Epoxides as Obligatory Intermediates in the Metabolism of Olefins to Glycols

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SUMMARY

In the presence of rat liver microsomes and NADPH n-1-octene, n-4-octene and 3-ethyl-2-pentene were converted to the glycols with no trace of epoxides. Increased substitution of ethylenic hydrogen atoms by alkyl groups was found to retard the rate of biological oxidation but to enhance that of epoxidation by perbenzoic acid in chloroform. Microsomes without cofactors hydrolyzed the monosubstituted ethylene oxide more rapidly than the di- or trisubstituted derivatives.

The presence of 20 mM 1,2-epoxy-n-octane, 1 mM n-4-octene and 3-ethyl-2-pentene were converted to 1,2-epoxy-n-octane, 4,5-epoxy-n-octane, and 2,3-epoxy-3-ethylpentane. However, in the presence of 20 mM 4,5-epoxy-n-octane, 1 mM n-4-octene yielded 4,5-epoxy-n-octane and no n-octane-4,5-diol. The complete replacement of n-octane-4,5-diol by 4,5-epoxy-n-octane in the presence of the inhibitor indicates that the epoxide is an obligatory intermediate in the conversion of n-4-octene to the glycol.

A few compounds containing a carbon-carbon double bond are metabolized to epoxides (1–3), but the usual products are glycols (4–6). At the beginning of the present investigation, the role of epoxides in the biological formation of glycols was uncertain. It seemed possible that the glycols might arise from either enzymatic or spontaneous hydrolysis of epoxides. However, direct dihydroxylation of the ethylenic moiety could not be ruled out. An attractive approach to this problem was discovered by focusing attention on a few series of olefins, epoxides, and glycols in which the epoxides are quite stable in water. The compounds selected for this purpose were derived from n-1-octene, trans-n-4-octene, and 3-ethyl-2-pentene.

METHODS

Chemical Syntheses—Commercial n-1-octene, trans-n-4-octene and 3-ethyl-2-pentene were purified by distillation. 1,2-Epoxy-

n-octane (b.p., 64–65°), 4,5-epoxy-n-octane (b.p., 57–58°), and 2,3-epoxy-3-ethylpentane (b.p., 53–54°) were prepared by the oxidation of the olefins with perbenzoic acid in chloroform (7). Their purity was confirmed by elemental analysis and by gas chromatography on 20% squalene and on 20% Apiezon L columns. Infrared spectrophotometry of liquid films exhibited absorption bands characteristic of the oxirane ring. n-Octane-1,2-diol (b.p., 158°), n-octane-4,5-diol (m.p. 115–125°), and 3-ethylpentane-2,3-diol (b.p., 92°) were synthesized by treatment of the olefins with 30% H₂O₂ in formic acid followed by alkaline hydrolysis (8). Their purity was confirmed by chromatography on 20% Apiezon L. Like the two other glycols, n-octane-4,5-diol appeared as a single peak, although its melting point suggested a mixture of the meso and racemic forms. Infrared spectrophotometry indicated the absence of absorption bands from carboxyl and olefinic groups in all three glycols. Heptachlor epoxide and dieldrin were supplied by Velsicol Corporation, Chicago, Illinois, through the courtesy of Dr. Milton Eisler.

Incubation Mixtures—The livers from male Sprague-Dawley (Holtzman) rats, weighing 180 to 200 g, were homogenized at 4° in 2 volumes of isotonic KCl. The conditions for sedimentation of the various subcellular fractions are specified elsewhere in the text. In all experiments the aliquots were equivalent to 2 g of liver. The composition of the incubation medium and other experimental details are presented in the legends of appropriate figures and tables.

Analytical Methods—The same analytical procedure was used for the quantification of the metabolites of the three olefins. The chilled reaction mixture plus 5 g of NaCl was extracted with 25 ml of ether. A 20-ml portion of the ether layer was removed after centrifugation and cautiously evaporated to minimize the loss of volatile metabolites. The chilled residue was dissolved in 1 ml of acetone, and aliquots of this solution were analyzed in a model 5000 Barber-Colman gas chromatograph equipped with an ionization detector. The composition of the column and other experimental conditions are specified in the legend of Fig. 1. When added to boiled subcellular fractions, the glycols (0.1 to 1 μ mole) were recovered almost quantitatively (90%). The recovery of 1,2-epoxy-n-octane or 2,3-epoxy-3-ethylpentane averaged 90%, but that of 4,5-epoxy-n-octane was only 70%. All data were corrected for recovery losses.

