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METABOLISM OF Δ^1-TETRAHYDROCANNABINOL BY THE RAT *IN VIVO* AND *IN VITRO**

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Abstract—The metabolism of [^{14}C]Δ^1-tetrahydrocannabinol (THC) was examined *in vitro* employing the 10,000 *g* supernatant (10 KS) of rat liver homogenates, after determination of apparent optimal conditions. The extent of metabolism was greater when THC was added as a suspension in rat serum than in a glycol or ethanol solution. The apparent K_m for THC metabolism by the 10 KS was found to be $1{\cdot}35 \times 10^{-4}$ M and the apparent v_{max} was 0·18 μg THC metabolized/mg of protein/min. Thin-layer chromatography indicated that at least seven metabolites were produced from THC, with the quantitative pattern of the metabolites changing dramatically with increasing duration of incubation. The major metabolite of 7-hydroxy-Δ^1-THC appeared to be 6,7-dihydroxy-Δ^1-THC. The dihydroxylated compound was slowly metabolized to more polar compounds. The bile was verified to be a major route of THC elimination in the rat, accounting for 60 per cent of an i.v. dose of THC (3 mg/kg) within 3 hr. Biliary excretion reached a maximum rate at THC doses of 6 and 12 mg/kg. Only traces of unchanged THC were found in the bile, and less than 5 per cent of a dose was excreted as 7-hydroxy-THC, the metabolites in the bile all being highly polar. The metabolism of THC, rather than the excretory process, was apparently the rate-limiting step in the elimination of the drug in the bile. When the biliary excretary mechanism was saturated, increase in the dose of THC resulted in increased urinary excretion of highly polar metabolites, but relative concentrations in tissues other than liver were unaffected.

The availability of radioactively labeled Δ^1- and $\Delta^{1,6}$-tetrahydrocannabinol (THC) has made possible the investigation of the biological disposition of these compounds at doses relevant to patterns of cannabis used by man. The literature on this subject has been reviewed recently [1–3]. Although studies *in vitro* have contributed much to our knowledge of the various pathways and products of cannabinoid metabolism, relatively little information on quantitative aspects is available.

None of the reports cited in recent reviews has presented evidence concerning the optimal conditions for metabolism *in vitro*. Despite the very low water solubility of THC, no report has dealt with the possible effect of the vehicle used *in vitro*. Incubation conditions have varied widely. In one study [4], the 10,000 *g* supernatant of 500 g pooled rat liver was incubated with 1 g Δ^1-THC. In another [5], the pooled supernatant from four rabbit livers was incubated with 120 mg Δ^1-THC. No data have been given to validate the suitability of these conditions. Therefore, these basic parameters were examined in the present study.

Furthermore, the early stages of hepatic metabolism of THC *in vivo* have not been explored in detail. The metabolite pattern in the excreta and in various tissues [6, 7] may have been modified by entero-hepatic circulation of THC and its metabolites [8] or by metabolism in organs other than the liver [9, 10] as well as by metabolism by the intestinal flora [11].

The apparent high levels of radioactivity detected in the bile and feces of rats [8, 12] after administration of radiolabeled THC indicated that an analysis of the biliary excretion of THC would provide a more direct measure of hepatic THC metabolism in the rat.

MATERIALS AND METHODS

Chemicals

[^{14}C](−)-3,4-trans-Δ^1-tetrahydrocannabinol ([^{14}C]THC, 1·45 to 1·50 μCi/mg) was synthesized as described elsewhere [13]. The radiochemical purity after preparative thick-layer chromatography (p.t.l.c.) was >98 per cent as determined by thin-layer chromatography (t.l.c.) plus radiochromatographic scanning (Actigraph III radiochromatograph scanner, Nuclear Chicago) and autoradiography of t.l.c. plates [14].

Nicotinamide, NADP, glucose 6-phosphate disodium salt, glucose 6-phosphate dehydrogenase (GPDH), NAD and uridine diphospho-glucuronic acid (UDPGA) were all purchased from Sigma Chemicals and rat serum from Pel Freez Bio-Animals. Ethyl acetate, acetone, methanol, hexanes and diethyl ether, all either pesticide or spectranalyzed grades, were obtained from Fisher Scientific. T.l.c. was carried out with 0·25 mm Silica gel G on 20 × 5 cm plastic sheets (Polygram Sil G, Brinkmann Instruments).

Animals

Male Wistar rats (285–305 g) were housed in metal cages without bedding material. Purina Chow and tap water were available *ad lib.* until the time of each experiment.

Assay of THC metabolism in vitro

Rats were decapitated and livers were rapidly excised, weighed and individually homogenized with

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4 vol. of 1·15% KCl in a glass Potter–Elvehjem homogenizer using a motor-driven teflon pestle. The homogenates were centrifuged at 10,000 *g* for 30 min at 5°, and supernatants were decanted and recentrifuged for an additional 20 min. The resulting supernatants (10 KS) were analyzed for protein concentration by the biuret method [15] and portions containing 50 mg protein, usually about 1·0 ml in volume, were used as the enzyme preparation. Preliminary experiments confirmed the observation of Burstein and Kupfer [16] that the metabolism of THC is effected by microsomal enzymes. However, the total metabolic activity of the 10 KS fraction was consistently slightly higher than that of the microsomes prepared from it by centrifugation at 200,000 *g* for 1 hr. The 10 KS fraction was, therefore, used in subsequent experiments.

The incubation mixtures also contained glucose 6-phosphate, disodium salt, 25 μmoles; nicotinamide, 10 μmoles; $MgCl_2$, 25 μmoles; and NADP and GPDH in varied amounts. Phosphate buffer (0·1 M, pH 7·4) was added to bring the final incubation volume, including the THC/vehicle preparation, to 2·5 ml. The 4 mM concentration of nicotinamide, which was used routinely, was well below the range of 13·4 to 40 mM that has been shown to inhibit hepatic microsomal metabolism of Type 1 binding substrates [17]. After 3 min of preincubation, [^{14}C]THC was added in an appropriate vehicle (see Results) to start the reaction.

