with the homologous antigen by IgM addition to soluble complexes of gs-1 antibody and antigen in antibody excess if we assumed that there was perhaps only one gs-3 site per antigen molecule, this cannot explain the heterologous gs-3 reaction. Here we must assume that both fractions have specific combining ability with antigen. Whether or not each fraction is specific for a distinct site or both have the same specificity can be decided by cross-absorption experiments.

STEPHEN OROSZLAN
DAVID BOVA
ROBERT TONI, RAYMOND V. GILDEN
Flow Laboratories,
Rockville, Maryland 20852

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13. Dr. W. Parks, National Cancer Institute, has provided us with a goat antiserum to FeLV which in his laboratory was found to contain gs-3 antibodies in the IgG fraction. We have confirmed this observation.
14. Supported by contract NIH-NCI-E-71-2097 from the Special Virus Cancer Program, National Cancer Institute, Bethesda, Maryland.

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Isolation and Characterization of Two Major Urinary Metabolites of Δ^1-Tetrahydrocannabinol

Abstract. *Two of the major metabolites which appear in rabbit urine after the administration of Δ^1-tetrahydrocannabinol have been isolated and their structures have been tentatively established. The available evidence indicates that they are 7-carboxy-Δ^1-tetrahydrocannabinols with an additional hydroxyl group on the side chain. The substances occur both free and as conjugates.*

While a good deal is known about the metabolism in vitro of the important cannabinoids (*1–4*), relatively little data has been reported on the metabolic fates of these substances in vivo (*5–7*). We have shown (*8*) that Δ^6-tetrahydrocannabinol (THC) injected into rabbits gives rise to a very small amount of 7-hydroxy-Δ^6-THC in the urine. Lemberger *et al.* (*7*) reported results which suggest that 7-hydroxy-Δ^1-THC occurs in the urine and feces of man after the administration of Δ^1-THC. Many of the above-mentioned studies also showed that most of the THC is metabolized to rather polar materials, with no unchanged drug being excreted. In addition, Agurell and his co-workers (*5*) observed that a large proportion of the urinary metabolites in the rabbit were acidic in nature. We have succeeded in isolating and identifying two of the principal metabolites of Δ^1-THC and now report the results of our work.

We administered ^{14}C-Δ^1-THC (*9*) to adult female New Zealand white rabbits by either a subcutaneous or intravenous route in propylene glycol–water mixtures. The animals were kept in metabolic cages, and urine was collected and frozen every 24 hours for a suitable period. The urine was pooled and acidified to *p*H 3.5. The radioactivity was extracted from the pooled samples via a column of XAD-2 resin (*10*). This treatment allowed the metabolites to be obtained in a concentrated form, free of inorganic salts and other highly polar materials.

The extract was then partitioned between water and chloroform. Only about 20 percent of the radioactivity was found in the organic phase; this chloroform-soluble material was further fractionated by chromatography on a Sephadex G-15 column, and the radioactivity was eluted in a broad band. Most of the nonradioactive material was concentrated in the early fractions. The later fractions, comprising about one-half of the radioactivity, were then combined and fractionated on a DEAE (diethylaminoethyl)-Sephadex column (Fig. 1). The major peak (fractions 150 to 165) was then methylated (*11*) and investigated further.

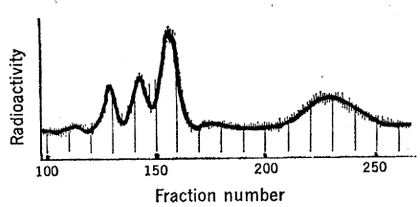


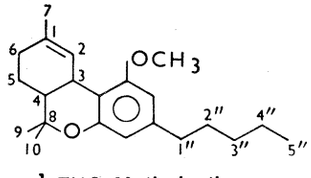
Fig. 1. The elution pattern of the free metabolites when chromatographed on a DEAE-Sephadex column with a NaCl gradient. The radioactivity of the column effluent was monitored by liquid scintillation counting with a Packard flow cell containing anthracene.

A final purification by thin-layer chromatography gave two barely separated zones which were located by radioautography. Most of the radioactivity was contained in these two areas, and their specific activities were close to that of the Δ^1-THC injected into the rabbits. Low-resolution mass spectrometry gave a molecular ion peak at 388 for both metabolites (*12*). This suggests isomeric methyl ester–methyl ether derivatives with a formula of $C_{23}H_{32}O_5$. The principal ions in the high mass end of the spectra of the methylated substances were: metabolite **1** [*m/e* 388 (relative intensity 58), 373 (53), 370 (17), 355 (21), 329 (100), 316 (12), 311 (21)] and metabolite **2** [388 (46), 373 (35), 370 (5), 329 (61), 316 (100), 311 (2)].

