Biochemical Pharmacology, Vol. 35, No. 5, pp. 839–845, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

SELECTIVE INHIBITION AND SELECTIVE INDUCTION OF MULTIPLE MICROSOMAL EPOXIDE HYDROLASES

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(*Received* 26 *March* 1985; *accepted* 15 *August* 1985)

Abstract—The inhibition *in vitro* and induction *in vivo* of microsomal *trans*-stilbene oxide hydrolase have been studied. This microsomal epoxide hydrolase activity is distinguishable from the previously well-defined microsomal arene oxide hydrolase by a number of catalytic criteria. Two substituted chalcone oxides, 4-phenylchalcone oxide and 4′-phenylchalcone oxide, are potent inhibitors of microsomal *trans*-stilbene oxide hydrolase, but have no apparent activity against benzo[*a*]pyrene 4,5-oxide hydrolase. Conversely, compounds that are potent inhibitors of benzo[*a*]pyrene 4,5-oxide hydrolase, including styrene oxide, cyclohexene oxide, and trichloropropene oxide, inhibit microsomal *trans*-stilbene oxide hydrolase only at very high (millimolar) concentrations. The chalcone oxides inhibit microsomal *trans*-stilbene oxide hydrolase noncompetitively, and have micromolar or nanomolar affinity constants for the enzyme. Attempts were made to induce microsomal *trans*-stilbene oxide hydrolase *in vivo*. Compounds that induced microsomal benzo[*a*]pyrene 4,5-oxide hydrolase levels in mice did not simultaneously induce *trans*-stilbene oxide hydrolase levels. Clofibrate was an exception; it induced levels of both enzymes to a small but statistically significant degree. The two microsomal hydrolase activities have, therefore, very different catalytic sites and appear to be under separate genetic control. 4-Phenylchalcone oxide and 4′-phenylchalcone oxide are selective inhibitors of microsomal *trans*-stilbene oxide hydrolase and may prove to be very useful in assessing the involvement of this enzyme in the metabolism of endogenous or xenobiotic epoxides.

Epoxidation by cytochrome P-450 is a common route of metabolism for the numerous xenobiotic olefins and aromatic compounds encountered by the cell [1–5]. Because these epoxides are often highly reactive electrophiles, and can readily form covalent bonds with cellular protein and DNA, they are common ultimate toxins, mutagens and carcinogens [3–9]. Epoxide hydrolases are cellular enzymes that catalyze the conversion of epoxides to dihydrodiols [5, 7–9]. Because dihydrodiols are much less reactive than their epoxide precursors, epoxide hydrolases are among the most important endogenous means of protection against reactive epoxide intermediates [7–9].

Several different epoxide hydrolases have been shown to exist in the cell. These enzymes can be distinguished on the basis of their substrate specificities and their abilities to bind to specific antibodies. The most widely studied epoxide hydrolase is a membrane-bound form that catalyzes the hydrolysis of arene oxides and of other *cis*-disubstituted and monosubstituted oxiranes [5, 8, 9]. This form of epoxide hydrolase has been found in almost every organ and tissue investigated and is distributed throughout the animal kingdom [10, 11]. The important role of this enzyme in the metabolism and detoxification of a large number of toxins and carcinogens has been well established [8, 9]. This epoxide hydrolase actively metabolizes the model substrates styrene oxide and benzo[*a*]pyrene 4,5-oxide (BPO)†, and is referred to in this report as microsomal BPO hydrolase.

More recently, several additional epoxide hydrolases have been identified and partially characterized. These enzymes also hydrate a number of endogenous and xenobiotic epoxides, but have catalytic and immunoreactive properties that demonstrate their non-identity with microsomal BPO hydrolase. One such epoxide hydrolase is found primarily in the cytosol and in the luminal fluid of the mitochondrion, and catalyzes the hydration of a number of alkene oxides, including *trans*-1,2-disubstituted as well as more highly substituted oxiranes [12–14]. This epoxide hydrolase has been shown to be different from microsomal BPO hydrolase by catalytic [15–17] and immunological [18] criteria. This enzyme has been characterized using several *trans*-substituted oxiranes including *trans*-stilbene oxide (TSO) [15, 19] as model substrates, and is referred to in this report as cytosolic TSO hydrolase.