After appropriate periods of incubation in an Eberbach shaker at 37°, in an oxygen atmosphere, the reaction was stopped by rapidly freezing the samples in liquid nitrogen. The samples were lyophilized and were extracted three times at 5° by vigorous shaking with 5 ml methanol for 30 min each time. The combined extracts were adjusted to 15 ml with methanol and the total radioactivity extracted was determined in 0·1-ml aliquots by using the channels ratio method with a Nuclear Chicago, model 725, liquid scintillation counter. The non-extractable residue from each sample was combusted in a Packard Tri-Carb Oxidizer, and the resulting $^{14}CO_2$ was trapped in ethanolamine and used for liquid scintillation counting with Spectrafluor Butyl-PBD in toluene. Thus, the whole of the ^{14}C activity added to the incubation mixture was accounted for.

After concentration of the extracts *in vacuo* on a rotary flash evaporator at 40°, aliquots of the extracts were separated by t.l.c. using hexanes–acetone–diethyl ether (4:3:2) as solvent system. Radioactive zones on the t.l.c. plates were detected by scanning and quantitated with a disc mechanical integrator.

Biliary excretion of THC

Surgical procedure. Rats were anesthetized with sodium barbital (450 mg/kg, i.p.; 10%, w/v, in 0·9% saline). Twenty-five min after the injection, anesthesia was supplemented with diethyl ether for 5 min. The bile duct was exposed and cannulated with 30 cm polyethylene tubing (PE-10) through a 2·5-cm midline incision. A second cannula was inserted through the incision into the peritoneal cavity to permit administration of 0·2 ml saline every 10 min after injection of THC. This fluid replacement was approximately equal to the volumes of bile and blood samples taken. The incision was closed with wound clips. Throughout the experiment, rectal temperature was maintained at 37·5 ± 0·5° with an electrical heating pad and heat lamp.

After the cannulation, the right external jugular vein was exposed through a 1 to 1·5-cm incision. Sixty min after barbital injection, the selected dose of [^{14}C]THC (1, 3, 6 or 12 mg/kg), dissolved in polyethylene glycol 200, 0·33 ml/kg, was injected through the pectoralis major muscle into the exposed vein during a 20-sec period. This incision was also clipped.

Analysis of bile samples. Serial bile samples were collected at specific time intervals (see Results) after injection of [^{14}C]THC. The amount of bile secreted/time period was determined gravimetrically, and the ^{14}C content was measured by scintillation counting of 0·05 ml of each sample in 10 ml Aquasol. The average rate/min of ^{14}C excretion during each sampling interval was calculated. One gram of bile was considered equal to 1 ml for calculation purposes. The remaining bile was kept frozen until it was extracted.

Aliquots of bile samples were adjusted to pH 2·2 with Sorenson's citrate buffer [18] and were extracted by shaking three times for 20 min at 5° with 15 ml ethyl acetate each time. The extracts were combined, 0·2 ml was counted in Aquasol and the remainder was concentrated under a stream of N_2. The concentrated extracts were chromatographed by t.l.c. as described above. Radioactive zones on the t.l.c. plates were detected and quantitated by radiochromatographic scanning.

Treatment of blood samples. Each time bile sampling vials were changed, 20-μl samples of tail tip blood were taken from the same animals with micropipettes. The blood was immediately applied to pieces of filter paper (15 × 15 mm) and dried. Each blood–paper sample was combusted and used for scintillation counting as described above for the liver incubation residues.

Tissue concentrations. The rats used in the bile duct cannulation experiments were killed by decapitation 3 hr after THC injection. Urine in the bladder was collected and weighed, and 0·2 ml was counted in Aquasol. In addition, the liver, lungs and kidneys were rapidly excised. All excess blood was removed from the surface, and the organs were weighed and lyophilized. Dried tissues were reweighed, ground in a mortar and 50 to 100-mg samples were oxidized and assayed for total ^{14}C content as described for the blood samples.

Dried, powdered tissues were extracted with ethyl acetate and the extracts were chromatographed as described for the bile samples.

Excretion of radioactivity in the bile of rats after i.v. injection of polar metabolites of [^{14}C]THC. This experiment was carried out to determine whether prior metabolism of THC or the secretory process itself was the rate-limiting step in the biliary excretion of THC metabolites in rats. Bile duct-cannulated rats (290-295 g) were injected i.v. with 0·1 ml of a polyethylene glycol 200 solution of the residue of an ethyl acetate extract of bile previously collected from other animals injected with [^{14}C]THC. The extract contained primarily highly polar metabolites of [^{14}C]THC, and less than 2 per cent unchanged THC. The dose was equivalent to 1 mg THC/kg based on the specific activity of the THC from which the metabolites were formed.

Serial bile samples were collected for 3 hr, as described earlier, and the ^{14}C content was determined.

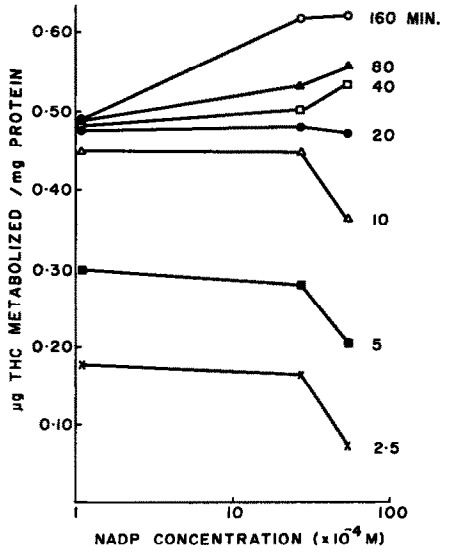

Fig. 1. Effect of NADP concentration on THC metabolism at incubation times of 2·5 to 160 min; initial THC concentration = 14·4 μg/ml; points are means of duplicate determinations from two rats.

