the C-terminal data presented here. Corresponding bovine data are also available and indicate it too is highly homologous to the human C isozyme.

Acknowledgment

We thank Mrs. Ya-Shiou L. Yu for her highly skilled research assistance.

References

Ambler, R. P. (1967), *Methods Enzymol. 11*, 436.
Babul, J., and Stillwagon, E. (1968), *Anal. Biochem. 28*, 216.
Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.
Carter, M. J. (1971), *Biochim. Biophys. Acta 235*, 222.
Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, p 371.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1*, 80.
Elias, H. G. (1970), Theory and Applications of Ultracentrifuge Techniques, Munich, Beckman Instruments GMBH.
Funakoshi, S., and Deutsch, H. F. (1968), *J. Biol. Chem. 243*, 6474.
Furth, A. (1968), *J. Biol. Chem. 243*, 4832.
Headings, V. E., and Tashian, R. E. (1971), *Biochim. Biophys. Acta 236*, 353.
Henderson, L. E., Henriksson, D., Nyman, P. O., and Strid, L. (1972), *Alfred Benzon Symp. IV* (in press).
Laurent, G., Charrel, M., Marriq, C., Garcon, D., and Derrien, Y. (1966), *Bull. Soc. Chim. Biol. 48*, 1125.
Moore, S., and Stein, W. (1963), *Methods Enzymol. 6*, 819.
Nyman, P. O. (1963), *Acta Chem. Scand. 17*, 429.
Nyman, P. O., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85*, 141.
Nyman, P. O., Strid, L., and Westermark, G. (1968), *Eur. J. Biochem. 6*, 172.
Pisano, J. J., and Bronzert, T. J. (1969), *J. Biol. Chem. 244*, 5597.
Reynaud, J., Luccioni, F., Bouthier, M., Savary, J., and Derrien, Y. (1970), *Biochim. Biophys. Acta 221*, 367.
Spencer, R. L., and Wold, F. (1969), *Anal. Biochem. 32*, 185.
Tanis, R. J., Tashian, R. E., and Yu, Y.-S. L. (1970), *J. Biol. Chem. 245*, 6003.
Tashian, R. E. (1969), *in* Biochemical Methods in Red Cell Genetics, Yunis, J. J., Ed., New York, N. Y., Academic Press, p 307.
Tashian, R. E., Goodman, M., Headings, V. E., DeSimone, J., and Ward, R. H. (1971), *Biochem. Genet. 5*, 183.
Tashian, R. E., Riggs, S., and Yu, Y.-S. L. (1966), *Arch. Biochem. Biophys. 117*, 320.
Tashian, R. E., Tanis, R. J., and Ferrell, R. E. (1972), *Alfred Benzon Symp. IV* (in press).
Tucker, E. M., Suzuki, Y., and Stromont, C. (1967), *Vox Sang. 13*, 246.
Yamada, S., and Itano, H. A. (1966), *Biochim. Biophys. Acta 130*, 538.

Hepatic Epoxide Hydrase. Structure–Activity Relationships for Substrates and Inhibitors*

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ABSTRACT: An epoxide hydrase preparation solubilized and partially purified from guinea pig liver microsomes catalyzes the hydration of a variety of epoxides to corresponding glycols. The activity of various epoxides as substrates or as inhibitors of this enzyme is dependent on the nature and stereochemistry of substituents on the oxirane ring. Oxiranes with a 1-aryl substituent (styrene oxides) or with certain 1-alkyl substituents (1-octene oxide, phenyl 2,3-epoxypropyl ethers) are among the best substrates for the enzyme and are *competitive* inhibitors for hydration of styrene-*t* oxide. 1,1-Disubstituted and cis-1,2-disubstituted oxiranes are less active as substrates and inhibitors. Trans-1,2-disubstituted, trisubstituted, and tetrasubstituted oxiranes are virtually inactive as substrates or inhibitors. Certain alicyclic oxiranes such as 1,2-epoxy-1,2,3,4-tetrahydronaphthalene and cyclohexene oxide are relatively inactive as substrates but very effective as inhibitors. Inhibition by these compounds appears to be *noncompetitive* with respect to substrate. 1,1,1-Trichloropropene 2,3-oxide is a very potent *uncompetitive* inhibitor of epoxide hydrase. Various analogs of epoxides such as azaridines, thiiranes, and oxaziridines, either do not inhibit the enzyme or are relatively weak inhibitors. Alcohols and certain ketones, such as metyrapone, activate the enzyme. Structure–activity correlations with the epoxide hydrase are distinctly different from those of squalene oxidocyclase.

Hepatic epoxide hydrase is an important enzyme in the metabolism of olefinic and aromatic substrates, since it controls one of two major enzymatic pathways for the further bioalteration of intermediate epoxides and arene oxides (Jerina *et al.*, 1968, 1970a; Leibman and Ortiz, 1968); the conversion of these compounds to glycols and dihydrodiols, respectively.

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The other pathway for the metabolism of oxiranes is by conjugation with glutathione (Boyland and Williams, 1965; Jerina *et al.*, 1968, 1970b). An epoxide hydrase activity has now been solubilized from guinea pig microsomes and partially purified (Oesch and Daly, 1971). This stable preparation catalyzes the hydration of a variety of epoxides. The purification factor (ratio of specific activity in final preparation to that in liver homogenate) for benzene oxide (factor of 4) contrasts markedly with that observed for all other epoxides studied (factors of 26–30), suggesting the presence of at least two isozymes in liver microsomes (Oesch *et al.*, 1971a). Correlations between the structure of various epoxides and their ability to serve as substrates or inhibitors of epoxide hydrase are presented below. In addition, epoxide analogs have been tested as inhibitors using an assay based on the hydration of styrene-*t* oxide (Oesch *et al.*, 1971b).

Experimental Section

Compounds. The substrates and inhibitors used were obtained from commercial sources or were prepared by standard procedures. Purity was established by nuclear magnetic resonance spectroscopy and thin-layer chromatography. When necessary, compounds were purified by preparative thin-layer chromatography, distillation, or recrystallization until at least 90% pure.

