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More acidic metabolites of Δ^1-tetrahydrocannabinol isolated from rabbit urine

MARIANNE NORDQVIST*, STIG AGURELL†, MARIA RYDBERG, LENA FALK AND TORSTEN RYMAN

Department of Pharmacognosy, Faculty of Pharmacy, BMC, Box 579, S-751 23 Uppsala, Sweden. †Also at Astra Läkemedel AB, S-151 85 Södertläje, Sweden

The in vivo metabolism of Δ^1-tetrahydrocannabinol (Δ^1-THC) was further investigated in the rabbit after i.v. administration. Nine acidic metabolites were isolated from a previously not investigated fraction of the urine and identified by gas chromatography-mass spectrometry and by proton magnetic resonance spectroscopy. The major metabolites were side-chain hydroxylated monocarboxylic acids. Three side-chain monocarboxylic acids hydroxylated in allylic positions in the isoprene moiety were also characterized. The metabolites 4″-hydroxy-Δ^1-THC-7-oic acid and 7-hydroxy-4″,5″-bisnor-Δ^1-THC-3″-oic acid were hitherto not identified. An earlier described dicarboxylic metabolite was present in high concentration. Further, the identity of an *O*-glucuronide as an in vivo urinary metabolite of Δ^1-THC was here for the first time unambiguously established by m.s. and p.m.r.

Studies in vitro on the metabolism of the major psychoactive compound of cannabis, Δ^1-THC, have shown the primary routes of biotransformation to be allylic hydroxylation (Nilsson et al 1970; Wall et al 1970; Wall 1971; Ben-Zvi et al 1974a,b; Jones et al 1974), epoxidation of the Δ^1-double bond (Gurny et al 1972; Ben-Zvi & Burstein 1975; Widman et al 1975a,b) and hydroxylation of the pentyl side chain (Widman et al 1975a,b; Wall & Brine 1976). Aldehydes (Ben-Zvi & Burstein 1974) and ketones (Gurny et al 1972; Jones et al 1974) as well as carboxylic acids result from further oxidative steps, which are more prominent in vivo.

Previous in vivo studies have revealed some structures of non-conjugated acidic metabolites of Δ^1-THC in the guinea-pig, man, mouse, rabbit and rat. They are mono- and dicarboxylic acids, unsubstituted as well as mono- and dihydroxylated compounds (Burstein et al 1972; Nordqvist et al 1974; Martin et al 1976b; Wall et al 1976; Harvey et al 1977a). Aromatized and hydrogenated monocarboxylic acids as metabolites of Δ^1-THC have also been found (Ben-Zvi et al 1974, 1976; Harvey et al 1977b). Recent results from our laboratory confirmed the predominance of dicarboxylic metabolites in rabbit urine after i.v. administration of Δ^1-THC (Nordqvist et al 1979). The side chain of Δ^1-THC was preferentially oxidized and shortened and the terpene moiety oxygenated in allylic position. An interesting metabolite with an unsaturated side chain was also characterized.

† Correspondence.
* Present address: Bldg 4, Rm 220, National Institutes of Health, Bethesda, Maryland 20014, U.S.A.

In this paper we report the structures of the remaining major metabolites of Δ^1-THC in rabbit urine. All but one were non-conjugated acids showing oxygenation both in allylic position to the Δ^1-double bond and in the side chain.

The occurrence of Δ^1-THC or its metabolites as glucuronides and/or sulphates in vivo has been suggested in man (Lemberger et al 1970; Woodhouse 1972; Kelley & Arnold 1976; Wall et al 1976) rabbit (Agurell et al 1970; Agurell et al 1972), rat (Mikes et al 1971; Widman et al 1974) and rhesus monkey (Ben-Zvi et al 1976) by indirect evidence based upon enzymatic hydrolysis. The detection of trace amounts of glucuronide conjugate of Δ^1-THC in mouse liver was recently reported by Harvey et al (1977c). The characterization was based upon some typical m.s. fragments of its silyl derivative. We now report the identification of a THC-O-glucuronide in rabbit urine based upon m.s. and p.m.r. evidence.

