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Liver Microsomal Epoxide Hydrase: Activation, Inhibition, and Immunological Properties of the Purified Enzyme

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SUMMARY

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Two modifiers of microsomal epoxide hydrase activity, metyrapone and cyclohexene oxide, are shown to exert differential effects on the catalytic activity of the purified enzyme from rat liver. The effects of these modifiers on rates of metabolism are dependent on the substrate studied. Metyrapone (6 mM) increases the rate of hydration of octene 1,2-oxide and benzo[a]anthracene 5,6-oxide by 210% and 40%, respectively, does not affect the rate of benzo[a]pyrene 4,5-oxide metabolism, and inhibits the rate of benzo[a]pyrene 11,12-oxide and dibenzo[a,h]-anthracene 5,6-oxide hydration by approximately 60%. At low concentrations (60-120 μM), cyclohexene oxide has no effect on the rate of metabolism of octene 1,2-oxide or benzo[a]anthracene 5,6-oxide, inhibits the rate of hydration of benzo[a]pyrene 4,5-oxide by 40%, and inhibits the rate of benzo[a]pyrene 11,12-oxide and dibenzo[a,h]-anthracene 5,6-oxide metabolism by 80%. Cyclohexene oxide is a competitive inhibitor of the metabolism of octene 1,2-oxide and is a noncompetitive inhibitor of benzo[a]pyrene 11,12-oxide metabolism. Anti-rat epoxide hydrase gamma globulin produced in goat, sheep, donkey, and rabbit forms an immunoprecipitate with rat liver epoxide hydrase but the immunoprecipitated enzyme retains full catalytic activity. Antibody produced in the goat appears to differentiate two sets of antigenic determinants in the rat enzyme. Antibody against rat liver epoxide hydrase does not cross-react with microsomal preparations of the enzyme from rabbit or human liver and shows poor cross-reactivity with guinea pig and hamster liver epoxide hydrase. These results indicate the presence of different forms of hepatic epoxide hydrase in several species and suggest that rat liver microsomal epoxide hydrase contains multiple regulatory sites. However, the possible existence of more than one form of the enzyme in rat liver cannot be excluded at the present time.

INTRODUCTION

Polycyclic aromatic hydrocarbons such as benzo[a]-pyrene have attracted attention due to their widespread occurrence in the environment (1) and their metabolic activation to ultimate carcinogens (2-4). The metabolism of aromatic compounds by mammals usually involves the epoxide-diol pathway localized in the endoplasmic reticulum of liver and other tissues. The first

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step of this pathway is the formation of intermediate arene oxides by the mixed function oxidase system (3-5). Certain of these arene oxides have toxic, mutagenic, and carcinogenic activity (3, 4, 6-9). Epoxide hydrase catalyzes the hydration of these reactive arene oxides to dihydrodiols via *trans* addition of water (3, 5). The dihydrodiols are usually biologically inactive per se but certain isomers of these compounds are further metabolized by the mixed function oxidase system to bay region diol epoxides (10, 11), which are ultimate carcinogens (12-14) and which are not substrates for epoxide hydrase (15-17). Thus, epoxide hydrase plays a dual role in the metabolic activation and inactivation of certain polycyclic aromatic hydrocarbons into mutagenic and carcinogenic metabolites.

An understanding of the role of metabolism in the carcinogenicity of polycyclic aromatic hydrocarbons requires a knowledge of the basic properties of the enzymes involved in these activation and inactivation processes. Rat liver epoxide hydrase has recently been highly purified (18-20) and the enzyme preparation is capable of hydrating a large number of arene and alkene oxides at widely differing rates (21, 22). Although the purified enzyme exhibits a single protein band on SDS[2] gels, with a molecular weight of approximately 50,000 daltons (18-20), the purification factor for the enzyme varies considerably depending on the substrate assayed (21, 22) and differences in the pH optima for metabolism by the purified enzyme have been observed with different substrates (21). As described by Lu *et al.* (21), however, there are several complexities in the assays for catalytic activity, such as the marked effect of lipid on the kinetics of hydration. The present investigation explores the immunological properties and the effects of chemical modifiers on the catalytic activity of the purified enzyme to determine whether the differences observed in the previous studies (21, 22) could be due to the presence of multiple forms of the enzyme or a single enzyme with multiple regulatory sites.

[2] *Abbreviations*: SDS, sodium dodecyl sulfate; BP 4,5-oxide, benzo[a]pyrene 4,5-oxide; BP 11,12-oxide, benzo[a]pyrene 11,12-oxide; BA 5,6-oxide, benzo[a]-anthracene 5,6-oxide; DBA 5,6-oxide, dibenzo[a,h]anthracene 5,6-oxide; IgG, immunoglobin G fraction purified from serum.

MATERIALS AND METHODS

Enzymes. Immature, male Long Evans rats (50-60 g) were injected intraperitoneally with sodium phenobarbital (75 mg/kg/day) for 3 days and liver microsomes were prepared as previously described (23). Epoxide hydrase was purified to apparent homogeneity according to the method of Lu *et al.* (18). The purified enzyme (capable of metabolizing styrene 7,8-oxide to styrene glycol at a rate of 680 nmol/min/mg of protein) showed a single protein band on SDS gels (24) with a molecular weight of 49,000 daltons. We had previously reported a molecular weight of 54,000 daltons for the purified rat liver epoxide hydrase, using a different SDS gel electrophoresis system and different molecular weight standards (18). These values are within the expected accuracy (± 10%) of various SDS gel systems (cf. 25).

Substrates. Six substrates including alkene and arene oxides were used in the present study. The specific activity of these tritium-labelled substrates, which had been used in a previous study (26), are as follows: [7-^{3}H]styrene 7,8-oxide, 0.5 μCi/μmol; [7,8-^{3}H]octene 1,2-oxide, 7.8 μCi/μmol; [6^{3}H]-benzo[a]pyrene 4,5-oxide, 5.9 μCi/μmol; [11,12-^{3}H]benzo[a]pyrene 11,12-oxide, 5.8 μCi/μmol; [7-^{3}H]benzo[a]anthracene 5,6-oxide, 9.5 μCi/μmol, and [5,6-^{3}H]dibenzo-[a,h]-anthracene 5,6-oxide, 5.9 μCi/μmol. Each substrate was dissolved in acetonitrile : NH_4OH (1000 : 1, v/v) and stored at −90° prior to use.