Enzyme Preparations. Male Hartley guinea pigs (250–300 g) were sacrificed by bleeding. Their livers were chilled, cut into small pieces, and homogenized in three volumes of freshly prepared cold 0.25 M sucrose. The homogenate was centrifuged at 600*g* for 15 min. The resulting supernatant, which contained 6.48 mg of N/ml, is referred to as "liver homogenate" while "microsomal preparations" refers to material obtained by centrifuging the liver homogenate at 8500*g* for 15 min followed by centrifugation of the resulting supernatant at 100,000*g* for 1 hr. The microsomal pellet was resuspended in sufficient phosphate buffer (0.01 M, pH 7.8) to restore the original volume of 8500*g* supernatant. These preparations contained 1.65–2.72 mg of N/ml. A microsomal preparation which contained 2.2 mg of N/ml was obtained from a New Zealand white rabbit (2.7 kg). The "purified epoxide hydrase preparation" is the final, stable preparation as described (Oesch and Daly, 1971) concentrated to 1.66 mg of N/ml. Percentages of nitrogen were determined by the Kjeldahl method in particulate fractions and by the Biuret method in solubilized fractions (Oesch and Daly, 1971).

Assays. Activity was measured as described with styrene-*t* oxide (Oesch *et al.*, 1971b).

Product Formation from Epoxides. Substrate (5 μmoles) was added in 0.1 ml of acetonitrile to a mixture of 0.7 ml of microsomal preparation and 0.2 ml of Tris buffer (0.5 M, pH 9.0 containing 0.2 mg of Tween 80). Following incubation at 37° for 20 min, products were extracted into four volumes of ethyl acetate (extracted twice). The combined extract was dried over Na_2SO_4, concentrated *in vacuo*, and chromatographed on thin-layer silica gel GF plates with benzene–chloroform–ethyl acetate (1:1:1, v/v) as eluent. Compounds were visualized with ultraviolet light, and the silica gel containing the product was removed and extracted with 1.0 ml of methanol. The extent of product formation was measured by ultraviolet spectroscopy. Allyl glycidyl ether and its hydration product, after separation by thin-layer chromatography, were visualized with 1% iodine in methanol.

Products which did not exhibit strong ultraviolet absorption were assayed by gas chromatography after derivatization with *n*-butylboronic acid (Oesch *et al.*, 1971a). The boronate derivatives of 1,2-dihydroxyoctane and of 1,2-dihydroxy-3-butoxypropanes emerged at 6.5 and 5 min, respectively, from a 2% QF-1 on 80–100 mesh gas Chrom Q column ($^1/_8$ in. × 6 ft) at 90°. Substrates emerged at 1 min and unreacted butylboronic acid at 4 min. The boronate of 2,3-dihydroxy-4-methylpentane emerged at 2 min from a 15% SE-30 on 80–100 mesh gas Chrom Q column ($^1/_8$ in. × 6 ft) at 170° with the epoxide substrate at 0.5 min and the unreacted reagent at 7 min. The boronate of 1,1,1-trichloro-2,3-dihydroxypropane emerged at 5 min from the SE-30 column at 170°, while the 1,1,1-trichloropropane 2,3-oxide emerged at 1 min. Quantitation was achieved by comparison to a plot of peak heights obtained with standard solutions of the derivatized dihydroxy products. Hydration of styrene-*t* oxide was assayed with each set of incubations, and the results normalized to conditions under which 30% hydration of styrene oxide would occur.

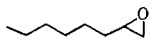
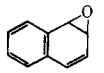
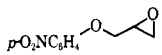
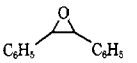
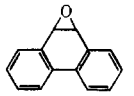
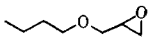
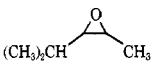
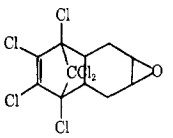
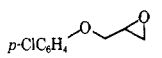
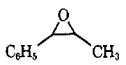
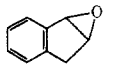
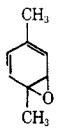
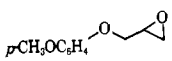
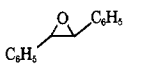
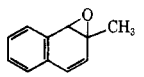
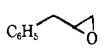
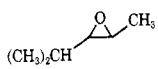
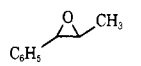
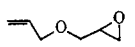
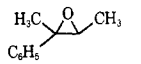
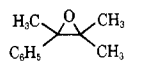
Results and Discussion

The importance of hepatic epoxide hydrase has been recognized for the past several years. However, extensive quantitative examination of the enzyme had not been carried out until after solubilization and purification (Oesch and Daly, 1971). Investigations with both liver homogenate and the purified preparation then provided quantitative data on rates of conversion (μmoles of product/mg of N per 5 min) of a variety of epoxides under conditions where enzyme activity was linear with respect to time, where enzyme was saturated with substrate, and where the validity of the assay in terms of recovery was established (Oesch *et al.*, 1971a).

Monosubstituted oxiranes such at 1-octene oxide, styrene oxide, and 4-chlorophenyl 2,3-epoxypropyl ether were found to be excellent substrates for epoxide hydrase with specific activities with the liver homogenate of 0.37, 0.12, and 0.11 μmole of product per mg of N per 5 min, respectively (Oesch *et al.*, 1971a). Another monosubstituted oxirane, bisnorsqualene oxide was, however, a nearly inactive substrate for hepatic epoxide hydrase. Alicyclic oxiranes varied considerably in their activity with epoxide hydrase. Thus, indene 1,2-oxide was an extremely active substrate (0.36 μmole/mg of N per 5 min), while cyclohexene oxide was a relatively inactive substrate (0.01 μmole/mg of N per 5 min). Arene oxides, such as naphthalene 1,2-oxide, phenanthrene 9,10-oxide, and benzene oxide, were extremely active substrates for epoxide hydrase in liver homogenates with activities of 0.53, 0.27, and 0.20 μmole/mg of N per 5 min, respectively. A relatively low purification factor for "benzene oxide hydrase" activity in the "purified preparation" of epoxide hydrase suggested that this substrate was acted upon by another epoxide hydrase present in liver microsomes, but largely lost during a purification procedure based on the hydration of styrene oxide. The foregoing data based on studies with a limited number of epoxides suggested that the structure of substituted oxiranes, alicyclic oxiranes, and arene oxides was of great importance to their activity with epoxide hydrase. The factors which influence substrate–enzyme interactions have now been investigated in some detail using more than 100 epoxides as substrates or inhibitors of epoxide hydrase.

