Biochemical Pharmacology, Vol. 29, pp. 1068–1071
Pergamon Press Ltd. 1980. Printed in Great Britain.

Metabolism of *d*-limonene by hepatic microsomes to non-mutagenic epoxides toward *Salmonella typhimurium*

(*Received* 24 *October* 1979; *accepted* 6 *December* 1979)

d-Limonene, a major component in many essential oils of *Citrus* fruits, has been demonstrated in man and in some other species of animals to be converted into two glycols, one of which is *d*-limonene-8,9-diol [2-(4′-methyl-3′-cyclohexene-1′-yl)-1,2-propanediol] as the major metabolite, and the other, *d*-limonene-1,2-diol[1-methyl-4-(1′-methylethenyl)-1,2-cyclohexanediol] as the minor one [1]. Both diols are reasonably supposed to arise from the corresponding 8,9-epoxide [1-methyl-4-(1′-methyl-1′,2′-epoxyethyl)-1-cyclohexene] and 1,2-epoxide [1-methyl-4-(1′-methylethenyl)-7-oxabicyclo(4.1.0)heptane] yielded from *d*-limonene by the catalytic action of hepatic microsomal P-450, as has already been established in the hepatic microsomal metabolism of a variety of olefins [2–8]. Attention has recently been focused on the genotoxic role of epoxides formed as obligatory or putative intermediates in the oxidative metabolism of naturally occurring ethylenic compounds, e.g. aflatoxins [9–11] and other mycotoxins [12,13], pyrrolizidine alkaloids [13,14] and safrole [15]. In connection with this, many synthetic ethylenic compounds, such as chlorinated ethylenes [16], *n*-1-hexadecene [17], and styrene [8,18], as well as aromatic hydrocarbons [19,20] have also been demonstrated to be metabolized by hepatic microsomal P-450 or P-448 to mutagenic or carcinogenic epoxides. We have had more chances to detect mutagenic compounds readily since the Ames method [21] using *Salmonella typhimurium* TA strains was introduced. Consequently, a large number of epoxides proved to be mutagenic toward these bacteria [22]. However, we have obtained still only poor evidence for the relationship between epoxide structures and their mutagenicities, although the work of Wade *et al.* [23] has very recently provided valuable information on this problem. In view of environmental toxicology of foods, therefore, that raises a fundamental question about the safety problem on *Citrus* fruits containing *d*-limonene, whether the predicted epoxy-precursors of the previously isolated urinary metabolites, *d*-limonene-1,2- and 8,9-diols, are mutagenic or not. In this communication, we wish to report the isolation of 1,2- and 8,9-epoxides of *d*-limonene and their conversion to the 1,2- and 8,9-diols by rat liver microsomes. Evidence will be provided that the epoxides are non-mutagenic toward several *Salmonella typhimurium* TA strains.

For the isolation and identification of the double bond-oxygenated metabolites of *d*-limonene in the liver, the terpene was incubated with microsomes prepared from

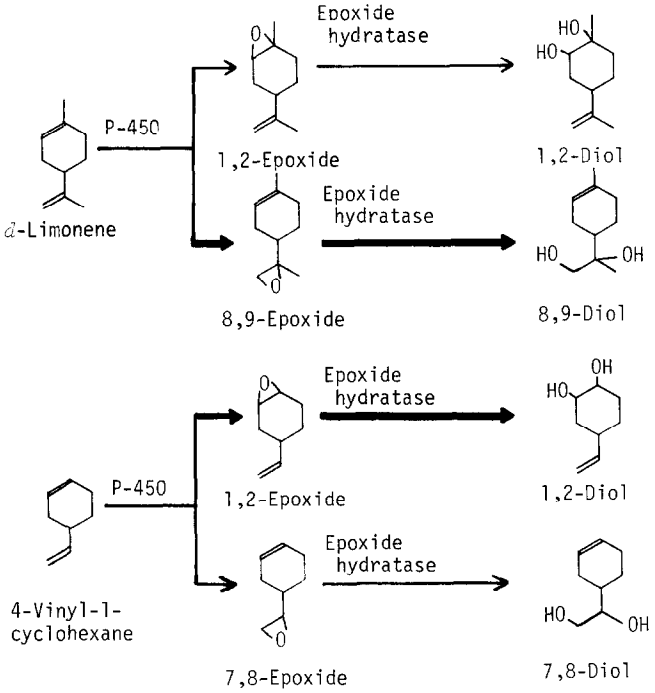
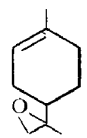
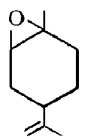
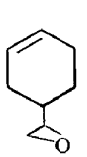
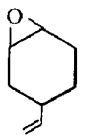
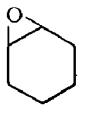
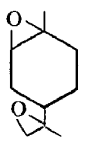
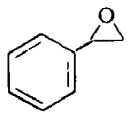


