Rothstein, M. (1977) *Mech. Ageing Dev. 6,* 241-257.
Rotman, M. B. (1970) in *The Lactose Operon* (Beckwith & Zipser, Eds.) pp 279-289, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
Ruth, R. C., Soja, D. M., & Wold, F. (1970) *Arch. Biochem. Biophys. 140,* 1-10.
Sage, H. J., & Singer, S. J. (1962) *Biochemistry 1,* 305-317.
Sarkar, P. K., & Doty, P. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55,* 981-989.
Sedlak, J., & Lindsay, R. H. (1968) *Anal. Biochem. 25,* 192-205.
Sharma, H. K., Gupta, S. K., & Rothstein, M. (1976) *Arch. Biochem. Biophys. 174,* 324-332.
Spande, T. F., & Witkop, B. (1967) *Methods Enzymol. 11,* 498-506.
Steinhagen-Thiessen, E., & Hilz, H. (1976) *Mech. Ageing Dev. 5,* 447-457.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., & Davidson, B. (1966) *Biochem. Biophys. Res. Commun. 23,* 163-169.
Weber, A., Gregori, C., & Schapira, F. (1976) *Biochim. Biophys. Acta 444,* 810-815.
Weiner, A. M., Platt, T., & Weber, K. (1972) *J. Biol. Chem. 247,* 3242-3251.
Winstead, J. A., & Wold, F. (1964) *Biochemistry 3,* 791-795.
Wrigley, C. W. (1971) *Methods Enzymol. 22,* 559-564.
Zeelon, P., Gershon, H., & Gershon, D. (1973) *Biochemistry 12,* 1743-1750.

Epoxide Hydrase: Structure–Activity Relationships of in Vitro Stimulators of the Microsomal Activity†

Vishwas S. Ganu‡ and William L. Alworth*

ABSTRACT: Benzophenone, 9-fluorenone, diphenylcyclopropenone, benzyl phenyl ketone, propyl phenyl ketone, chalcone, and chalcone oxide are in vitro stimulators of the activity of microsomal epoxide hydrase (EC 4.2.1.63). Chalcone oxide and 9-fluorenone, the more effective of these stimulators, were found to be more effective than metyrapone, a previously reported stimulator of this enzyme. Other aryl ketones, such as acetophenone, benzyl ethyl ketone, dibenzyl ketone, and phenylmethylcylopropenone, do not stimulate microsomal epoxide hydrase activity. Kinetic analyses established that, while chalcone, 9-fluorenone, diphenylcyclopropenone, and chalcone oxide all increase the observed V_{max} of the hydrase reaction, diphenylcyclopropenone and chalcone epoxide increase V_{max} and K_M by comparable factors. At higher concentrations of the styrene oxide, substrate inhibition is observed. The observed kinetics of microsomal epoxide hydrase with styrene oxide as substrate are consistent with the formation of an inactive ES_2 complex at high substrate concentrations. One in vitro effect of chalcone oxide is to largely eliminate the inhibition caused by high styrene oxide concentrations. It is concluded that compounds containing an aryl carbonyl substituted with an additional hydrophobic group are effective in vitro stimulators of epoxide hydrase. The data suggest that such compounds bind at a site which is distinct from the catalytic site where the styrene oxide substrate binds.

It is now accepted that most chemical carcinogens must be metabolically converted into reactive, electrophilic derivatives before they are capable of initiating tumors. In particular, it is believed that the carcinogenicity and mutagenicity of polycyclic aromatic hydrocarbons such as benzo[*a*]pyrene are the result of in vivo metabolism which produces reactive intermediates (ultimate carcinogens) that bind covalently with macromolecules within the cells (Miller & Miller, 1966; Sims & Grover, 1974; Jerina & Daly, 1974; Heidelberger, 1975). Recent experimental evidence, involving investigations of both the binding of benzo[*a*]pyrene derivatives to DNA and the relative mutagenicity of benzo[*a*]pyrene derivatives, indicates that diol epoxide derivatives (7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene 9,10-oxides) function as major ultimate carcinogenic metabolites of this polycyclic hydrocarbon (Thakker et al., 1977a, and references therein; Yang et al., 1977b, and references therein). The recent investigations have also established that the metabolic conversion of benzo[*a*]pyrene to the reactive diol epoxide derivatives requires microsomal monooxygenase (EC 1.14.14.1) and microsomal epoxide hydrase (EC 4.2.1.63) activities (Booth & Sims, 1976; Yang et al., 1976, 1977a, b; Thakker et al., 1976, 1977a).