RESULTS

Metabolism of THC in vitro

Effect of THC vehicle. During 10 min of incubation, the 10 KS fraction of rat liver metabolized 42–47 per cent ($n = 4$) of added THC (initial concentration 50 μg/ml) when this was added suspended in rat serum; 28–35 per cent when dissolved in ethanol, polyethylene glycol 200 or propylene glycol; and 3–7 per cent when added without vehicle. The final rat serum concentration in the incubation mixture was 4%, while the organic vehicles were all present at 1%. Accordingly rat serum was selected as vehicle for all experiments *in vitro*.

Protein concentration. The effect of varying the concentration of 10 KS protein in the incubation mixture was tested over the range of 5–30 mg/ml. THC metabolism during the first 10 min of incubation was proportional to the protein concentration over the whole range. Accordingly a protein concentration of 20 mg/ml was used routinely.

Cofactors. The NADP concentration significantly influenced the rate of THC metabolism with the effects being dependent on the duration of incubation (Fig. 1). In incubations lasting up to 20 min, an increase in the NADP concentration from $1{\cdot}1 \times 10^{-4}$ M to $2{\cdot}75 \times 10^{-3}$ M resulted in very little change, while an increase from $2{\cdot}75 \times 10^{-3}$ M to $5{\cdot}5 \times 10^{-3}$ M consistently decreased the amount of THC metabolized. On the other hand, in samples incubated from 40 to 160 min, THC metabolism was increased by the higher levels of NADP.

In another experiment, increasing the NADP concentration from $1{\cdot}1 \times 10^{-4}$ M to $2{\cdot}2 \times 10^{-3}$ M did not alter the amount of metabolism at three different THC concentrations (6·4, 16·2 and 32·4 μg/ml) during 10 min of incubation. These results agreed with the result obtained for the 10-min incubation in the previous experiment.

GPDH concentration was varied from 0 to 6 units/2·5-ml sample. Amounts greater than 0·5 unit supported the maximum rate of THC metabolism as determined after either 10 or 30 min of incubation; 1·0 unit/flask was chosen for routine use.

Since glucuronide conjugates of THC metabolites had been reported to occur in small concentrations in the feces of humans [19] and in the urine of rabbits [20], UDPGA disodium salt, 8×10^{-4} and $3{\cdot}2 \times 10^{-3}$ M, was added to incubation samples *in vitro* in an attempt to induce the production of polar glucuronides. UDPGA did not influence the amount of THC metabolized. However, at the higher concentration of UDPGA, the radioactivity in a t.l.c. band with R_f = 0·29 to 0·36 was significantly reduced ($P < 0{\cdot}025$), while the activity in the non-extractable fraction was slightly increased ($P < 0{\cdot}10$). NAD, 5×10^{-3} M, was found to have no effect on the metabolism of THC in the same experiment. In view of these findings, neither UDPGA nor NAD was used in subsequent experiments.

Time course of metabolism. In a typical experiment, THC, at initial concentrations of 15·2 and 32·4 μg/ml, was incubated for increasing periods of time to determine the time course for THC metabolism. The amount of THC metabolized during the first 20 min of incubation was dependent on the duration of incubation as well as on the initial THC concentration (Fig. 2). Although the rate of THC disappearance decreased rapidly beyond 30 min, the THC metabolite pattern continued to change quantitatively for the duration of the experiment (Fig. 3). Examples of autoradiographs and radiochromatographic scans of t.l.c. plates depicting metabolite patterns after 10, 20 and 160 min of incubation are shown in Fig. 4.

Zone 6 was tentatively identified as 7-hydroxy-Δ^1-THC by t.l.c. and g.l.c. comparison with a pure standard. It appeared immediately after the start of the incubation and reached a peak level of 40 per cent of the total activity after 10 min of incubation. Thereafter, its concentration fell to about 25 per cent after 20 min and to less than 5 per cent after 80 min. On the other hand, zones 5 and 2 and the non-extractable fraction increased in concentration as zone 6 decreased. The rates of appearance and disappearance of zone 3, tentatively identified as 6,7-dihydroxy-Δ^1-THC on the basis of

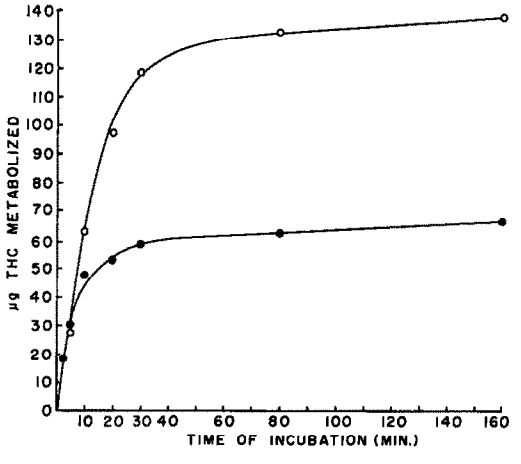

Fig. 2. THC metabolism *in vitro* at apparent optimal conditions: initial THC concentrations: 15·2 μg/ml, ●; 32·4 μg/ml, ○; points represent mean values of duplicate determinations from two rats/experiment; NADP, $2{\cdot}5 \times 10^{-3}$ M.

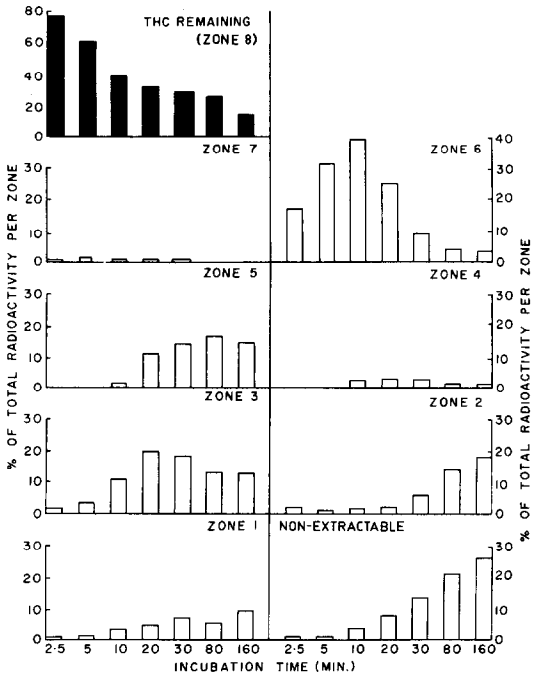

Fig. 3. Concentrations of THC and its metabolites after different periods of incubation in a typical experiment; initial THC concentration = 15·2 μg/ml; bars indicate mean values of duplicate determinations from two rats.

published R_f values [21], tended to be intermediate between those of zones 5 and 6.