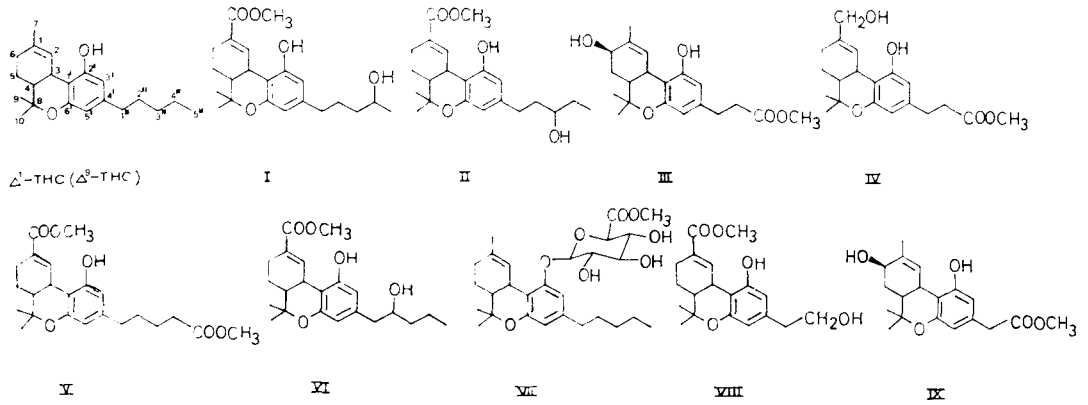
MATERIALS AND METHODS

(−)-Δ^1-Tetrahydro-[3′, 5′-^{14}C]cannabinol was administered i.v. to four female rabbits to a total dose of 110 mg (specific activity 14·6 μCi mmol^{-1}) as previously reported by Nordqvist et al (1979).

Isolation of metabolites

The initial purification of collected urine and the separation of extracted acidic urinary metabolites into the 'ammonia' and 'methanol fraction' (Fig. 1) have also been described (Nordqvist et al 1979).

The 'methanol fraction' was dried in vacuo, dissolved in the eluant 0·1 M ammonium bicar-



Structures of Δ^1-THC and methyl ester derivatives of isolated metabolites. The monoterpenoid (Δ^1-THC) system of nomenclature is illustrated.

