Ichikawa and Yamano [12] who estimated the hemoprotein content by subtracting from the total protoheme content that contributed by cytochrome b_5 and P_{450}.

The catecholamine present in the microsomal fraction (Table 2) indicated contamination by intact chromaffin granules; their occurrence, along with disrupted chromaffin granules which lost their amine content during fractionation, might have partially contributed to the level of Cyt b_{561} found in the microsomal preparation used in these studies.

It is our estimate, however, that contamination by chromaffin granules, both intact and disrupted, never exceeded 25 per cent of the total protein in the microsomal fraction.

It is thus clear that Cyt b_{561} is a constituent of both adrenomedullary microsomes and chromaffin granules, a reflection of the origin of chromaffin granules in the endoplasmic reticulum from which microsomes are derived [14].

Since the studies of Flatmark *et al.* [15] revealed the presence of Cyt b_{561} in chromaffin granules as well as in the norepinephrine-storing granules found in adrenergic nerves, it was considered of interest to investigate the occurrence of the cytochrome in the amine-storing organelles of the rabbit blood platelets which have a mechanism for amine uptake strikingly similar to the one of adrenergic nerve organelles.

However, Cyt b_{561} could not be detected by light absorption studies on suspensions of serotonin (5-HT) organelles isolated from rabbit blood platelets, even when high concentrations of organelle membrane protein were used. In view of the well known similarities in amine uptake mechanism of chromaffin granules and rabbit blood platelet organelles, the presence of Cyt b_{561} in the latter organelles would have provided some evidence for the involvement of Cyt b_{561} in the amine uptake process. The negative finding is inconsistent with a participation of Cyt b_{561} in this process; however, it is possible that in the platelet organelle another mechanism might subserve this function.

In analogy with the classic interactions between drugs and the microsomal cytochrome P_{450}, the ability of various inhibitors of amine uptake in chromaffin granules to alter the spectral properties of Cyt b_{561} was investigated.

Since changes of the light absorption spectra (recorded between 350 and 600 nm) of a suspension of chromaffin granule membranes could not be detected by adding reserpine, guanethidine or *N*-ethylmaleimide in concentrations varying from 10^{-6} M to 10^{-3} M, we conclude that the interactions between granule membranes and these drugs are not amenable to the same type of spectral analysis applied to microsomal cytochrome P_{450}.

The data presented in this paper demonstrate that membranes of bovine chromaffin granules possess enzymes which may be involved in electron transport. The electron transfer activity of Cyt b_{561} does not appear to be integrated with the biosynthetic step of catecholamines occurring at the membrane level, i.e. the hydroxylation of dopamine by DBO.

Thus the physiological role of the electron transfer chain in the chromaffin granule membrane remains, at present, obscure.

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The biotransformation of 1-hexadecene to carcinogenic 1,2-epoxyhexadecane by hepatic microsomes

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n-1-Olefins with eight, ten or twelve carbon atoms are common sources for plasticizers and with fourteen, sixteen or eighteen carbon atoms for sodium alkyl sulfate detergents. One of the major metabolic pathways of these compounds in mammalian liver is now assumed to be oxidation to the corresponding epoxides by microsomal epoxidase followed by conversion of the products to glycols by hepatic microsomal epoxide hydrolase (epoxide hydrase) [1–3] or to α-hydroxyalkyl-*S*-glutathione conjugates by soluble exposide-*S*-glutathione transferase [3]. However, except for a few cases such as epoxides of polychlorinated cyclodiene insecticides, heptachlor and related toxicants [4, 5], cyclohexene [6], indene [6], styrene [6], and stilbene [7], epoxides formed *in vitro* are labile to the hydrolase and are rapidly converted to glycols, so that it is very difficult to detect them without using epoxide hydrolase inhibitors, such as various epoxides [1–3] and certain aziridines [8]. The initial evidence for epoxide intermediates in the microsomal metabolism of ole-

fins to the corresponding glycols was presented by Watabe and Maynert [1] using other olefin oxides as epoxide hydrolase inhibitors and by Jerina *et al.* [9] using arene with isotope-labeled naphthalene and non-labeled naphthalene oxide as an epoxide pool for trapping the biologically formed radioactive oxide.

Olefin oxides have recently been used to trap epoxy intermediates in the microsomal metabolism of carcinogenic polycyclic aromatic hydrocarbons to K-region dihydrodiols, e.g. cyclohexene oxide for benz[a,h]anthracene oxide [10] and 7-methylbenz[a]anthracene oxide [11] and styrene oxide for benzo[a]pyrene oxide [12]. These arene oxides which are suggested to be the active forms of carcinogens are covalently bound to nucleic acids and proteins in cells in culture [13, 14] and produce malignant transformations of cells [10, 15, 16]. Similar evidence for covalent bonding with DNA has been obtained with propylene oxide [17].

