then rinsed and processed for electron microscopy. Cationic ferritin is seen to bind to the membranes (Fig. 4), indicating the intactness of the negatively charged surface coat. The surface coat is an integral part of the membrane and has been recognized as important in the determination of specific functional properties of the cell (*3, 8*).

A number of enzymatic markers were measured in the cell homogenate and in the membrane preparation. The cell homogenate is prepared by washing two culture dishes twice with homogenizing solutions [sucrose, 250 m*M*; histidine, 30 m*M*; EDTA, 5 m*M*; sodium deoxycholate, 0.01 percent (weight to volume); and tris buffer, 5 m*M* (*p*H 6.8)]. The cells are scraped and homogenized in 2.0 ml of homogenizing solution with ten strokes of a Teflon-glass Elvehjem-Potter homogenizer. Protein is analyzed by the method of Lowry *et al.* (*9*), Na^+,K^+-adenosinetriphosphatase according to Philipson and Edelman (*10*), and succinic dehydrogenase (SDH) and 5′-nucleotidase according to Wong and Zull (*11*).

Membrane enzymes are assayed by adding the reagents directly to the culture dishes on which the membranes were prepared and incubating at 37°C for 30 minutes. Reagents are then poured from the plates and assayed for reaction products. To determine Na^+,K^+-adenosinetriphosphatase activity this procedure is first done with 20 m*M* KCl in the reaction medium; it is then repeated with the same dish but in the absence of KCl, and the difference between the results of the two assays is taken as the enzyme activity.

To determine protein, the membranes on the plates are washed with distilled H_2O; 6 ml of H_2O is then added and the H_2O is frozen. The ice is allowed to thaw slightly and the disk of ice is rubbed vigorously against the bottom of the dish. This leaves the membranes floating as the ice melts. The H_2O and membranes are poured into a centrifuge tube, the membranes are precipitated with 10 percent trichloracetic acid and spun down, and protein is determined (*3, 8*). Protein and enzyme activities are shown in Table 1.

The membrane markers are increased 7- to 15-fold in specific activity. The mitochondrial marker (SDH) is undetectable, and there is more than 100-fold reduction in protein in the course of preparation. To further document the small amount of intracellular residuum in the membrane preparation, cultures are grown on scintillation disks. The cultures are labeled with ^{42}K for 40 to 60 minutes, the disks are inserted into a flow cell, and isotopic washout is continued for 10 minutes, at which time all the remaining ^{42}K is intracellular (*2, 3*). The ^{42}K activity is recorded, the disks are rapidly removed from the flow cell, and membranes are prepared. The disks, with attached membranes, are then reinserted in the flow cell and counted. The residual activity, which represents retained intracellular material, in six preparations was 0.85 ± 0.49 percent—that is 99 percent of the intracellular material labeled by ^{42}K was eliminated by the procedure. The reverse procedure—labeling the membrane of the cells by using a lactoperoxidase iodination technique (*12*)—was not successful because of heavy nonspecific binding of the radioactive iodine to the plastic disks. This obscured the signal from the membranes.

Although by DePierre and Karnovsky's definition (*13*) the technique applies the preparative rather than the analytic approach, it is unique in a number of important respects. The cells are never exposed to any chemical agents that might modify the basic membrane characteristics. Exposure to a critically oriented high-velocity N_2 jet disrupts and fractionates the tissue in a single operation that requires less than 1 second. The membranes remain in a sheetlike configuration and adhere to the surface on which the cells were grown. They are amenable to enzymatic, ionic binding, histochemical, and ultrastructural studies. Such studies can be preceded by a variety of studies of the same cells before isolation of their membranes. The technique should be applicable to any tissue grown in monolayer culture.

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Identification of a *C*-Glucuronide of Δ^6-Tetrahydrocannabinol as a Mouse Liver Conjugate in vivo

Abstract. *Δ^6-Tetrahydrocannabinol-C-4′-glucuronide was found in the livers of mice that had been administered Δ^6-tetrahydrocannabinol. Thus, C-glucuronidation of a compound that contains a free hydroxyl group has been demonstrated in vivo.*

Glucuronidation of foreign compounds is a major metabolic pathway in mammals. Although *O*-, *N*-, and *S*-glucuronidation are well known (*1*), to our knowledge *C*-glucuronidation in vivo has been observed only in a group of pyrazolidine-containing drugs that have a strongly acidic hydrogen on a carbon atom but no hydroxyl, amino, or thiol groups (*2*).

In several chemical reactions, including glucosidation, the C-4′ aromatic position in some cannabinoids is substituted in preference to the free phenolic group (*3*). On this basis we assumed (*4*) that *C*-glucuronidation could take place on that carbon atom. We found that enzymatic conjugation of Δ^6-tetrahydrocannabinol (Δ^6-THC) (**1**) with uridine 5′-diphosphoglucuronic acid in the presence of UDP-glucuronyltransferase led to *C*-glucuronides (*4*). The identification was made by comparison with synthetic substances.

It seemed of importance to determine whether *C*-glucuronidation of Δ^6-THC could also take place in vivo. We report here that Δ^6-THC does undergo *C*-glucuronidation in vivo.