Two additional different microsomal epoxide hydrolases have also been identified recently. One of these catalyzes the conversion of cholesterol 5,6-oxide to the corresponding diol. No xenobiotic epoxides are known to be metabolized by this enzyme; cholesterol 5,6-oxide and other Δ^5-steroid epoxides are its only known substrates to date [20–22]. This enzyme appears to be unrelated either catalytically or structurally to any other known epoxide hydrolase [20, 22] and probably plays a very

* Supported by PHS Grant CA 34455.

† Abbreviations: BPO, benzo[*a*]pyrene 4,5-oxide; TSO, *trans*-stilbene oxide; 4PCO, 4-phenylchalcone oxide; 4′PCO, 4′-phenylchalcone oxide; 2AAF, 2-acetylaminofluorene; BHA, butylated hydroxyanisole; TCPO, 3,3,3-trichloropropene 1,2-oxide; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance spectroscopy; HPLC, high performance liquid chromatography; and I_{50}, inhibitor concentration resulting in 50% inhibition of enzyme activity.

specific and limited physiological role. A second different microsomal epoxide hydrolase which appears to be very similar to cytosolic TSO hydrolase has also been described. This epoxide hydrolase is an integral microsomal protein, but it has the same apparent substrate specificity, pH optimum, and kinetic properties as cytosolic TSO hydrolase [23]. Its substrate specificity is quite different from that of microsomal BPO hydrolase, and the complete absence of cross-reactivity of the enzyme responsible for TSO hydration in the microsomal membrane with antiserum specific to microsomal BPO hydrolase demonstrates that this activity is not attributable to nonspecific reactions of microsomal BPO hydrolase [23]. This microsomal epoxide hydrolase is referred to in this report as microsomal TSO hydrolase.

The purpose of this report is to further characterize the catalytic properties of microsomal TSO hydrolase using a series of inhibitors and activators of epoxide hydrolase activity, and to investigate the possibility of independent modulation of microsomal BPO and TSO hydrolase activities by these compounds *in vitro*. The inducibility *in vivo* of microsomal TSO hydrolase by a series of compounds which are known to induce microsomal BPO hydrolase was also investigated. These studies should be useful in defining and characterizing the differences between microsomal TSO hydrolase and other microsomal epoxide hydrolases. Furthermore, the identification of compounds that selectively induce or inhibit this or other single forms of microsomal epoxide hydrolase should provide useful tools for studying the physiological and/or toxicological roles of these individual epoxide hydrolases.

MATERIALS AND METHODS

4-Phenylchalcone oxide (4PCO) and 4′-phenylchalcone oxide (4′PCO) were gifts from Dr. Bruch Hammock, Departments of Entomology and Environmental Toxicology, University of California at Davis. 2-Acetylaminofluorene (2AAF), butylated hydroxyanisole (BHA), clofibrate, clotrimazole, ellipticine, harmane, metyrapone, and trichloropropene oxide (TCPO) were purchased from the Sigma Chemical Co., St. Louis, MO. Benzofuran, isoquinoline, styrene oxide, desyl chloride, unlabeled sodium borohydride, and unlabeled TSO were obtained from the Aldrich Chemical Co., Milwaukee, WI. Tritiated sodium borohydride was purchased from New England Nuclear, Boston, MA. Sodium phenobarbital was obtained from J. T. Baker, Phillipsburg, NJ.