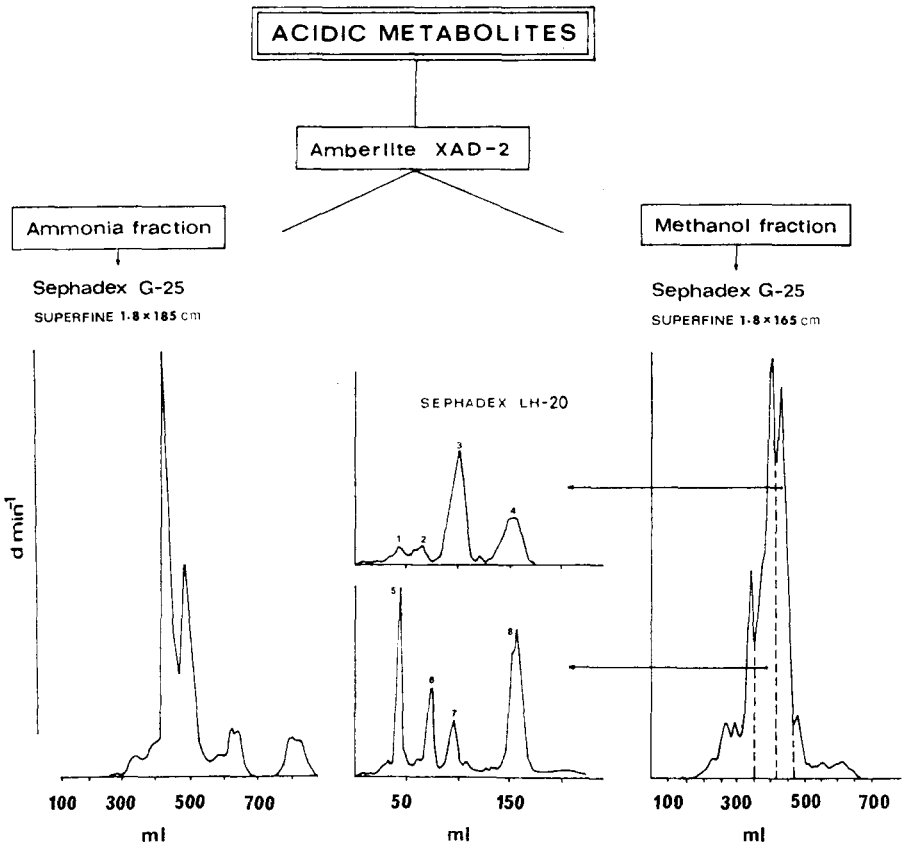


FIG. 1. Scheme for separation of acidic metabolites of Δ^1-THC extracted from rabbit urine into diethyl ether at pH 3. The acids were eluted from a column of Amberlite XAD-2 resin (2 × 40 cm) with 10% aqueous ammonia (350 ml) followed by methanol (500 ml). The 'methanol fraction' was further purified on a Sephadex G-25 column (1·8× 165 cm) with 0·1 M ammonium bicarbonate as eluant. Column chromatography on Sephadex LH-20 (1 × 70 cm) after esterification separated the acids as their methyl esters into peaks 1 to 8, which are discussed in this paper.

bonate (1% butanol added) and applied to a column of Sephadex G-25 Superfine. The elution rate was 4·5 ml h^{-1}. Fractions were collected and assayed for radioactivity. Those of high radioactivity (Fig. 1) were extracted with diethyl ether after adjusting the pH to 3 with phosphoric acid. Further isolation and purification was after esterification accomplished on Sephadex LH-20 columns (Nilsson et al 1973) and by t.l.c. Alkaline hydrolysis was performed at 70 °C with 0·5 M sodium hydroxide in 70% aqueous ethanol for 30 min under nitrogen. Enzymatic hydrolysis was carried out by incubation for 18 h at 37 °C with excess of β-glucuronidase type L-1 in 0·1 M citrate-phosphate buffer pH 3·8 or with excess of sulphatase type III in 0·1 M citrate buffer pH 4·8 (Sigma Chemical Co.).

Identification methods

T.l.c. was carried out on precoated Silica gel F plates (Merck, 0·25 mm) developed in diethyl ether–light petroleum (7 : 3), unless stated otherwise. Metabolites were visualized with 0·1% Fast Blue B salt in 2M sodium hydroxide or with *p*-anisaldehyde in sulphuric acid followed by heating (El-Feraly et al 1977). For preparative use the plates were prewashed with benzene–methanol (1 : 1) and the metabolites extracted from the Silica gel with methanol–dichloromethane (1 : 9). All solvents used for t.l.c. purification were redistilled. Radioactivity was assayed in a Packard Tri Carb Model B 2450 spectrometer with external standardization. Instagel (Packard) and a solution of Permablend III (Packard) in toluene were used as scintillation fluids. G.l.c. was accomplished with a Varian Aerograph Model 2100 (2% SE-30 column/Gas-Chrom Q, 270 °C). Before g.l.c. analysis all samples were esterified with diazomethane (20° C for 15 min) and silylated with *N,O*-bis-(trimethylsilyl)-acetamide in dry acetonitrile (60 °C for 15 min). Mass spectra (m.s.) were recorded at 20 eV with an LKB 9000 gas chromatograph-mass spectrometer (3% SE-30 column/Gas-Chrom Q). ^{1}H-nuclear magnetic resonance (p.m.r.) spectra were recorded with a Varian 100 MHz instrument (Fourier transform, $CDCl_3$).

RESULTS AND DISCUSSION

Metabolites of the methanol fraction

The Sephadex G-25 column separated the acids from the methanol fraction (18% of urinary metabolites) mainly into two groups (Fig. 1). The metabolites of both these groups were esterified with diazomethane and applied to Sephadex LH-20 columns. Elution was performed with light petroleum-chloroform-ethanol (10 : 10 : 1). The peaks 3 to 8 (Fig. 1) were further purified by t.l.c. before structure elucidation by p.m.r. and m.s.