Of the *n*-1-olefin oxides, 1,2-epoxyhexadecane has been shown to cause skin carcinoma in the mouse [18]. Very recently, their inhibitory effects on epoxide hydrolase activity and their susceptibility to enzymatic hydrolysis to glycols have been investigated by Watabe and Akamatsu [19], who found that 1,2-epoxydecane is the most potent inhibitor and the poorest substrate for the hydrolase. The present paper deals with the formation of the carcinogenic olefin oxide, 1,2-epoxyhexadecane, as a labile intermediate in the hepatic microsomal metabolism of 1-hexadecene to 1,2-dihydroxyhexadecane.

1-Hexadecene dissolved in acetone and suspended in 0·1 M phosphate buffer, pH 7·4, was incubated with rabbit liver microsomes in the presence of an NADPH-generating system. The reaction was terminated by the addition of sodium hydroxide, and the mixture extracted with ether containing 1,2-epoxytetradecane or 1,2-dihydroxytetradecane as the internal reference for the quantitative determination of metabolites. The ethereal extract was subjected to preparative silica gel thin-layer chromatography developed in benzene–acetone (5 : 1). Authentic 1,2-dihydroxytetradecane and 1,2-dihydroxyhexadecane or 1,2-epoxytetradecane and 1,2-epoxyhexadecane co-chromatographed as single bands at R_F 0·2 or 0·7, respectively. Each zone of the chromatogram was eluted with ethanol. The eluate from the R_F 0·2 zone was trimethylsilylated after the evaporation of the solvent and analyzed by gas-chromatography–mass spectroscopy. Gas-chromatographic data (retention time: 7·4 min on a 2% OV-17 column at 210°) and the mass spectrum were identical with those of authentic 1,2-dihydroxyhexadecane di-trimethylsilyl ether; a molecular ion peak with *m/e* 402 appeared together with fragment ion peaks characteristic of the glycol-TMS derivative at *m/e* 103 (strong intensity, TMS—O—CH_2^+) and 299 (strong intensity, TMS—O—CH^+—$(CH_2)_{13}CH_3$). The eluate from R_F 0·7 zone was concentrated and directly analyzed by gas-chromatography–mass spectroscopy. Gas-chromatographic data (retention time: 4·5 min under the above conditions) and the mass spectrum obtained were identical with those of authentic 1,2-epoxyhexadecane; a molecular ion peak with *m/e* 240 appeared together with fragment ion peaks with *m/e* 57 (strong intensity, $CH_2\langle O \rangle CH—CH_2^+$) and 43 ($CH_2\langle O \rangle CH^+$).

The formation of the epoxide was observed only when the olefin was incubated in the presence of the epoxide hydrolase inhibitor 1,2-epoxydecane (10 mM) (Table 1). These results indicate that 1-hexadecene is metabolized to 1,2-dihydroxyhexadecane via 1,2-epoxyhexadecane since enzymatic conversion of the epoxide to the glycol by rabbit liver microsomes has previously been reported [19]. Neither the glycol nor the epoxide was formed when 1-hexadecene was incubated with a boiled microsomal preparation.

Either the inhibitor or its enzymatically hydrolyzed product, 1,2-dihydroxydecane, may have an inhibitory effect on microsomal epoxidation of 1-hexadecene since addition of the inhibitor to the incubation system resulted in the formation of smaller amounts of 1,2-epoxyhexadecane than those of the corresponding glycol formed in the absence of the inhibitor. Gas-chromatographic analysis indicated that 55 per cent of the inhibitor (10 mM) was hydrolyzed to the glycol by the microsomes during the 30-min incubation. Both 1,2-epoxydecane and 1,2-dihydroxydecane had inhibitory effects on microsomal epoxidase activity which was assayed as previously reported by using heptachlor (1 mM) as the substrate [8], the enzymatically formed epoxide of which is completely stable in the microsomal system [4, 5]. Microsomal heptachlor epoxide formation was decreased by 30 and 85 per cent by the addition of 1 and 10 mM of 1,2-epoxydecane, respectively, and by 12 and 95 per cent by 1 and 10 mM of the corresponding glycol, respectively. Microsomal epoxide hydrolase activity was not affected to any extent by 1,2-dihydroxydecane.

Table 1. Accumulation of the epoxy intermediate in the hepatic microsomal metabolism of 1-hexadecene by the inhibitory effect of 1,2-epoxydecane on epoxide hydrolase

Inhibitor (10 mM)	Metabolites formed (μg) 1,2-Epoxyhexadecane	1,2-Dihydroxyhexadecane
None	n.d.	16·1
Added	2·4	n.d.