Assay for epoxide hydrase activity. The reaction mixture contained 25 μl of 0.5 M Tris-HCl buffer, 50 μl of water and enzyme, and 5 μl of substrate solution to reach a final volume of 80 μl (21, 26). All assays were performed in duplicate. For microsomal studies, the pH of the buffer was 8.7 at 37°. With purified epoxide hydrase, 15 μg of dilauroyl phosphatidylcholine were included in the incubation mixture (21) and the pH optima varied depending on the substrate assayed (21). Incubations were performed for 1 min at 37°, and the reac-

tions were stopped by the addition of 25 μl of tetrahydrofuran. One-third of the total mixture (35 μl) was chromatographed on silica gel thin layer plates and the radioactive dihydrodiol or glycol products were separated and quantitated as previously described (26). The apparent K_m and V_{max} values were calculated by means of a computer program written by Cleland (27), except that each substrate concentration was weighted by the inverse of the local variance as described by Ottaway (28).

Preparation of antibodies to purified epoxide hydrase. Animals (donkey, sheep, goat, and rabbits) were immunized intradermally along the flanks at 20 or more sites with 200 μg per animal of purified rat liver epoxide hydrase. The immunogen was diluted in 0.9% NaCl to 1 ml (for each animal) and a water-in-oil emulsion was produced with Freund's complete adjuvant as previously described (29). Six weeks after immunization, animals were boosted with an additional intradermal injection of 40 μg of purified enzyme in Freund's complete adjuvant. An intravenous injection of 20 μg of epoxide hydrase in the absence of adjuvant was given 4–5 weeks later and the animals were bled 1 week after the final boost (29). Thereafter, the animals were boosted every 4 weeks with 20 μg of adjuvant-free antigen and bled 1 week later. The IgG fraction from sera with the highest antibody titre was purified as described by Thomas *et al.* (30).

Ouchterlony double-diffusion analysis. The immunodiffusion medium contained 0.9% agarose, 1.0 M glycine-NaOH (pH 7.4), 0.2% Emulgen 911, 0.08 M NaCl, and 0.015 M sodium azide. The gel thickness in the 50 mm polystyrene petri dishes was approximately 1.3 mm with wells of 4 mm diameter. After filling the wells with the appropriate protein solution (12 μl), the plates were incubated at 23° in a humid atmosphere for 2–3 days.

Chemicals. Agarose (Sea Kem) was purchased from Marine Colloids, Inc., Rockland, Maine. Emulgen 911 was obtained from Kao-Atlas Ltd., Tokyo, Japan. Freund's complete adjuvant was purchased from Difco Laboratories, Detroit, Michigan. Cyclohexene oxide and 2-bromo-4′-nitroacetophenone were purchased from Aldrich Chemical Co. Phenylmethylsulfonylfluoride was obtained from Sigma Chemical Co. Dilauroyl phosphatidylcholine was purchased from Serdary Research Laboratories, Ontario, Canada. Metyrapone was a gift from Dr. J. Daly.

RESULTS

Effect of metyrapone and cyclohexene oxide on epoxide hydrase activity. In a recent study (21) with purified rat liver epoxide hydrase, we observed significant differences in the pH optima of the purified enzyme and in purification factors, which vary from 15 to 45-fold for 11 alkene and arene oxide substrates. Five of these substrates were further studied with the hope of establishing whether differences in purification factors and pH optima were due to a) the complexity of the assays, b) the existence of multiple forms of the enzyme, or c) a single form of epoxide hydrase with multiple regulatory sites. Several investigators have reported that metyrapone stimulates both membrane-bound and purified epoxide hydrase when styrene oxide is used as substrate (18, 31–33). Figure 1 (top) shows the effect of metyrapone on the hydration of five different substrates by the purified enzyme. The conversion of octene 1,2-oxide to the corresponding glycol was stimulated approximately 3-fold at concentrations of 3–15 mM metyrapone. Similar results were obtained when styrene 7,8-oxide was used as substrate (data not shown). At these same concentrations of metyrapone, the activity of epoxide hydrase for BP 11,12-oxide and DBA 5,6-oxide was inhibited by 40–80%. No stimulation of metabolism of these substrates could be observed even when the concentration of metyrapone was decreased to 10 μM (data not shown). When BA 5,6-oxide was used as a substrate for epoxide hydrase, metyrapone produced a small but significant stimulation (40%) at 3–6 mM but did not affect enzymatic activity when the concentration was increased to 15 mM. The activity of the enzyme for BP 4,5-oxide was unaffected at concentrations of metyrapone between 1.2 and 9 mM but slight inhibition was observed at 15 mM. The markedly different effects of