Peak 1 (0·2%* of the radioactivity in urine) and peak 2 (0·4 %) were identical to peak 5 and 6, respectively, according to t.l.c.

* The percentage figure is calculated after purification on Sephadex LH-20.

Peak 3 contained the methyl esters of three metabolites. The two major ones (I, II) were separated by preparative t.l.c. (Silica gel, diethyl ether-light petroleum, 7 : 3, developed twice). The most polar metabolite (I, 1·4%, R_F = 0·19, Rt = 3·95 min) was assigned to 4″-hydroxy-Δ^1-THC-7-oic acid from the m.s. of the silylated methyl ester [*m/e* 518 (M^+, 100%), 503 (44), 475 (3), 459 (78), 428 (50), 413 (16), 374 (13), 117 (31)] and p.m.r. (δ7·98 d, 1 H, C-2; δ6·29 and δ6·17 d, 1 H, C-5′ and C-3′; δ3·78 m, C-4″; δ3·75 s, 3 H*, C-7; δ3·37 br.d., 1 H, C-3; δ2·50 t, C-1″; δ1·45 s, 3 H, C-9; δ1·19 d, 3 H, C-5″; δ1·12 s, 3 H, C-10). The triplet of the side chain terminal methyl protons in Δ^1-THC was replaced by a sharp doublet at a slightly downfield shift. A single proton on C-4″ appeared as a multiplet centered at δ3·78 in consistency with the p.m.r. data of 4″-hydroxylated tetrahydrocannabinoids (Binder 1976; Vidic et al 1976). Further, the singlet of the allylic methyl protons had disappeared. A 3 H-singlet at δ3.75 and the downfield shift of the vinylic proton from δ6·33 in Δ^1-THC (Archer et al 1970) to δ7·98 in metabolite I indicated a 7-oic methyl ester. The m.s. exhibited benzylic cleavage including loss of the hydroxy function [$(M-144)^+$] and further the ion *m/e* 117 (31%) characteristic for compounds hydroxylated in the penultimate (ω-1) position of the side chain (Binder et al 1974). The assignment of I was additionally supported by c.m.r. data (Nishida et al to be published).

* Methyl ester protons.

Metabolite II (1·2%, R_F = 0·21, Rt = 3·75 min) was shown to be 3″-hydroxy-Δ^1-THC-7-oic acid from the m.s. of its silylated methyl ester [(*m/e* 518 (M^+, 7%), 503 (3), 489 (1), 487 (1), 459(1), 428 (1), 387 (3), 374 (100)] and p.m.r. (δ8·00 d, 1 H, C-2; δ6·30 and δ6·20 d, 1 H, C-5′ and C-3′; δ3·75 s, 3 H*, C-7; δ3·62 m, 1 H, C-3″; δ3·37 br.d., 1 H, C-3; δ2·7–2·4 m, C-1″; δ1·45 s, 3 H, C-9; δ1·13 s, 3 H, C-10; δ0·94 t, 3 H, C-5″). The predominant benzylic cleavage of the side chain with McLafferty rearrangement [$(M-144)^+$], including loss of the hydroxy function (Binder et al 1974) as well as p.m.r. data (Binder et al 1974; Vidic et al 1976) strongly indicated 3″-hydroxylation. In addition, the presence and position of the carboxyl group could be established as in I. Further evidence for the structural assignment was obtained by c.m.r. (Nishida et al to be published).

The least polar metabolite isolated from peak 3 by preparative t.l.c. (III, 0·3%, R_F = 0·37, Rt = 2·05 min) showed after derivatization the m.s. fragments *m/e* 490 (M^+, 38%), 475 (6), 459 (5), 400 (94), 385 (100), 359 (15), 357 (11), indicative of a monohydroxylated monocarboxylic acid carrying a propyl side chain. A base peak deriving from loss of both $—CH_3$ and —HOT* was earlier found in 6β-hydroxylated side-chain acids (Nordqvist et al 1979). Comparison on t.l.c. and g.l.c. confirmed the identity with 6β-hydroxy-4″, 5″-bisnor-Δ^1-THC-3″-oic acid.