With each olefin the identity of the epoxide and glycol metabolites was checked by both gas-liquid and thin layer chromatography. After development with a 5:1 benzene-acetone mixture and spraying with concentrated H₂SO₄ on silica gel plates, 1,2-epoxy-n-octane, 4,5-epoxy-n-octane, and 2,3-epoxy-3-ethylpentane had Rp values of 0.75, 0.68, and 0.80, respectively.

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After development with a 2:1 benzene-ethyl acetate mixture, the $R_f$ values of $n$-octane-1,2-diol, $n$-octane-4,5-diol, and 3-ethylpentane-2,3-diol were 0.40, 0.33, and 0.42, respectively.

**RESULTS**

**Nonenzymatic Synthesis and Hydrolysis of Epoxides—**Prior to the initiation of the biochemical work it seemed worthwhile to obtain some data on the influence of branching on the nonenzymatic conversion of olefins to epoxides. Table I shows that the rate constants for perbenzoic acid oxidation increased strikingly with increased substitution of the ethylenic hydrogen atoms. Thus, in this reaction, the inductive effects of the alkyl groups outweigh steric hindrance. This finding is in agreement with the observations reviewed by Swern (9).

### TABLE I

| Olefin              | Rate Constant $k$ (m$^{-1}$min$^{-1}$) |
|---------------------|----------------------------------------|
| $n$-1-Octene        | 0.07                                   |
| trans-$n$-4-Octene  | 3.30                                   |
| 3-Ethyl-2-pentene   | 18.00                                  |

Nonenzymatic hydrolysis of epoxides was not investigated beyond demonstrating that at pH 3, 7, or 10 saturated aqueous solutions of 1,2-epoxy-$n$-octane, 4,5-epoxy-$n$-octane, or 2,3-epoxy-3-ethylpentane were stable at 37°. Incubation of 0.5 mM solutions of these epoxides in 40% acetone for 6 hours resulted in less than 0.01% conversion to the glycols. Information on the behavior of epoxides in neutral solutions has been scarce, but both acids and bases have been shown to catalyze cleavage of the ring. The study of Prichard and Long (10) indicates that electron-releasing substituents, including alkyl groups, facilitate acid-catalyzed hydrolysis of ethylene oxides. On the other hand, alkaline hydrolysis appears to be influenced by steric as well as inductive effects, and generalizations are not yet possible.

**Enzymatic Oxidation of Olefins—**Incubation of $n$-1-octene, trans-$n$-4-octene, and 3-ethyl-2-pentene with the NADPH-enriched 9000 × g supernatant of a rat liver homogenate produced the corresponding glycols, but the epoxides could not be detected (Fig. 1). The limit for the gas chromatographic detection of the epoxides was 0.3 nmoles or a 0.06% yield based on the initial amount of olefin. The identity of the glycols was confirmed by thin layer and gas cochromatography. The relative yields of the glycols (11.3%, 4.0%, 0.12%) indicate that increasing substitution of the ethylenic moiety by alkyl groups decreases the rate of the reaction. Thus, here, steric hindrance from the alkyl groups appears to outweigh their inductive effects.

The product from $n$-1-octene contained a trace of an unknown metabolite with a retention time ($R_f$) lower than that of the epoxide. Both of the other olefins yielded at least three or four compounds in addition to the glycols. The most abundant metabolite from $n$-4-octene ($R_f = 10.7$ min), like the glycol ($R_f = 14.9$ min), increased linearly with time of incubation. The following evidence suggests that this compound is an octenol. First, its $R_f$ is similar to that of 2-octanol (10.9 min). Second, treatment of the extract with an alcohol reagent (acetyl chloride) caused this substance to disappear and a new compound with a shorter $R_f$ to appear. Third, the addition of bromine to the extract converted the metabolite to a derivative with lower volatility. The formation of the other two unknown products of $n$-4-octene was not clearly related to incubation time. No attempt was made to identify these substances or the unidentified metabolites of 3-ethyl-2-pentene.