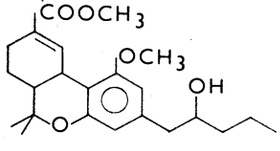
The water-soluble metabolites remaining after the chloroform partitioning were next isolated by the following procedure. Alkaline hydrolysis was used to liberate the metabolite from what appeared to be a conjugate with an amino acid. We have not determined the optimum conditions for this reaction, but 2 hours in a refluxing methanol–0.2*N* NaOH solution led to extensive hydrolysis. The hydrolysis mixture was then acidified to *p*H 3.5 and extracted with chloroform; 56 percent of the radioactivity could now be removed from the aqueous phase. The chloroform extract was then partitioned with dilute sodium bicarbonate to separate acidic materials. About 82 percent of the radioactivity was found in the bicarbonate solution, which was then acidified and chloroform extracted. All of the radioactive material soluble in bicarbonate was found in the chloroform phase. These partitioning steps resulted in a large increase in specific activity, so that after methylation (*11*) thin-layer chromatography could be utilized for the final purification. As

was described above for the unconjugated metabolites, two major, closely running zones of radioactivity were observed by radioautography. The overall recovery of radioactivity from the urine just prior to thin-layer chromatography was about 15 to 20 percent, and most of this was concentrated in the zones containing the two metabolites that were roughly equivalent. The low-resolution mass spectrum of each substance gave a molecular ion of 388, and the fragmentation patterns compared well with each of the respective isomers isolated from the chloroform extract.

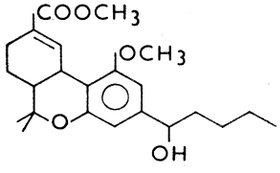
A tentative assignment of positions for the carboxyl and hydroxyl functions of each metabolite could be made from their nuclear magnetic resonance spectra (*13*), provided that no rearrangement of the carbon skeleton had occurred (Fig. 2). The signal at 1.64 ppm, which was seen in Δ^1-THC methyl ether because of the 7-methyl group, was absent from the spectra of both metabolites, an indication of metabolism at this position. This was supported by a downfield shift of the vinyl proton signal in both metabolites of about 1.5 ppm when again compared with Δ^1-THC methyl ether. The size of the shift was considerably larger than was reported for 7-hydroxy-Δ^1-THC (*2*), suggesting that the carboxylic group is located at position 7. The metabolites each showed a triplet ($J = 6$ hz) at 0.85 to 0.87 ppm for the side chain methyl group, thus eliminating this and the 4″-position as points for oxygenation. The gem-dimethyl group at position 8 was also eliminated since a strong signal at 1.19 to 1.20 ppm was present in each spectrum which could be assigned to these methyls.



Δ^1-THC Methyl ether



Methylated metabolite 1



Methylated metabolite 2

Fig. 2. The structures of derivatives of two major metabolites obtained from rabbit urine after administration of Δ^1-THC. The methyl ether–methyl ester of **1** is slightly less mobile than **2** on silica gel thin-layer chromatography in an acetone-hexane (2 : 3) system.

The phenolic methoxyl group and the methyl ester signal both appeared as sharp singlets; the former was close to that for Δ^1-THC methyl ether, suggesting that no changes in the pattern of aromatic substitution had occurred. In both of the metabolites the aromatic hydrogens gave superimposed singlets strong enough to account for both protons. The tertiary carbons at 3 and 4 could be discarded as possible points of hydroxylation since each metabolite was readily acylated by acetic anhydride in pyridine to give a monoacetate ($m/e = 430$). Consideration of all of the above data led us to conclude that the compounds we had isolated were derivatives of 7-carboxy-Δ^1-THC with an additional hydroxyl group located on the side chain on carbons 1″ through 3″.

From the nuclear magnetic resonance data we can also say with reasonable certainty that one of the metabolites has a hydroxy function at 1″. This is due to the complete absence of any signal in the 2.4 ppm region such as the triplet at 2.44 ppm ($J = 7.5$ hz) shown by Δ^1-THC methyl ether. This isomer was also the more mobile one on silica gel thin-layer chromatography (acetone-hexane, 1 : 4). By contrast, the second metabolite exhibited a doublet at 2.48 ppm ($J = 8$ hz), which suggests hydroxylation at 2″ for this less mobile isomer.

The presence of a 7-carboxyl function in both metabolites is not entirely unexpected since hydroxylation of the 7 position is a well-established process. The further transformation to an acid likely involved an aldehyde (with or without the side chain hydroxyl) as an intermediate. Such an aldehyde may well have an important role in the biological activity of Δ^1-THC.

Hydroxylation of the side chain in the cannabinoids is not unprecedented. Maynard *et al.* (*4*) have shown that dog liver microsomes incubated with Δ^6-THC give rise to 1″-hydroxy- and 3″-hydroxy-Δ^6-THC. The lack of 7-hydroxylation is exceptional since this reaction has been demonstrated in liver preparations from both the rat and the rabbit. Nilsson *et al.* (*14*) have reported that cannabidiol is monohydroxylated at the 1″-position in vitro, and finally Wall (*3*) has given tentative evidence that cannabinol gives rise to 2″-hydroxylation when incubated with rat liver homogenate. A metabolic study in man has been initiated in our laboratory where tritiated Δ^1-THC was administered sublingually. The preliminary results indicate that the urinary metabolites are similar to those we report for the rabbit.

SUMNER BURSTEIN
JACK ROSENFELD
THOMAS WITTSTRUCK
Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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