The properties of epoxide hydrase have been the subject of several investigations (for a review of the early work, see Oesch, 1972). Recognition that epoxide hydrase is involved in the conversion of carcinogenic polycyclic hydrocarbons such as benzo[*a*]pyrene into their ultimate carcinogenic forms has led to increased interest in this enzyme. Recent papers, for example, have described additional investigations of the substrate specificity of both membrane-bound and purified epoxide hydrase (Bentley et al., 1976; Lu et al., 1977), a simplified method of purifying microsomal epoxide hydrase (Knowles & Burchell, 1977), new, sensitive assay procedures (Schmassmann et al., 1976; Jerina et al., 1977), additional information regarding the mechanism of the enzymatic ca-

† From The Laboratory of Chemical Biology, Department of Chemistry, Tulane University, New Orleans, Louisiana 70118. *Received January 13, 1978.* This work will be submitted by V.S.G. to Tulane University in partial fulfillment of the Ph.D. requirements in Chemistry. This work was supported, in part, by research grants from the American Cancer Society and from the Cancer Association of Greater New Orleans, Inc.

‡ Recipient of a Teagle Foundation Fellowship. Present address: Biology Division Brookhaven National Laboratory, Upton, N.Y. 11973.

0006-2960/78/0417-2876$01.00/0

talysis (Hanzlik et al., 1976; Yang et al., 1977b), the effects of epoxide hydrase inhibitors upon the microsomal metabolism of benzo[*a*]pyrene (Fahl et al., 1977), and studies of the stereospecificity of microsomal and purified epoxide hydrase (Thakker et al., 1977b).

During the initial investigations of structure–activity relationships of epoxide hydrase, over 100 compounds were screened as potential substrates or inhibitors (Oesch et al., 1971b, 1973; Oesch, 1972, 1974). In the course of these, and related investigations (Liebman & Ortiz, 1973a), a few compounds were observed to act as in vitro stimulators of epoxide hydrase activity with styrene oxide as substrate. Such compounds included cyclohexanol, glycidol, cyclododecene oxide, α-tetralone, 1,2-dimethyl-3,4-dihydronaphthalene 1,2-oxide, 2,2,3-trimethyl-3-phenyloxirane, and the aziridine analogue of 3,4-dihydronaphthalene 1,2-oxide (Oesch et al., 1971b). The most potent in vitro stimulators of epoxide hydrase activity, however, reported from these investigations are metyrapone [2-methyl-1,2-bis(3-pyridyl)-1-propanone], 1-(2-isopropylphenyl)imidazole, and 1-(2-cyanophenyl)imidazole (see especially, Oesch, 1974). These three compounds have also been found to be potent inhibitors of microsomal monooxygenase enzymes (Liebman, 1969; Liebman & Ortiz, 1973a,b; Wilkinson et al., 1974; Napoli & Counsell, 1977; Rogerson et al., 1977).[1]

Since the recent experimental evidence indicates that both epoxide hydrase and monooxygenase activities are involved in converting benzo[*a*]pyrene into its major ultimate carcinogenic metabolite, we concluded that additional investigation of the structure–activity relationships of in vitro stimulators of epoxide hydrase was warranted. Our investigations have established that benzophenone (**2**), 9-fluorenone (**8**), benzyl phenyl ketone (**6**), propyl phenyl ketone (**4**), diphenylcyclopropenone (**9**), chalcone (**11**), and chalcone oxide (**12**) all significantly stimulate the in vitro activity of the microsomal epoxide hydrase from rat liver when styrene oxide is the substrate. The more effective of these compounds, chalcone oxide and 9-fluorenone, were found to be more effective than metyrapone which was utilized as a reference standard in the studies. Several of these newly identified stimulators of epoxide hydrase possess structural features that have not been present in the previously investigated substrates, inhibitors, or stimulators of epoxide hydrase. An analysis of those chemical compounds found to have a significant stimulatory effect and the related compounds found to have no stimulatory effect (dibenzyl ketone (**7**), benzyl ethyl ketone (**5**), acetophenone (**3**), phenylmethylcyclopropenone (**10**)) provides new information regarding the structure–activity relationships of in vitro stimulators of microsomal epoxide hydrase. An analysis of the changes in the kinetic parameters of the epoxide hydrase reaction produced by the presence of the investigated stimulators suggests that these compounds bind at a site which is distinct from the active site where the styrene oxide substrate binds.

Materials and Methods

Chemicals. The [8-^{14}C]styrene oxide used in these studies as the substrate was synthesized and purified according to the procedures of Ganu et al. (1977). The diphenylcyclopropenone and the phenylmethylcyclopropenone were synthesized according to the procedures described by Breslow et al. (1959) and by Dehmlow & Dehmlow (1975), respectively, and were purified by recrystallization. Chalcone was prepared by the base catalyzed condensation of benzaldehyde and acetophenone (Vogel, 1956) and converted into chalcone oxide with hydrogen peroxide in sodium hydroxide. The chalcone oxide was recrystallized and its structure confirmed by NMR. Benzophenone, propyl phenyl ketone, benzyl ethyl ketone, and 9-fluorenone were purchased from Aldrich Chemical Co., dibenzyl ketone was from Eastman Organic Chemicals, and benzyl phenyl ketone was from Pfaltz and Bauer, Inc.