Peak 4 contained methyl esters of mainly two metabolites which were separated by preparative t.l.c. (Silica

gel, diethyl ether-light petroleum, 4:1). The most polar metabolite (IV, 0·8%, R_F = 0·16, Rt = 2·50 min) was 7-hydroxy-4″, 5″-bisnor-Δ^1-THC-3″-oic acid according to the m.s. of its silylated methyl ester [*m/e* 490 (M^+, 7%), 475 (3), 459 (2), 418 (3), 416 (2), 403 (2), 400 (5), 387 (100), 357 (2)] and p.m.r. (δ6·67 d, 1 H, C-2; δ6·28 and δ6·16 d, 1 H, C-5′ and C-3′; δ4·04 br.s., 2 H, C-7; δ3·69 s, 3 H**, C-3″; δ2·70 m, C-1″; δ1·43 s, 3 H, C-9; δ1·10 s, 3 H, C-10). The m.s. base peak (*m/e* 387, M^+-CH_2OT) strongly indicated hydroxylation at C-7 (Harvey & Paton 1976; Wall & Brine 1976). This was further proven by p.m.r. of its methyl ester (Nilsson et al 1970; Wall et al 1970). The side chain terminal methyl protons of Δ^1-THC did not appear but a 3 H-singlet at δ3·69 indicated the methyl ester of an oxidized side chain.

* $Si(CH_3)_3$ is designated as T.
** Methyl ester protons.

The least polar metabolite from peak 4 (0·3%) was identical to III (m.s. and t.l.c.).

Peak 5 contained the methyl ester of a major metabolite (V, 1·0%, R_F = 0·53, Rt = 6·30 min) with m.s. and p.m.r. data identical to those of Δ^1-THC-7,5″-dioic acid (Nordqvist et al 1979).

Peak 6 was methyl 2″-hydroxy-Δ^1-THC-7-oate (VI, 0·7%, R_F = 0·30, Rt = 2·75 min) identified by m.s. after silylation [*m/e* 518 (M^+, 3%), 503 (1), 446 (21), 374 (4), 145 (100)] and by p.m.r. (δ8·01 d, 1 H, C-2; δ6·31 and δ6·24 s, 1 H, C-5′ and C-3′; δ3·8 m, C-2″; δ3·76 s, 3 H*, C-7; δ3·38 br.d., 1 H, C-3; δ2·7–2·4 m, C-1″; δ2·0 m, C-5; δ1·45 s, 3 H, C-9; δ1·13 s, 3 H, C-10; δ0·94 t, C-5″). The m.s. base peak (*m/e* 145) and the pronounced loss of 72 m.u. (benzylic cleavage with transfer of the trimethylsilyl group to the aromatic ring) are typical for a 2″-hydroxylated cannabinoid (Binder et al 1974; Harvey & Paton 1976). The location of a hydroxy function on C-2″ was further confirmed by p.m.r. (cf. Binder et al 1974; Vidic et al 1976). The absence of the allylic methyl protons, the downfield shift of the vinylic proton on C-2 and the appearance of a 3 H-singlet at δ3·76 placed the methyl ester function on C-1.

* Methyl ester protons.