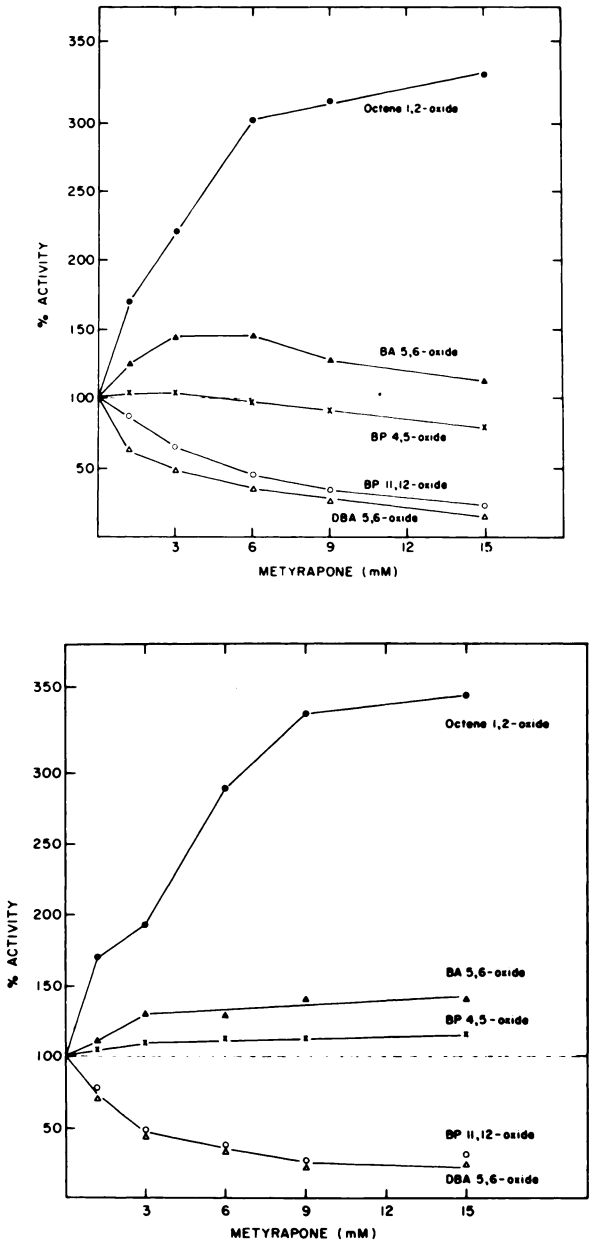

FIG. 1. *Effect of metyrapone on the catalytic activity of purified (top) and liver microsomal (bottom) epoxide hydrase from phenobarbital-treated male rats*

The substrate concentrations were as follows: octene 1,2-oxide, 1250 μM; BP 4,5-oxide, 250 μM; BP 11,12-oxide, 150 μM; BA 5,6-oxide, 300 μM; DBA 5,6-oxide, 250 μM. The amount of purified or microsomal protein used in the 80 μl incubation volumes were 5 μg or 150 μg for octene 1,2-oxide, 2 μg or 50 μg for BP 4,5-oxide, 10 μg or 150 μg for BP 11,12-oxide, 1 μg or 20 μg for BA 5,6-oxide and 20 μg or 150 μg for DBA 5,6-oxide. Incubations were performed as described in METHODS, except that metyrapone (dissolved in H_2O) was added just prior to addition of substrate. The turnover numbers (nmol/min/mg protein) for the various substrates in the absence of metyrapone with liver microsomal and purified epoxide hydrase were: 56 and 1140 for octene oxide, 1.4 and 26 for BP 11,12-oxide, 1.2 and 11 for DBA 5,6-oxide, 46 and 1360 for BA 5,6-oxide, and 27 and 611 for BP 4,5-oxide.

metyrapone on the catalytic activity of purified epoxide hydrase were also observed with liver microsomes from phenobarbital-treated rats (Fig. 1, bottom), indicating that purification did not lead to any significant change in the response of the enzyme to metyrapone.

Cyclohexene oxide has been reported to be a noncompetitive inhibitor of guinea pig hepatic epoxide hydrase with styrene 7,8-oxide as substrate (32). Figure 2 (top) shows the effects of cyclohexene oxide on the hydration of five substrates by purified epoxide hydrase. More than 50% inhibition of the enzymatic activity toward BP 11,12-oxide and DBA 5,6-oxide was observed with 60 μM cyclohexene oxide while the metabolism of octene oxide and BA 5,6-oxide was unaffected. The inhibition of hydration of BP 4,5-oxide appeared to be intermediate

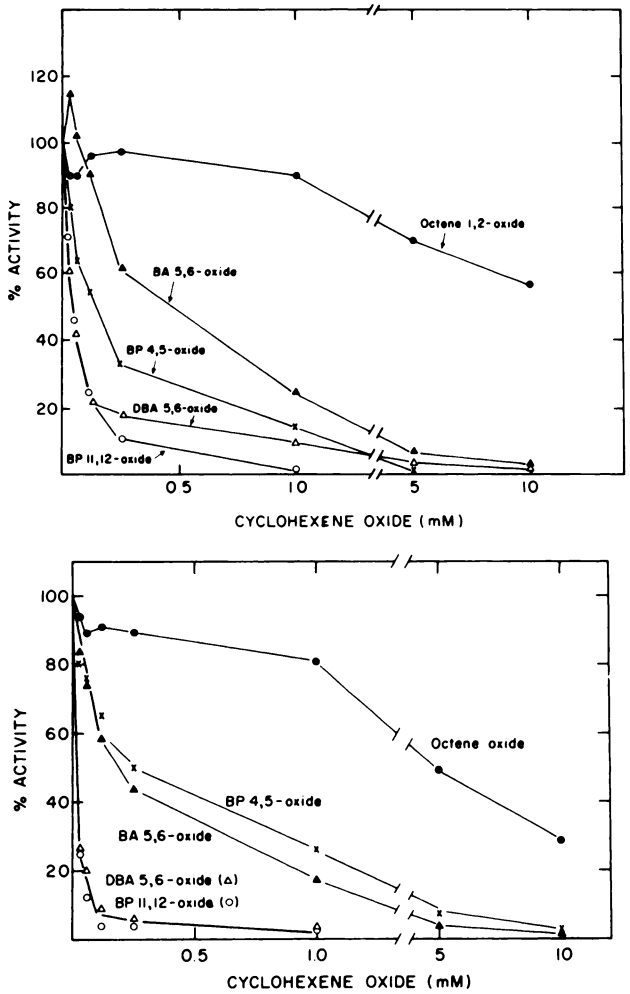

FIG. 2. *Effect of cyclohexene oxide on the catalytic activity of purified (top) and liver microsomal (bottom) epoxide hydrase from phenobarbital-treated male rats*

The amount of purified or microsomal protein used in the 80 μl incubation volumes were 20 μg or 300 μg for octene 1,2-oxide, 2 μg or 50 μg for BP 4,5-oxide, 20 μg or 150 μg for BP 11,12-oxide, 2 μg or 20 μg for BA 5,6-oxide and 20 μg or 150 μg for DBA 5,6-oxide. Substrate concentrations were as described in the legend to Fig. 1. Cyclohexene oxide in 2 μl of acetonitrile was added just prior to the substrate (in 4 μl of acetonitrile : NH_4OH). Incubations were performed as described in METHODS. The turnover numbers for the various substrates in the absence of cyclohexene oxide were similar to those reported in the legend to Fig. 1.