Pathway of THC metabolism. During the course of the experiments, *in vitro*, sufficient amounts of radiolabeled zones 3 and 6 were produced to permit their elution and purification by p.t.l.c. The apparent radiochemical purity of each compound was >98 per cent as determined by t.l.c. in four different solvent systems described by Ho *et al.* [21]. Subsequently the metabolism of each compound was examined in duplicate under the conditions *in vitro* used for THC. Samples containing THC were incubated in the same experiment to permit a comparison of the relative rates of metabolism. The metabolite patterns produced from THC, zone 6 and zone 3 are shown in Table 1. Degradation did not occur in control incubation samples not containing the 10 KS fraction.

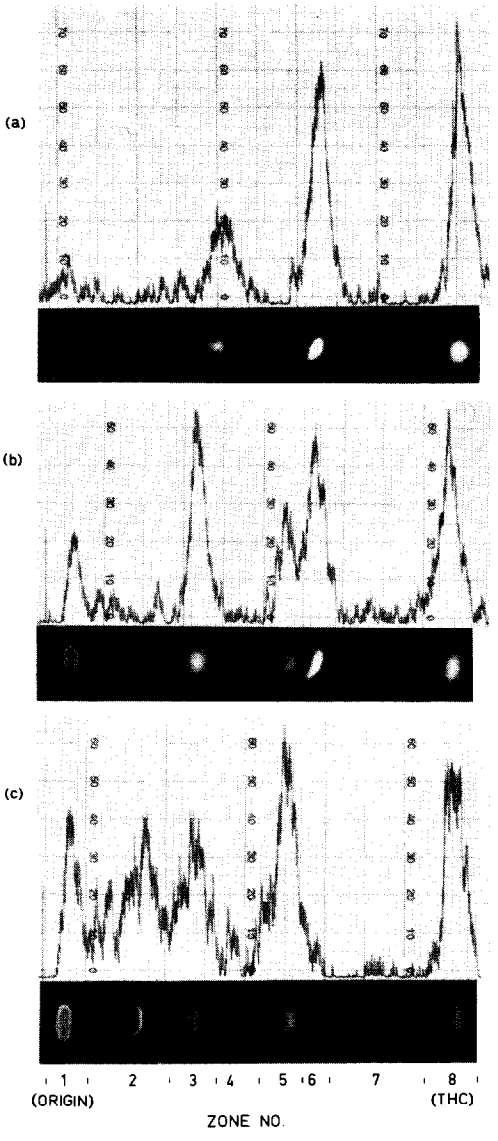

Fig. 4. Radiochromatographic scans and corresponding autoradiographs of t.l.c. plates showing THC metabolite patterns after different periods of incubation; initial THC concentration = 15·2 μg/ml for all samples; (a) 10-min incubation; (b) 20-min incubation; and (c) 160-min incubation.

Table 1. Metabolites produced from THC, 7-OH-THC and 6,7-di-OH-THC *in vitro*

Compound incubated*	Non-extractable	Per cent of total radioactivity found in zone 1†	2	3 (di-OH-THC)	4	5	6 (OH-THC)	7	8 (THC)
THC, 32 μg	7·3‡	4·8	1·9	19·8	3·0	10·7	25·6	0·8	33·3
THC, 80 μg	11·3	2·0	0·8	12·5	0·4	2·6	41·3	2·0	27·2
Zone 6 (7-OH-THC) 80 μg	3·3	1·4	0·3	11·6	2·2	2·0	79·2		
Zone 3 (6,7-di-OH-THC) 32 μg	6·2	1·5	8·7	78·4	3·1	2·2			

* Twenty-min incubation.
† Zone numbers used in previous studies of THC metabolism *in vitro*.
‡ Values represent means of duplicate determinations.

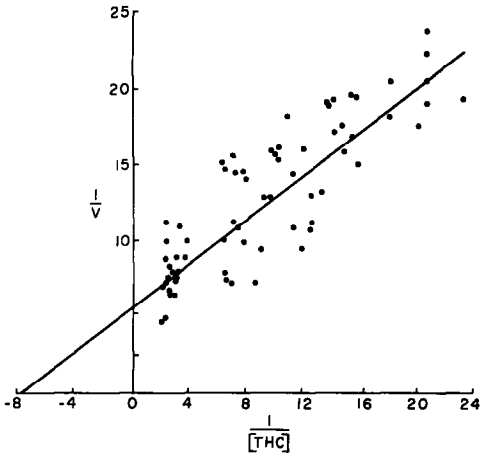

Fig. 5. Lineweaver–Burk plot of THC metabolism *in vitro*; values were obtained from experiments with 50 rats, employing three to four different concentrations of THC/10 KS liver preparation; plotted points indicate the mean values from individual experiments, and do not correspond to the same number of animals in all cases; 50 mg protein/2·5-ml sample; 10-min incubation; V = μg THC metabolized/mg of protein/min; THC = initial mM concentration of THC.