Substrate Efficacy. The results for epoxides as substrates of microsomal epoxide hydrase are presented in Table I. Monosubstituted oxiranes with either aryl or fairly large alkyl substituents are excellent substrates. Octene 1-oxide has been previously reported to be an excellent substrate for micro-

TABLE I: Diol Formation from Various Epoxides with Microsomal Epoxide Hydrase.[a]

Monosubstituted Oxiranes	% Conversion to Diol[b]	Di-, Tri-, and Tetrasubstrated Oxiranes	% Conversion to Diol[b]	Alicyclic Oxiranes	% Conversion to Diol[b]	Arene Oxide	% Conversion to Diol[b]
	80		24		3*		90[c]
	69		35		2*		60[c]
	55		5		6*		45[c]
	53		2*		80[c]		Not detectable
	46		Not detectable		2[c]		Not detectable
	34		Not detectable				
	30		Not detectable				
	5						
	Not detectable		Not detectable				
Bisnorsqualene oxide	1[c]		Not detectable				

[a] Conducted with guinea pig liver microsomes. For conditions, see Experimental Section. Results have been normalized to an average 30% conversion of styrene-*t* oxide to diol. [b] Nonenzymic hydration was <10% of enzymic conversion except as shown by asterisk and has been subtracted from all values shown. [c] Calculated value from earlier studies. See text.

somal epoxide hydrase (Oesch *et al.*, 1971a; Maynert *et al.*, 1970; Watabe and Kanehira, 1970). A 1,1-disubstituted oxirane, 1-methylstyrene oxide, is only slightly less active as a substrate than the parent monosubstituted oxirane, styrene oxide. The isomeric cis-1,2-disubstituted oxirane, 2-methylstyrene oxide, is, however, much less active. Another cis-1,2-disubstituted oxirane, *cis*-4-methylpentene 2-oxide, is relatively inactive as a substrate, while *cis*-stilbene oxide is as active as styrene oxide. Product formation could not be detected from trans-1,2-disubstituted, tri-, or tetrasubstituted oxiranes. Squalene oxidocyclase has the reversed order of substitution requirements for optimal substrate activity: trisubstituted > trans-disubstituted > cis-disubstituted ≫ monosubstituted oxirane (Corey *et al.*, 1968; Clayton *et al.*, 1968).

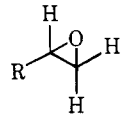
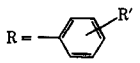
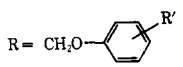
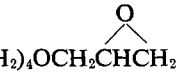
Alicyclic oxiranes differ greatly in activity. Thus, cyclohexene oxide (Oesch *et al.*, 1971a) and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene (Table I) are poor substrates, while indene oxide (Oesch *et al.*, 1971a) is an excellent substrate. The insecticide, 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene, was found to be a rather poor substrate for epoxide hydrase (Table I). Brooks and coworkers (1969, 1970) have published an extensive study on the structure–activity relationships of this and related compounds with microsomal epoxide hydrase. HEOM[1] was one of the more active substrates for epoxide hydrase in this class of compounds (Brooks *et al.*, 1970).

The three "arene oxides," naphthalene oxide, benzene oxide, and phenanthrene oxide, are all active substrates for microsomal epoxide hydrase (Oesch *et al.*, 1971a; Jerina *et al.*, 1968, 1970b). By contrast, neither 2,5-dimethylbenzene 1,2-oxide nor 2-methylnaphthalene 1,2-oxide (for synthesis, see Kaubisch *et al.*, 1972)[2] was converted with liver homogenates to

[1] Abbreviation used is: HEOM, 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene.

[2] Manuscript in preparation.

TABLE II: Monosubstituted Oxiranes as Inhibitors of Epoxide Hydrase with Styrene-*t* Oxide as Substrate.[a]

Monosubstituted Oxiranes 	% Inhibn at a Concn of Inhibitor	
	Equal to Substrate[b]	One-Half of Substrate[b]
		
R′ = 2,6-Dichloro	ns	ns
3-Acetamido	14	11
4-Nitro	14	11
3-Nitro	31	25
4-Methoxy	31	20
4-Methyl	48	31
4-Hydrogen (styrene oxide)	48	33
4-Carbomethoxy	49	31
3-Methoxy	57	41
4-Phenyl	64	49
4-Oxirane	69	50
4-Chloro	69	52
4-Bromo	74	59
3-Chloro	79	65
3-Bromo	84	68
		
R′ = 4-Methoxy	17	ns
4-Hydrogen	21	14
4-*tert*-Butyl	24	11
4-Chloro	28	22
2-Chloro	30	21
4-Methyl	35	23
4-Ethyl	35	27
2-Methyl	36	24
3-Methyl	40	28
4-Nitro	47	39
R = CH_3	ns	ns
CH_2CH_3	ns	ns
$CH{=}CH_2$	ns	ns
		
$(CH_2)_4CHCH_2$	21	14
$C(CH_3)_3$	36	22
$(CH_2)_5CH_3$	43	32
$(CH_2)_8COOCH_3$	58	44
$CH_2C_6H_5OCH_3$	18	ns
$CH_2C_6H_5$	27	16
CH_2Cl	10	ns
CCl_3	98	97
CH_2F	ns	ns
CF_3[c]	68	34
$OC(=O)CH_2(CH_3)_3$	18	ns
$OC(=O)CH_3$	25	13
$CH_2N(C_2H_5)_2$	ns	ns
CH_2OH	Stimulates (+12)	ns
$CH_2O(CH_2)_3CH_3$	ns	ns
$CH_2OCH_2CH{=}CH_2$	ns	ns
		
$CH_2O(CH_2)_4OCH_2CHCH_2$	ns	ns

[a] Significant effects with P <0.001 are given (ns = not significant). [b] Substrate (styrene-*t* oxide) concentration 2×10^{-3} M. The purified epoxide hydrase preparation was used (40 μl per incubation). The inhibitor was added in 20 μl of appropriate solvent (in most cases acetonitrile) at zero time with no preincubation. The same amount of solvent was added to the controls. Incubation mixture, conditions, and assay as described (Oesch *et al.*, 1971b). [c] Incubation in sealed tubes at 27°.

detectable amounts of dihydrodiols (Table I). Pandov and Sims (1970) reported that phenanthrene oxide was an active substrate for the hydrase, but that dibenz[*a,h*]anthracene 5,6-oxide containing two additional aromatic rings was an extremely inactive substrate.