Peak 7 contained the methyl ester of a metabolite (VII, 0·4%, R_F = 0·04, Rt = 10·35 min), which could not be visualized on t.l.c. with Fast Blue B salt in alkaline solution. Instead, the spraying agent *p*-anisaldehyde in sulphuric acid was used to detect cannabinoids with the phenolic group blocked (El-Feraly et al 1977). Metabolite VII appeared as a faint spot after heating. It was purified by preparative t.l.c. (Silica gel, diethyl ether). According to p.m.r. (δ6·62 and δ6·44 d, 1 H, C-5′ and C-3′; δ6·31 br.s., 1 H, C-2; δ4·78 d, J = 8 Hz, 1 H; δ4·0–3·7 m; δ3·87 s, 3 H; δ3·67 s, 1 H; δ3·20 br.d., 1 H, C-3; δ2·50 t, 2 H, C-1″; δ1·42 s, 3 H, C-9; δ1·08 s, 3 H, C-10; δ0·87 t, 3 H, C-5″) the compound contained a methyl ester function, the protons of which resonanced at δ3·87. The main feature is otherwise that of Δ^1-THC with similar shifts for the protons on C-2, C-3, C-9, C-10, C-1″ and C-5″, respectively, but with the aromatic protons at pronounced higher shifts than in Δ^1-THC (Archer et al 1970; Yagen et al 1977). The failure to detect VII with a phenol sensitive spray and the p.m.r. data suggested a conjugate, where Δ^1-THC was bound via the phenolic oxygen to a molecule containing a methyl ester. Further, the multiplet at δ4·0–3·7 indicated a sugar moiety to be the attached molecule. The linkage should be β (J = 8 Hz; Yagen et al 1977). The m.s. of the silylated derivative [*m/e* 720 (M^+, 1%), 705 (1), 615 (1), 406 (25), 386 (30), 371 (3), 317 (96), 314 (52), 313 (100), 275 (13), 227 (4), 217 (16)] showed the M^+-ion at *m/e* 720 in agreement with that expected for a glucuronide of THC. Typical m.s. fragments of the sugar moiety were obtained at *m/e* 406, 317, 275 and 217 (Billets et al 1973; Lyle et al 1977). Aglycone ions appeared at *m/e* 386, 371, 314 and 313 proving the structure of Δ^1-THC. Phenolic linkage was confirmed by the fragments at *m/e* 406 (Miyazaki et al 1976) and at *m/e* 386 (Billets et al 1973). The predominant fragment ion at *m/e* 313 is also typical of an O-glucuronide of Δ^1-THC (Lyle et al 1977). The structural assignment of metabolite VII as a β-glucuronide of Δ^1-THC was supported by enzymatic hydrolysis with β-glucuronidase type L-1 leading to free Δ^1-THC according to m.s. and t.l.c. (diethyl ether–light petroleum 1:19, developed three times). No hydrolysis was detectable after treatment with sulphatase type III or alkaline ethanol. On g.l.c. the silyl derivative of the methyl ester was reasonably stable in contrast to the methyl ester itself, which decomposed to yield Δ^1-THC.

Peak 8 contained the methyl esters of mainly two metabolites. The most polar (VIII, 2·3%, R_F = 0·12, Rt = 2·15 min) was identical to 2″-hydroxy-3″, 4″, 5″-trisnor-Δ^1-THC-7-oic acid (Nordqvist et al 1979) according to m.s. and p.m.r.

The methyl ester of the least polar metabolite from peak 8 (IX, 0·2%, R_F = 0·41, Rt = 1·95 min) exhibited after silylation the m.s. [*m/e* 476 (M^+, 42%), 461 (17), 417 (7), 403 (7), 386 (84), 371 (100), 345 (42), 305 (26)] which was in agreement with that of 6β-hydroxy-3″,4″,5″-trisnor-Δ^1-THC-2″- oic acid (Nordqvist et al 1979).

CONCLUSIONS

Early in vivo investigations indicated an extensive metabolism of Δ^1-THC in the rabbit leading to polar compounds such as carboxylic acids and conjugates (Agurell et al 1970, 1972; Burstein et al 1972).

Allylic hydroxylation of Δ^1-THC at C-7 is as reported earlier (Nordqvist et al 1979) a major metabolic pathway in the rabbit. In vivo this initial step is followed by oxidation to 7-oic acids. A predominant portion of the identified metabolites of Δ^1-THC in the rabbit (16·5% of urinary metabolites) carries a carboxyl group on C-1. The next carbon in preference of oxidation is C-3″. The major metabolite was shown to be a dicarboxylic acid with both C-7 and C-3″ oxidized, 4″,5″-bisnor-Δ^1-THC-7,3″-dioic acid (5·8%; Nordqvist et al 1979). Other dicarboxylic acids were also present in high amounts. Δ^1-THC-7,5″-dioic acid was isolated both from the 'ammonia' and the 'methanol fraction' (1·5%). Acids with pentyl side chains were eluted in the 'methanol fraction' and identified as 4″-hydroxy-Δ^1-THC-7-oic acid (I), 3″-hydroxy-Δ^1-THC-7-oic acid (II) and 2″-hydroxy-Δ^1-THC-7-oic acid (VI). The last metabolite was earlier isolated from rabbit urine and characterized by Burstein et al (1972). They also reported the occurrence of 1″-hydroxy-Δ^1-THC-7-oic acid, which was not detected in this study. Harvey et al (1977a) more recently reported the occurrence of 2″- and