Tritiated TSO was synthesized by reacting desyl chloride with sodium boro[^{3}H]hydride and cyclizing the resulting chlorohydrin with base [17]. The *trans*-epoxide was purified from the product mixture by silica gel column chromatography and preparative TLC on silica gel plates, yielding a product that was judged by analytical HPLC and NMR to be 99.9% free of either *cis*-stilbene oxide or diphenylethanediol. The labeled TSO was diluted with unlabeled TSO that had been purified by silica gel chromatography and recrystallization. The final product used in the enzyme activity assays had a specific activity of 7200 cpm/mole. Tritiated BPO was obtained from the National Cancer Institute's Chemical Carcinogen Reference Standard Repository, and was diluted for use in the enzyme assays with unlabeled BPO, obtained from the same source. The specific activity of the final product was 1050 cpm/mole.

Six-week-old male C57B1/6 mice, obtained from Harlen Sprague Dawley, Inc., Indianapolis, IN, were used in all experiments. The mice were kept under strict temperature and light–dark regulation on hardwood bedding in plastic cages. They were allowed free access to tap water and Purina Rat Chow. For induction experiments, the putative inducers were dissolved in either corn oil or normal saline and injected intraperitoneally at 24-hr intervals for 4 days. Mice treated with corn oil were the source of control livers in induction experiments. In the inhibition experiments, livers from untreated mice were used. Preliminary experiments showed that enzyme activities measured in livers obtained from corn oil-treated or saline-treated mice did not differ from activitities in untreated animals. The following treatment regimens, which had been shown in other studies to produce maximal induction of microsomal BPO hydrolase in rats, were used: 2AAF, 100 mg/kg/day [24]; benzofuran, 150 mg/kg/day*; BHA, 300 mg/kg/day [25]; phenobarbital, 80 mg/kg/day [26]; and TSO, 400 mg/kg/day [26]. Clofibrate was injected at a dose of 250 mg/kg/day.

Mice were killed, 24 hr after the last injection when pretreated, and liver microsomes were prepared by published methods [23]. Microsomal fractions could be stored at −75° at least 2 weeks with no loss of BPO hydrolase or TSO hydrolase activities. BPO hydrolase and TSO hydrolase activities were measured by published methods [15]; the substrates were removed by solvent extraction, and the remaining diol products were quantitated by liquid scintillation chromatography. Under the conditions employed, reaction rates were linear with respect to time and protein concentration. Radiolabeled substrates were added in 1 or 3 μl of acetonitrile. Incubation blanks contained either boiled microsomes or buffer in place of active microsomes, but always contained the same amount of substrate as the samples to which they were compared. In the inhibition studies, "uninhibited" samples contained 5 μl of methanol alone, and all "inhibited" samples contained 5 μl of methanol containing varied concentrations of inhibitors. Substrate concentrations of 50 μM TSO and 25 μM BPO were used in the determination of I_{50} values as well as in the induction studies.

I_{50} values were calculated by probit analysis of percent inhibition of enzyme activity as a function of the log of the inhibitor concentration, using a published computer program [27]. The inhibition constants K_{ii} and K_{is} were estimated using a computer program published by Cleland [28], which calculates the best fit of the data shown in Figs. 2 and 3 to the equation corresponding to a noncompetitive inhibition model:

$$v = VA/[K(1 + I/K_{is})\, A\, (1 + I/K_{ii})]$$

* Personal communication, with permission of J. W. Bridges.

where v = initial velocity; V = maximal velocity; K = Michaelis constant; I = inhibitor concentration; A = substrate concentration; and K_{is} and K_{ii} are the inhibitor constants. A weighted least squares method was used to determine the best fit. The induction of BPO hydrolase and TSO hydrolase activities was judged by using analysis of variance to compare enzyme activities obtained after treatment with those from control animals.

