1, 7·4 g; exp. 2, 10·4 g.

Although extensively bound in plasma,[1,2] the rapid accumulation of THC in tissues after intravenous administration[3] suggested that it is not strongly bound in plasma. Additional support for this view was provided by studies on the distribution of THC between plasma and liver homogenates. Rat livers were homogenized in 9 vol. of isotonic sucrose, preincubated at 37° for 30 min and chilled to 4°. Aliquots of the homogenate obtained from 1 g of liver were resuspended in 10 ml of isotonic sucrose containing 4 ml of rat serum and 50 μg of THC and the mixtures centrifuged for 1 hr

at 100,000 *g*. In these experiments about 60 per cent of the THC was sedimented with the particulate components; if, however, the liver homogenate was omitted, the concentration of THC was constant throughout the solution after centrifugation. When liver microsomes obtained from 1 g of liver were substituted for the homogenate, about 30 per cent of the THC was sedimented with the particulate material. Interestingly, the transfer of the THC from plasma to the particulate material was not changed in preparations which contained homogenates or microsomes which had been heated for 5 min at 90°.

The localization of THC in hepatic nuclei and microsomes prompted the investigation of the effects of this binding on the metabolism of THC and other drugs by the microsomal enzymes.

TABLE 3. METABOLISM OF THC BY LIVER FRACTIONS*

	THC metabolized (μg)
Homogenate	26
600 *g* Supernatant	97
9000 *g* Supernatant	135
Microsomes	106
Soluble	7
9000 *g* Supernatant + nuclei	41
9000 *g* Supernatant + denatured nuclei†	67
Microsomes + nuclei	21
Microsomes + denatured nuclei	70

* ^{14}C-THC (200 μg) was incubated for 30 min with preparations of rat liver equivalent to 333 mg of liver, as described in Methods. The results are the average of two incubation mixtures and are representative of at least two experiments.

† Nuclei were resuspended in isotonic KCl, heated in a boiling water bath for 5 min and after homogenization were chilled to 4°.

Effects of intrahepatic binding on the metabolism of THC. The hydroxylation of THC has been shown to be catalyzed by NADPH-dependent enzymes which are localized in hepatic microsomes.[12,13] In the present studies over three times as much THC was metabolized by the supernatant fractions or microsomes than by the whole homogenate of rat liver (Table 3). Upon first consideration, these findings suggested that an inhibitor of the metabolism of THC was present in the nuclei and evidence is indeed available to support this conclusion. For example, nicotinamide has long been known to stimulate several pathways of drug metabolism, *in vitro*, by blocking the destruction of NAD by nucleosidases.[14,15] More recently, Sasame and Gillette[16] have shown that NADPH–pyrophosphatase in rat liver converts NADPH to products including 2′-AMP which inhibit not only NADPH-cytochrome *c* reductase but also the *N*-demethylation of aminopyrine, the hydroxylation of aniline and the *O*-demethylation of *p*-nitroanisole by hepatic microsomes. Since the specific activity of NADPH–pyrophosphatase was particularly high in the nuclei of rat liver, it was actually suggested that the presence of this enzyme was responsible for the marked differences between the rates of metabolism of several drugs by homogenates and microsomal preparations of rat liver. However, the finding that THC is extensively bound by the

nuclei suggests that its strikingly impaired metabolism in homogenates results in part from binding to nuclei which reduces the concentration of the drug which is available to the microsomes. This hypothesis is supported by the findings that the ability of nuclei to inhibit the metabolism of THC is only partially blocked by heating (Table 3), and the activity of the homogenate was not enhanced by increasing the NADPH generating system 3-fold (Table 4).

TABLE 4. INTERACTION OF NUCLEI WITH THE METABOLISM OF THC*

Incubation conditions	THC metabolized (μg)
Experiment 1	
Homogenate	30
+ increased NADPH†	39
under oxygen	86
9000 *g* Supernatant	101
+ increased NADPH	98
under oxygen	158
Experiment 2	
Homogenate	28
9000 *g* Supernatant	82
+ nuclei	29
+ dialyzed nuclei‡	30
Experiment 3	
Homogenate (200 μg THC)	26
(500 μg THC)	32
(1000 μg THC)	5
9000 *g* Supernatant (200 μg THC)	126
(500 μg THC)	138
(1000 μg THC)	112

* ^{14}C-THC (200 μg) was incubated for 30 min with preparations of rat liver equivalent to 333 mg of liver as described in Methods. The results are the average of two incubation mixtures and are representative of two experiments.