(35%) at this concentration of cyclohexene oxide. At high concentrations of cyclohexene oxide (5–10 mM), the inhibition of the catalytic activity of epoxide hydrase was greater than 90% for all substrates except octene oxide, which was only inhibited by 30–40%. Again, a comparison of the inhibition of membrane-bound epoxide hydrase with the purified enzyme (Fig. 2, bottom) showed similar although not identical results with these five substrates.

The differential effects of metyrapone and cyclohexene oxide on the hydration of octene oxide and BP 11,12-oxide were further evaluated by kinetic analyses. Lineweaver-Burk plots for the hydration of these two substrates in the presence and absence of metyrapone (fig. 3) revealed that metyrapone is a noncompetitive inhibitor of the hydration of BP 11,12-oxide. Metyrapone significantly increased both the V_{max} and apparent K_m values for epoxide hydrase with octene oxide as substrate, as has been shown for the hydration of styrene oxide (32). Cyclohexene oxide was found to be a noncompetitive inhibitor of epoxide hydrase with BP 11,12-oxide as substrate but a competitive inhibitor of the hydration of octene oxide (fig. 4). A 9-fold increase in the apparent K_m for octene oxide was observed in the presence of 250 μM cyclohexene oxide without significant change in the V_{max} of the reaction. The differential effects of metyrapone and cyclohexene oxide on

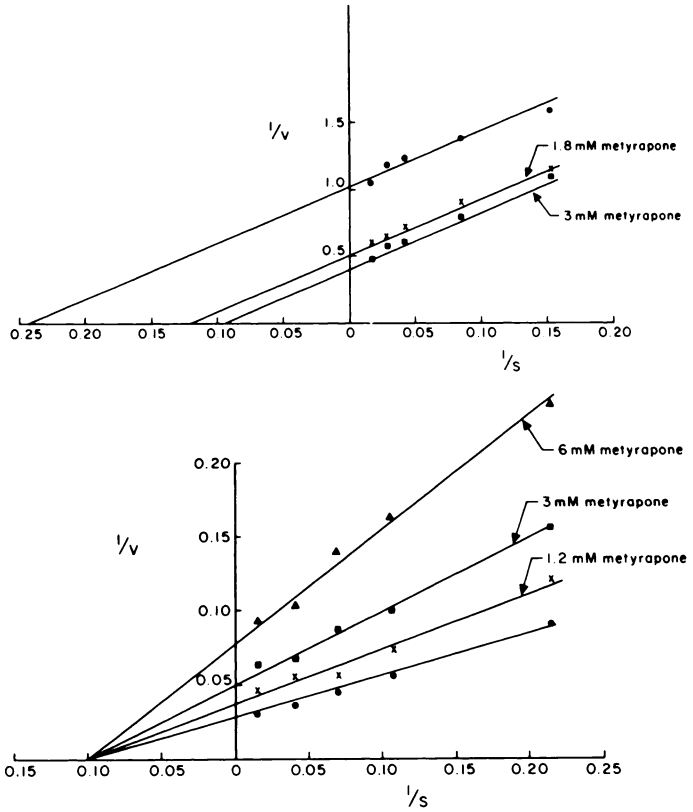

FIG. 3. *Lineweaver-Burk plots for the hydration of octene 1,2-oxide (top) and BP 11,12-oxide (bottom) in the absence or presence of metyrapone*

Assay conditions were as follows: octene 1,2-oxide, 0.05 μg of purified epoxide hydrase, 1 min incubation and BP 11,12-oxide, 15 μg of purified epoxide hydrase, 1 min incubation. Dilauroyl phosphatidylcholine was not included in the incubation mixtures since this compound is a competitive inhibitor of epoxide hydrase at low substrate concentrations (21). S, substrate concentration in μM; V, rate in μmol/min/mg protein for octene 1,2-oxide, and nmol/min/mg protein for BP 11,12-oxide. Values represent the mean of duplicate determinations at each substrate concentration.