Inhibition by Substrates. A great variety of epoxides were tested as inhibitors of the enzymatic hydration of styrene-*t* oxide with the purified epoxide hydrase preparation. The results are presented in Tables II–IV. Ring-substituted styrene oxides inhibited to differing extents (0–84%) the hydration of styrene-*t* oxide; presumably as competitive substrates (Table II). No correlations between the electronic effects of substituents and the efficacy of the substituted styrene oxide as an inhibitor were apparent. Where the isomeric 3- and 4-substituted styrene oxides were tested, the 3 isomer was always somewhat more active as an inhibitor. Monosubstituted oxiranes with small substituents such as methyl, ethyl, or vinyl did not significantly inhibit the hydration of styrene-*t* oxide, while those with large substituents such as *tert*-butyl, benzyl, or *n*-hexyl did inhibit the reaction (Table II). This might reflect a greater affinity of the latter epoxides for the enzyme because of hydrophobic interactions of the substituent with enzyme residues near the active site. Inhibition of styrene-*t* oxide hydration with glycidyl ethers (2,3-epoxypropyl ethers) was significant only with the various phenyl 2,3-epoxypropyl ethers (Table II). The extent of inhibition varied somewhat (17–47%) with the nature of the substituent on the aromatic ring; the 4-nitro compound was the most potent inhibitor and the 4-methoxy compound, the least potent. The former compound was the most active substrate and the latter was the least active substrate of the phenyl 2,3-epoxypropyl ethers tested (Table I). Butyl 2,3-epoxypropyl ether, while not a significant inhibitor, was surprisingly a fairly active substrate (Table I). Inhibition by 4-chlorophenyl 2,3-epoxypropyl ether

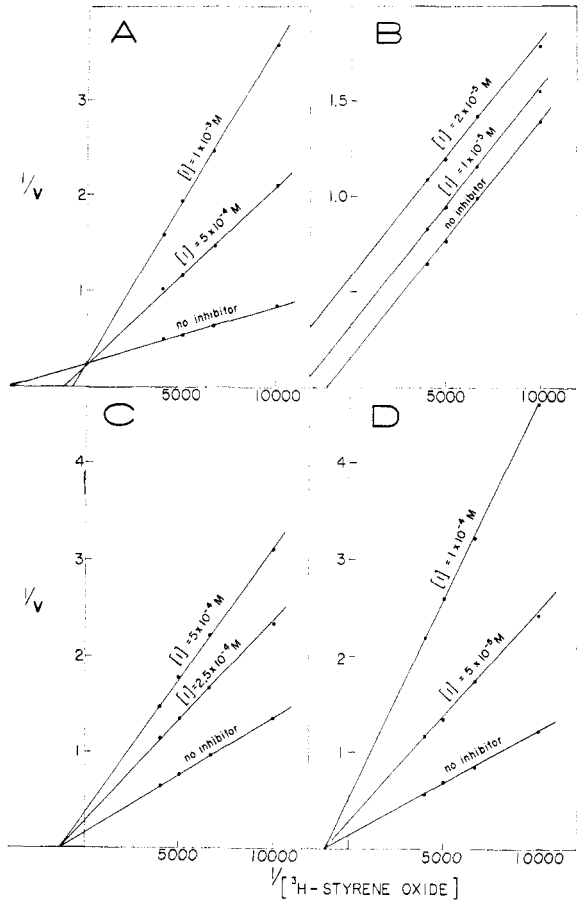


FIGURE 1: Inhibition of purified preparations of epoxide hydrase with styrene-*t* oxide as substrate. Inhibitor: (A) 4-chlorophenyl 2,3-epoxypropyl ether, (B) 1,1,1-trichloropropene 2,3-oxide, (C) cyclohexene oxide, and (D) 1,2-epoxy-1,2,3,4-tetrahydronaphthalene. Double-reciprocal plot of velocity (μmoles of styrene-*t* glycol formed per mg of N per 5 min) *vs.* concentration of substrate (M). Incubation conditions and assay as described (Oesch *et al.*, 1971b). Inhibitor was added in 20 μl of acetonitrile at zero time with no preincubation. Either 10 μl (A) or 20 μl (B–D) of the purified epoxide hydrase preparation was used per incubation.

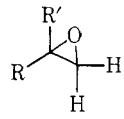
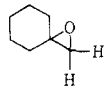
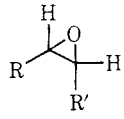
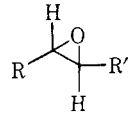
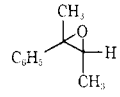
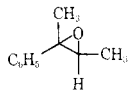
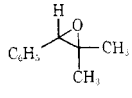
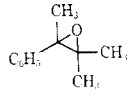
was competitive with respect to substrate styrene-*t* oxide (Figure 1A) as expected.

Two acyloxy oxiranes were found to inhibit hydration of styrene-*t* oxide (Table II). Because of the low levels of inhibition, these compounds were not investigated further.

The most potent inhibitor of epoxide hydrase was a monosubstituted oxirane with a bulky, strongly electron-withdrawing substituent, the trichloromethyl group (Table II). This compound completely inhibited the hydration of styrene-*t* oxide at concentrations one fifth of substrate. Analogous epoxides with either the bulky *tert*-butyl group or the strongly electron-withdrawing trifluoromethyl group were not as effective. Quite surprisingly, the inhibition with 1,1,1-trichloropropene 2,3-oxide proved, on kinetic analysis, to be uncompetitive with respect to substrate (Figure 1B). This compound will be discussed below and in a forthcoming publication (Oesch *et al.*, 1972).[2] It is a rather inactive substrate for the hydrase (Table I).