Fig. 1. Oxidation of *d*-limonene double bonds by rat liver microsomes. *d*-Limonene and 4-vinyl-1-cyclohexene (2 mM each) were incubated at 37° for 20 min with rat liver microsomes (7 mg protein/ml) in the presence of an NADPH-generating system consisting of NADP (0.5 mM), glucose 6-phosphate (5 mM), glucose 6-phosphate dehydrogenase (1 IU/ml), nicotinamide and magnesium chloride (5 mM each). The substrates were added as acetone (2%) solutions. TCPO (2 mM) was dissolved in this solution when added.

Table 1. Mutagenic activities of epoxides of *d*-limonene and olefins toward *Salmonella typhimurium* TA 100

Dose* (μmoles/plate)	His$^+$ revertant colonies/plate†									
	None								$CH_3(CH_2)_3CH(O)CH_2$	$CH_3(CH_2)_{13}CH(O)CH_2$
0	133									
0.05			64							
0.1		53	61	51	66	47		62	49	51
1.0		L‡	L	49	57	60	43	101	66	25
10.0				133	94	488	70	918	219	38
20.0				L	L		68		435	

* Solutions of the epoxides in dimethyl sulfoxide (100 μl each) were mixed with the bacterial overnight culture (0.1 ml, 2×10^9 cells/ml) suspended in molten top agar and 0.1 M phosphate buffer, pH 7.4, and poured onto Vogel-Bonner medium E agar plates.

† After the incubation of the plate for 48 hr at 37°, numbers of colonies were counted. Data are arithmetic mean values from at least three experiments.

‡ L represents lethal.

male Wistar rat liver. The reaction was terminated by the addition of sodium hydroxide, and the mixture extracted with *n*-pentane for the isolation of epoxides. Following saturation with sodium chloride, the residual aqueous phase was extracted with ethyl acetate for the isolation of diols. The extracting solvents, *n*-pentane and ethyl acetate, contained β-methyl styrene and biphenyl, respectively, as internal standards for subsequent analysis of metabolites by gas chromatography–mass spectrometry (g.c.–m.s.). The petroleum extract contained both position isomers of *d*-limonene epoxides which appeared at retention times of 7 and 9 min in the gas chromatogram obtained on a 2% OV-1 column (3 mm × 2 m; 90°; 36 ml/min He) and were assigned by their mass spectra as *d*-limonene 1,2-epoxide and 8,9-epoxide, respectively. The epoxides were identified with authentic specimens synthesized by the method of Carlson *et al.* [24], which were separated and isolated on an octadecylsilicone column in methanol–water (11:9) by high performance liquid chromatography (h.p.l.c.). Under the h.p.l.-chromatographic conditions used, *d*-limonene 1,2-epoxide and 8,9-epoxide appeared as sharp single peaks at retention times of 16 and 19 min, respectively. The solvent was evaporated very mildly from the ethyl acetate extract, and the residue obtained was trimethylsilylated with hexamethyldisilazane and trimethylsilyl chloride in dry pyridine. The trimethylsilylated extract was also analyzed by g.c.–m.s. under the above-mentioned conditions, except that the column temperature was raised to 110°. In the chromatogram, an intense single peak appeared at 22 min, which was classified as di-trimethylsilyl ether of *d*-limonene-8,9-diol by the mass spectrum and identified with the authentic specimen derived from *d*-limonene 8,9-epoxide by acid hydrolysis, followed by trimethylsilylation. The isomeric 1,2-diol which had been recognized as a minor metabolite *in vivo* [1] also appeared as a very weak peak at 10 min in the gas chromatogram. Rates of formation ($\times 10^{-2}$nmoles/mg microsomal protein/min) of four *d*-limonene metabolites were 13.3, 16.5, 0.5 and 36.1 for 1,2-epoxide, 8,9-epoxide, 1,2-diol and 8,9-diol, respectively. The intermediacy of the 1,2- and 8,9-epoxides in the microsomal transformation of *d*-limonene into the 1,2- and 8,9-diols was confirmed by the addition of the microsomal epoxide hydratase inhibitor, 3,3,3-trichloropropene 1,2-oxide (TCPO) to the incubation mixture, i.e. TCPO inhibited the formation of both diols to an undetectable extent by g.l.c. and accumulated the 1,2- and 8,9-epoxides in the biological reaction mixture.

