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Enzymatic Hydration of [^{18}O]Epoxides. Role of Nucleophilic Mechanisms

Robert P. Hanzlik,* Marshall Edelman, William J. Michaely, and Georganna Scott

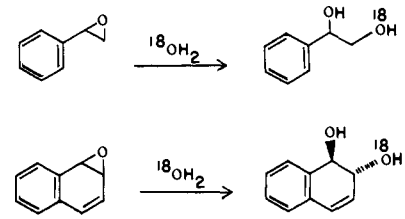
Contribution from the Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045. *Received July 18, 1975*

Abstract: The hydration of five mono- and 1,1-disubstituted oxiranes by enzymes in rat liver microsomes has been shown, by means of ^{18}O-tracer studies, to be extremely regiospecific, involving cleavage of the C(2)–O bond of the oxirane ring. Base-catalyzed hydration of these epoxides also involved C(2)–O bond cleavage but was much less regiospecific, whereas acid-catalyzed hydration involved mainly C(1)–O bond cleavage. Both the enzymatic and metal ion catalyzed hydration of [^{18}O]-2-pyridyloxirane were found to involve ≥95% C(2)–O bond cleavage. In the presence of large concentrations of added nucleophiles and metal binding agents the enzymatic hydration of 1,2-epoxytetradecane was not inhibited nor were any products other than diol formed. These results preclude the involvement of (1) a metal ion at the active site of epoxide hydrase and (2) a simple enzyme–acid-catalyzed hydration in which water or hydroxide from solution attacks a protonated epoxide and suggest that epoxide hydrase activates a water molecule per se for nucleophilic attack at the less hindered oxirane carbon. A *hypothetical* active site mechanism for epoxide hydrase is discussed.

The metabolism of aromatic or olefinic compounds by mammals often involves the epoxide–diol pathway, the enzymes of which are localized in the microsomal fraction of liver and other tissues.[1] The first step of this pathway, epoxide formation, is catalyzed by various nonspecific cytochrome P_{450}-dependent mixed function oxygenases. Epoxides produced by these enzymes, especially the arene oxides, have been shown to have various toxic and deleterious effects upon the organism. These effects are presumably associated with the alkylation of critical cellular constituents by the chemically reactive epoxides. The second enzyme of the pathway, epoxide hydrase, is thus thought to play a protective role by converting reactive epoxides into less toxic diol products which may be metabolized further and excreted.

Oesch and co-workers have shown that epoxide hydrase is a relatively nonspecific enzyme, both *d* and *l* enantiomers of monosubstituted and 1,1-disubstituted oxiranes bearing at least one large lipophilic substituent being among the best substrates, while highly substituted oxiranes do not serve as substrates and fail to inhibit the hydration of styrene oxide.[1c,2,3] Stereochemical studies have shown that enzymatic hydration of epoxides of cyclic olefins, including arene oxides, produces *trans*-1,2-diols.[4,5]

The direction of epoxide opening has been determined in two cases using $^{18}OH_2$ and unlabeled epoxides. Acid dehydration of the dihydrodiol obtained by incubation of naphthalene with microsomes in buffers containing $^{18}OH_2$ gave a mixture of 1- and 2-naphthol; only the 2-naphthol contained ^{18}O.[4] Similarly, diol obtained from the enzymatic hydration of styrene epoxide in $^{18}OH_2$ media contained ^{18}O, 90% of which was in the β position.[5] The result obtained with styrene oxide suggests the possible importance of nucleophilic as opposed to acid-catalyzed mechanisms. Unfortunately, it was difficult to evaluate the relative importance of steric hindrance and carbonium ion stability in the case of the naphthalene oxide. Therefore we decided to examine the question of carbonium ion vs. nucleophilic pathways and to test the *generality* of the styrene oxide result with a group of epoxides representing the *range of structural types* known to be good substrates for epoxide hydrase. For reasons of economy, and to complement the labeling method used in the above cases, we decided to study the enzymatic hydration of [^{18}O]epoxides in ordinary buffers. During our study, Jeffrey et al. reported that C, N, and S nucleophiles attack preferentially at C(2) of naphthalene 1,2-dioxide.[6]