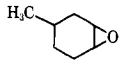
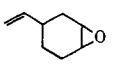
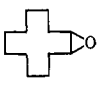
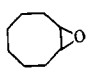
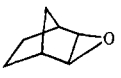
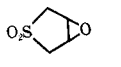
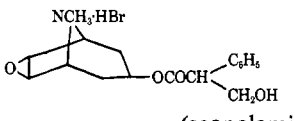
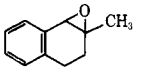
1,1-Disubstituted oxiranes usually inhibit the hydration of styrene-*t* oxide slightly less effectively (Table III) than the parent monosubstituted compound (Table II). The effectiveness of this group of oxiranes as inhibitors appears to decrease with increasing bulk of the two substituents. In the

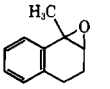
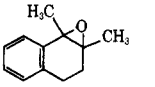
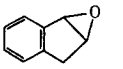
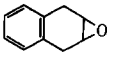
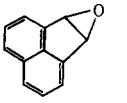
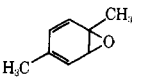
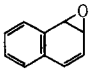
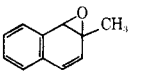
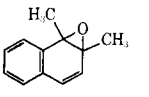
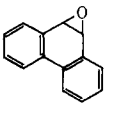
TABLE III: Di-, Tri-, and Tetrasubstituted Oxiranes as Inhibitors of Epoxide Hydrase with Styrene-*t* Oxide as Substrate.[a]

	% Inhibition at a Concn of Inhibitor	
	Equal to Substrate[b]	One-Half of Substrate[b]
1,1-Disubstituted Oxiranes, 		
R = C_6H_5		
R′ = $COOC_2H_5$	ns	ns
R′ = C_6H_5	19	ns
R′ = $CH(CH_3)_2$	27	13
R′ = CH_3	36	20
R = $CH_2C_6H_5$, R′ = CH_3	13	ns
R = R′ = C_2H_5	17	ns
	45	28
1,2-Cis-Disubstituted Oxiranes, 		
R = R′ = C_6H_5	12	ns
R = C_6H_5, R′ = CH_3	68	51
R = $C(CH_3)_3$, R′ = C_2H_5	ns	ns
R = $CH(CH_3)_2$, R′ = CH_3	25	19
1,2-Trans-Disubstituted Oxiranes, 		
R = R′ = C_6H_5	ns	ns
R = C_6H_5, R′ = CH_3	ns	ns
R = C_6H_5, R′ = $COOCH_3$	ns	ns
R = R′ = C_3H_7	ns	ns
R = $C(CH_3)_3$, R′ = C_2H_5	ns	ns
R = $CH(CH_3)_2$, R′ = CH_3	ns	ns
R = CH_3, R′ = $CO_2C_2H_5$	ns	ns
Tri- and Tetrasubstituted Oxiranes		
	ns	ns
	ns	ns
	ns	ns
	Stimulates (+11)	ns

[a] Significant effect with $P < 0.001$ (ns = not significant). [b] Substrate (styrene-*t* oxide) concentration 2×10^{-3} M. Incubation conditions, with purified epoxide hydrase, see Table II.

TABLE IV: Alicyclic Oxiranes and Arene Oxides as Inhibitors of Epoxide Hydrase with Styrene-*t* Oxide as Substrate.[a]

Alicyclic Oxiranes	% Inhibn at a Concn of Inhibitor: Equal to Substrate[b]	% Inhibn at a Concn of Inhibitor: One-Half of Substrate[b]
	13	ns
 [c]	56	38
	68	48
	72	57
	75	59
	ns	ns
	Stimulates (+13)	Stimulates (+11)
	Stimulates (+12)	ns
	ns	ns
	ns	ns
 (scopolamine·HBr)	ns	ns
	88	60
	ns	ns

Alicyclic Oxiranes	% Inhibn at a Concn of Inhibitor: Equal to Substrate[b]	% Inhibn at a Concn of Inhibitor: One-Half of Substrate[b]
	ns	ns
	ns	ns
	42	25
	19	11
	17	ns
Arene Oxides		
	ns	ns
	ns	ns
	35	18
	15	ns
	Stimulates (+24)	Stimulates (+12)
	21	17

[a] Significant effect with P <0.001. A variety of polyhalogenated epoxides such as 1,2,3,4,9,9-hexachloro-*exo*-5,6-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene, 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,4-methanonaphthalene (HEOM), and chlordene epoxide, were inactive (ns = not significant). [b] Substrate (styrene-*t* oxide) concentration 2×10^{-3} M. Incubation conditions, with purified hydrase, see Table II. [c] Cyclopentene oxide inactive.

case of a small substituent such as ethyl, the 1,1-diethyl-substituted oxirane is a more potent inhibitor than the monoethyl analog (Table II). The nature of inhibition by 1,1-disubstituted oxiranes was not examined. Since substantial product formation could be demonstrated (Table I) from the 1,1-disubstituted oxiranes, these compounds are presumably at least partially competitive with respect to substrate.

1,2-Cis-disubstituted oxiranes inhibit the hydration of styrene-*t* oxide (Table III). Indeed, *cis*-2-methylstyrene oxide is a better inhibitor than the parent monosubstituted oxirane, styrene oxide. However, it appears that, as the combined bulk of the two substituents is increased, the efficacy as an inhibitor is greatly decreased (Table III). The same is not necessarily true with respect to activity of the same compounds as substrates; *i.e.*, *cis*-2-methylstyrene oxide which contains one small and one large substituent is a much poorer substrate than *cis*-2-phenylstyrene oxide (*cis*-stilbene oxide) which has two large substituents. The nature of the inhibition of epoxide hydrase by 1,2-cis-disubstituted oxiranes has not been determined, but on the basis of the above discussion, might be

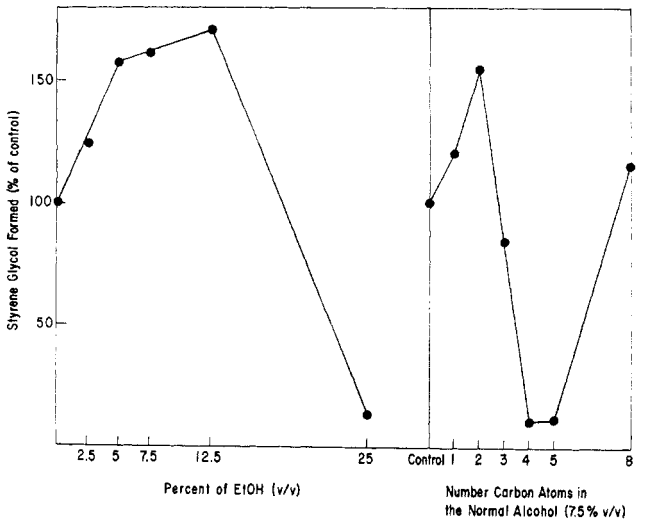


FIGURE 2: Effect of concentration and chain length of 1-alkanols upon epoxide hydrase activity with styrene-*t* oxide as a substrate. Experiments were carried out as described in Table II with purified preparations of epoxide hydrase and were corrected for boiled enzyme controls.

anticipated to be only partially competitive with substrate. Maynert and coworkers (1970) have reported that 4-hexene oxide (presumably cis) is a relatively inactive substrate and inhibitor of microsomal epoxide hydrase.