The very low yield of *d*-limonene-1,2-diol in the microsomal metabolism of *d*-limonene was reasonably interpreted by the remarkable difference in rates of hydrolysis of the 1,2- and 8,9-epoxides by liver microsomal epoxide hydratase. Incubation of *d*-limonene 1,2- and 8,9-epoxides (2 mM each) with rat liver microsomes (0.075 mg protein/ml) in 0.1 M phosphate buffer, pH 7.4, without any fortifying agents yielded the corresponding diols. Rates of formation of the 1,2-diol and 8,9-diol were 0.6 and 75 nmoles/mg protein/min, respectively. The results are summarized in Fig. 1.

It is of interest that epoxidation of the *d*-limonene double bonds by hepatic microsomal P-450 occurs preferentially at the 8,9-position, although the 1,2-position is known to be epoxidized much more readily by chemical epoxidizing agents such as organic peracids [24]. This fact would be attributable to the steric hindrance effect of the 7-methyl group attached to the 1,2-double bond on the interaction of the *d*-limonene molecule with the active site of P-450. In order to confirm this assumption, racemic 4-vinyl-1-cyclohexene, whose 1,2-double bond is also known to be more susceptible to chemical epoxidation that its vinyl group, was used as the substrate for the microsomal epoxidation. After the incubation under the same conditions as used in the microsomal metabolism of *d*-limonene, followed by g.c.–m.s. of *n*-hexane and ethyl acetate extracts, the latter of which was trimethylsilylated before analysis, 4-vinyl-1-cyclohexene was found to yield 43.9×10^{-2} and 18.5×10^{-2} nmoles/mg protein/min of 4-vinylcyclohexane-1,2-diol and 4-(1′,2′-dihydroxyethyl)-1-cyclohexene, respectively. However, no detectable amount of its epoxides was found in the mixture (Fig. 1). Addition of TCPO (2 mM) completely inhibited the formation of both glycols and accumulated 4-vinyl-1-cyclohexene 1,2-epoxide and 4-(1′,2′-epoxyethyl)-1-cyclohexene. This indicates the glycols from 4-vinyl-1-cyclohexene by microsomes to arise from its epoxides as intermediate precursors.

The epoxides used as substrates were hydrolyzed by microsomal epoxide hydratase to the corresponding glycols: rates of hydrolysis were 11.6 and 144.5 nmoles/mg microsomal protein/min for 4-vinyl-1-cyclohexene 1,2-epoxide and 4-(1′,2′-epoxyethyl)-1-cyclohexene, respectively. Therefore, it appears that in the less hindered bifunctional olefin, 4-vinyl-1-cyclohexene, the more oxidizable double bond is the more susceptible to the enzymatic epoxidation. The fact that the epoxycyclohexane moieties in the epoxides of *d*-limonene and 4-vinyl-1-cyclohexene are less susceptible to enzymatic hydrolysis than their vinylidene epoxide ones is coincident with the previously established rule that ethylene oxides with more alkyl substituents undergo less enzymatic hydrolysis [5,6].

Mutagenicity of synthetic *d*-limonene 1,2- and 8,9-epoxides were examined in the absence of a rat liver 9000 *g* supernatant fraction by the method of McCann *et al.* [25] using *Salmonella typhimurium* TA 100, TA 98, TA 1535, TA 1537 and TA 1538. Both the 1,2-epoxide and 8,9-epoxide showed no mutagenic activity toward *S. typhimurium* TA 100 at any dose, but only cytotoxic effects at higher concentrations, whereas cyclohexene oxide, 1,2-epoxy-*n*-hexane and styrene oxide had mutagenic activities under these conditions (Table 1). 4-Vinyl-1-cyclohexene diepoxide, a potent carcinogen to mouse skin [26], was a weak mutagen only to strain TA 100. *n*-Hexadecene 1,2-epoxide, also a skin carcinogen [27], however, showed no mutagenic activity. 4-Vinyl-1-cyclohexene 1,2-epoxide and 4-(1′,2′-epoxyethyl)-1-cyclohexene were also non-mutagenic to this bacterial strain. None of these epoxides, however, showed any mutagenic activities toward *S. typhimurium* TA 98, TA 1535, TA 1537 and TA 1538 at doses of 0.1–20 μmoles/plate, although 0.025 μmoles/plate of benzo[*a*]pyrene 4,5-oxide induced the formation of 6064 His^+ revertant colonies/plate in strain TA 98 under the same conditions. These results indicate it very difficult to predict the bacterial mutagenicity of olefin oxides from their structures.