1-Hexadecene (10 μmoles), dissolved in acetone (0·2 ml), was incubated at 37° for 30 min in air with rabbit liver microsomes (equivalent to 2 g of tissue) suspended in 0·1 M phosphate buffer, pH 7·4 (9·8 ml), containing NADP (4 μmoles), glucose 6-phosphate (50 μmoles), glucose 6-phosphate dehydrogenase (4 units), magnesium chloride (50 μmoles), and nicotinamide (50 μmoles). The inhibitor, 1,2-epoxydecane, was dissolved in the acetone together with the substrate. The reaction was terminated by the addition of 2 ml 5N NaOH, and the mixture extracted with 30 ml ether, containing 1,2-epoxy- and 1,2-dihydroxytetradecanes (2 μg and 20 μg, respectively) as internal references for gas-chromatographic analysis. After concentration of the extract, the residual solution was subjected to silica gel thin-layer chromatography (Wakogel as the adsorbent and benzene–acetone (5 : 1) as the developing solvent). Elution of epoxides and glycols from chromatograms was carried out separately with ethanol. After evaporation of the solvent the glycols were converted to acetonides by the previously reported method [19]. A 10% Apiezon L column (coated on Chromosorb W, 60–80 mesh, 180 cm × 4 mm) was used at 220° for the epoxides and at 230° for the glycol acetonides at a flow rate of 35 ml/min of nitrogen as the carrier gas. Under these conditions, 1,2-epoxydecane, 1,2-epoxytetradecane, and 1,2-epoxyhexadecane were eluted in less than 1·5 min, at 4·4 and 6·7 min, respectively, and acetonides of 1,2-dihydroxydecane, of 1,2-dihydroxytetradecane, and of 1,2-dihydroxyhexadecane in less than 1·5 min, at 4·6 and 9·0 min, respectively. n.d.: not detectable (less than 0·02 μg).

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Prostaglandins and cannabis—III. Inhibition of biosynthesis by essential oil components of marihuana

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In previous publications [1, 2], we reported that several cannabinoids were inhibitors *in vitro* of the conversion of precursors into prostaglandins E_1 and E_2 (PGE_1 and PGE_2). These findings have since been confirmed and extended by Crowshaw and Hardman [3], who also found that PGF production was stimulated by certain cannabinoids. Since this activity was not confined to Δ^1-tetrahydrocannabinols (THC), the major psychoactive principle of cannabis, we thought it worthwhile examining other fractions of the plant for possible effects on PG synthetase activity.

Marihuana contains an essential oil fraction, which can be obtained from it by steam distillation, which consists of a complex mixture of terpenes and other volatiles [4]. Neither Δ^1-THC nor any other cannabinoids are found in this fraction and, aside from odor, it is not believed to contribute to the properties of the drug. Nevertheless, we decided to examine the volatile fraction, since previous conclusions on its inactivity were based on gross pharmacological measurements [5].

A botanically characterized sample of 50 g marihuana* designated as "Mexican Variant" was subjected to steam distillation until 1·5 litres condensate was collected. This was extracted with ether (500 ml) and concentrated by evaporation of the solvent to yield 120 mg pungent oil. Gas–liquid chromatographic analysis (g.l.c.) showed that no Δ^1-THC or other cannabinoids were present in amounts that would interfere with the inhibitor assay.

The oil was then resolved into four crude fractions by chromatography on Silica gel plates.† Each of these was recovered and assayed for inhibitory activity. The conditions for the assay were the same as those previously

Table 1. Inhibition of prostaglandin E_1 biosynthesis by essential oil constituents of *Cannabis sativa* and related substances

Substance	Occurrence		ID_{50}* (μg/ml)
Fraction 1	*C. sativa*		2·3
Fraction 5	*C. sativa*		†
Eugenol	*C. sativa*	*M. fragrans*	5·6
Safrole		*M. fragrans*	47
Myristicin		*M. fragrans*	170
Methoxy eugenol		*M. fragrans*	2·6
Borneol	*C. sativa*		> 100
Fenchyl alcohol	*C. sativa*		> 100
Linalool	*C. sativa*	*M. fragrans*	NI‡
α-Terpineol	*C. sativa*	*M. fragrans*	NI
Limonene	*C. sativa*	*M. fragrans*	NI
p-Cymeme	*C. sativa*	*M. fragrans*	NI
Carvacrol			4·1
2-Cymidine	*C. sativa*		12·5
β-Caryophyllene	*C. sativa*		910
Δ^1-THC	*C. sativa*		100
Cannabigerol	*C. sativa*		95

* ID_{50} = dose which causes 50 per cent inhibition (see also Ref. 2).

† Fraction 5 was a mixture; therefore, an ID_{50} could not be calculated. However, it appears to be of the same order of activity as fraction 1.

‡ NI = non-inhibitory.

* The marihuana used in this study was kindly provided by Dr. Carlton E. Turner, University of Mississippi, under a program of the National Institute on Drug Abuse. The material was grown on campus and was about 18 weeks old when harvested. Thin-layer chromatographic assay was reported to show a 1·56% Δ^1-THC content.

† Thin-layer chromatography was done on 0·5 mm Merck EM Silica gel plates with fluorescence indicator. Hexane–ether (1:1) was used as the developing mixture, and the zones were visualized with a u.v. lamp. R_f values were: 0·3, 0·4, 0·5 and 0·8.

‡ High pressure liquid chromatography was done on a Waters liquid chromatograph using a 2 ft Corasil column with u.v. monitor (280 nm). The elution mixture was hexane–tetrahydrofuran (200:1) at 1·0 ml/min.