RESULTS

A series of compounds, all previously shown to either inhibit or activate epoxide hydrolases *in vitro*, were tested to determine their effects on microsomal TSO hydrolase activity. Table 1 shows the results of these experiments. The two substituted chalcone oxides, 4-PCO and 4'-PCO (Fig. 1), which were shown previously to be potent inhibitors of cytosolic TSO hydrolase [17], were also potent inhibitors of microsomal TSO hydrolase. The I_{50} values obtained here, 68 nM for 4PCO and 3.8 μM for 4'PCO, are similar to the reported values of 64 nM (4PCO) and 0.76 μM (4'PCO) for cytosolic TSO hydrolase [17].

In addition, three epoxides that are potent inhibitors of microsomal BPO hydrolase [29] were examined as inhibitors of microsomal TSO hydrolase. These three compounds, styrene oxide, cyclohexene oxide, and TCPO all inhibited microsomal TSO hydrolase. However, the concentrations required for 50% were all in the millimolar range, over 10^5-fold higher than the concentration of substrate. It should be noted that these three compounds inhibit microsomal BPO hydrolase at concentrations in the micromolar range [29].

Also, the effects on microsomal TSO hydrolase of five compounds that have been shown to activate microsomal BPO hydrolase *in vitro* [30, 31] were

Table 1. *In vitro* inhibition of microsomal TSO hydrolase*

Inhibitor	Concentration	% of Control activity†	I_{50}‡
4'-Phenylchalcone oxide	380 nM	94§	3.8 μM
	760 nM	65	
	2 μM	43	
	23 μM	29	
4-Phenylchalcone oxide	32 nM	84	68 nM
	64 nM	36	
	640 nM	18	
Trichloropropene oxide	200 μM	90	18 mM
	6 mM	71	
	20 mM	56	
	60 mM	25	
Cyclohexene oxide	50 μM	98	16 mM
	500 μM	94	
	5 mM	76	
	50 mM	25	
Styrene oxide	50 μM	93	4 mM
	500 μM	75	
	5 mM	52	
	50 mM	17	
Ellipticine	5 μM	97	>50 mM
	500 μM	96	
	50 mM	86	
Isoquinoline	50 μM	98	13 mM
	500 μM	86	
	5 mM	71	
	50 mM	27	
Metyrapone	500 μM	91	>50 mM
	5 mM	85	
	50 mM	76	
Harmane	500 μM	80	>50 mM
	5 mM	87	
	50 mM	70	
Clotrimazole	500 μM	91	>50 mM
	5 mM	89	
	50 mM	51	

* Substrate (TSO) concentration = 50 μM.

† "Control activity" refers to TSO hydrolase activity in the presence of 50 μl methanol.

‡ Calculated by log-probit analysis.

§ Mean of three determinations.

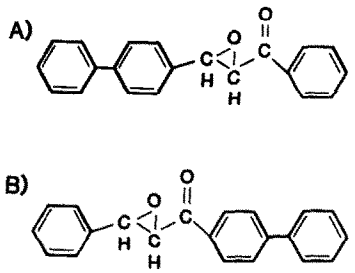

Fig. 1. (A) 4-Phenylchalcone oxide (4PCO), and (B) 4'-phenylchalcone oxide (4'PCO).

investigated (Table 1). None of these compounds, ellipticine, clotrimazole, metyrapone, harmane, or isoquinoline, increased microsomal TSO hydrolase activity. In fact, all five compounds decreased the enzyme activity, albeit at concentrations 10^6-fold greater than that of the substrate. Isoquinoline inhibited the enzyme with an I_{50} of 13 mM, while 50% inhibition with the other four compounds could not be achieved at 50 mM concentrations.

The effects of the chalcone oxides on microsomal BPO hydrolase activity *in vitro* were also measured (Table 2). No significant inhibition was seen even at the highest concentration of inhibitor used, 90 μM. Insolubility of the chalcone oxides precluded their testing at concentrations higher than this.