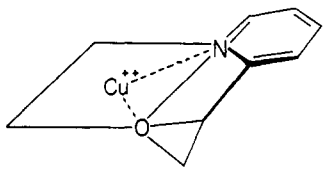
Results and Discussion

The epoxide substrates **1a–5a** were chosen for this study for several reasons. First, they represent close structural analogs of compounds previously shown[3] to be good substrates for epoxide hydrase, i.e., lipophilic mono- and 1,1-disubstituted oxiranes.[7] Second, they were chosen to allow unambiguous evaluation of the relative importance of carbonium ion formation vs. nucleophilic attack in the hydration process. 2-Pyridyloxirane (**2a**) is apparently the first example of an oxirane with a heterocyclic substituent to be studied but was included to allow comparison of the enzymatic results to those obtained with a regiospecific "chemical model" for epoxide hydrase.[8] Epoxides **1a–5a** were all found to be excellent substrates for epoxide hydrase.

Mass spectral data indicating the ^{18}O content of epoxides **1a–5a**, as well as the content and distribution of ^{18}O in the corresponding diols **1b–5b**, are given in Tables I and II. Differences in ^{18}O content in the starting epoxides are attributed to the presence of small and variable amounts of extraneous ordinary water in solvents and/or reagents used in their synthesis. Results obtained from multiple mass spec-

tral determinations on materials from several individual runs under each different experimental condition generally gave quite good agreement.

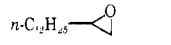
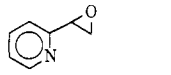
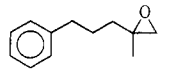
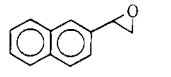
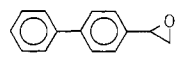
Except for the acid hydration of **2a,** the results of both acid and base hydration of the epoxides generally agree quite well with predictions based on relative carbonium ion stability and steric hindrance to nucleophilic attack by solvated HO^-, respectively.[9] In the case of **2a,** acid hydration occurs with much more C(2)–O bond breaking than expected for an aryloxirane (cf. **4a**), and with Lewis acid catalysts such as Cu^{2+}, hydration and "methanolysis" occur with virtually 100% selectivity for C(2)–O bond cleavage. There are two probable causes for this behavior. In the case of acid (and possibly metal ion) catalysis, the positive charge on the pyridine ring greatly reduces the likelihood of carbonium ion formation at C(1), so that nucleophilic attack at the least hindered carbon of the protonated (or metal-chelated) oxirane predominates over SN1-type carbonium ion formation. In agreement with this the rate of hydration of **2a** is only twice as fast at pH 1.9 as at the pH 5–8.3 plateau.[8] In the case of metal (and possibly proton) catalyzed hydration, the formation of a chelate ring fixes the C(1)–O bond in the plane of the pyridine ring, out of conjugation with the aromatic π system, and the great stability of the five-membered chelate ring probably stabilizes the C(1)–O bond at the expense of further strain in the C(2)–O bond.



Comparison of the results obtained with the enzymatically formed diols **1b–5b** shows that in all five cases ≥96% of the oxirane oxygen is retained at C(1) of the diol, which implies C(2)–O bond cleavage during hydration. In terms of enzymatic mechanisms these results are clearly inconsistent with anything like a *simple* acid-catalyzed mechanism involving carbonium ion formation. In contrast to the nonenzymatic base hydration of epoxides the enzymatic hydration is essentially *completely* regiospecific. Therefore the lack of occurrence of *any* significant amount of nucleophilic attack by water (or hydroxide) at C(1) of the oxirane can be due only in part to the steric hindrance contributed by the oxirane substituents, and one or both of the following conditions must obtain. The enzyme could be protecting C(1) from attack by nucleophiles through hydrophobic binding interactions. Alternatively, or concurrently, the hydrase enzyme could actively direct the incoming water (or hydroxide) toward C(2) of the oxirane, perhaps by means of some type of general base catalysis.