The enzymatic conversion of the olefins to glycols was found to require microsomes and an NADPH-generating system (Table II). Twice-washed microsomes plus NADPH produced...
the glycols in about the same amounts as did the 9000 × g supernatant. Moreover, the products from the individual olefins did not differ appreciably from those shown in Fig. 1. Thus, it would appear that soluble enzymes are not responsible for the formation of any of the unidentified metabolites.

**Enzymatic Hydrolysis of Epoxides**—The absence of epoxides and the presence of glycols in the products from the biological oxidation of the olefins invited an examination of the action of liver homogenates on 1,2-epoxy-n-octane, 4,5-epoxy-n-octane, and 2,3-epoxy-3-ethylpentane, for these compounds had been shown to be quite stable in aqueous solution. The data in Table III show that incubation of 10 μmoles of 1,2-epoxy-n-octane for 15 min with a 9,000 × g supernatant equivalent to 2 g of rat liver resulted in a quantitative conversion to n-octane-1,2-diol.

**Inhibition of Epoxide Hydrolyase**—Recognition that microsomes contain an epoxide hydrolase suggested that inhibition of this enzyme would provide useful information on the role of epoxides in the conversion of olefins to glycols. Among a number of epoxides and glycols examined for such inhibitory action, only 4,5-epoxy-n-octane was found capable of revealing 1,2-epoxy-n-octane as a metabolite of n-1-octene in a system containing microsomes and NADPH (Table IV). In this particular experiment, the epoxide appeared in somewhat greater quantity than the glycol (0.40 versus 0.23 μmole). The sum of the two compounds was practically the same as the amount of glycol formed in the absence of the inhibitor (0.63 versus 0.64 μmole). Control experiments established the requirement of NADPH and gaseous oxygen for the appearance of the epoxide. Although apparently ineffective as inhibitors of epoxide hydrolase, heptachlor epoxide and dieldrin reduced the formation of n-octane-1,2-diol. It seems possible that these epoxides may have an inhibitory effect on the epoxidase involved in the conversion of n-1-octene to 1,2-epoxy-n-octane. However, this question was not investigated.

The indication that 4,5-epoxy-n-octane inhibited microsomal epoxide hydrolases, whereas 2,3-epoxy-3-ethylpentane did not,

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**Table II**

| Fraction                  | n-Octane-1,2-diol | n-Octane-4,5-diol | 3-Ethylpentane-2,3-diol |
|---------------------------|-------------------|-------------------|-------------------------|
| 9,000 × g supernatant +   | 0.50              | 0.11              | 0.01                    |
| NADPH                     | 0.00              | 0.13              | 0.03                    |
| Microsomes                | 0.00              | 0.13              | 0.03                    |
| Microsomes + NADPH        | 0.00              | 0.13              | 0.03                    |
| Microsomes + NADPH        | 0.00              | 0.13              | 0.03                    |
| Microsomes + NADPH        | <0.01             | 0.13              | 0.03                    |
| Boiled microsomes + NADPH | 0.00              | 0.13              | 0.03                    |
| 165,000 × g supernatant   | 0.00              | 0.13              | 0.03                    |
| NADPH                     | 0.00              | 0.13              | 0.03                    |

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When boiled 9,000 × g supernatant fluid was used, the epoxide was recovered completely unchanged. About 85% of the hydrolytic activity was localized in the microsomes; the remainder could not be sedimented at 165,000 × g. The microsomal reaction did not require the presence of either NADPH or MgCl₂, and the activity of the 165,000 × g supernatant was not diminished by dialysis. Inasmuch as the conversion of an epoxide to a glycol must be formulated as hydrolysis, the enzyme responsible for this reaction may be called epoxide hydrolase.