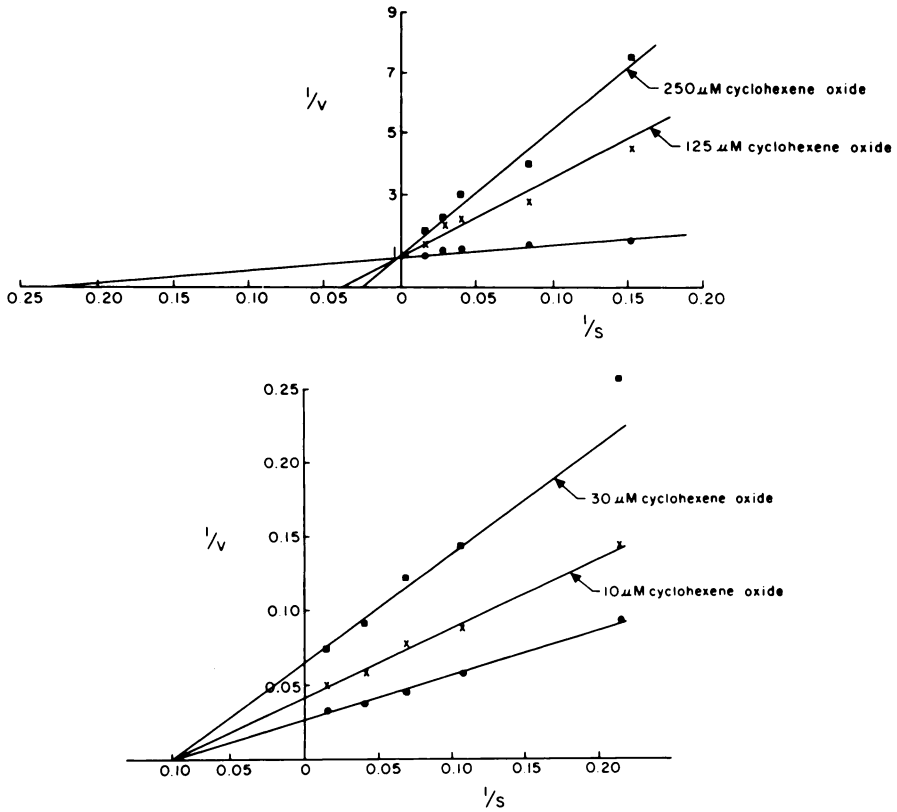

FIG. 4. *Lineweaver-Burk plots for the hydration of octene 1,2-oxide (top) and BP 11,12-oxide (bottom) in the absence or presence of cyclohexene oxide*

Assay conditions were as described in the legend to Fig. 3. Values represent the mean of duplicate determinations at each substrate concentration.

the kinetic parameters (K_m and V_{max}) were verified in repeated experiments and from a statistical analysis of the data. Thus, the type of inhibition of epoxide hydrase activity by cyclohexene oxide is dependent on the substrate used in the assay.

Effect of 2-Bromo-4'-nitroacetophenone on epoxide hydrase activity. Recent studies (34) with an active site-directed inhibitor (2-bromo-4'-nitroacetophenone) have indicated that an essential histidine is involved in the catalytic activity of rat liver epoxide hydrase. The inhibitory effect of 2-bromo-4'-nitroacetophenone on the hydration of five substrates by epoxide hydrase was very similar (fig. 5). These results suggest that a single form of epoxide hydrase catalyzes the hydration of these substrates, although the possible existence of multiple forms of epoxide hydrase with similar catalytic sites cannot be excluded.

Immunological properties of liver microsomal epoxide hydrase. Antibody produced in donkey, sheep, and rabbits against purified rat liver epoxide hydrase reacts with the enzyme to give a single immunoprecipitin band in Ouchterlony double diffusion plates (fig. 6). The single immunoprecipitin band is also obtained when either excess antibody or excess antigen is used in the plates (data not shown). In contrast to these results, antibody prepared in the goat against the same preparation of purified rat epoxide hydrase reveals the presence of two immunoprecipitin bands with the purified enzyme. Similar results were obtained with four different preparations of purified epoxide hydrase as well as with solubilized rat liver microsomes. The antibodies from the goat appear to differentiate two separate sets of antigenic determinants in rat epoxide hydrase. The difference in goat anti-

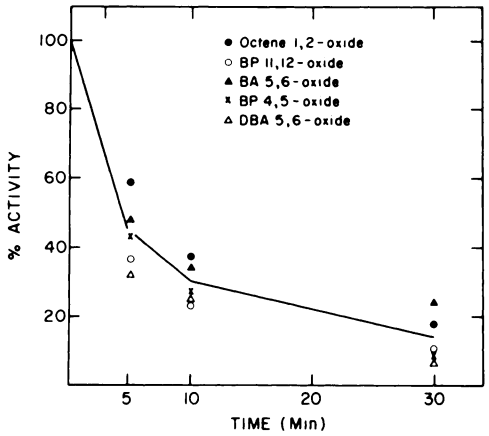

FIG. 5. *Effect of 2-bromo-4'-nitroacetophenone on epoxide hydrase activity*

Purified epoxide hydrase (400 μg protein) was incubated in a total volume of 200 μl consisting of 10 μl of 1 M potassium phosphate (pH 9) containing 0.6% Emulgen 911 (34) and 4 μl of acetonitrile containing 12.2 μg of 2-bromo-4'-nitroacetophenone (250 μM final concentration). Incubations were performed at 30° for 5, 10, and 30 min. The reactions were stopped by placing the samples in ice. Control samples were treated in an identical manner in the absence of 2-bromo-4'-nitroacetophenone. Aliquots of the incubation mixtures were then assayed for epoxide hydrase activity as described in METHODS. Protein and substrate concentrations were as described in the legend to Fig. 1.

body titre against the two sets of antigenic determinants is most pronounced when the antibody is isolated from sera early in the immunization schedule (fig. 6), i.e., early goat antibody recognizes predominantly one of the two sets of antigenic determinants.

Antibodies from donkey, sheep, goat, and rabbit against purified rat liver epoxide hydrase produced immunoprecipitates with the enzyme but did not inhibit catalytic activity toward any of the five substrates studied, i.e., the immunoprecipitates retain full catalytic activity. The immune globulin G fraction from the sheep or rabbit was incubated with the purified enzyme at 4° for 16 hours and catalytic activity was determined on the supernatant after removal of the immunoprecipitate by centrifugation. The results presented in Table 1 indicate that immunoprecipitation of the enzyme results in a parallel loss of catalytic activity from the supernatant for all five substrates. Similar results were obtained with the goat antibody, although immunoprecipitation of the enzyme was more gradual with increasing antibody concentration compared to the sheep and rabbit antibody (data not shown). Since antibodies prepared against rat epoxide hydrase apparently bind to antigenic sites which do not alter the catalytic site of the enzyme, the response of the immunoprecipitated enzyme to the differential effects of metyrapone and cyclohexene oxide was determined. The results presented in Table 2 indicate that the effects of metyrapone and cyclohexene oxide on the enzyme-antibody complex and the native enzyme are the same. These results indicate that metyrapone and cyclohexene oxide bind to sites on the enzyme which are distinct from the antigenic sites.