d-Limonene showed no mutagenic activity to any of the above-mentioned bacterial strains in the presence as well as in the absence of a 9000 *g* supernatant fraction of a liver homogenate from rats pretreated with PCB.

Laboratory of Drug Metabolism & Toxicology, Department of Hygienic Chemistry, Tokyo College of Pharmacy, Hachioji, Tokyo 192-03, *Japan*

TADASHI WATABE
AKIRA HIRATSUKA
MASAKAZU ISOBE
NAOKI OZAWA

REFERENCES

1. R. Kodama, K. Noda and H. Ide, *Xenobiotica* **4**, 85 (1974).
2. B. Davidow and J. L. Radomski, *J. Pharmac. exp. Ther.* **107**, 259 (1953).
3. K. C. Leibman and E. Ortiz, *Molec. Pharmac.* **4**, 201 (1968).
4. K. C. Leibman and E. Ortiz, *J. Pharmac. exp. Ther.* **173**, 242 (1970).

5. T. Watabe and E. W. Maynert, *Pharmacologists* **10**, 203 (1968).
6. E. W. Maynert, R. L. Foreman and T. Watabe, *J. biol. Chem.* **245**, 5234 (1970).
7. T. Watabe and K. Akamatsu, *Biochem. Pharmac.* **23**, 1079 (1974).
8. T. Watabe, M. Isobe, K. Yoshikawa and E. Takabatake, *J. Pharm.-Dyn.* **1**, 98 (1978).
9. J. A. Miller, *Cancer Res.* **30**, 559 (1970).
10. R. G. Croy, J. M. Essigmann, V. N. Reinhold and G. N. Wogan, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1745 (1978).
11. A. A. Stark, J. M. Essigmann, A. L. Demain, T. R. Skopek and G. N. Wogan, *Proc. natn. Acad. Sci. U.S.A.* **76**, 1343 (1979).
12. Y. Ueno, K. Kubota, T. Ito and Y. Nakamura, *Cancer Res.* **38**, 536 (1978).
13. R. Schoental, in *Chemical Carcinogens* (Ed. C. E. Searle), pp. 626–689. American Chemical Society, Washington (1976).
14. R. Schoental, *Nature, Lond.* **227**, 401 (1970).
15. P. Borchert, J. A. Miller, E. C. Miller and T. K. Shires, *Cancer Res.* **33**, 590 (1973).
16. H. Bartsch and R. Montesano, *Mutation Res.* **32**, 93 (1975).
17. T. Watabe and N. Yamada, *Biochem. Pharmac.* **24**, 1051 (1975).
18. T. Watabe, M. Isobe, K. Yoshikawa and E. Takabatake, *Scand. J. Work environ. Hlth* **4**, 142 (1978).
19. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20**, 165 (1974).
20. H. V. Gelboin and P. O. P. Ts'o (Eds.), in *Polycyclic Hydrocarbons and Cancer; Environment, Chemistry, and Metabolism*, Vol. 1. Academic Press, New York (1978).
21. B. N. Ames, F. D. Lee and W. E. Durston, *Proc. natn. Acad. Sci. U.S.A.* **70**, 782 (1973).
22. J. McCann, E. Choi, E. Yamasaki and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **72**, 979 (1975).
23. D. R. Wade, S. C. Airy and J. E. Sinsheimer, *Mutation Res.* **58**, 217 (1978).
24. R. G. Carlson, N. S. Behn and C. Cowles, *J. org. Chem.* **36**, 3832 (1971).
25. J. McCann, E. Choi, E. Yamasaki and B. N. Ames, *Proc. natn. Acad. Sci. U.S.A.* **72**, 5135 (1975).
26. B. L. Van Duuren, L. Langseth, L. Orris, E. D. Palmes and F. L. Schmitt, *J. natn. Cancer Inst.* **31**, 41 (1963).
27. B. L. Van Duuren, L. Langseth, B. M. Goldschmidt and L. Orris, *J. natn. Cancer. Inst.* **39**, 1217 (1967).