Enzyme Preparations. Male Sprague-Dawley rats (90–150 g), grown on rat chow and not injected or otherwise purposely exposed to inducers of microsomal enzymes, were sacrificed by a blow on the neck. The liver was removed quickly, sliced into small pieces, and homogenized in cold 0.25 M sucrose (3 mL per g) in a VirTis Model 23 homogenizer equipped with a "Turbo-Shear" blade assembly. The homogenate was filtered through cheese cloth and then centrifuged at 600*g* for 15 min. The resulting supernatant was recentrifuged at 8000*g* for 15 min, the pellet discarded, and the supernatant centrifuged a final time at 80 000*g* for 90 min. The resulting pellet was washed with 0.25 M sucrose, resuspended in 0.25 M sucrose, and frozen. All of the above isolation procedures were carried out at 3–6 °C; the frozen microsomal preparations were stored at −60 °C until used.

Assay Procedures. A radiometric assay using [8-^{14}C]styrene oxide (Ganu et al., 1977), based upon the standard radiometric assay developed by Oesch et al. (1971a), was used to determine the microsomal epoxide hydrase activity. The incubation medium was composed of 0.1 mL of 0.5 M Tris buffer, pH 9.0, containing 0.1% w/v of Tween 80, 0.1 mL of deionized water, 10 μL of an acetonitrile solution of the substrate (70 126 dpm, 804.5 nmol), 10 μL of an acetonitrile solution of the compound to be investigated, an additional 10 μL of acetonitrile, 0.1 mL of 0.25 M sucrose, and 0.1 mL of the microsomal preparation containing 1.9–3.5 mg of total protein (biuret determination). Each assay was initiated by addition of the substrate and terminated by addition of 1 mL of light petroleum ether (bp 30–60 °C) and rapid mixing. All incubations were carried out at 37 °C.

The amount of product produced in the assays was determined by counting an aliquot of the ^{14}C-labeled glycol product in a Beckman series 200 liquid scintillation counter with external standardizations. Experimentally established blanks, combining both counting background and nonenzymatic product formation, were subtracted to establish the net product resulting from the enzyme catalyzed hydrolysis of styrene oxide. Blanks with both high and low substrate concentrations were determined and subtracted from the appropriate assay values. The experimental blanks were established by treating the assay medium, minus the substrate and compound under investigation, at 100 °C for 15 min, cooling the solution, adding the appropriate amount of substrate and additional compound as acetonitrile solutions, and then completing the assay and analysis in the usual manner.

In the absence of added stimulators the formation of product was linear with time for at least 5 min at 37 °C within the range of protein concentrations utilized (1.9–3.5 mg/assay). In the presence of stimulators of epoxide hydrase activity the incubation period was decreased to maintain linearity of product formation; incubation times were varied from 1 to 5 min depending upon substrate concentration tested and extent of stimulation observed.

The effect of Triton X-100 (Sigma Chemical Co.) on the stimulation of epoxide hydrase was investigated by preparing buffer containing 2% Triton X-100 instead of the usual 0.1%

[1] It is worth noting that we have found that each of the in vitro stimulators of microsomal epoxide hydrase in Table I is also an in vitro inhibitor of the microsomal monooxygenase when assayed with [4′-^{3}H]benzenesulfonanilide (Alworth, W. L., Fingerman, S. W., Temple, T. T., & Branyas, N. A., 1977, unpublished observations).

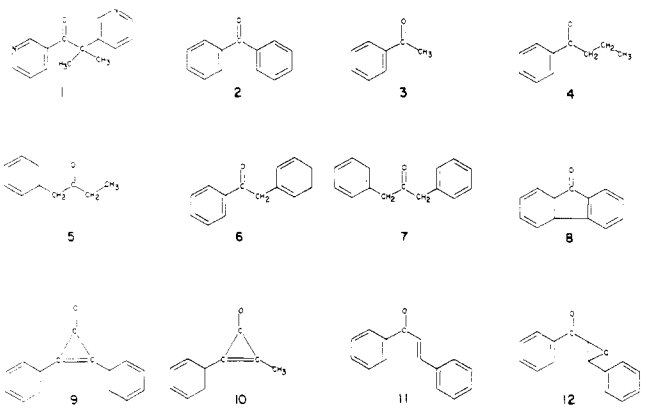

FIGURE 1: Compounds tested as in vitro stimulators of the activity of microsomal epoxide hydrase.

TABLE I: Stimulators of Microsomal Epoxide Hydratase.

stimulators[a]	obsd activation (%)[b] styrene oxide (substrate) concn 0.122 mM	0.49 mM	1.87 mM
metyrapone (**1**)	43	91	50
benzophenone (**2**)	40	93	71
propyl phenyl ketone (**4**)	68	49	34
benzyl phenyl ketone (**6**)	56	53	60
9-fluorenone (**8**)	110	128	82
diphenylcyclopropenone (**9**)	14	79	76
chalcone (**11**)	44	56	60
chalcone oxide (**12**)	32	140	310

[a] All stimulators were tested at 2 mM concentration except chalcone oxide which, for solubility reasons, was tested at 1.5 mM. [b] (Activity in presence of tested compound − activity in absence)/(activity in absence of tested compound) times 100. All values represent the average of a least two independent determinations in which the experimental variation was ≤10%. Microsomal protein concentrations used in all these determinations were within the range 4.47–8.14 mg/mL.