1,2-Trans-disubstituted oxiranes showed no significant activity as substrates (Table I) or as inhibitors (Table III) of epoxide hydrase. Apparently, such *trans*-disubstituted oxiranes do not bind effectively to the enzyme. The trans isomer of norsqualene oxide was an extremely inactive substrate for microsomal epoxide hydrase, while the cis isomer was much more active (Clayton *et al.*, 1968). A recent paper (Watabe *et al.*, 1971) reports that the *cis*-stilbene oxide is 700 times more active as a substrate for epoxide hydrase than the trans isomer.

Tri- and tetrasubstituted oxiranes did not significantly inhibit hydrase activity (Table III) and could not be demonstrated as substrates for the enzyme (Table I). Other studies have shown squalene 2,3-oxide as an extremely inactive substrate for epoxide hydrase (Oesch *et al.*, 1971a) and 3-ethyl-2-pentene oxide (Maynert *et al.*, 1970) as a relatively inactive substrate for epoxide hydrase and a weak inhibitor toward hydration of octene 1-oxide.

Among a variety of alicyclic oxiranes tested as inhibitors of epoxide hydrase (Table IV), the cyclohexene oxide and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene were rather remarkable inhibitors, in view of their low activities as substrates (Table I, Oesch *et al.*, 1971a). Inhibition with cyclohexene oxide and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene appeared to be noncompetitive with respect to the substrate, styrene-*t* oxide (Figure 1C,D); thus providing an explanation for the lack of relationship between activities as substrate or as inhibitor for this class of oxiranes. Inhibition was neither increased nor decreased by preincubation of enzyme with cyclohexene oxide before adding the substrate, styrene-*t* oxide. Determination of kinetic parameters for cyclohexene-*t* oxide hydration had indicated a K_m of $>10^{-3}$ M (Oesch *et al.*, 1971a). This is in substantial agreement with its K_i of approximately 2×10^{-3} M for inhibition of hydration of styrene-*t* oxide. This agreement may, however, be fortuitous since the *noncompetitive* nature of the inhibition suggests that two sites on the enzyme are involved in binding of cyclohexene oxide as substrate or as inhibitor. *Trisubstituted* alicyclic oxiranes such as 1-methylcyclohexene oxide were *much* less potent as inhibitors than the parent alicyclic compound (Table IV).

Benzene oxide was inactive as an inhibitor of the purified epoxide hydrase with styrene-*t* oxide as substrate (Table IV). This lack of inhibition was independent of the organic solvent (acetone, acetonitrile, or ethanol) used to add the benzene oxide, of the source of the enzyme (guinea pig or rabbit) or of the physical state (particulate or solubilized) of the enzyme, even at concentrations of benzene oxide fourfold higher than styrene-*t* oxide. Since purification factors (Oesch *et al.*, 1971a) for hydrase activity with styrene oxide and benzene oxide are quite different (28 and 4, respectively), the lack of cross inhibition with these two compounds is not surprising. Rather it appears that homologous hydrase enzymes in liver microsomes are differentially concerned with metabolism of benzene oxide and other oxides. The properties of the apparent "benzene oxide hydrase" are under investigation.

Naphthalene oxide, another arene oxide, is by contrast to benzene oxide, an effective inhibitor of hydration of styrene oxide (Table IV). The converse is also true, since styrene oxide effectively inhibits the hydration of naphthalene oxide in microsomal preparations (Jerina *et al.*, 1970b). In the arene oxides as in other classes of epoxides, the tri- and tetrasubstituted oxiranes were much less effective inhibitors.

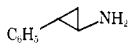
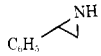
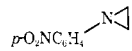
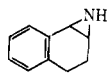
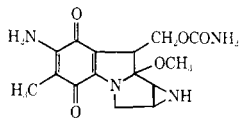
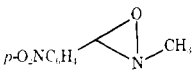
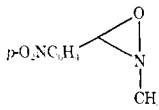
One of the objectives of the present investigation was to develop a potent inhibitor of epoxide hydrase acitivty for *in vivo* studies on the role of epoxides in drug toxicity (*cf.* Brodie *et al.*, 1971) and polycyclic hydrocarbon carcinogenicity (*cf.* Grover *et al.*, 1971). The only truly potent oxirane inhibitor of the enzyme uncovered in the present study was 1,1,1-trichloropropene 2,3-oxide (Table I). This compound causes virtually complete inhibition of the hydration of styrene oxide at one-fifth substrate concentration and was uncompetitive with substrate (Figure 1B). Unfortunately, the compound did not cause measurable inhibition of epoxide hydrase *in vivo* even at toxic dosages (Oesch *et al.*, 1972).[2]

Substrate Analogs and Inhibitors. A variety of structural analogs of epoxides were examined as potential inhibitors for the purified epoxide hydrase. These included thiiranes, aziridines, oxaziridines, and cyclopropanes (Table V). Styrene episulfide was a moderately active inhibitor, while cyclohexene episulfide was inactive. The former compound is an analog to a competitive epoxide inhibitor (styrene oxide), while the latter is an analog to a potent noncompetitive epoxide inhibitor (cyclohexene oxide), suggesting differing structural requirements for the sites involved in competitive and noncompetitive inhibition.