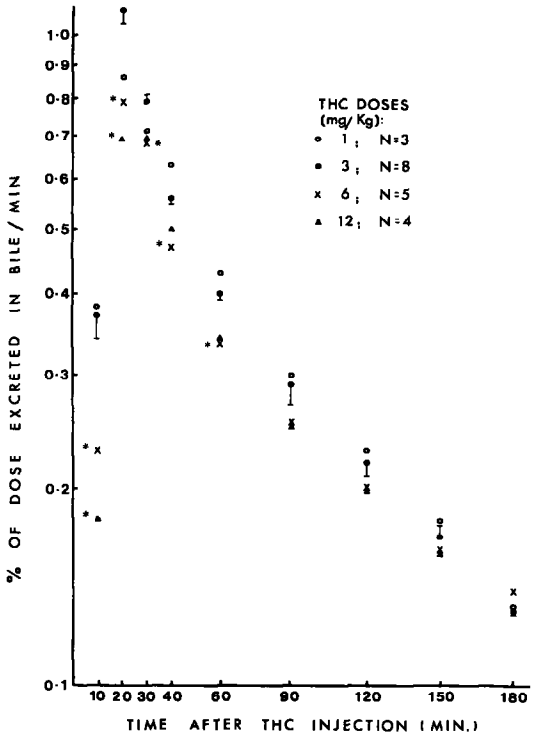

Fig. 6. Proportional rates of ^{14}C excretion in the bile of rats after different doses of [^{14}C]THC; points and bars represent mean and S. E. M. The asterisk (*) = significantly different from rate at the 3 mg/kg dose of THC ($P < 0.05$).

The presumed 7-hydroxy-THC (zone 6), the major metabolite of THC, was metabolized to more polar compounds with its major metabolite being presumed 6,7-dihydroxy-THC (zone 3). Furthermore, the major metabolites of 6,7-dihydroxy-THC appeared in zone 2 and in the non-extractable fraction. Apparently small amounts of less polar metabolites (zones 4 and 5) were also produced from zone 3 (Table 1).

Kinetics of THC metabolism. Preliminary experiments demonstrated that the rate of THC metabolism was dependent on the initial THC concentration. Accordingly the Michaelis–Menten kinetics of THC metabolism were studied using initial THC concentrations ranging from 15·6 to 140 μg/ml, i.e. $4{\cdot}96 \times 10^{-5}$ to $4{\cdot}45 \times 10^{-4}$ M. Incubations were carried out for 10 min under the previously described conditions.

The apparent V_{max} for THC metabolism was found to be $0{\cdot}180 \pm 0{\cdot}008$ μg/mg of protein/min, while the apparent K_m was $1{\cdot}35 \pm 0{\cdot}09 \times 10^{-4}$ M. The kinetic parameters were determined for each animal from Lineweaver–Burk plots obtained by linear regression by the method of least squares [22]. The composite Lineweaver–Burk plot, representing values from 50 animals, is shown in Fig. 5. The solid line shown in the graph was drawn to pass through the calculated mean $1/V_{max}$ and $-1/K_m$ values.

Metabolism of THC in vivo

Biliary excretion of ^{14}C after different doses of [^{14}C]THC. The proportional rates of biliary excretion of THC and its metabolites after increasing doses (1, 3, 6 and 12 mg/kg) of THC are shown in Fig. 6, in terms of the per cent of the injected dose excreted/min. The apparent maximum rates of excretion were attained at about 20 min after injection of all doses. With the lower doses, the rate then declined quickly, but after the 12 mg/kg dose this maximum rate was maintained for the next 10 min. Furthermore, during the first 20 min, the proportional rates of excretion after both the 6 and 12 mg/kg doses were significantly lower ($P < 0.05$) than after the 3 mg/kg dose. At 30–60 min, the excretion rate was lower ($P < 0.05$) only for the 6 mg/kg dose. These relatively lower rates observed for a short time after the two highest doses may indicate saturation of the metabolic and/or biliary excretory processes. However, the excretion of total radioactivity

Table 2. Effect of different doses of [^{14}C]THC on the bile flow and biliary excretion of total ^{14}C

I.v. dose of [^{14}C]THC (mg/kg)	% Total ^{14}C excreted in 3 hr	THC equivalent of ^{14}C excreted (μg)	Total bile flow (g/3 hr)	THC (μg/100 mg bile)
1	56·93 ± 0·77 (3)*	569	3·9271 ± 0·1451	14·5
3	60·10 ± 1·43 (8)	1803	3·6719 ± 0·1319	49·1
6	51·34 ± 2·23 (5)†	3080	3·1815 ± 0·3545	96·8
12	52·43 ± 4·03 (4)	6292	3·6134 ± 0·2041	174·9

* Values represent mean ± S.E.M.; number of rats is given in parentheses.
† Significantly less than after the 3 mg/kg dose ($P < 0.02$).

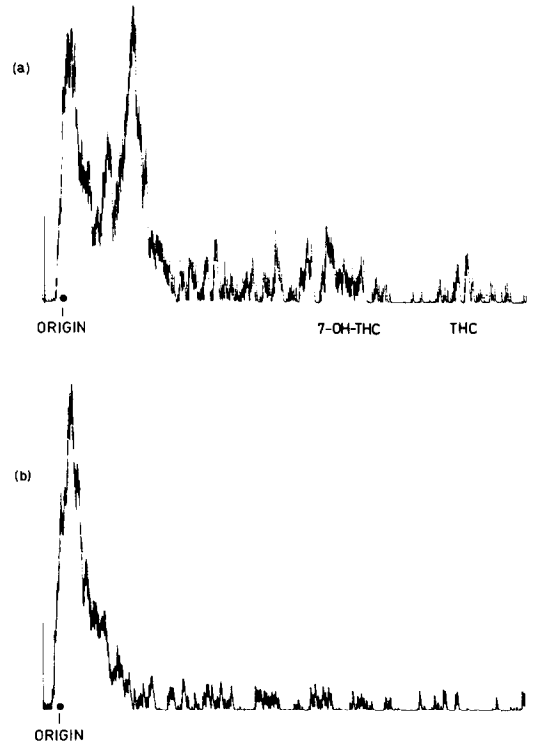

Fig. 7. Radiochromatograms of THC metabolites extracted from bile with ethyl acetate at pH 2·2; (a) extract of the bile sample from the 10 to 20-min interval; (b) extract of the bile sample from the 90 to 120-min interval.

during the 3-hr experimental period was clearly independent of bile flow as is indicated in Table 2.