The two chalcone oxides were by far the most potent inhibitors of microsomal TSO hydrolase investigated. Therefore, a more thorough examination of the inhibition by these compounds was undertaken. Lineweaver–Burk analyses of inhibition by 4PCO and 4'PCO are shown in Figs. 2 and 3. Inhibition by both compounds appeared to be noncompetitive, that is, not due to direct competition with the substrate at the substrate binding site. Fitting the data in Figs. 2 and 3 to the equation derived by Cleland for noncompetitive inhibition (see Materials and Methods) yielded values for K_{is} and K_{ii} of 440 nM and 2.3 μM, respectively, for 4'PCO and a value for K_{is} of 28 nM for 4PCO. A reliable value for K_{ii} for this compound could not be calculated, due primarily to the large variance introduced by the scatter of the points corresponding to the highest (160 nM) concentration of inhibitor.

Table 2. *In vitro* effects of substituted chalcone oxides on microsomal BPO hydrolase activity*

Compound	Concn. (μM)	% of Control activity†
4-Phenylchalcone oxide	90	97§
	9	124
	0.9	121
4'-Phenylchalcone oxide	90	110
	9	122
	0.9	113

* Substrate (BPO) concentration = 25 μM.

† "Control activity" refers to BPO hydrolase activity in the presence of 5 μl methanol.

§ Mean of two determinations.

* Personal communication, with permission of J. W. Bridges.

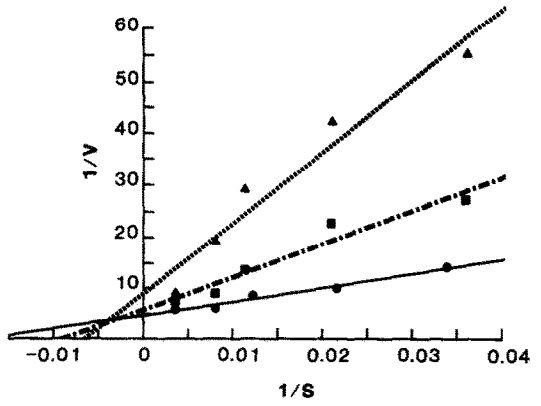

Fig. 2. Inhibition of microsomal TSO hydrolase by 4-phenylchalcone oxide (4PCO). Lineweaver–Burk plots of enzyme activity in the presence of (1) no inhibitor (●); (2) 64 nM 4PCO (■); and (3) 120 nM 4PCO (▲). Each point represents the mean value of three determinations.

The possibility that microsomal TSO hydrolase could be induced by compounds that are known to be inducers of other epoxide hydrolases was also investigated. Mice were treated with high doses of compounds that are known to induce microsomal BPO hydrolase, including 2AAF [24], benzofuran,* BHA [25], phenobarbital [26], and TSO [26], or with clofibrate, which has been shown to induce cytosolic TSO hydrolase activity in mice [32]. The effects of pretreatment on enzyme activities are shown in Table 3. The only compound that induced microsomal TSO hydrolase in these mice was clofibrate, which increased the enzyme levels by 83%. When these data were analyzed by analysis of variance, it was determined that an induction of 30% above control values was significant at the $P = 0.05$ level. At the same time, induction by these compounds of microsomal BPO hydrolase was measured. Five of the six

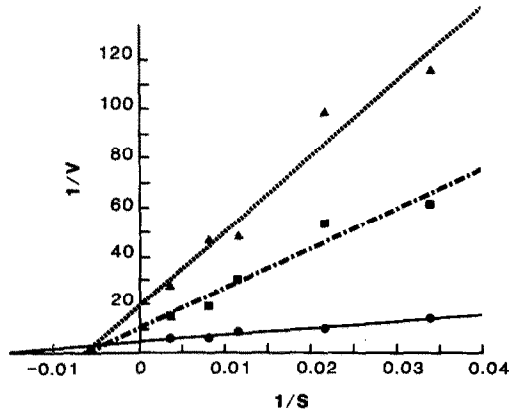

Fig. 3. Inhibition of microsomal TSO hydrolase by 4'-phenylchalcone oxide (4'PCO). Lineweaver–Burk plots of enzyme activity in the presence of (1) no inhibitor (●); (2) 2.3 μM 4'PCO (■); and (3) 5.7 μM 4'PCO (▲). Each point represents the mean value of three determinations.