Two types of mechanisms are known for general base catalysis in enzymatic reactions. The more commonly encountered type involves deprotonation of a water molecule by a basic group of the enzyme such as a histidine imidazole or glutamate carboxylate group. The second type, less commonly encountered, involves deprotonation of a water molecule via coordination to an enzyme-bound Lewis acid such as a zinc ion. This results in the formation of a metal-coordinated hydroxide which is considerably more nucleophilic than *water.* This type of "general base" catalysis is seen in the action of carbonic anhydrase[11] and aconitase[12] and in the hydrolysis of metal-coordinated amino acid esters and amides[13] and may be involved in the hydrolysis of an acyl enzyme (anhydride) intermediate in carboxypeptidase action.[14] With regard to a possible role of a metal ion in epoxide hydrase action, Oesch and Daly could detect neither a metal ion cofactor requirement nor inhibition of epoxide hydrase by EDTA.[15] However, the fact that the metal-catalyzed hydration and methanolysis[8] of **2a** mimic the enzymatic hydration process in terms of both rate acceleration and regiospecificity suggested that further enzymatic studies along these lines would be useful.

Table I. Structures and Labeling of Oxirane Derivatives[7]

1a		26.7 atom % ^{18}O
2a		18.8 atom % ^{18}O
3a		29.6 atom % ^{18}O
4a		23.6 atom % ^{18}O
5a		18.6 atom % ^{18}O

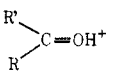
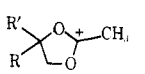
Carbonic anhydrase is known to hydrolyze esters and sultones and to hydrate CO_2 and various aldehydes by virtue of the hydroxozinc group at its active site.[11] In particular, Pocker and Meany have shown that the hydration of pyridine-2-carboxaldehyde, a close structural analogue of epoxide **2a,** is strongly catalyzed by carbonic anhydrase as well as by metal ions.[16,17] We therefore assumed that carbonic anhydrase might be a useful "model" for epoxide hydrase and tested epoxides **1a, 2a, 4a,** and **5a** as substrates for the former. However, despite many attempts under various conditions hydration of epoxides was never observed, even with **2a** as the substrate.[18]

In another group of experiments we studied the enzymatic hydration of [^{3}H]-**1a** in the presence of various nucleophiles and metal binding agents, including cyanide, azide, iodide, fluoride, and mercaptoethanol (each at 0.2 M in the incubation medium), and 2-(1′-hydroxyethyl)pyridine and 2-(2′-hydroxyethyl)pyridine (each at 100 μg/ml in the incubation medium). In no case was there any significant inhibition of epoxide hydration, and in *no case was any product other than diol obtained.* The former observation casts serious doubt upon the possible involvement of a metal ion Lewis acid at the active site of epoxide hydrase, whether for oxirane activation or water activation. More importantly, the latter observation implies that *nucleophiles other than water (or hydroxide) do not compete for the opportunity to attack an enzymatically activated epoxide,* which may be taken as evidence that the hydrase enzyme activates water per se for attack on the oxirane ring. The apparent lack of metal ion involvement leaves general base catalysis as the only reasonable alternative consistent with the ^{18}O distribution studies and the lack of competition by other nucleophiles. However, these results do not exclude the *possibility* that an acidic group on the enzyme cooperatively facilitates C–O bond cleavage by protonation of the oxirane oxygen.

Experimental Section

Synthesis of Epoxide Substrates. Epoxides **1a–5a** were synthesized by treating the corresponding olefins with *N*-bromosuccinimide (NBS) in aqueous tetrahydrofuran for 2–24 h at room temperature.[19] The bromohydrins were isolated by column chromatography or preparative TLC and were immediately converted to the epoxides by stirring with a threefold excess of finely powdered K_2CO_3 in anhydrous methanol for 2–6 h. The same procedure was used for ^{18}O-labeled epoxides, except that the proportions of reactants were adjusted to optimize yields based on $^{18}OH_2$ (30 atom % ^{18}O, Bio-Rad). The usual mole ratios were olefin–NBS–$^{18}OH_2$ = 1:1.1:8; with less water little reaction occurred. A further modifi-