Tween 80. Each assay in this series of investigations contained a total of 2 mg of protein (Bradford, 1976), thus producing a detergent:protein ratio of 1 g:1 g. The assays in the presence of Triton X-100 were incubated for 5 min at 37 °C.

Results

The structures of the compounds tested in this investigation are shown in Figure 1.

The experimental data summarized in Table I established that benzophenone (**2**), diphenylcyclopropenone (**9**), benzyl phenyl ketone (**6**), propyl phenyl ketone (**4**), 9-fluorenone (**8**), chalcone (**11**), and chalcone oxide (**12**), when added to the incubation medium, all stimulate the activity of microsomal epoxide hydrase as measured by a standard radiometric assay with styrene oxide substrate. Table I also includes our data regarding the effect of added metyrapone (**1**), a previously reported stimulator of epoxide hydrase which was utilized in these investigations as a reference standard. Figure 2 illustrates the alterations in the observed kinetic parameters that are produced by adding chalcone oxide. Table II summarizes the changes in the observed kinetic parameters produced by the additions of chalcone oxide, diphenylcyclopropenone, 9-fluorenone, and chalcone to the incubation medium.

The plot in Figure 2 corresponding to the reaction rate with styrene oxide in the absence of added chalcone oxide is based upon a computer calculated fit of the kinetic expression $v = V_{max}S/(K_M + S + (S^2/K_i))$. All other K_M and V_{max} values

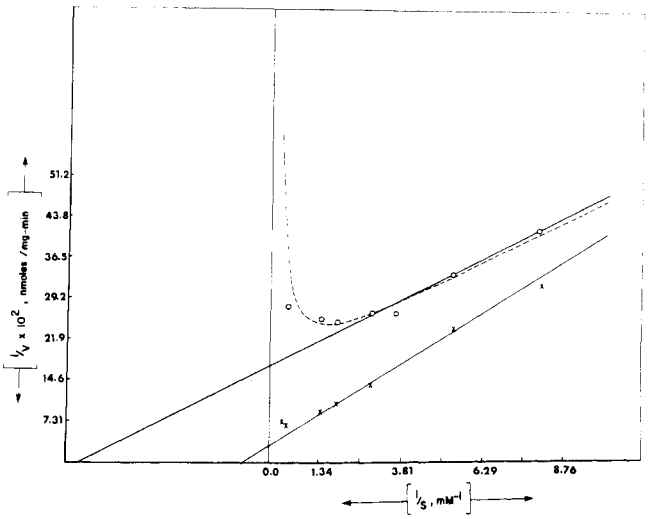

FIGURE 2: Activities of microsomal epoxide hydrase (6.05 mg/mL) with styrene oxide as substrate in the absence (O) and in the presence (X) of 1.5 mM chalcone oxide. Each point represents the average of a least two independent determinations in which the observed experimental variation was ≤10%. The dashed line is a computer drawn curve based upon the assumption that an inactive ES_2 complex forms at high styrene oxide concentrations.

TABLE II: Kinetic Parameters for Epoxide Hydrase Catalyzed Hydrolysis of Styrene Oxide in the Presence of Selected Stimulators.

stimulator added[a]	obsd K_M[b] (mM)	obsd V_{max}[b] (nmol of product/(mg min))
none	0.19[c]	6.2[c]
chalcone oxide	1.17	32.3
diphenylcyclopropenone	0.61	17.0
9-fluorenone	0.12	10.2
chalcone	0.25	10.4

[a] All stimulators were present at 2 mM concentration except chalcone oxide, for solubility reasons, was present at 1.5 mM. [b] At least two independent values of the velocity at four different substrate concentrations within the linear range (0.089 to 0.336 mm, see Figure 2) were obtained and the kinetic parameters determined from this data by the direct linear method of Eisenthal & Cornish-Bowden (1974). [c] The parameters for the epoxide hydrase reaction in the absence of added stimulators are average values based upon control values obtained in the investigation of the effect of each of the stimulators in Table II. Separate values of K_M and V_{max} determined in the independent experiments differ from the average values reported in Table II by ≤10%.

in Figure 2 and in Table II were determined by the direct linear graphical method of Eisenthal & Cornish-Bowden (1974). Cornish-Bowden & Eisenthal (1974) discussed why their graphical method of determining K_M and V_{max} is more statistically valid than the graphical methods customarily used. In the present investigation, since the enzyme reaction is inhibited by high concentrations of the styrene oxide substrate and thus shows significant departure from Michaelis–Menten kinetics (see Figure 2), only velocity values determined in the linear region of the kinetic plots (0.089–0.336 mM styrene oxide) were utilized in the determination of K_M and V_{max} by the Eisenthal–Cornish-Bowden graphical method. The dashed line in Figure 2 represents a calculated fit of the kinetic expression $v = V_{max}S/(K_M + S + (S^2/K_I))$. A least-squares analysis established that the best calculated fit occurred at K_M = 0.185 mM, K_i = 2.88 mM, and V_{max} = 6.15 nmol/(mg min). The Eisenthal–Cornish-Bowden analysis yielded values

of K_M = 0.17 mM and V_{max} = 5.7 nmol/(mg min), and these values were used to draw the appropriate straight line in Figure 2.