Antibody prepared in sheep against pu-

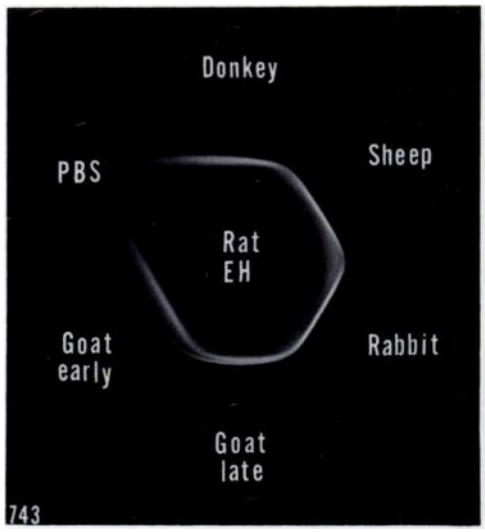

FIG. 6. *Ouchterlony immunodiffusion plate with antibodies against purified epoxide hydrase raised in donkey, sheep, rabbit, and goat*

The donkey, sheep, rabbit and goat IgG concentrations were 50 mg/ml, 7.5 mg/ml, 10 mg/ml and 20 mg/ml, respectively. The "goat early" IgG was prepared from antisera collected at the first bleeding, 11 weeks after immunization. The "goat late" IgG was prepared from sera collected 17 weeks after immunization. The well marked "PBS" contained phosphate buffered saline. The center well contained purified epoxide hydrase (270 μg/ml).

TABLE 1

Immunoprecipitation of purified epoxide hydrase by antibody produced in sheep and rabbits

Purified epoxide hydrase (300 μg) was incubated in a total volume of 300 μl of phosphate-buffered saline at pH 7.4 with the appropriate amount of anti-epoxide hydrase at 4° for 16 hr. The incubation mixtures were made up to the same final antibody concentration (10 mg/mg epoxide hydrase) with the IgG fraction from preimmune sera. The incubation mixtures were centrifuged at 5,000 × *g* for 10 min and the supernatant was carefully removed from the immunoprecipitates. Aliquots (2–20 μl) of the supernatant were analyzed for epoxide hydrase activity as described in METHODS. The turnover numbers (nmol/min/mg protein) for the various substrates in the absence of antibody were: octene oxide, 1310, BP 4,5-oxide, 540, BP 11,12-oxide, 27, BA 5,6-oxide, 1020, and DBA 5,6-oxide, 13.

Antibody (*mg/mg epoxide hydrase*)	Substrate				
	Octene oxide	BP 4,5-oxide	BP 11,12-oxide	BA 5,6-oxide	DBA 5,6-oxide
	(*% of control activity*)				
None	100	100	100	100	100
Sheep—1 mg/mg	103	110	103	111	116
3 mg/mg	74	84	79	90	94
5 mg/mg	0	5	1	4	6
Rabbit—4 mg/mg	95	92	104	90	105
5 mg/mg	60	59	60	57	57
7.5 mg/mg	19	19	18	20	24
10 mg/mg	0	1	0	1	6

TABLE 2

Effect of metyrapone and cyclohexene oxide on the catalytic activity of immunoprecipitated epoxide hydrase

Purified epoxide hydrase (640 μg) was incubated with 6.4 mg of rabbit anti-epoxide hydrase in a final volume of 600 μl of phosphate-buffered saline (pH 7.4) for 16 hr at 4°. The sample was centrifuged at 5,000 × *g* for 10 min and the immunoprecipitate was analyzed for epoxide hydrase activity as described in METHODS. Control enzyme was treated in a similar manner in the absence of antibody. The turnover numbers (nmol/min/mg protein) for the control enzyme were 1230 and 24 for octene oxide and BP 11,12-oxide, respectively.

Substrate	Addition	Control Enzyme	Enzyme-Antibody Complex
		(*% of control activity*)	
Octene oxide	None	100	111
	Cyclohexene oxide (5 mM)	47	52
	Cyclohexene oxide (10 mM)	29	34
	Metyrapone (3 mM)	227	227
	Metyrapone (9 mM)	246	238
BP 11,12-oxide	None	100	109
	Cyclohexene oxide (60 μM)	28	28
	Cyclohexene oxide (250 μM)	7	18
	Metyrapone (3 mM)	46	49
	Metyrapone (9 mM)	20	36

rified epoxide hydrase from phenobarbital-treated rats was used to study the effects of different inducers on the immunological properties of epoxide hydrase. Figure 7 shows that liver microsomal epoxide hydrase from control rats or rats treated with phenobarbital, 3-methylcholanthrene or pregnenolone 16α-carbonitrile are antigenically identical, giving a single immunoprecipitin band with a line of identity with purified epoxide hydrase from phenobarbital-treated rats. Interestingly, this same antibody preparation did reveal significant species differences in hepatic epoxide hydrase from guinea pig, hamster, rabbit, and human (Fig. 8). Human and rabbit liver microsomes did not cross-react with the antibody to rat epoxide hydrase while

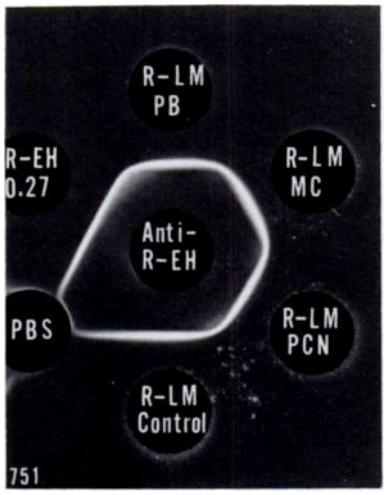

FIG. 7. *Ouchterlony immunodiffusion plate with rat liver microsomes and antibodies prepared in sheep against purified epoxide hydrase*

The antibody against rat epoxide hydrase ("Anti-R-EH") was present at a concentration of 7.5 mg IgG/ml. The rat liver microsomes ("R-LM") were obtained from rats treated with either 75 mg/kg of phenobarbital (PB), 25 mg/kg of 3-methylcholanthrene (MC), or 25 mg/kg of pregnenolone 16α-carbonitrile (PCN) for 3 days. The microsomes (5-15 mg protein/ml) were solubilized with sodium cholate and Emulgen 911 at a concentration of 1 mg and 0.2 mg per mg microsomal protein, respectively. The purified epoxide hydrase ("R-EH") was present at a concentration of 270 μg/ml. The well marked "PBS" contained phosphate-buffered saline.