Biochemical Pharmacology, Vol. 29, pp. 1071–1073.

0006-2952/80/0401–1071 $02.00/0

β-Diethylaminoethyldiphenylpropylacetate (SKF 525-A) and 2,4-dichloro-6-phenylphenoxyethylamine·HBr (DPEA) inhibition of fatty acid conjugation to 11-hydroxy-Δ^9-tetrahydrocannabinol by the rat liver microsomal system*

(*Received* 2 *February* 1979; *accepted* 5 *November* 1979)

An *in vitro* rat liver coenzyme A fortified microsomal enzyme system that could conjugate certain long-chain fatty acids to 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC) was developed recently by our laboratory [1]. We [2] had previously identified these fatty acid conjugated cannabinoids from both *in vitro* and *in vivo* studies as primarily palmitic, stearic and lesser amounts of C_{18}-unsaturated fatty acid conjugates of Δ^9-tetrahydrocannabinol (Δ^9-THC) and Δ^8-tetrahydrocannabinol (Δ^8-THC). In earlier *in vivo* studies [3], we also found that these fatty acid conjugated cannabinoic metabolites comprised at least 80 per cent of the radioactive cannabinoids retained in the liver, spleen, fat and bone marrow of rats 15 days after an acute intravenous or chronic intraperitoneal injections of [^{14}C]-Δ^8-tetrahydrocannabinol or [^{14}C]-Δ^9-tetrahydrocannabinol. *In vivo* metabolites of cannabinol have also been identified as fatty acid conjugates [4]. Other studies [5, 6] have shown that cholesterol can be conjugated to fatty acids *in vitro* by a similar coenzyme A microsomal system. Thus, the same metabolic pathway in microsomes may be involved in the esterification of cholesterol and 11-hydroxy-Δ^9-THC.

The present studies evaluate the effects of β-diethylaminoethyldiphenylpropylacetate (SKF 525-A) and 2,4-dichloro-6-phenylphenoxyethylamine·HBr (DPEA) on the *in vitro* microsomal fatty acid conjugating system. These compounds are known inhibitors of the classical hepatic microsomal mixed-function oxidase system which is involved in the metabolism of a variety of drugs. For each evaluation, 1 ml of 0.1 M sodium phosphate buffer (pH 7.0), containing 2 μmoles coenzyme A, 10 μmoles ATP and 10 μmoles $MgCl_2 \cdot 6H_2O$, was added to a 16 × 125 mm glass test tube. To this was added 0.5 ml of twice washed microsomes (4 mg protein/ml), 1 μmole [^{3}H]-11-OH-Δ^9-THC in 20 μl ethanol, and 0.5 ml buffer containing either 2, 4 or 8 μmoles SKF 525-A or DPEA. The tubes were incubated in a 37° metabolic shaker for 1 hr and then lyophilized, extracted and evaluated for [^{3}H]-11-palmitoyloxy-Δ^9-THC ([^{3}H]-11-palm-Δ^9-THC) by thin-layer chromatography (t.l.c.) separation and counting of the radioactive t.l.c. sections as described previously [1]. A buffer control, containing everything except microsomes, was also evaluated at the same time as the test samples.

Table 1 shows that both SKF 525-A and DPEA inhibit, at approximately the same percentage, the production of 11-palm-Δ^9-THC from 11-OH-Δ^9-THC. The 2×10^3 M concentrations of SKF 525-A and DPEA, needed for approximately 50 per cent inhibition of this coenzyme A fortified rat microsomal system, were much higher than that usually needed for 50 per cent inhibition of substrates metabolized *in vitro* in the commonly used NADP fortified rat microsomal system [7]. The concentrations were, however, close to that used in microsomes of rabbits and mice for 50 per cent inhibition of aromatic hydroxylation of aniline [7]. It has been reported [8–10] that many drugs, including SKF 525-A, bind nonspecifically to liver microsomal proteins or phospholipids and that effective concentrations and inhibitory potencies of the drugs depend on experimental conditions. The increased concentrations of SKF 525-A and DPEA needed for 50 per cent inhibition of conjugation of fatty acids to 11-OH-Δ^9-THC in our system may be valid or may be due to differences in volumes, microsomal pro-

* This investigation was supported by U.S. Public Health Service Grant DA00793.