It is apparent from the summarized results in Tables I and II that effectiveness of in vitro stimulators of epoxide hydrase activity must be measured at several substrate concentrations before valid comparisons are possible. We find, for example, that chalcone oxide is the most effective stimulator tested at 1.87 mM styrene oxide concentration, but that 9-fluorenone is the most effective at 0.122 mM styrene oxide concentration (Table I). Unfortunately, the data in the literature report the measured activation of epoxide hydrase at a single concentration of styrene oxide (at saturating 2 mM concentration) and is therefore of limited value for comparative purposes.

In addition to the data presented in Table I, dibenzyl ketone (**7**), acetophenone (**3**), benzyl ethyl ketone (**5**), and phenylmethylcyclopropenone (**10**) were tested as stimulators of epoxide hydrase activity. The effects of these compounds at 2 mM concentrations were tested in the presence of both 1.87 mM and 0.179 mM styrene oxide. No stimulation of the epoxide hydrase activity by these compounds was detected.

Burchell et al. (1976) reported that microsomal epoxide hydrase can be stimulated 30–60% by high concentrations of neutral or anionic detergents (1–2 g:1 g protein). We therefore investigated the effect of added Triton X-100 upon the in vitro stimulation of epoxide hydrase by various aryl ketones. Our investigations were carried out at higher protein concentrations than the studies of Burchell et al., and the total Triton X-100 concentration in our investigations was therefore about ten times that which produced the optimum stimulation in the studies of Burchell et al. Under these conditions, we did not observe a stimulation of the microsomal epoxide hydrase by Triton X-100 alone. We did observe, however, that, even in the presence of high Triton X-100 concentration (1 g:1 g protein), 2 mM metyrapone, 2 mM 9-fluorenone, and 1.5 mM chalcone oxide continue to effect a significant stimulation of microsomal epoxide hydrase activity. The observed stimulations were 129%, 82%, and 162%, respectively, when 1.87 mM styrene oxide was the substrate.

Discussion

Our investigations were prompted by an initial observation that diphenylcyclopropenone was an effective activator of microsomal epoxide hydrase. We had anticipated that the molecular structure of diphenylcyclopropenone with its strongly polarized carbonyl group (Breslow et al., 1965) might resemble the structure of an arene oxide substrate bound to the catalytic site of epoxide hydrase, and that diphenylcyclopropenone would therefore serve as a competitive inhibitor of the enzyme. Since the experimental results were directly opposite to our predictions, we reviewed the data in the literature regarding the in vitro stimulation of epoxide hydrase activity. We found that, while this phenomenon had been described in several papers, and that, although a number of compounds had been reported to function as stimulators of epoxide hydrase (Oesch, 1972; Oesch et al., 1971b, 1973; Liebman & Ortiz, 1973a; Oesch, 1974; Lu et al., 1975), no systematic investigation of structure–activity relationships of this phenomenon had been described. A series of compounds with structural elements common to both diphenylcyclopropenone and metyrapone was therefore chosen for study.

Liebman & Ortiz (1973a) reported stimulations of microsomal epoxide hydrase from rat liver of 200% with 0.1 mM 1-(2-isopropylphenyl)imidazole and about 130% with 1 mM metyrapone. With purified epoxide hydrase from rat liver, Oesch (1974) reported stimulations of 76% by 2 mM 1-(2-isopropylphenyl)imidazole and of 87% by 2 mM metyrapone, while Lu et al. (1975) observed stimulations of 220% with 1.5 mM metyrapone and 1.5 mM 1-(2-isopropylphenyl)imidazole. In all three investigations styrene oxide was the substrate. Most recently Salmona et al. (1976) have reported that 1 mM metyrapone *inhibits* the activity of rat liver epoxide hydrase with styrene oxide as substrate by 40%.[2]

The data summarized in Tables I and II demonstrate that the observed in vitro stimulation of epoxide hydrase is a complex phenomenon. The data emphasize the fact that determining the true relative effectiveness of a series of stimulators with assays at a single, saturating level of styrene oxide is not possible. Furthermore, Lu et al. (1977) recently noted that kinetic parameters for membrane-bound epoxide hydrase are affected by the amount of microsomes used, while the parameters for purified epoxide hydrase are affected by added phospholipid. In initial experiments we noted that the amount of microsomal protein used appeared to affect the extent of the stimulation of epoxide hydrase produced by a given compound. For our comparison studies, therefore, the amount of microsomal protein was kept within the limits 4.47–8.14 mg/mL.