Table 3. Effects of *in vivo* inducers on microsomal epoxide hydrolase activities

Inducer (dose)	TSO hydrolase activity (% of control)*	BPO hydrolase activity (% of control)*
Benzofuran (150 mg/kg/day × 4 days)	81 ± 15†	203 ± 23‡
Clofibrate (250 mg/kg/day × 4 days)	183 ± 24‡	271 ± 43‡
BHA (300 mg/kg/day × 4 days)	100 ± 11	263 ± 27‡
2AAF (100 mg/kg/day × 4 days)	104 ± 20	202 ± 18‡
Phenobarbital (80 mg/kg/day × 4 days	86 ± 25	178 ± 27‡
trans-Stilbene oxide (400 mg/kg/day × 4 days)	127 ± 17	139 ± 8

* "Control" refers to activity from livers of corn oil-treated mice.
† Mean ± S.E. of activities from four animals.
‡ Different from control values at P = 0.05 level.

compounds produced a significant increase in BPO hydrolase activity, but TSO did not. Analysis of variance for these data showed that a 76% induction above control levels was significant at the P = 0.05 level.

DISCUSSION

Table 1 shows the effects on microsomal TSO hydrolase of a series of compounds that have been shown previously to directly modulate the activity of other epoxide hydrolases *in vitro* [30, 31]. The I_{50} concentrations of these compounds were determined against 50 μM TSO; the two substituted chalcone oxides 4PCO and 4'PCO inhibited the enzyme activity at concentrations one to three orders of magnitude lower than that of the substrate, whereas the other epoxides (styrene oxide, cyclohexene oxide, and TCPO) were effective inhibitors only at millimolar concentrations. Probit analysis of the inhibition curves obtained for 4PCO and 4'PCO shows that the slopes of the probit lines for the two compounds are similar (1.04 for 4PCO, 1.01 for 4'PCO), suggesting that they may both inhibit the enzyme by interacting at the same site.

More detailed analysis of the inhibition kinetics, shown in Fig. 2 and 3, provide additional evidence that these two compounds are inhibiting the enzyme by a common mechanism. Lineweaver–Burk plots for inhibition by the two compounds show a pattern consistent with noncompetitive inhibition for both. According to this theoretical model, the inhibitor is interacting with the enzyme or the enzyme–substrate complex at a site distinct from the substrate binding site [33]. Noncompetitive inhibitors affect both the slope and intercept of the double-reciprocal plot, and therefore two inhibition constants should be calculable. K_{is}, the constant determined by the effect of the inhibitor on the slope, is an approximation of the affinity constant of the inhibitor for the unoccupied enzyme [34]. K_{ii}, the constant determined by the effect of the inhibitor on the y intercept, is an approximation of the affinity constant of the inhibitor for the enzyme–substrate complex [34]. These values represent true inhibitor constants, unlike the I_{50} value, which varies with substrate concentration. Computer-assisted analysis of this data yielded values for K_{is} and K_{ii} of 440 nM (standard error 130 nM) and 2.3 μM (standard error 1.4 μM), respectively, for 4'PCO, and a value for K_{is} of 28 nM (standard error 10 nM) for 4PCO. A value for K_{ii} for 4PCO could not be accurately estimated because of the large standard error, primarily due to scatter of the data obtained at high inhibitor and low substrate concentrations.

While these inhibitor constants may permit the simple conclusion that 4PCO and 4'PCO have a fairly high (submicromolar) affinity for the enzyme, further interpretation of their absolute accuracy or mechanistic significance must proceed with caution. The enzyme exists in a lipid millieu, which may result in large differences between the inhibitor (or substrate) concentration in the medium and that at the site of interaction with the enzyme. Furthermore, TSO as used exists in two enantiomeric forms, as do the *trans*-isomers of 4PCO and 4'PCO employed. Differences in enzyme affinities for the various enantiomers will complicate any kinetic interpretations. Nevertheless, these data do lend support to the conclusion reached in the I_{50} studies, namely that these chalcone oxides have high affinity for, and are potent inhibitors of, microsomal TSO hydrolase.