The rate of hydrolysis of 4,5-epoxy-n-octane by microsomes was slower than that of 1,2-epoxy-n-octane but faster than that of 2,3-epoxy-3-ethylpentane. Thus, this reaction differs from the acid-catalyzed conversion of epoxides to glycols in which increasing substitution by alkyl groups facilitates hydrolysis. It may be presumed that alkyl groups hinder the approach of the substrate to the surface of the enzyme. In the experiments in which the NADPH-generating system and MgCl₂ were omitted, the gas chromatograms of the products contained only two peaks representing the glycol and unchanged epoxide. However, when the cofactors were added, the yields of n-octane-4,5-diol and 3-ethylpentane-2,3-diol were lower, and the chromatograms revealed additional peaks. The retention time on 20% Apiezon L of the new product from 4,5-epoxy-n-octane was 13.1 min, and from 2,3-epoxy-3-ethylpentane, 14.0 min (cf. Fig. 1). In the former case, the peak height was 90% as large as that of the glycol, but in the latter it was 80% as large. These new compounds did not appear to be isomers of the pure glycols, because they could not be detected in crude synthetic products expected to contain all possible isomers. They may represent alcoholic derivatives of the epoxides. In respect to its relative activity in hydrolyzing 1,2- and 4,5-epoxy-n-octane, the enzyme in the 165,000 × g supernatant behaved much the same as that in the microsomes. This observation indicates that the two enzymes may be the same.
The substrate was 10 μmoles of the olefin added to the incubation mixture in 0.2 ml of ethanol in which the inhibitor was also dissolved or, in the case of heptachlor epoxide and dieldrin, suspended. The medium contained the NADPH-generating system. The incubation time was 30 min. Other methods and conditions were the same as those described in Fig. 1 and Table II. In several other experiments employing the 4,5-epoxide, the yields of the 1,2-epoxide and the 1,2-diol equalled that of the glycol in the absence of the inhibitor.

![Fig. 2: Gas chromatogram showing the inhibitory effect of 1,2-epoxy-n-octane on the metabolism of n-4-octene by rat liver microsomes. The left arrow indicates the peak from 4,5-epoxy-n-octane, and the right arrow, the expected position of n-octane-4,5-diol. Reading from left to right, the other large peaks represent acetone, n-4-octene, 1,2-epoxy-n-octane, and n-octane-1,2-diol. In separate experiments all the peaks specified were identified by cochromatography of authentic compounds and the extract. Methods and conditions were the same as those in Table IV. The slight differences in retention times from those in Fig. 1 can be attributed to aging of the chromatographic columns.](http://www.jbc.org/)

| Inhibitor (2 × 10^−3 M) | 1,2-Epoxide-n-octane formed | n-Octane-1,2-diol formed | % Inhibition of glycol formation |
|--------------------------|-----------------------------|-------------------------|---------------------------------|
| None                     | 0.00                        | 0.64                    | 0%                              |
| 4,5-Epoxy-n-octane       | 0.40                        | 0.23                    | 64%                             |
| 2,3-Epoxy-3-ethylpentane | 0.00                        | 0.63                    | 2%                              |
| Heptachlor epoxide       | 0.00                        | 0.50                    | 22%                             |
| Dieldrin                 | 0.00                        | 0.54                    | 16%                             |
| Ethylene glycol          | 0.00                        | 0.64                    | 0%                              |
| Propylene glycol         | 0.00                        | 0.61                    | 0%                              |

![Fig. 3: Alternative pathways for the biological conversion of an olefin to a glycol. Experiments described in the text appear to obviate the possibility of a direct dihydroxylation enzyme (E1) in the transformation of n-4-octene to n-octane-4,5-diol.](http://www.jbc.org/)

Direct evidence that these results should be attributed to inhibition of epoxide hydrolase was obtained by employing an epoxide as the substrate. A low concentration (0.3 mM) of 1,2-epoxy-n-octane inhibited completely for 10 min the hydrolysis of 5 mM 4,5-epoxy-n-octane by washed microsomes at a concentration of 25 mg of liver per ml.