In spite of the complexities, we believe the data reported here support the following conclusions. First, the lack of a stimulatory effect of dibenzyl ketone (**7**) or benzyl ethyl ketone (**5**), in comparison with the active stimulators in Table I, indicates the critical importance of an aryl ketone grouping within the series of stimulators related to metyrapone.[3] This conclusion is consistent with the observation of Oesch et al. (1971b) that reduction of metyrapone to the corresponding alcohol eliminated the stimulatory effect. Second, a comparison of compounds such as phenylmethylcyclopropenone (**10**), and acetophenone (**3**), which fail to stimulate epoxide hydrase, with such active stimulators as diphenylcyclopropenone (**9**), metyrapone (**1**), benzophenone (**2**), 9-fluorenone (**8**), and propyl phenyl ketone (**4**), emphasizes the additional importance of a second, relatively large hydrophobic substituent within this series.[4] Third, comparison of the extent of stimulation produced by chalcone (**11**) with that produced by chalcone oxide (**13**) (Table I) clearly establishes that functionality in addition to the critical ketone grouping discussed above can serve to increase the stimulatory effect. In this case,

---

[2] The experiments of Salmona et al. (1976) were performed at pH 7.4 in the presence of 150 mM KCl and 5 mM $MgCl_2$. The microsomes were also preincubated with metyrapone for 15 min prior to the introduction of the styrene oxide substrate. These assay conditions differ from those generally used (Oesch et al., 1971a). We have found that metyrapone (1 mM), when added in acetonitrile solutions, continues to stimulate epoxide hydrase by 45% at pH 7.4 in the presence of 150 mM KCl and 5 mM $MgCl_2$. Preincubation also had no significant effect upon the stimulation normally produced by metyrapone. We did find, however, that the addition of metyrapone in acetone solution as described by these workers lowered the percent activation by metyrapone to 15% (Ganu, V. S., & Alworth, W. L., 1977, unpublished observations).

[3] The conclusions of this study regarding the structure–activity relationships of epoxide hydrase activators only apply to the series of compounds (aryl ketones) related to metyrapone. As noted above, additional compounds of seemingly unrelated structure, such as 1-arylimidazoles, are also known to stimulate the in vitro activity of epoxide hydrase. At present the relationship of these compounds to the aryl ketone stimulators is unclear. We have recently established that 1-(2-isopropylphenyl)pyrrole is not a stimulator of epoxide hydrase (Ganu, V. S., & Alworth, W. L., unpublished data). It thus appears that the N-3 nitrogen function of 1-(2-isopropylphenyl)imidazole is required for activity. The nucleophilic N-3 position of the 1-arylimidazole may therefore be fulfilling the function of the necessary carbonyl grouping of the aryl ketone activators.

[4] We have recently observed that analogues of metyrapone with phenyl rings replacing the pyridyl rings possess some stimulatory effect. Thus, the pyridyl rings of metyrapone are not a critical feature of this stimulator (Ganu, V. S., Bonner, M., & Alworth, W. L., 1977, unpublished data).

the presence of an oxirane group α to the aryl ketone markedly enhances the observed stimulation at the higher concentration of styrene oxide. Indeed, at saturating levels of styrene oxide substrate, we find that 1.5 mM chalcone oxide is about *six times* as effective at stimulating epoxide hydrase activity as is 2 mM metyrapone.

Although the effective stimulators of epoxide hydrase reported here appear to possess structural features related to epoxide hydrase substrates, analysis of the changes in kinetic parameters that occur in the presence of stimulators of epoxide hydrase (Table II) indicates that these compounds bind to the enzyme at a site that is distinct from the catalytically active binding site for the styrene oxide substrate. We find that the addition of diphenylcyclopropenone or of chalcone oxide to the epoxide hydrase assay medium leads to an uncompetitive type activation plot in which V_{max} and K_M are increased to the same extent. The stimulation of epoxide hydrase by metyrapone has previously been reported to yield an uncompetitive type kinetic plot (Oesch et al., 1971b). In contrast, the increased V_{max} observed when chalcone is added to the incubation is accompanied by only a slight increase in the apparent K_M and the increased V_{max} in the presence of 9-fluorenone is accompanied by a decrease in apparent K_M. All these experimental observations can be interpreted by a single general scheme of equilibria for nonessential activation involving an enzyme, substrate, activator (ESA) complex (see, for example, Scheme A1, General Scheme for Nonessential Activation as described by Segel, 1975). The type of uncompetitive type activation observed in the presence of chalcone oxide or diphenylcyclopropenone is readily explained by postulating that the activator molecule A binds more tightly to E than to the ES complex and that the ESA ternary complex is more reactive than the ES complex (Segel's Scheme A1 with $\alpha = \beta > 1$). Changes in the relative binding constants of the activator molecule to E and to ES and changes in the relative catalytic rate constants for the ESA complexes can be used to explain the variations in kinetic parameters observed in the presence of both chalcone and 9-fluorenone. Overall, therefore, these observations imply that the enzyme must possess two distinct binding sites, one for S and one for A.