† Concentration of components of NADPH generating system were three times those described in Methods.

‡ Preparation was dialyzed overnight against isotonic KCl (three changes, 40 vol.).

The decreased metabolism of THC in the presence of nuclei may result in part from an increased requirement for oxygen. Incubation under oxygen enhances the metabolism of the drug to a greater extent in homogenates, but even under these conditions almost twice as much THC is metabolized by microsomal preparations (Table 4).

Interestingly, increasing the concentration of THC in the incubation mixtures two and one-half times did not appreciably enhance its metabolism by either homogenates or microsomal preparations, but a 5-fold increase in the concentration of the substrate resulted in a substrate inhibition which was most striking with the homogenate. Since the ability of the nuclei to impair the metabolism of THC was unaffected by dialysis, these findings suggest the possibility that, at high concentrations, THC might displace a lipid soluble inhibitor from binding sites on the nuclei.

Interaction of THC with the metabolism of drugs by hepatic microsomes. The finding

that THC is extensively bound by hepatic microsomes suggested the possibility of its interaction with the metabolism of various substrates by these organelles. THC was added to incubation mixtures dissolved in 0·1 ml of propylene glycol. Since propylene glycol itself caused a slight inhibition of the microsomal drug-metabolizing enzymes, 0·1 ml of the solvent was also added to control incubation mixtures. The most striking actions of THC are on those metabolic pathways which are linked with the microsomal cytochrome P-450. At concentrations less than 10^{-4} M THC markedly inhibited the oxidative metabolism of both aminopyrine and hexobarbital but caused only a slight impairment of the conjugation of estradiol and *p*-nitrophenol with glucuronic acid (Table 5). Interestingly, THC actually stimulated the reduction of *p*-nitrobenzoic acid.

TABLE 5. INHIBITION OF HEPATIC DRUG METABOLISM BY THC*

Pathway	Relative activity THC (M)		
	1×10^{-4}	5×10^{-5}	1×10^{-5}
Aminopyrine demethylation	50	55	80
Hexobarbital oxidation	42	45	90
p-Nitrobenzoic acid reduction	133	133	120
Estradiol conjugation	75	87	96
p-Nitrophenol conjugation	82	84	93

* The results are the mean relative activities, control 100, obtained with at least two incubation mixtures and are representative of two experiments. Substrate concentrations: aminopyrine, 1×10^{-3} M; hexobarbital, 2×10^{-4} M; *p*-nitrobenzoic acid, 2×10^{-3} M; estradiol, $3{\cdot}3 \times 10^{-5}$ M; *p*-nitrophenol, $6{\cdot}6 \times 10^{-5}$ M.

Inhibition of the metabolism of THC, in vitro. Like THC, the tricyclic antidepressant desipramine (DMI) is also extensively bound by hepatic microsomes[17] and disappears from the tissues of rats with a long half-life.[18] Since DMI has been shown to inhibit hepatic metabolism of a number of drugs including tremorine, oxotremorine,[19] guanethidine,[20] propranolol[21] and amphetamine,[22] it was of interest to determine if DMI and related antidepressants also inhibit the metabolism of THC. In contrast to their potent action on the metabolism of other drugs, the tricyclic antidepressants were poor inhibitors of the metabolism of THC (Table 6). DMI, iprindole and nortriptyline strikingly inhibited the metabolism of THC only when they were added to the incubation mixtures in concentrations which were several times that of the substrate. In contrast to the action of the tricyclic antidepressants, SKF-525A, a classical inhibitor of drug metabolism, at concentrations as low as 10^{-5} M markedly inhibited the metabolism of THC, *in vitro*.

DISCUSSION

Although THC is extensively bound to the lipoproteins in plasma,[2] the rapid distribution of the drug into tissues after intravenous administration[3] suggested that this binding is neither irreversible nor particularly strong. The results of the present studies suggest that the rapid transfer of THC from plasma to tissues may be facilitated by extensive binding to subcellular components.

TABLE 6. INHIBITION OF THE METABOLISM OF THC*

	Conc (M)	Inhibition (%)
DMI	1×10^{-3}	66
	1×10^{-4}	20
	1×10^{-5}	10
Iprindole	1×10^{-3}	65
	1×10^{-4}	10
	1×10^{-5}	0
Nortriptyline	1×10^{-3}	84
	1×10^{-4}	31
	1×10^{-5}	3
SKF-525A	1×10^{-3}	81
	1×10^{-4}	76
	1×10^{-5}	58

* ^{14}C-THC, $1{\cdot}3 \times 10^{-4}$ M, was incubated for 30 min with rat liver microsomes equivalent to 333 mg of liver. Results are the mean values obtained with two incubation mixtures and are representative of at least two experiments.