**DISCUSSION**

The ability of hepatic microsomes, an NADPH-generating system, and oxygen to convert n-1-octene, trans-n-4-octene, and 3-ethyl-2-pentene to the corresponding glycols was anticipated, for earlier work had demonstrated the formation of 5-(2,3-dihydroxypropyl)-5-(1-methylbutyl)barbituric acid from 5-allyl-5-(1-methylbutyl)barbituric acid (secobarbital) under the same conditions. Recently, Leibman and Ortiz (11) reported the dihydroxylation of indene and styrene by a similar microsomal preparation. Also, Jerina et al. (12) have mentioned similar requirements for the transformation of naphthalene to trans-1,2-dihydro-1,2-dihyroxynaphthalene. Thus, it would appear that hepatic microsomes contain the necessary enzymes for the conversion of both aliphatic and aromatic double bonds to diols. The capacity of microsomes and NADPH to form stable epoxides from cyclohexene insecticides such as aldrin and heptachlor has been recognized for several years (13, 14).

Our preliminary report (15) that 1,2-epoxy-n-octane was readily hydrolyzed to n-octane-1,2-diol by microsomes coincided with an announcement by Leibman and Ortiz (16) that the same behavior had been observed with indene, cyclohexene, and styrene epoxides. More recently (17), the enzymatic formation of 1,2-dihydro-1,2-dihyroxynaphthalene from 1,2-naphthalene oxide was recorded. Inasmuch as the conversion of an epoxide to a glycol has classically been formulated as a hydrolytic reaction, the appropriate name for the enzyme would appear to be epoxide hydrolase. However, other workers (17, 18) have called it epoxide hydrase. Like other hydrolytic enzymes such as esterases and amidases, it does not require NADPH or magnesium ion as a cofactor.

The systematic study of 1,2-epoxy-n-octane, 4,5-epoxy-n-octane, and 2,3-epoxy-3-ethylpentane revealed that increasing the number of alkyl groups in the oxirane ring retards the rate of cleavage by microsomes, whereas acid-catalyzed hydrolysis is enhanced. Presumably, the bulk of the alkyl groups hinders the approach of the epoxide to the surface of the enzyme. The facile cleavage of the oxirane ring by the hydrolase could account for the fact that epoxides have only rarely been detected as end products of metabolism. Heptachlor epoxide and dieldrin, which are stable enough to be excreted in substantial amounts,

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may be presumed to be poor substrates for the enzyme, although rats can convert dieldrin to the trans form of the corresponding glycol (19). In the present investigation, the metabolism of heptachlor epoxide and dieldrin was not examined beyond demonstrating that they did not inhibit the action of microsomal epoxide hydrolase on 1,2-epoxy-n-octane.

The discovery that high concentrations of some epoxides could inhibit the microsomal hydrolase provided the means for examining the role of epoxides in the conversion of olefins to glycols. In the presence of 20 mM 4,5-epoxy-n-octane, the product from n-1-octene contained both 1,2 epoxy n-octane and n-octane-1,2-diol, whereas in the absence of the inhibitor only the glycol could be detected. This result could be explained in two ways. (a) The only route from the olefin to the glycol involves the epoxide, but inhibition of the hydrolase was incomplete. (b) Glycol may be formed by a mechanism other than epoxidation, for example, by direct dihydroxylation (Fig. 3). A similar experiment in which the substrate was n-4-octene and the inhibitor was 20 mM 1,2-epoxy-n-octane gave unequivocal results. In the presence of the inhibitor, the product contained the epoxide but not the glycol, whereas, in the absence of the inhibitor, the glycol was present, but the epoxide was not. This observation appears to prove that the epoxide is an obligatory intermediate in the conversion of n-4-octene to the corresponding glycol.

It now seems likely that the biological conversion of both aliphatic and aromatic carbon-carbon double bonds proceeds through epoxides. In connection with aromatic compounds, the evidence of Holtzman, Gillette, and Milne (20) is strong, although indirect. They found that trans-1,2-dihydro-1,2-dihydroxynaphthalene formed from naphthalene in the presence of microsomes, NADPH, and gaseous $^{18}O_2$ contained only 1 atom of heavy oxygen. Recently, Jerina et al. (17) reported the detection of 1,2-naphthalene oxide as a metabolite of naphthalene and suggested its role as an obligatory intermediate in the formation of the dihydrodiol as well as 1-naphthol and a premercapturic acid. Whether a single microsomal hydrolase acts on both aliphatic and aromatic epoxides is not yet clear. Studies involving inhibitors such as 1,2-epoxy-n-octane should answer this question.

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J. Biol. Chem. 1970, 245:5234-5238.

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