Figure 2 demonstrates the marked substrate inhibition produced by higher concentrations of styrene oxide. Oesch (1972) has previously reported substrate inhibition by high concentrations of styrene oxide and recently Lu et al. (1977) have reported that nonlinear Lineweaver–Burk plots were obtained when styrene oxide was utilized as a substrate for membrane-bound epoxide hydrase. We have assumed that the observed inhibition is caused by the binding of a second molecule of styrene oxide to form an unreactive ternary ES_2 complex and have analyzed the kinetics of styrene oxide hydration by microsomal epoxide hydrase in terms of the expression $v = V_{max}S/(K_M + S + (S^2/K_i))$. As shown by the dashed curve in Figure 2, the computer calculated plot fits the experimental data reasonably well. Unfortunately, solubility problems made it impossible to obtain experimental data points at even higher styrene oxide concentrations.

Figure 2 shows that the addition of chalcone oxide to the assay medium largely abolishes the substrate inhibition observed at higher concentrations of styrene oxide. Chalcone oxide is unique among the investigated stimulators in this regard; diphenylcyclopropenone, chalcone, or fluorenone do not abolish the substrate inhibition observed at high styrene oxide concentrations. This effect of chalcone oxide, when combined with the observed uncompetitive activation by chalcone oxide, with the indicated formation of an unreactive ES_2 complex, and finally with a comparison of the structures of styrene oxide and chalcone oxide, lead us to conclude that a major feature of the stimulation of epoxide hydrase activity by chalcone oxide involves binding to the ES complex in a manner that prevents formation of the unreactive ES_2 complex. We believe it is reasonable to propose that chalcone oxide, as an activator A, occupies the same site as does the styrene oxide when it binds as an inhibitor to form the ES_2 ternary complex. Overall, therefore, we conclude that compounds possessing such structural features as are represented by the series of active stimulators in Table I bind at a site on the enzyme that is distinct from the catalytic site where styrene oxide binds as substrate.

The data in this paper and data in the literature (see especially, Oesch et al., 1971b) clearly establish that only a relatively small group of compounds possessing specific structural features are capable of stimulating epoxide hydrase activity. In addition, it has been established that the in vitro stimulation of epoxide hydrase activity occurs with both the membrane-bound and the highly purified (lipid-depleted) enzyme (Oesch et al., 1971b; Oesch, 1974; Lu et al., 1975). We have now established that metyrapone, 9-fluorenone, and chalcone oxide continue to stimulate the in vitro activity of epoxide hydrase even in the presence of high concentrations of Triton X-100. We believe, therefore, that the data establish that the stimulation phenomenon reflects a true property of the epoxide hydrase enzyme and that the observed modification of the enzyme activity is of some, as yet undetermined, physiological significance. It must be noted, however, that the stimulation of epoxide hydrase activity by various compounds in vivo has not yet been demonstrated.

With the aid of the structure–activity relationships described in this paper, it should be possible to design significantly more effective stimulators of epoxide hydrase. Such stimulators may then be utilized to investigate the possible physiological significance of this phenomenon. Investigations by Levin et al. (1977) have demonstrated that the *relative levels* of epoxide hydrase and monooxygenase activities are of more critical importance than are the *absolute levels* of these enzymatic activities in determining the distribution among the types of benzo[*a*]pyrene metabolites produced by different microsomal preparations. In vitro experiments carried out by Oesch and co-workers (Oesch, 1976; Oesch et al., 1976) have shown that the addition of epoxide hydrase inhibitors to microsomal preparations metabolizing benzo[*a*]pyrene results in increased mutagenicity of the benzo[*a*]pyrene products toward *Salmonella typhimurium* TA 1537. These workers also found that the addition of homogeneous epoxide hydrase to the microsomal preparations results in significantly decreased mutagenicity of the benzo[*a*]pyrene metabolites. Wood et al. (1976) have reported that the addition of highly purified epoxide hydrase to a purified and reconstituted cytochrome P-448-dependent monooxygenase system also markedly decreased the number of mutations produced when such systems are used to metabolically activate benzo[*a*]pyrene. These results indicate that the effect of stimulators of epoxide hydrase upon the metabolism of carcinogenic arylhydrocarbons such as benzo[*a*]pyrene may be of great potential significance.

Acknowledgments

We wish to acknowledge that this research effort has benefited from several helpful discussions with L. D. Byers. M. H. Nodine provided the computer programs used to calculate the enzyme velocity in the presence of inhibiting levels of substrate.

References

Bentley, P., Schmassmann, H., Sims, P., & Oesch, F. (1976) *Eur. J. Biochem. 69,* 97–103.

Booth, J., & Sims, P. (1976) *Biochem. Pharmacol. 25,* 979–980.

Bradford, M. M. (1976) *Anal. Biochem. 72,* 248–254.

Breslow, R., Haynie, R., & Mirra, J. (1959) *J. Am. Chem. Soc. 81,* 247–248.

Breslow, R., Eicher, T., Krebs, A., Peterson, R. A., & Posner, J. (1965) *J. Am. Chem. Soc. 87,* 1320–1325.

Burchell, B., Bentley, P., & Oesch, F. (1976) *Biochim. Biophys. Acta 444,* 531–538.

Cornish-Bowden, A., & Eisenthal, R. (1974) *Biochem. J. 139,* 721–730.

Dehmlow, S. S., & Dehmlow, E. V. (1975) *Z. Naturforsch. B 30,* 404–408.