3″-hydroxy-Δ^1-THC-7-oic acid in the guinea-pig, mouse and rat. In addition, they detected 6α-hydroxylated Δ^1-THC-7-oic acids. Only Δ^1-THC-7-oic acids oxygenated in various positions of the side chains were identified in our study. The occurrence of 4″-hydroxy-Δ^1-THC-7-oic acid (I) has not been established in any other species but recent in vivo studies on cannabidiol (CBD) in the mouse (Martin et al 1977) and on cannabinol (CBN) in the rat (Yisak et al 1978) have revealed the formation of all the equivalent side-chain hydroxylated acids as found in this study (I, II, VI). Side-chain monocarboxylic acids with a chain length of 2 to 4 carbons have been reported as in vivo metabolites of Δ^1-THC in the liver of guinea-pig (Harvey et al 1977a). In the rabbit only the occurrence of 4″, 5″-bisnor-Δ^1-THC-3″-oic acid has been established so far (Martin et al 1976b). The primary neutral side-chain hydroxylated metabolites of Δ^1-THC are of considerable interest because of their presumed psychotomimetic activity (Agurell et al 1976). In vitro studies have shown C-4″ to be the most favoured carbon for side-chain hydroxylation of Δ^1-THC in the dog (Widman et al 1975), of CBD in the rat (Martin et al 1976a,c) and of CBN in the rat (Widman et al 1975b; Fonseka & Widman 1977) and most significantly in the rabbit (Widman et al 1975b).

2″-Hydroxy-3″,4″,5″-trisnor-Δ^1-THC-7-oic acid (VIII) was the major metabolite in the 'methanol fraction. This unique metabolite carrying a shortened side chain with a terminal hydroxy function was the second most abundant identified metabolite of Δ^1-THC excreted in rabbit urine (totally 2·6% of urinary metabolites). Its formation can not easily be explained, but its presence as a chemical artefact should be ruled out because of the mild separation systems used. It might, however, originate by microbial reduction of an intermediate metabolite in the gut of the rabbit.

Δ^1-THC was partly excreted as 6β-and/or 7-hydroxylated side-chain acids in the rabbit urine. From the 'methanol fraction' we isolated the methyl ester of a new metabolite of Δ^1-THC, 7-hydroxy-4″, 5″-bisnor-Δ^1-THC-3″-oic acid (IV), a tentative precursor of the major metabolite 4″, 5″-bisnor-Δ^1-THC-7, 3″-dioic acid. It might also be oxidized to the earlier identified 7-hydroxy-2″, 3″, 4″, 5″-tetranor-Δ^1-THC-1″-oic acid by β-oxidation. Martin et al (1977) have reported the occurrence of 6-oxygenated and 7-hydroxylated side-chain monocarboxylic metabolites of CBD in the mouse liver, among them the equivalent acids with a side chain of one and three carbons, respectively, and hydroxylated at C-7.

The presence of the THC-glucuronide in the ether extract may indicate a higher amount of it in the residual aqueous phase, but according to earlier studies on rabbit urine it is a minor constituent (Agurell et al 1970). It has been detected as a very minor metabolite in mouse liver after i.p. administration of Δ^1-THC (Harvey et al 1977c) in contrast to the corresponding glucuronides of CBD and CBN, which were formed as major metabolites. Experiments in man suggest that detoxification via conjugation is more prevalent after Δ^1-THC than after CBD or CBN administration (Wall et al 1976), but it does not seem to be a major route.

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