It has been observed in earlier studies that microsomal TSO hydrolase has many properties in common with cytosolic TSO hydrolase [23]. The inhibition of microsomal TSO hydrolase by low levels of 4PCO and 4'PCO shown here is further evidence of the similarity between this enzyme and cytosolic TSO hydrolase. The I_{50} concentrations for inhibition of cytosolic TSO hydrolase by 4PCO and 4'PCO were determined by Mullin and Hammock [17] as 64 and 760 nM respectively. These values compare favorably to the I_{50} values determined here for microsomal TSO hydrolase of 68 nM for 4PCO and 3.8 μM for 4'PCO. These authors also determined the constants K_{ii} and K_{is} for inhibition of cytosolic TSO hydrolase by 4-isopropylchalcone oxide and found these values to be in the nanomolar range as well [17]. The similarities in kinetic and catalytic properties of cytosolic and microsomal TSO hydrolase are

striking, and all studies to date indicate that these two enzymes are quite probably very similar or identical proteins [23].

The two inhibitors 4PCO and 4'PCO may prove to be extremely useful tools for evaluating the physiological and toxicological role of microsomal TSO hydrolase. Their value lies in their selectivity towards microsomal TSO hydrolase and their lack of activity against other microsomal epoxide hydrolases. As Table 2 shows, microsomal BPO hydrolase was not inhibited significantly by either 4PCO or 4'PCO at concentrations up to 90 μM. The use of higher concentrations of the inhibitors was precluded by their limited solubility. In an earlier study, the inhibition by 4PCO of microsomal *cis*-stilbene oxide hydrolase activity was measured. *cis*-Stilbene oxide is a substrate for the same microsomal enzyme that catalyzes BPO hydrolase activity. The inhibition of this activity by 200 μM 4PCO was less than 20% [17]. It appears that the chalcone oxides are very selective inhibitors of microsomal TSO hydrolase and have little or no activity toward microsomal BPO hydrolase. In fact, unsubstituted chalcone oxide was seen in an earlier study to actually stimulate microsomal BPO hydrolase activity [31].

An alternative means of selectively modifying the individual epoxide hydrolase activities in a given preparation would be to selectively inhibit BPO hydrolase activity, while leaving TSO hydrolase activity intact. This can be done quite effectively, as Table 1 shows. Styrene oxide, cyclohexene oxide, and TCPO are all inhibitors of microsomal BPO hydrolase at micromolar concentrations [29]. The inhibition of microsomal TSO hydrolase by these compounds at micromolar concentrations is negligible, and only at millimolar concentrations is significant inhibition seen. These three compounds, then, when used at micromolar concentrations, will completely inhibit BPO hydrolase activity, leaving TSO hydrolase activity unaffected. Further studies, shown in Table 1, examined the effects on TSO hydrolase activity of a number of compounds that normally activate BPO hydrolase activity *in vitro* [31, 32]. The absence of any activation of TSO hydrolase activity by these compounds may present another possibility for selective modulation of individual hydrolase activities by selective activation. In fact, isoquinoline, a compound that activates microsomal BPO hydrolase activity [31], is seen here to inhibit microsomal TSO hydrolase activity with about the same potency as TCPO or cyclohexene oxide.