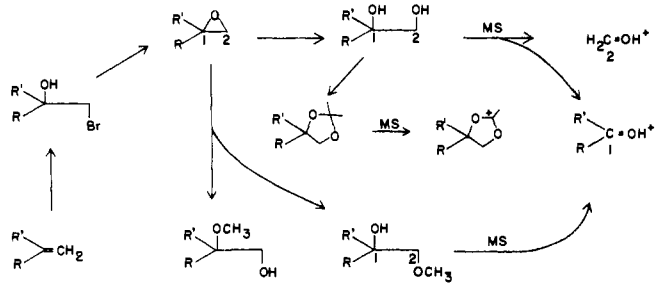
Table II. Content and Distribution of ^{18}O in Diols and Their Derivatives and Calculated Amount of C(1)–O vs. C(2)–O Bond Breaking during Diol Formation[a]

Oxirane precursor	Conditions	Atom % ^{18}O in fragment ion (±1.5%)		Percent bond cleavage (±5%)	
				C(1)–O	C(2)–O
1a	Microsomes	26.5 ± 1.2			>98
	Bu_4NOH	26.8			>98
	$HClO_4$	7.3 –15.8	26.4	40–73	27–60
	TCA	16.8 ± 1.2	25.9	37	63
	MeO^-	27.8			>98
2a	Microsomes	18.1		4	96
	Cu^{2+}–H_2O	17.9		5	95
	Cu^{2+}–MeOH	18.8[b]			>99[b]
	NaOH	13.5		28	72
	$HClO_4$	11.9		37	63
3a	Microsomes	28.7	29.6	3	97
	$HClO_4$	1.1		96	4
	Bu_4NOH	24.5		18	82
4a	Microsomes	22.8		3	97
	$HClO_4$	3.8	23.6	84	16
	Bu_4NOH	19.6		17	83
5a	Microsomes	18.1	18.6	3	97

[a] See Scheme I for numbering of oxiranes and diol derivatives and for their synthesis and mass spectral fragmentation patterns. [b] See ref 8.

cation was used for the synthesis of [^{18}O]-**2a**; 1.0 equivalent of anhydrous CF_3CO_2H was added prior to the addition of the NBS. For large-scale preparations of unlabeled **2a** 20 g (0.19 mol) of 2-vinylpyridine was treated in 500 ml of dioxane–water (3:7) with 10 ml of glacial acetic acid, and 36 g (0.202 mol) of NBS was added over a 30-min period. After stirring for 1 h 40 g of Na_2CO_3 was added in portions and the reaction was stirred overnight. Extraction with ethyl ether (4 × 100 ml) followed by distillation gave 10.3 g of **2a** [bp 58° (0.2 Torr)] (Scheme I). Tritiated (as well as

Scheme I. Synthesis and Mass Spectral Fragmentation of Epoxides and Derivatives[7]



unlabeled) **1a**, **4a**, and **5a** were also prepared by peracetic acid epoxidation of the corresponding olefins in CH_2Cl_2 over NaOAc. The unlabeled, tritiated, and ^{18}O-labeled epoxides were compared and checked for purity by NMR, TLC, and GLC; radiopurity was confirmed by both TLC and GLC (DC550, Carbowax 20M).

Key NMR spectral characteristics and physical properties for the epoxides as well as the intermediate bromohydrins (i.e., mono- or 1,1-disubstituted 2-bromoethanols) are given below; NMR values and δ ppm from internal Me_4Si in the solvent indicated; R_f values are for the back and front edge of the spot or band on 0.25-mm silica layers developed in hexane containing the indicated amount of ethyl acetate (v/v %).

Synthesis of 1a: bromohydrin, mp 36–38°; R_f 0.18–0.39 (15); NMR (CCl_4) 3.6–3.3 (3 H, m). **1a**, an oil: R_f 0.42–0.60 (15); NMR (CCl_4) 2.56 (1 H, t, J = 3 Hz), 2.32 (2 H, m).

Synthesis of 2a: bromohydrin, an oil; R_f 0.17–0.23 (40); NMR ($CDCl_3$) 4.95 (1 H, t, J = 6 Hz) and 3.70 (2 H, d, J = 6 Hz). **2a**, an oil: R_f 0.20–0.31 (40); NMR ($CDCl_3$) 4.00 (1 H, dd, J = 4, 2 Hz), 3.95 (1 H, dd, J = 5, 2 Hz), and 3.15 (1 H, dd, J = 5, 4 Hz).