Δ^6-Tetrahydrocannabinol (50 mg) was suspended in 1 ml of a 5 percent solution of Tween 80 in isotonic saline solution. It was administered (100 μl per mouse; 140 mg/kg per dose) intraperitoneally to ten male mice (~ 35 g each) 26 and 2 hours before the animals were killed. The livers (17 g) were removed and were homogenized in a glass homogenizer for 3 minutes in 30 ml of saline solution (*p*H 7). The homogenate was extracted with

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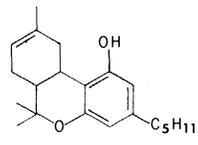
ethyl acetate (6 × 50 ml) and the emulsion obtained was separated by centrifugation. The extract was dried over sodium sulfate and evaporated. It was then treated with diazomethane, acetylated with acetic anhydride (5 ml) and pyridine (5 ml), and separated on silica gel preparative plates (Merck, Darmstadt; eluent, 30 percent ether in petroleum ether). The zone corresponding to that of the synthetic (*4*) Δ^6-THC-C-4′-glucuronide methyl ester tetraacetate (**2**) was extracted with ether and the solution was injected into a gas chromatograph–mass spectrometer (*5*). A major peak with a retention time equivalent to that of synthetic **2** was observed on gas chromatography. The mass spectrum at this peak contained all the significant mass ions exhibited by synthetic **2** under identical conditions (Table 1). No major ion peaks in addition to those due to the *C*-glucuronide derivative (**2**) were observed above a mass-to-charge ratio (*m/e*) of 350. Of particular interest is the molecular ion of compound **2**, *m/e* 672, which is 42 mass units higher than the molecular ion of Δ^6-THC-*O*-glucuronide methyl ester triacetate (**3**).

A mass fragmentographic determination (selected ion monitoring) of the in vivo *C*-glucuronide derivative (**2**) at mass ions *m/e* 672, 630, and 511 indicated a ratio of mass ion intensities close to that found in synthetic **2** (Table 2). This method is regarded as specific because of the choice of specific mass ions and because the signals due to these ions reach their maximum values simultaneously at the expected retention time (*6*).

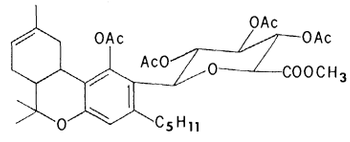
These results clearly indicate the formation of Δ^6-THC-C-4′-glucuronide from Δ^6-THC in mouse liver. In humans and other mammals cannabinoids are excreted after extensive initial oxidative metabolism, in part as water-soluble conjugates (*7, 8*). Until recently, little was known about the nature of these conjugates, although many reports on cannabinoid metabolism indicated that the extracts were initially treated with glucuronidase, sulfatase, or acid or base. A more quantitative investigation was undertaken by Widman *et al.* (*9*), who found that in a cannulated rat about 60 percent of the biliary excretion of Δ^1-THC was in the form of water-soluble conjugates. No sulfates were present and only about 30 percent of these cannabinoids were released by glucuronidase treatment. Alkaline hydrolysis of the remaining material released a similar amount of mainly neutral cannabinoids (*9*).

Harvey *et al.* (*10*), identified *O*-glucuronides of several cannabinoids as liver conjugates in mice in vivo. Only trace amounts of such conjugates of Δ^6-THC were detected. However, these conjugates were not the only type of water-soluble metabolites present (*11*).

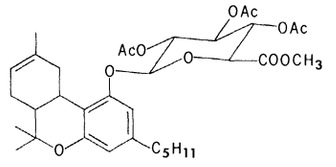
How widespread is *C*-glucuronidation in nature? We believe that it is not as rare as results up to now have indicated. Most *O*-glucuronides have been identified by their enzymatic hydrolysis with glucuronidase. As this enzyme presumably does not attack C-C bonds, *C*-glucuronides would not have been identified. Numerous plants are known to produce *C*-glycosides, particularly of phenols (*12*). It is reasonable to assume that animals can metabolize foreign compounds by a similar pathway. As phenols are consumed by humans, both in food and as drugs, a *C*-glucuronidation excretion route may be of general importance.



1



2



3

Table 1. Mass ions of synthetic and metabolic Δ^6-THC-C-4′-glucuronide methyl ester tetraacetate (**2**) as a percentage of *m/e* 630.

Mass ion (*m/e*)	Synthetic **2**	Metabolic **2**
672	22	25
630	100	100
612	11	12
547	11	12
511	37	37
493	14	12
451	30	50
450	28	45
449	11	10
409	22	35
367*	78	N.D.†
349	60	40

*Because of a typographical error, the peak at *m/e* 367 was given as *m/e* 366 in our synthetic paper (*4*). †Not determined because of a very strong background effect.

Table 2. Multiple ion detection in synthetic and metabolic Δ^6-THC-C-4′-glucuronide methyl ester tetraacetate (**2**), expressed as a percentage of *m/e* 630.

Mass ion (*m/e*)	Synthetic **2**	Metabolic **2**
672	33	24
630	100	100
511	30	29

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5. The instrument used was LKB-2091. For gas chromatography we employed a 6 foot by 1/8 inch glass column packed with 3 percent SE-30 on 100–120 mesh Gas Chrom Q. Helium at 25 ml/min was used as the carrier gas, and the column oven temperature was programmed at 5°C/min from 250° to 300°C. The following mass spectrometric operating conditions were employed: separator, 250°C; ion source, 240°C; electron energy, 70 eV; and accelerating voltage, 3500 V. To obtain the authentic mass spectrum at a gas chromatographic peak, the background mass spectrum (taken at the base of the peak) was subtracted from the mass spectrum taken at the top of the peak.
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