The differences in the responses of microsomal TSO hydrolase activity and BPO hydrolase activity to *in vitro* modulation, as demonstrated above, offer persuasive evidence that these activities are attributable to different enzymes. Previous evidence has also shown that these enzymes are structurally distinct and are, therefore, probably different gene products rather than the result of post-translational modification of a common precursor [23]. Table 3 shows the results of an experiment wherein the *in vivo* effects on TSO hydrolase of a number of compounds that are known inducers of BPO hydrolase [24–26] were measured, with two aims in mind. The first was to determine whether TSO hydrolase activity could be modulated by any of a number of known enzyme inducers. Also, these experiments were designed to determine whether TSO hydrolase and BPO hydrolase were simultaneously inducible, or whether the tested compounds could induce one enzyme without affecting the other. If the absence of any linkage of the induction of these two proteins could be shown, the presumption that they are not only separate gene products, but are also controlled by totally distinct genetic mechanisms, would be strengthened. These experiments show that those compounds that induce BPO hydrolase do so without any effect on TSO hydrolase. BPO hydrolase activity was induced 2- to 3-fold after treatment, but no significant increases in TSO hydrolase activity were seen. The exception was treatment by clofibrate, which induced both enzyme activities about 2-fold. The degree of induction of BPO hydrolase by these various compounds is not as high here as has been reported in other studies [24–26, 32]. Indeed, TSO, which readily induces this activity in the rat [26], albeit at very high concentrations (400 mg/kg/day), had no effect on either enzyme measured here. This may be due in some part to the much higher levels of TSO hydrolase activity in the mouse than in the rat [12], which should cause rapid deactivation or excretion of this compound. The fact that the levels of enzyme seen after induction were lower here than has been shown elsewhere [24–26, 32] may simply indicate that these mice are inherently less responsive than the animals used in other studies. Nevertheless, a difference in the response of TSO hydrolase and BPO hydrolase to the various compounds is definitely demonstrable, and it is clear that the induction of the two enzymes, at least by the compounds used here, is not linked. Simultaneous induction of both activities was seen with clofibrate. This compound also induces cytosolic TSO hydrolase [32]. The degree of linkage of the induction of the cytosolic and microsomal forms of TSO hydrolase may eventually provide clues as to how these two forms are similar and how they differ.

In conclusion, the studies presented here offer further evidence that TSO hydrolase activity in microsomes is due to an enzyme with catalytic properties very different from those of BPO hydrolase. The high affinity of chalcone oxides for TSO hydrolase and their ability to inhibit it at nanomolar concentrations contrast with their negligible activity toward BPO hydrolase, even at 10^4-fold higher concentrations. Conversely, the other epoxides tested, which inhibit BPO hydrolase activity at micromolar concentrations, were effective inhibitors of TSO hydrolase only at millimolar concentrations. When used at the proper concentrations, these two sets of compounds should allow the selective inhibition of a single form of microsomal epoxide hydrolase, leaving activity due to the second from extant. Investigation of *in vivo* modulation of hydrolase activity showed that those compounds that induce high levels of BPO hydrolase activity do not simultaneously induce TSO hydrolase activity, indicating that the two enzymes are probably not regulated at the same genetic locus. The exception is clofibrate, which induces both enzymes, and is probably acting by a mechanism very different to that of the other

compounds. This study has identified a number of compounds that will be useful tools for separating and identifying the participation of individual forms of epoxide hydrolase in metabolic pathways of physiological and toxicological interest. These compounds may prove to be particularly useful in investigations with human tissue, in light of recent observations showing relatively high levels of microsomal TSO hydrolase activity in human liver and lung.*

Acknowledgements—The author wishes to acknowledge the National Cancer Institute's Chemical Carcinogen Reference Standard Repository, a function of the Division of Cancer Cause and Prevention, NCI, NIH, Bethesda, MD 20205, for supplies of labeled and unlabeled BPO, and Dr. Bruce Hammock, Departments of Entomology and Environmental Toxicology, University of California at Davis, for his generosity in providing the substituted chalcone oxides as well as valuable discussion. The participation of Ronald Schoenbeck, Minu Patel, and Fun-Yun Wu in this study is also gratefully acknowledged.

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* T. M. Guenthner and T. A. Karnezis, manuscript in preparation.