The qualitative and quantitative THC metabolite patterns detected in the bile did not differ after administration of the different THC doses. Between 80 and 90 per cent of the total radioactivity present in the bile collected at each time interval was extracted with ethyl acetate at pH 2·2. Preliminary experiments showed a progressive decrease in recovery at higher pH values, to only 30 per cent at pH 8·0. Unchanged [^{14}C]THC could not be detected in more than trace amounts. Only 2–4 per cent of the total radioactivity present in bile samples collected during the first 20 min after THC injection was tentatively identified as 7-hydroxy-THC. Up to three polar radioactive zones (R_f = 0, 0·10 and 0·15 respectively) could be separated by t.l.c. from ethyl acetate extracts of bile collected during the first 20 min. However, in later samples, the only major zone was located at the origin (Fig. 7).

Disappearance of total radioactivity from the blood. The disappearance of total radioactivity from the blood of the same rats occurred in at least two first-order phases after all doses of THC. The pattern resembled that already described in the literature [23]. An initial rapid fall was observed during the 30 min after THC injection, followed by a slower fall from 30 to 180 min. More extensive experiments on blood curves will be described elsewhere.*

Tissue concentrations of THC and its metabolites. The distribution of total ^{14}C was determined 3 hr after

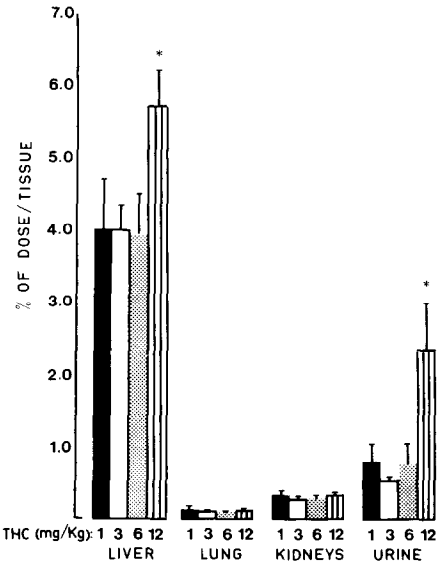

Fig. 8. Tissue distribution of ^{14}C after different doses of [^{14}C]THC; values represent mean; bars indicate S. E. M. The asterisk (*) = significantly ($P < 0.05$) greater than for 3 mg/kg; not significantly different from levels at other doses.

the injection of the different THC doses in the organs which are major sites of THC metabolism and excretion (Fig. 8). The proportion of the injected ^{14}C in the liver and in the urine did not change for the three lowest doses, but was slightly but significantly higher after the 12 mg/kg dose.

Attempts to chromatograph solvent extracts of the dried tissues were unsuccessful because of the low levels of radioactivity relative to the contaminant lipid material present in the extracts. The urine extracts also

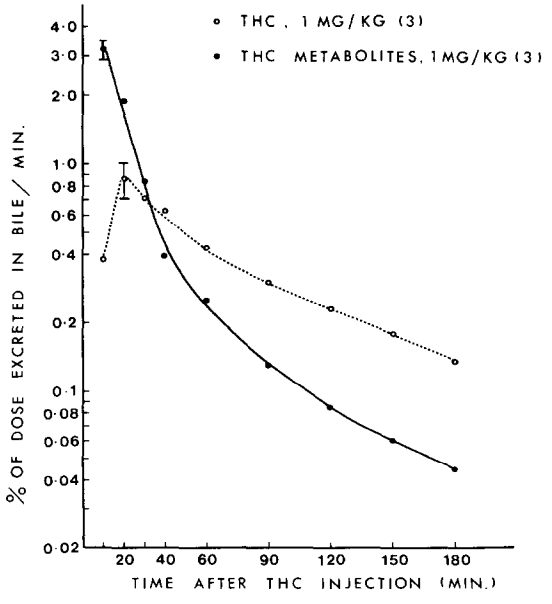

Fig. 9. Comparison of biliary excretion rates of total radioactivity after equal doses of [^{14}C]THC and [^{14}C]THC metabolites; points represent mean values from three animals for each of the two treatments; bars indicate the range of values observed at time periods of greatest variation in excretion rates.

* A. J. Siemens and H. Kalant, *Can. J. Physiol. Pharmac.*, in press.

contained relatively low levels of activity, all of which was highly polar, as determined by t.l.c.

Biliary re-excretion of polar metabolites of [^{14}C]THC. The rate of excretion of total ^{14}C after the i.v. injection of polar metabolites of [^{14}C]THC (Fig. 9) was much greater than that after injection of the same dose of THC. Moreover, the highest excretion rate was observed during the first 10 min. About 51 per cent of the metabolite dose was excreted during the first 20 min compared to 12 per cent after THC injections. During 3 hr, 79 per cent of the metabolite dose was excreted, compared to only 57 per cent of the THC dose.

DISCUSSION

The studies *in vitro* essentially agree with the conclusions of other investigators that THC is metabolized by a NADPH-dependent hepatic microsomal system [1]. However, the choice of vehicle has a significant influence on the quantitative results. Polyethylene glycol 200 [13, 23], propylene glycol [8] and ethanol [19, 24] have all been used successfully *in vivo*, because the small volume of vehicle is immediately diluted in a large volume of blood. *In vitro*, however, the smallest practical amount of organic solvent caused a slight but appreciable inhibition of total THC metabolism. Our results do not indicate whether the vehicle affected the proportions of the various metabolites formed.

The cofactor concentrations which yielded the highest initial rates of THC metabolism are, in general, similar to those commonly reported for the hepatic microsomal mixed function oxidase system [25–27]. However, if incubations are carried on for longer than 10 min, some of the cofactor concentrations become critical.