Synthesis of 3a: bromohydrin, an oil; R_f 0.13–0.26 (15); NMR (CCl_4) 1.20 (3 H, s) and 3.28 (2 H, s). **3a**, an oil: R_f 0.30–0.42 (15); NMR (CCl_4) 1.22 (3 H, s) and 2.38 (2 H, s).

Synthesis of 4a: bromohydrin, an oil; R_f 0.26–0.31 (25); NMR (CCl_4) 5.0 (1 H, m), 3.65 (1 H, s), and 3.60 (1 H, d, J = 3 Hz). **4a**, a solid: mp 54–56°; R_f 0.27–0.33 (15); NMR (CCl_4) 3.85 (1 H, dd, J = 4, 2 Hz), 3.05 (1 H, dd, J = 3, 6 Hz), and 2.73 (1 H, dd, J = 6, 2 Hz).

Synthesis of 5a: bromohydrin, an oil which solidified eventually; R_f 0.15–0.21 (15); NMR ($CDCl_3$) 4.95 (1 H, m), 3.65 (1 H, s), and 3.60 (1 H, d, J = 3 Hz). **5a**, a solid: mp 85–86.5°; R_f 0.33–0.39 (15); NMR ($CDCl_3$) 3.75 (1 H, dd, J = 4, 2 Hz), 3.00 (1 H, dd, J = 3, 6 Hz), and 2.68 (1 H, dd, J = 6, 2 Hz).

Chemical Synthesis of Diols and Their Derivatives. Acid hydration of [^{18}O]epoxides was accomplished by dissolving 1–4 mg of epoxide in 1–2 ml of THF and adding an equal volume of 3% $HClO_4$ or 10% Cl_3CCO_2H. After 2–24 h the mixtures were treated with excess $NaHCO_3$ and partitioned between ether and water, and the ether was dried over Na_2SO_4 prior to concentration and TLC to isolate the diol. For base hydration, no *homogeneous* aqueous–cosolvent system could be found which was satisfactory for bases such as NaOH, KOH, or K_2CO_3. However, this problem was avoided by dissolving 1–2 mg of epoxide in 1–2 ml of THF, adding 50–100 mg of crystalline $Bu_4NOH{\cdot}33H_2O$, sealing the homogeneous solution in a 13 × 100 mm culture tube with a Teflon-lined screw cap, and heating at 77° for 12–36 h. Concentration and TLC gave excellent yields of diol. **2a** was hydrated by dissolving 5 mg directly in 2 ml of 0.1 N NaOH or 3% $HClO_4$, incubating at 37° for 48 h, and extracting with CH_2Cl_2. When 5 mg of **2a** was dissolved in 2 ml of 0.1 M $CuSO_4$, the color changed from pale blue to deep blue during 30 min, at which time liquid chromatography showed the reaction to be complete (see below). Addition of excess Na_2S followed by extraction with CH_2Cl_2 or $CHCl_3$ recovered diol in high yield. Diol monomethyl ethers were prepared by dissolving the epoxide in 1 M $NaOCH_3$ in methanol for 3 h. Partitioning between ether and water, followed by TLC of the concentrated ether extract, gave the separated monomethyl ethers from **1a** and **2a**, whose structures were confirmed by their NMR and mass spectra. Acetonides of diols were formed conveniently by dissolving the diols in anhydrous acetone (1–5 mg/ml) and adding finely powdered *anhydrous* $CuSO_4$ (50–150 mg); conversion was quantitative within several hours at room temperature.

Preparation of Rat Liver Microsomes and Incubation of Substrates. Livers of male Holtzman rats (6–8 weeks) were homogenized in 0.1 M phosphate buffer (pH 7.4, 2.5 ml/g of liver). The homogenate was centrifuged at 10 000g for 30 min, and the resultant supernatant was further centrifuged at 105 000g for 2 h. The microsomal pellets were resuspended in phosphate buffer (0.1 M, pH 8.0) for immediate use in incubations. Alternatively, microsomes could be stored at −20° for weeks with no appreciable loss of hydrase activity. For preparative scale incubations 5–8 ml of microsome suspension (30 mg/ml) was added to a 25 × 100 mm culture tube containing 2–4 mg of epoxide which had been deposited with an equal weight of Tween-80 from 200 μl of acetone by evaporation under a gentle stream of nitrogen. Prior to incubation the