guinea pig and hamster liver microsomes showed poor cross-reactivity, indicating that hepatic epoxide hydrase from these species are different proteins. Liver microsomes from C57BL/6J and DBA/2J mice showed strong reactivity with anti-rat epoxide hydrase suggesting that these mouse strains have epoxide hydrase antigenically identical to that of the rat. The lack of cross-reactivity of anti-rat epoxide hydrase with human and rabbit liver microsomes was not due to incomplete solubilization of the microsomes since 1) antibody reacts as well with rat liver microsomes as the highly purified enzyme and 2) solubilized and purified human liver epoxide hydrase also failed to react with the antibody prepared against rat epoxide hydrase (data not shown).

DISCUSSION

Despite wide interest in the possible roles of epoxide hydrase in the metabolism-dependent mutagenicity and carcinogenicity of polycyclic aromatic hydrocarbons, sufficient evidence has not been obtained to establish definitively whether tissues contain a single epoxide hydrase with broad substrate specificity or a family of enzymes with a more limited substrate specificity. Evidence for more than one liver epoxide hydrase include differential loss of catalytic

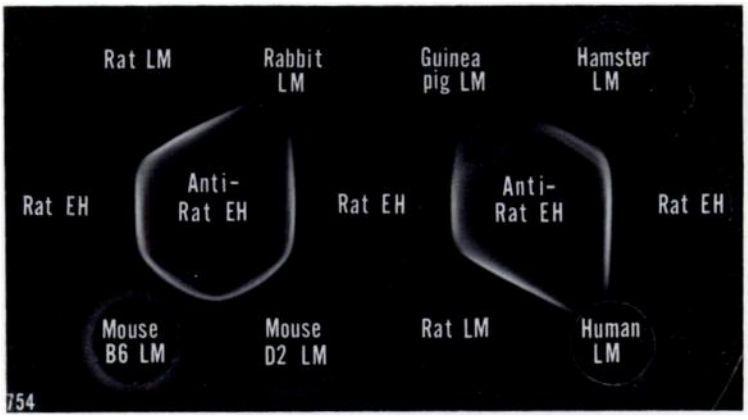

FIG. 8. *Ouchterlony immunodiffusion plate with liver microsomes from rat, hamster, guinea pig, rabbit, mouse (C57BL/6J or DBA/2J) and human*

D2 are DBA/2J mice and B6 are C57BL/6J mice. The sheep antibody ("Anti-Rat EH") was present at a concentration of 7.5 mg IgG/ml. The liver microsomes ("LM") were solubilized (15-25 mg protein/ml) as described in the legend to Fig. 7. "Rat EH" represents the highly purified enzyme (270 μg/ml).

activity toward benzene oxide compared to styrene oxide on storage of liver microsomes, different ratios of epoxide hydrase activity toward various substrates in preparations from different species, and the inability of benzene oxide to inhibit the hydration of styrene or naphthalene oxide in microsomal and partially purified epoxide hydrase preparations (cf. 35, 36). Recently, rat liver epoxide hydrase has been purified to apparent homogeneity as judged by a single protein band on SDS gels (18, 19). This purified enyme preparation is capable of hydrating a large number of alkene and arene oxides at widely differing rates (21, 22). Studies by Bentley *et al.* (22) and Lu *et al.* (21) have shown that the purification factor for the metabolism of different substrates varies by a factor of 3–4 fold. However, Bentley *et al.* (22) concluded that a single form of purified epoxide hydrase was responsible for the metabolism of the substrates assayed because these investigators were unable to establish a linear relationship between metabolism and time of incubation for the various arene oxides. They apparently considered the differences in purification factors to be within the experimental error of the assay. Lu *et al.* (21) were successful in establishing a linear relationship but cautioned against conclusions on the existence of multiple forms of the enzyme because of the complexities of the assay. Oesch and Bentley (37) also provided evidence for a single form of rat liver epoxide hydrase catalyzing the hydration of styrene oxide and BP 4,5-oxide based on immunological properties of the enzyme and similar inhibition patterns by 1,1,1-trichloropropene 2,3-oxide and cyclohexene oxide.

Several investigators have reported an *in vitro* stimulation of epoxide hydrase activity by metyrapone with styrene oxide as substrate (18, 31–33). This increase in enzymatic activity has been shown with hepatic microsomes and purified epoxide hydrase from the guinea pig, human, and rat. The results of the present study indicate that the effect of metyrapone on epoxide hydrase is substrate dependent. In the presence of metyrapone, styrene oxide, octene oxide, and to a lesser extent BA 5,6-oxide, are hydrated at an enhanced rate while the metabolism of BP 4,5-oxide is essentially unaffected and the hydration of BP 11,12-oxide and DBA 5,6-oxide are markedly inhibited. Similar results are obtained with the membrane-bound or highly purified epoxide hydrase. A kinetic analysis of the activation of epoxide hydrase by metyrapone when octene oxide is used as substrate revealed a change in both the V_{max} and apparent K_m of the enzyme, as was reported with styrene oxide as substrate. Metyrapone is a noncompetitive inhibitor of the hydration of BP 11,12-oxide. Apparently, different sites on the same enzyme or different forms of the enzyme are involved in activation and inhibition of metabolism of various substrates by metyrapone.