In addition to THC, a number of drugs including imipramine,[18] chlorpromazine and diaminodiphenyl sulfide[23] as well as guanoxan, guanethidine and debrisoquin[20] are metabolized several times more rapidly by hepatic microsomes than they are by homogenates of liver, and it has been postulated that nuclei may contain unidentified inhibitory factors.[23] Recently, Sasame and Gillette[16] have demonstrated that NADPH–pyrophosphatase, which is particularly active in the nuclei of rat liver, can convert NADPH to products which inhibit both NADPH-cytochrome *c* reductase and the mixed function oxidases in hepatic microsomes. Although this interaction offers a most interesting explanation for the reduced drug metabolism in homogenates, it appears that other mechanisms may be involved as well. In the present studies, the ability of nuclei to inhibit the metabolism of THC was only partially blocked by heating or incubation under oxygen and was unaffected by dialysis or by increasing the concentration of the NADPH generating system. Since the inhibitory action of the nuclei cannot be attributed solely to their enzymatic activity or requirement for oxygen, it appears likely that the reduced metabolism of drugs such as THC, imipramine and chlorpromazine[17] in homogenates results in part from binding to nuclei which reduces the availability of the drug to the microsomes.

The binding of THC by liver microsomes was reflected by its ability to interact with several pathways of drug metabolism. The fact that THC primarily altered oxidation and reduction rather than conjugation with glucuronic acid clearly implicated an interaction with cytochrome P-450. Moreover, the ability to stimulate nitroreductase, *in vitro*, is not a unique property of THC but rather is characteristic of a variety of compounds which through an interaction with cytochrome P-450 cause a characteristic type I change in the absorption spectrum of liver microsomes.[24] It is noteworthy

that Cohen *et al.*[25] have recently shown that THC indeed combines with hepatic microsomes to give a typical type I difference spectrum.

Since pretreatment with THC has been shown to prolong the duration of action of hexobarbital and pentobarbital as well as that of barbital,[26,27] a non-metabolized barbiturate, it appears that the enhancement of the depressant action of barbiturates by THC is a consequence of their interaction at receptor sites, rather than the result of an inhibition of metabolism. The recent findings of Cohen *et al.*,[25] as well as unpublished observations from this laboratory, indeed support this view. For example, the pretreatment of rats with THC (10 mg/kg, i.p.) 30 min before the administration of hexobarbital (80 mg/kg, i.v.) strikingly prolonged the sleeping time but did not alter the levels of barbiturate in the brains or bodies at various times after its administration. It thus appears that the concentration of THC in liver after the administration of a single dose is not sufficient to inhibit the metabolism of the barbiturate, *in vivo*. In this regard, it is noteworthy that we have previously observed[3] that the concentration of THC in the livers of rats 15 min after its intravenous administration (4 mg/kg) is approx. 4×10^{-5} M. Although this concentration of THC is sufficient to inhibit the metabolism of hexobarbital *in vitro*, it would probably be ineffective *in vivo*, because of the rapid initial rate of metabolism of the drug and its binding by hepatic nuclei.

The observation that desipramine, iprindole and nortriptyline were potent inhibitors of the metabolism of THC only when their concentrations were several times that of the substrate is consistent with the extensive binding of THC by hepatic microsomes and the high affinity of the drug for microsomal hemoprotein which has been described by Cohen *et al.*[25] Preliminary studies on the kinetic aspects of the metabolism of THC, in this laboratory, have confirmed the high affinity of THC for the microsomal enzymes; the apparent Michaelis constant for its metabolism by rat liver microsomes is $5{\cdot}40 \pm 1{\cdot}03 \times 10^{-5}$ M (mean of four determinations $\pm$ S.D.).

Although the present studies have been concerned with some of the effects of the binding of THC in liver, it is noteworthy that even after intravenous administration the concentration of THC in lung was not only the highest of all tissues examined but also was several times that measured in the liver.[3] Since THC, a component of marihuana, is usually self-administered by inhalation, these findings suggest the potential importance of the investigation of the interactions of THC with biochemical systems in the lung.

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