The apparent inhibition of THC metabolism at NADP concentrations of $2{\cdot}75 \times 10^{-3}$ and $5{\cdot}5 \times 10^{-3}$ M may conceivably have been due either to substrate inhibition of GPDH by NADP, leading to a reduced rate of NADPH formation during the first 20 min of incubation, or to inhibition of NADPH oxidase. Longer periods of incubation may have resulted in a decreased concentration of available NADP, thus decreasing the possibility of either type of inhibition and promoting a higher rate of THC metabolism. A $2{\cdot}5 \times 10^{-3}$ M NADP concentration was considered optimal for subsequent experiments.

These studies clearly indicated that the duration of incubation significantly influences the ultimate pattern of THC metabolite concentrations. Some metabolite levels (zones 1 and 2) increased steadily, while others, notably in t.l.c. zones 6 and 3 and possibly 5, reached peak concentrations and then fell. Various investigators [5, 6, 28] have attempted to increase yields of THC metabolites, particularly 7-hydroxy-THC, by incubating THC with liver preparations for up to 3 hr. This would appear to be inappropriate for 7-hydroxy-THC, since incubations longer than 10 min led to a progressive reduction in yield of the metabolite in zone 6 and a relatively high yield of zone 5 and the most polar THC metabolites (Fig. 3).

Previous estimates of K_m for THC metabolism by rat liver microsomes are $1{\cdot}5 \times 10^{-5}$ M [29] and $2{\cdot}8 \times 10^{-5}$ M [30]. The 10-fold increase to $1{\cdot}35 \times 10^{-4}$ M in the present work is probably due to non-specific binding of THC to non-microsomal protein in the 10 KS fraction. Only about 14 per cent of the 10 KS protein could be recovered as microsomal protein after centrifugation at 200,000 *g*.

Experiments designed to determine the primary pathway of THC metabolism supported the earlier suggestion [31] that the production of 7-hydroxy-THC is indeed the first and most rapid step in the metabolism of THC *in vitro*. The subsequent hydroxylation to form 6,7-dihydroxy-THC, is probably the rate-limiting metabolic step in the disappearance of the pharmacological activity of THC, since the 7-hydroxy-THC is pharmacologically active [10]. The rate-limiting step in the over-all metabolic sequence may be either the production or the subsequent oxidation of the 6,7-dihydroxy-THC, since equal percentages of the mono- and dihydroxy compounds were metabolized during a 20-min incubation (Table 1).

The finding that comparable amounts of zones 4 and 5 were produced during a 20-min incubation with either 7-hydroxy-THC or 6,7-dihydroxy-THC suggests that these unidentified metabolites are not precursors of the 6,7-dihydroxy-THC despite their lower polarity, but may represent a side-route of metabolism from either of the hydroxy derivatives. The results also suggest that the most polar metabolites, appearing in zones 1 and 2, probably originate from 6,7-dihydroxy-THC rather than directly from 7-hydroxy-THC.

It is not clear whether the non-extractable portion of ^{14}C represents the final and most highly polar metabolites, or includes bound non-polar compounds. THC may contribute directly to the non-extractable fraction, since the ^{14}C-concentration of the latter was consistently higher after incubation of THC than of the two hydroxylated derivatives. Indeed a small portion of the non-extractable radioactivity may have been unchanged THC; in control extractions, only 97–98 per cent of the added THC could be extracted from incubation mixtures which were frozen in liquid nitrogen 5 sec after THC addition.

In the presence of UDPGA, the concentration of the metabolite in zone 3 was significantly decreased, while the more polar components tended to be increased in concentration, although not significantly. Thus, if conjugation was occurring, zone 3 appeared to represent the compound which was being conjugated.

NAD did not appear to contribute to the metabolism of THC or to the ultimate metabolite levels. This could be expected, since NAD may act as a primary electron acceptor only when the concentration of NADP is less than 1 μM [32]. The lack of effect of NAD also indicates that the hydroxylated metabolites of THC, unlike those of tolbutamide [33] and pentobarbital [34], are not oxidized by alcohol dehydrogenase (ADH) which is normally present in the cytoplasm.

The results of the experiments *in vivo*, in general, demonstrated that the rates of biliary excretion, and the blood disappearance of THC and/or its metabolites, were proportional to the initial dose of the drug. However, the finding that the proportional rates of biliary excretion could be decreased by increasing the THC dose suggested that the metabolic and/or excretory processes could be saturated. Which of the two processes was rate-limiting is not definitely known. However, since the apparent decrease in the relative rates was already observed at 10 min after injection of

the two highest doses of THC, well before the maximum absolute rates were achieved, it seems probable that the metabolic system, rather than the subsequent secretory system, was saturated. According to Willinsky *et al.* [13], the concentration of ^{14}C in rat liver after i.v. injection of 4·5 mg [^{14}C]THC/kg was 35–40 per cent higher at 10 min than at 20 min. However, the present experiments showed that the maximum rates of biliary excretion were attained at about 20 min after 1–12 mg THC/kg. Therefore, the delay in achieving these maximum rates may represent the time required for the formation of excretable metabolites. Only about 10 per cent of the apparent delay was attributable to dilution by bile present in the cannula before THC was injected. Chromatography of bile extracts demonstrated that very little or none of the total ^{14}C excreted was unchanged THC.

The injection of THC metabolites, which should not have required further hepatic metabolism before they could enter the bile, resulted in a much greater initial rate of ^{14}C excretion in the bile than after THC injection. This further supports the suggestion that metabolism was the rate-limiting step. Therefore, a measure of the biliary excretion of THC appears to provide a direct indication of THC metabolism.

Since the ^{14}C concentration ratios of bile/blood ranged from 386 after 1 mg THC/kg to 505 after 12 mg THC/kg, determined at 20 min after THC injection, the observed over-all excretory process appeared to be active in nature.

Klausner and Dingell [8] found that about 77 per cent of 1 mg [^{14}C]THC which had been added to the perfusion fluid of an isolated rat liver was excreted in the bile within 2 hr after administration. In the present experiments, only 60 per cent of a comparable dose was excreted in 3 hr. This apparent slower rate of excretion *in vivo* was likely due to a dilution effect of distribution of THC and metabolites in the rest of the body. Consequently, a smaller amount of drug would be presented to the liver/unit time.