Eisenthal, R., & Cornish-Bowden, A. (1974) *Biochem. J. 139,* 715–720.

Fahl, W. E., Nesnow, S., & Jefcoate, C. R. (1977) *Arch. Biochem. Biophys. 181,* 649–664.

Ganu, V. S., Nelson, S. O., Verlander, L., & Alworth, W. L. (1977) *Anal. Biochem. 78,* 451–458.

Hanzlik, R. P., Edelman, M., Michaely, W. J., & Scott, G. (1976) *J. Am. Chem. Soc. 98,* 1952–1955.

Heidelberger, C. (1975) *Annu. Rev. Biochem. 44,* 79–121.

Jerina, D. M., & Daly, J. W. (1974) *Science 185,* 573–582.

Jerina, D. M., Dansette, P. M., Lu, A. Y. H., & Levin, W. (1977) *Mol. Pharmacol. 13,* 342–351.

Knowles, R. G., & Burchell, B. (1977) *Biochem. J. 163,* 381–383.

Levin, W., Lu, A. Y. H., Ryan, D., Wood, A. W., Kapitulnik, J., West, S., Huang, M.-T., Conney, A. H., Thakker, D. R., Holder, G., Yagi, H., & Jerina, D. M. (1977) in *Origins of Human Cancer, Book B* (Hiatt, H. H., Watson, J. D., & Winsten, J. A., Eds.) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p 668.

Liebman, K. C. (1969) *Mol. Pharmacol. 5,* 1–9.

Liebman, K. C., & Ortiz, E. (1973a) *Drug Metab. Dispos. 1,* 775–779.

Liebman, K. C., & Ortiz, E. (1973b) *Drug Metab. Dispos. 1,* 184–190.

Lu, A. Y. H., Ryan, D., Jerina, D. M., Daly, J. W., & Levin W. (1975) *J. Biol. Chem. 250,* 8283–8288.

Lu, A. Y. H., Jerina, D. M., & Levin W. (1977) *J. Biol. Chem. 252,* 3715–3723.

Miller, E. C., & Miller, J. A. (1966) *Pharmacol. Rev. 18,* 805–838.

Napoli, J. L., & Counsell, R. E. (1977) *J. Med. Chem. 20,* 762–766.

Oesch, F. (1972) *Xenobiotica 3,* 305–340.

Oesch, F. (1974) *Biochem. J. 139,* 77–88.

Oesch, F. (1976) *J. Biol. Chem. 251,* 79–87.

Oesch, F., Jerina, D. M., & Daly, J. W. (1971a) *Biochim. Biophys. Acta 227,* 685–691.

Oesch, F., Kaubisch, N., Jerina, D. M., & Daly, J. W. (1971b) *Biochemistry 10,* 4858–4866.

Oesch, F., Jerina, D. M., Daly, J. W., & Rice, J. M. (1973) *Chem.-Biol. Interact. 6,* 189–202.

Oesch, F., Bentley, P., & Glatt, H. R. (1976) *Int. J. Cancer 18,* 448–452.

Rogerson, T. D., Wilkinson, C. F., & Hetnarski, K. (1977) *Biochem. Pharmacol. 26,* 1039–1042.

Salmona, M., Pachecka, J., Cantoni, L., Belvedere, G., Mussini, E., & Garattini, S. (1976) *Xenobiotica 6,* 585–591.

Schmassmann, H. U., Glatt, H. R., & Oesch, F. (1976) *Anal. Biochem. 74,* 94–104.

Segal, I. H. (1975) in *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems,* Wiley-Interscience, New York, N.Y., Chapter 5.

Sims, P., & Grover, P. L. (1974) *Adv. Cancer Res. 20,* 166–262.

Thakker, D. R., Yagi, H., Lu, A. Y. H., Levin, W., Conney, A. H., & Jerina, D. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 3381–3385.

Thakker, D. R., Yagi, H., Akagi, H., Koreeda, M., Lu, A. Y. H., Levin, W., Wood, A. W., Conney, A. H., & Jerina, D. M. (1977a) *Chem.-Biol. Interact. 16,* 281–300.

Thakker, D. R., Yagi, H., Levin, W., Lu, A. Y. H., Conney, A. H., & Jerina, D. M. (1977b) *J. Biol. Chem. 252,* 6328–6334.

Vogel, A. I. (1956) *A Textbook of Practical Organic Chemistry,* Longman Green and Co. Ltd., London, p 718.

Wilkinson, C. F., Hetnarski, K., & Hicks, L. J. (1974) *Pesticide Biochem. Physiol. 4,* 299–312.

Wood, A. W., Levin, W., Lu, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M., & Conney, A. H. (1976) *J. Biol. Chem. 251,* 4882–4890.

Yang, S. K., McCourt, D. W., Roller, P. R., & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 2594–2598.

Yang, S. K., McCourt, D. W., Leutz, J. C., & Gelboin, H. V. (1977a) *Science 196,* 1199–1200.

Yang, S. K., Roller, P. R., & Gelboin, H. V. (1977b) *Biochemistry 16,* 3680–3687.