Cyclohexene oxide has been reported to be a noncompetitive inhibitor of guinea pig liver microsomal epoxide hydrase when styrene oxide is used as substrate (32). We chose to study this inhibitor with a variety of substrates since noncompetitive inhibition should be independent of substrate concentration which varies considerably for the different substrates assayed. Marked differences in the inhibition of metabolism of five substrates by cyclohexene oxide are observed. Low concentrations (30–120 μM) of cyclohexene oxide do not inhibit the metabolism of octene oxide and BA 5,6-oxide but significantly inhibit the hydration of BP 4,5-oxide, BP 11,12-oxide and DBA 5,6-oxide. At high concentrations of cyclohexene oxide (5–10 mM), the metabolism of all substrates is inhibited by greater than 90% except for octene oxide which is only inhibited by 30–40%. The inhibition of octene oxide is competitive while that of BP 11,12-oxide is noncompetitive. Oesch and Bentley (37) proposed a single form of epoxide hydrase based on similar inhibition of BP 4,5-oxide and styrene oxide hydration by one concentration of cyclohexene oxide. The results obtained with cyclohexene oxide in the present study point out the necessity for using a variety of substrates and inhibitor concentrations to evaluate the effects of this inhibitor.

Reaction of the purified enzyme with the donkey, sheep, or rabbit antibody yields a single immunoprecipitin band on Ouchter-

lony double diffusion plates. Two immunoprecipitin bands are observed, however, when rat liver epoxide hydrase reacts with goat antibody. None of the antibody preparations inhibit the catalytic activity of epoxide hydrase although immunoprecipitates are clearly formed. An analysis of the residual epoxide hydrase activity in the supernatants after removing the immunoprecipitates by centrifugation revealed no difference in catalytic activity toward the five substrates assayed. Oesch and Bentley (37) reported similar results using BP 4,5-oxide and styrene oxide as substrates and antibody produced in rabbits[3]. These authors interpreted the results as evidence for a single form of epoxide hydrase. Although the immunological data of Oesch and Bentley (37) as well as our results with the rabbit, donkey, and sheep antibodies are consistent with the concept of a single form of rat liver epoxide hydrase, they *do not* exclude the presence of multiple forms having similar antigenicities. The two immunodiffusion bands obtained with the goat antibody could be due to two forms of rat epoxide hydrase which have structural similarities but also possess unique antigenic regions. If antibody raised in the goat were directed against only the unique antigenic determinants of each form of the enzyme, then two immunoprecipitin bands would be expected. Similar immunological relationships have been obtained with multiple forms of other enzyme (29, 38, 39). If the other three species (donkey, sheep, and rabbit) produced antibody to antigenic determinants common to both forms of the enzyme, regardless of whether antibody was made to unique determinants as well, then immunodiffusion techniques would not differentiate between two forms of epoxide hydrase. It is highly unlikely that a contaminating protein in the purified epoxide hydrase preparation is responsible for one of the two immunoprecipitin bands observed with the goat antibody since unrelated proteins would not be expected to share antigenic determinants. Immunodiffusion techniques are capable of resolving two dissimilar proteins by varying the antigen-to-antibody ratio. In the present study, only a single immunoprecipitin band was observed with sheep, rabbit, and donkey antibodies when the antigen-antibody ratio was varied.

It is unlikely that proteolytic degradation of epoxide hydrase is responsible for the catalytic and immunological properties of the rat enzyme described above. When rat liver was homogenized and fractionated into subcellular fractions in the presence of 0.4 mM phenylmethylsulfonylfluoride, a known protease inhibitor (40), the effects of metyrapone and cyclohexene oxide on the hydration of octene oxide and BP 11,12-oxide were identical to those observed in the absence of the protease inhibitor. In addition, no change in the minimum molecular weight of a microsomal protein which comigrated with the purified enzyme in SDS-gels was seen when the microsomes were prepared in the presence of phenylmethylsulfonylfluoride (data not shown). These results, together with the similar catalytic and immunological properties of the purified enzyme compared to microsomal epoxide hydrase, indicate that the enzyme has not been altered by proteolytic digestion during the isolation procedure.

Liver microsomal epoxide hydrase from the human and rabbit do not cross-react with the sheep antibody to the rat enzyme while guinea pig and hamster epoxide hydrase show poor cross-reactivity and microsomal epoxide hydrase from C57BL/6J and DBA/2J mice show strong reactivity and a line of identity with rat epoxide hydrase. The lack of cross-reactivity of human liver epoxide hydrase deserves further mention. Preliminary results with a partially purified human liver preparation (>75% pure) showed that the human enzyme has a subunit molecular weight identical to that of the rat enzyme on SDS-gels. In addition, a comparative study on the metabolism of 11 arene and alkene oxides by human and rat

[3] Oesch and Bentley (37) indicated that antibody produced in rabbits to rat liver epoxide hydrase inhibited catalytic activity, although they only presented data on the residual activity in the supernatant after removal of the immunoprecipitate. No explanation is presently available for our lack of inhibition of catalytic activity by antibody produced in four different species compared to the antibody reported by Oesch and Bentley (37).

liver microsomes revealed a strikingly similar, although perhaps not identical, substrate specificity even though the rates of metabolism of the 11 substrates by liver microsomes vary by more than 100-fold (41). The effects of metyrapone and cyclohexene oxide on the human enzyme are also similar to those observed with the rat enzyme (data not shown). Similar observations have been made by Oesch (33, 42) with a variety of inhibitors and activators when using styrene oxide as substrate. These results indicate that there are subtle differences in the human and rat liver enzymes.

Metyrapone and cyclohexene oxide display the same effects on catalytic activity of the native enzyme and the immunoprecipitated enzyme indicating that the antigenic, stimulatory and inhibitor sites are on different portions of the enzyme molecule. A recent study by DuBois *et al.* (34) provided evidence that an essential histidine is located at or near the active site of the purified rat liver enzyme. The site-directed inhibitor used in that study and the present study (2-bromo-4′-nitroacetophenone) showed a parallel inhibition of the hydration of several substrates for the enzyme. These data suggest a single enzyme in the purified preparation but do not exclude the possibility of more than one enzyme with very similar active sites. Although more detailed studies on epoxide hydrase are needed to establish the number of forms of epoxide hydrase in a given tissue, the results presented here clearly demonstrate that rat liver epoxide hydrase contains multiple regulatory sites and that different molecular forms of the enzyme exist in the liver of different species.

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