The proportion of the administered ^{14}C excreted in the urine during 3 hr was exceedingly small, but similar after the three lowest THC doses. The reason for the higher per cent excreted after 12 mg/kg is not known. However, it is possible that since the excretory route in the bile may have been saturated for a portion of the time, a relatively higher proportion of the drug may have been presented to the kidneys for excretion. The distribution of ^{14}C activity to the lung and kidneys was found to be proportional to the dose injected.

The 3 mg/kg dose of THC appears to be an optimal dose for studies of biliary excretion. It is in the range of direct proportionality, and highly consistent excretory rates were obtained in all animals after this dose, while at higher doses variability increased. In addition, 3 mg/kg was well tolerated by all animals, whereas two of seven and four of eight animals died within 10 min after the 6 and 12 mg/kg doses respectively. Moreover, 3 mg THC/kg i.v. is a reasonable dose in the rat since it produces no overt motor impairment [35] but a clear effect on operant behaviour [36].

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REFERENCES

1. W. D. M. Paton and R. G. Pertwee, in *Marihuana: Chemistry, Pharmacology, Metabolism and Clinical Effects* (Ed. R. Mechoulam), p. 191. Academic Press, New York (1973).
2. S. H. Burstein, in *Marihuana: Chemistry, Phamacology, Metabolism and Clinical Effects* (Ed. R. Mechoulam), p. 167. Academic Press, New York (1973).
3. G. G. Nahas, *N.Y. State J. Med.* **72,** 856 (1972).
4. M. E. Wall, D. E. Brine, G. A. Brine, C. G. Pitt, R. I. Freudenthal and H. D. Christensen, *J. Am. chem. Soc.* **92,** 3466 (1970).
5. I. M. Nilsson, S. Agurell, J. L. G. Nilsson, A. Ohlsson, F. Sandberg and M. Wahlqvist, *Science, N.Y.* **168,** 1228 (1970).
6. M. E. Wall, *Ann. N.Y. Acad. Sci.* **191,** 23 (1971).
7. S. Burstein, J. Rosenfeld and T. Wittstruck, *Science, N.Y.* **176,** 422 (1972).
8. H. A. Klausner and J. V. Dingell, *Life Sci.* **10,** Pt. 1, 49 (1971).
9. K. Nakazawa and E. Costa, *Nature. Lond.* **234,** 48 (1971).
10. H. D. Christensen, R. I. Freudenthal, J. T. Gidley, R. Rosenfeld, G. Boegli, L. Tistino, D. R. Brine, C. G. Bitt and M. E. Wall, *Science, N.Y.* **172,** 165 (1971).
11. J. R. Gillette, *Ann. N.Y. Acad. Sci.* **179,** 43 (1971).
12. R. F. Turk, *Ph.D. Thesis,* Indiana University School of Medicine (1970).
13. M. D. Willinsky, H. Kalant, O. Mercsz, L. Endrenyi and N. Woo, *Eur. J. Pharmac.* **27,** 106 (1974).
14. A. J. Siemens, *Ph.D. Thesis,* University of Toronto (1973).
15. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177,** 751 (1949).
16. S. H. Burstein and D. Kupfer, *Ann. N.Y. Acad. Sci.* **191,** 61 (1971).
17. H. A. Sasame and J. R. Gillette, *Biochem. Pharmac.* **19,** 1025 (1970).
18. K. Diem (Ed.), *Documenta Geigy Scientific Tables,* 6th Edn, p. 314. Geigy Pharmaceuticals, Ltd., Montreal (1962).
19. L. Lemberger, S. D. Silberstein, J. Axelrod and I. J. Kopin, *Science, N.Y.* **170,** 1320 (1970).
20. S. Agurell, I. M. Nilsson, A. Ohlsson and F. Sandberg, *Biochem. Pharmac.* **19,** 1333 (1970).
21. B. T. Ho, V. Estevez, L. F. Englert and W. M. McIsaac, *J. Pharm. Pharmac.* **24,** 414 (1972).
22. H. Lineweaver and D. Burk, *J. Am. chem. Soc.* **56,** 658 (1934).
23. K. O. Fehr and H. Kalant, *Eur. J. Pharmac.* **25,** 1 (1974).
24. S. H. Burstein, F. Menezes, E. Williamson and R. Mechoulam, *Nature, Lond.* **225,** 87 (1970).
25. R. L. Dixon, L. G. Hart, L. A. Rogers and J. R. Fouts, *J. Pharmac. exp. Ther.* **142,** 312 (1963).
26. A. Rubin, T. R. Tephly and G. J. Mannering, *Biochem. Pharmac.* **13,** 1007 (1964).
27. D. Kupfer, E. Levin and S. H. Burstein, *Chem. Biol. Interact.* **6,** 59 (1973).
28. E. W. Gill, W. Jones and D. K. Lawrence, *Biochem. Pharmac.* **22,** 175 (1973).
29. C. M. Cohen, D. W. Peterson and G. J. Mannering, *Life Sci.* **10,** Pt. 1, 1207 (1971).
30. D. Kupfer, I. Jansson and S. Orrenius, *Chem. Biol. Interact.* **5,** 201 (1972).
31. E. B. Truitt, Jr., *Pharmac. Rev.* **23,** 273 (1971).
32. B. S. Cohen and R. W. Estabrook, *Archs. Biochem. Biophys.* **143,** 54 (1971).
33. H. G. McDaniel, H. Podgainy and R. Bressler, *J. Pharmac. exp. Ther.* **167,** 91 (1969).
34. R. Kuntzman, M. Ikeda, M. Jacobsson and A. H. Conney, *J. Pharmac. exp. Ther.* **157,** 220 (1967).
35. H. Kalant and A. E. LeBlanc, *Can. J. Physiol. Pharmac.* **52,** 291 (1974).
36. C. D. Webster, A. E. LeBlanc, J. A. Marshman and J. M. Beaton, *Psychopharmacologia* **30,** 217 (1973).