tubes were capped and shaken for 10 s on a vortex mixer. After incubation for 1 h at 37° the incubation mixtures were cooled and extracted four times with ether by vigorous shaking on the vortex mixer followed by brief centrifugation to separate the emulsion. The extracts were dried over Na_2SO_4, concentrated, and analyzed by TLC on 5 × 20 × 0.025 cm silica plates using 40% ethyl acetate in hexane (60% for diol from **2a**). The diol band (generally R_f 0.05–0.15) was visualized under uv light except for diol **1b** which was visualized after spraying the developed plate with Rhodamine 6-G (0.05% in 95% EtOH) and allowing it to dry for 30 min. Preparative incubations of [^{18}O]-**2a** with microsomes were run as above omitting Tween-80; analytical incubations were monitored by liquid chromatography as discussed below.

Analytical incubation of tritiated **1a, 4a,** and **5a** with microsomes was run and analyzed by minicolumn chromatography as discussed for the carbonic anhydrase testing or was analyzed by TLC and scanned for radioactive areas. The latter were scraped off the plate, eluted, and counted for ^{3}H.

Testing of Carbonic Anhydrase for Epoxide Hydrase Activity. Carbonic anhydrase (Worthington) was dissolved (1 mg/ml) in various buffers including phosphate, Tris, and Veronal, of pH 7.4–8.6. Enzyme (1 ml) (or boiled enzyme) was added to a 16 × 100 mm culture tube containing 50–100 μg of [^{3}H]epoxide[19] (**1a, 4a,** or **5a**) with an equal weight of Tween-80. Tubes were shaken briefy on a vortex mixer and incubated at 25–37° for 1 h. They were extracted with toluene (4 × 2 ml) by vortexing and centrifuging. For a given incubation tube each successive 2-ml toluene extract was placed on top of a 1-cm column packed in toluene with 6 cm of silica gel [deactivated with 10% (w/w) H_2O] on top of which was placed 2 cm of anhydrous granular Na_2SO_4. After four 2-ml extractions more toluene was run through the column until a total of 15 ml was collected for the first fraction. A second 15 ml of toluene was then run through the column, followed by 15 ml of ether. Aliquots of these fractions were counted for ^{3}H. Tests with epoxide–diol mixtures indicated 95–100% recovery from boiled enzyme solutions and complete separation of epoxide (fraction 1) from diol (fraction 3), with no detectable decomposition of the epoxides during the processing.

2-Pyridyloxirane (**2a**) was dissolved (0.75 mg/ml) directly in carbonic anhydrase solution (native or boiled, 1 mg/ml at pH 8 in 0.02 M Veronal or 0.1 M phosphate), and the mixture was incubated at 30° for 2 h. The incubations were monitored by direct liquid chromatography of 2-μl aliquots on a 600 × 2 mm Vydac C-18 bonded reverse phase column eluted with 1.5 ml/min of a solution containing 40 ml of MeCN, 0.1 mol of K_2HPO_4, and 0.1 mol of KH_2PO_4 per liter.

Mass Spectral Analysis for ^{18}O. Mass spectra were obtained by direct sample introduction using a Varian CH-5 instrument. At least seven scans of the peak doublets of interest were averaged for each sample, and in most cases samples were run several times on different days. The ^{18}O content of a fragment ion was calculated as the ratio of peak intensities (M + 2)/M + (M + 2) and was corrected by subtracting the corresponding value observed for unlabeled material. The ^{18}O content of the epoxides could not be determined directly; their molecular ion was of very weak intensity due to facile loss of O, OH, and H_2O. Therefore the values in Table I were estimated from mass spectra of derivatives of the epoxides formed in ways in which loss of ^{18}O would be extremely unlikely (see Scheme I). These usually involved a nucleophilic opening of the oxirane. Interestingly, the *enzymatically* formed diols and their acetonides were also useful in this respect. The H_2COH^+ fragment ion from the diols was also of very low intensity, which in most cases precluded reliable measurement of its ^{18}O content from *m/e* 31:33 intensity ratios. Therefore, the distribution of ^{18}O in the various diols was determined by comparing the ^{18}O content of the $R'RCOH^+$ fragment ion to that of the precursor epoxide.

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