A *cis*-oxaziridine was moderately active (~40% inhibition), while the corresponding isomeric *trans*-oxaziridine was without effect. This dependency of activity on cis *vs.* trans stereochemistry parallels the behavior of 1,2-disubstituted oxiranes (Table III). Cyclopropanes and aziridines were without inhibitory activity. Another aziridine, 2,3-iminosqualene, does not inhibit hydration of bisnorsqualene oxide, but *is* a potent inhibitor of squalene oxidocyclase (Corey *et al.*, 1967, 1968).

Styrene and 1,2-dialin, the olefins corresponding to the substrate, styrene oxide, and the potent inhibitor, 1,2-epoxy-1,2,3,4-tetrahydronaphthalene, were tested as inhibitors and substrates. Weak inhibitions of 11 and 23%, respectively, were observed at 2×10^{-3} M. Hydration of these olefins to alcohols by epoxide hydrase in analogy to a bacterial enzyme which adds water to either olefins or epoxides (Niehaus *et al.*, 1970) could not be detected by thin-layer chromatography.

TABLE V: Epoxide Analogs as Inhibitors of Purified Epoxide Hydrase with Styrene-*t* Oxide as Substrates.[a]

Inhibitor	% Inhibn at a Concn of Inhibitor Equal to Substrate[b]
	ns
	ns
	41
	ns
	ns
	ns
	Stimulates (+45)
	ns
	ns
	43

[a] Significant effect with $P < 0.001$ (ns = not significant). Cyclic ethers (tetrahydrofuran, dihydropyran, 1,8-epoxy-*p*-menthane, and furfuryl alcohol) had no significant effect on epoxide hydrase activity. [b] Substrate (styrene-*t* oxide) concentration 2×10^{-3} M. Incubation conditions, with purified epoxide hydrase, see Table II.

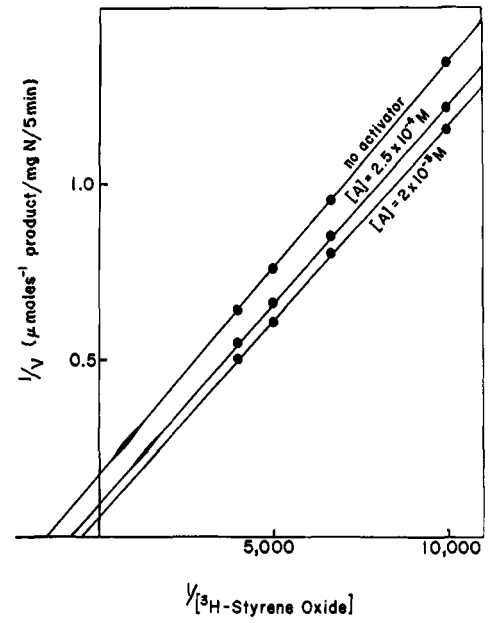


FIGURE 3: Activation of epoxide hydrase by metyrapone. Double-reciprocal plot of velocity (μmoles of styrene-*t* glycol/mg of N per 5 min) *vs.* concentration of substrate, styrene-*t* oxide (M). Incubation conditions and assay as described (Oesch *et al.*, 1971b). Metyrapone [A] was added in 20 μl of acetonitrile at zero time and 20 μl of the "purified epoxide hydrase preparation" was used per incubation.

Activation. Compounds which stimulate hydrase activity would be useful in studies on the role of this enzyme in drug metabolism. A number of simple alcohols were found to activate epoxide hydrase. These included cyclohexanol and 2-cyclohexen-1-ol which caused 20 and 9% stimulation of enzyme activity at 2×10^{-3} M. Glycidol (Table II) caused a 12% stimulation of activity at this concentration, while β-phenethanol and a variety of diols which corresponded to the products from the hydration of naphthalene 1,2-oxide, styrene oxide, phenanthrene 9,10-oxide, 4-chlorophenyl 2,3-epoxypropyl ether, 1,2-epoxy-1,2,3,4-tetrahydronaphthalene, and 1,2,3,4,9,9-hexachloro-6,7-epoxy-1,4,4a,5,6,7,8,8a-octahydro-1,2-methanonaphthalene had no effect on enzyme activity. The activation by 1-alkanols was a function of chain length and was maximal at a concentration of 1–2 M with ethanol (Figure 2). Other solvents, such as methylene chloride, methylene iodide, acetonitrile, and acetone, had no effect on enzyme activity. Certain ketones, α-tetralone, and the ketonic bispyridine, metyrapone, stimulated the enzyme, 15 and 116%, respectively, at 2×10^{-3} M, while others such as β-tetralone, 1- and 2-indanone, acetophenone, cyclohexanone, and the aldehyde, phenylacetaldehyde, had no effect. The alcohol analog of metyrapone was virtually inactive. Pyridine and nicotinamide were without effect. One of the aziridines of Table V caused 45% stimulation. Previously, Leibman and Ortiz (1970) reported that both acetophenone and metyrapone stimulate epoxide hydrase activity toward styrene oxide in microsomal preparations. Only the latter result could be confirmed with solubilized preparations or with liver homogenate. Kinetic analysis of the activation of epoxide hydrase by metyrapone indicates negative cooperativity with substrate (Figure 3). Attempted *in vivo* stimulation of epoxide hydrase with metyrapone was unsuccessful (Oesch *et al.*, 1972).[2]

Enzyme Kinetics. Detailed kinetic analysis of purified epoxide hydrase has been considerably hindered by the lack of a rapid continuous assay method for product formation. Nonetheless, point by point analysis of the inhibition of styrene-*t* oxide hydration by four different oxiranes (Figure 1) has been extremely rewarding in that competitive 4-chlorophenyl 2,3-epoxypropyl ether (A), uncompetitive 1,1,1-trichloropropene 2,3-oxide (B), and noncompetitive cyclohexene oxide (C) and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene, (D) inhibitions were demonstrated. The kinetic parameters, K_m and V_{max}, for hydration of styrene oxide are a function of protein concentration, incubation temperature, and age of the enzyme preparation after storage at −15° (Figure 4). Earlier values for K_m of 5.3×10^{-4} M and V_{max} of 2.7 μmoles/mg of N per 5 min at 0.2 mg of protein/ml (Oesch and Daly, 1971) may be compared with present values for K_m of 2.3×10^{-4} M and V_{max} of 3.8 μmole/mg of N per 5 min at 0.26 mg of protein/ml (Figure 4) obtained after storage of the enzyme preparation at −15° for 6 months.

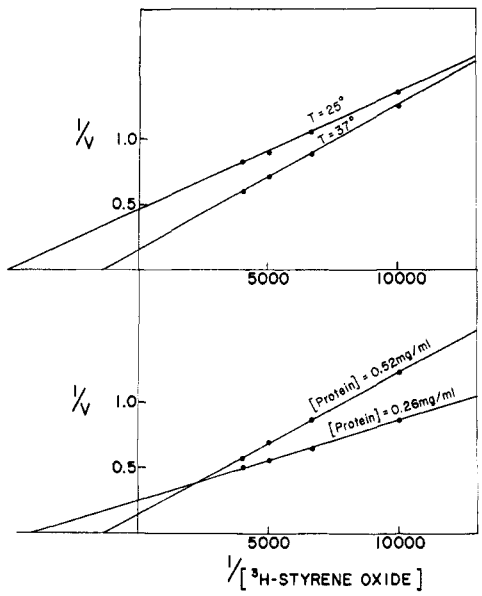


FIGURE 4: Influence of temperature and protein concentration on K_m and V_{max} of a purified preparation of epoxide hydrase with styrene-*t* oxide as substrate. (A) Constant protein concentration (0.52 mg/ml) and (B) constant temperature (37°). Double-reciprocal plot of velocity (μmoles of styrene-*t* glycol formed per mg of N per 5 min) *vs.* concentration of substrate (M). Incubation conditions and assay as described (Oesch *et al.*, 1971b). Either 20 or 10 μl, respectively, of the purified epoxide hydrase preparation was used per incubation.

Conclusions

The present studies delineate many of the factors involved in the hydration of epoxides by a purified hepatic epoxide hydrase preparation. The results indicate that monosubstituted oxiranes with a fairly large hydrophilic substituent are excellent substrates of epoxide hydrase and fairly potent competitive inhibitors of hydration of styrene oxide. Both 1,1- and 1,2-cis-disubstituted oxiranes are fairly effective inhibitors of hydration of styrene oxide. Their potency as inhibitors and as substrates do not correlate, suggesting that the inhibition may be only partially competitive with respect to substrate. Trans-1,2-disubstituted and tri- and tetrasubstituted oxiranes are extremely inactive as substrates and inhibitors. Alicyclic oxiranes vary considerably and independently in their efficacy as inhibitors and substrates for epoxide hydrase. Two of the more potent alicyclic inhibitors of styrene oxide hydration, cyclohexene oxide and 1,2-epoxy-1,2,3,4-tetrahydronaphthalene are extremely inactive substrates for the enzyme, and indeed inhibition of the enzyme by these oxiranes proved to be noncompetitive with respect to styrene oxide as substrate. Kinetic analysis of another potent inhibitor, 1,1,1-trichloropropene 2,3-oxide, demonstrated the inhibition to be uncompetitive with respect to substrate. The relative activity of various oxiranes as substrates for the hydrase enzyme does not appear to correlate well with that expected from either a mechanism involving an initial acid-catalyzed opening of the oxirane ring or with one involving an initial nucleophilic attack of water. The lack of inhibition of styrene oxide hydration by benzene oxide provided further evidence for the presence of isozymes of epoxide hydrase in liver microsomes. Nitrogen, sulfur, and carbon analogs of epoxides did not prove to be effective inhibitors of epoxide hydrase. A *cis*-oxizaridine inhibited the enzyme, while the trans isomer did not. Further studies on the role of epoxide hydrases in drug metabolism will provide information regarding the significance of arene oxides in the toxicity and carcinogenicity of aromatic compounds.

References

Boyland, E., and Williams, K. (1965), *J. Biochem. (Tokyo) 94*, 190.

Brodie, B. B., Reid, W. D., Cho, A. K., Sipes, G., Krishna, G., and Gillette, J. R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 160.

Brooks, G. T. (1969), *Proc. Brit. Insectic. Fungic. Conf., 1969*, 472.

Brooks, G. T., Harrison, A., and Lewis, S. E. (1970), *Biochem. Pharmacol. 19*, 255.

Clayton, R. B., Van Tamelen, E. E., and Nadeau, R. G. (1968), *J. Amer. Chem. Soc. 90*, 820.

Corey, E. J., Kang, L., and Jautelat, M. J. (1968), *J. Amer. Chem. Soc. 90*, 2724.

Corey, E. J., Ortiz de Montellano, P. R., Link, and Dean, P. D. (1967), *J. Amer. Chem. Soc. 89*, 2797.

Grover, P. L., Sims, P., Huberman, E., Marquardt, H., Kuroki, T., and Heidelberger, C. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1098.

Jerina, D. M., Daly, J., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S. (1968), *Arch. Biochem. Biophys. 128*, 176.

Jerina, D. M., Daly, J. W., Witkop, B., Zaltzman-Nirenberg, P., and Udenfriend, S. (1970b), *Biochemistry 9*, 147.

Jerina, D. M., Ziffer, H., and Daly, J. W. (1970a), *J. Amer. Chem. Soc. 92*, 1056.

Leibman, K. C., and Ortiz, E. (1968), *Mol. Pharmacol. 4*, 201.

Leibman, K. C., and Ortiz, E. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 346.

Maynert, E. W., Foreman, R. L., and Watabe, T. (1970), *J. Biol. Chem. 245*, 5234.

Niehaus, W. G., Jr., Kisic, A., Torkelson, A., Bednarczyk, D. J., and Schroepfer, G. J. (1970), *J. Biol. Chem. 245*, 3802.

Oesch, F., and Daly, J. W. (1971), *Biochim. Biophys. Acta 227*, 692.

Oesch, F., Jerina, D. M., and Daly, J. W. (1971a), *Arch. Biochem. Biophys. 144*, 253.

Oesch, F., Jerina, D. M., and Daly, J. W. (1971b), *Biochim. Biophys. Acta 227*, 685.

Pandov, H., and Sims, P. (1970), *Biochem. Pharmacol. 19*, 299.

Watabe, T., Akamatsu, K., and Kiyonaga, K. (1971), *Biochem. Biophys. Res. Commun. 44*, 199.

Watabe, T., and Kanehira, S. (1970), *Chem. Pharm. Bull. 18*, 1295.