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Human Liver Microsomal Epoxide Hydrolase

Correlation of Immunochemical Quantitation with Catalytic Activity

PAUL E. THOMAS,[1] DENE E. RYAN,[1] CHRISTER VON BAHR,[2] HANS GLAUMANN,[2] AND WAYNE LEVIN[1]

Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, New Jersey 07110, and Departments of Clinical Pharmacology and Pathology, Karolinska Institute, Huddinge Hospital, Huddinge, Sweden

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SUMMARY

Human liver microsomal epoxide hydrolase was purified to apparent homogeneity as judged by a single protein-staining band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sheep antibody to human liver epoxide hydrolase reacted with the enzyme in detergent-solubilized human liver microsomes, giving a single immunoprecipitin band which formed a line of identity with the pure human enzyme. Antibody against the human enzyme reacted well with epoxide hydrolase in detergent-solubilized monkey liver microsomes by the Ouchterlony test but less strongly cross-reacted with the enzyme from rat liver and several other species. The antibody produced against purified human liver epoxide hydrolase precipitated the enzyme but did not inhibit catalytic activity, reminiscent of the relationship of rat liver epoxide hydrolase to its antibody. The absolute level of epoxide hydrolase in liver microsomal samples from 11 human subjects, as measured by a radial immunodiffusion assay, varied 3.4-fold whereas the rate of hydration of octene oxide varied 2.9-fold. The excellent correlation of the amounts of epoxide hydrolase determined catalytically or immunochemically ($r = 0.99$) indicated that interindividual variation in octene oxide hydration rates by human liver microsomes is a consequence of differences in amount of epoxide hydrolase protein which is present, and not the result of differences in levels of endogenous modulators of catalytic activity.

INTRODUCTION

Hepatic microsomal epoxide hydrolase (EC 3.3.2.3) catalyzes the *trans* addition of water to alkene and arene oxides which are formed by the cytochrome P450-dependent monooxygenase system (1). Some of these alkene and arene oxides are reactive electrophiles which bind covalently to macromolecules, producing toxic and mutagenic effects (1, 2). Hydration of certain alkene and arene oxides by microsomal epoxide hydrolase to chemically less reactive *trans* diols is, in general, a detoxification pathway (1, 2). However, the carcinogenicity of many polycyclic aromatic hydrocarbons is now known to result from their metabolic activation via the cytochrome P450-dependent monooxygenase system and epoxide hydrolase to a bay-region diol epoxide (3, 4). Thus, microsomal epoxide hydrolase, like the cytochrome P450-dependent monooxygenase system, participates in both activation and detoxification of xenobiotics.

As a result of the critical role of epoxide hydrolase in the metabolic activation and inactivation of toxic xenobiotics, a basic understanding of the properties and reaction mechanisms of this enzyme is of great importance. In the past several years, significant progress has been made in the biochemical and biophysical characterization of hepatic microsomal epoxide hydrolase from experimental animals and its regulation by physiological and environmental factors (cf. ref. 5). Purified human liver epoxide hydrolase has been shown to be very similar to the rat liver enzyme with respect to substrate specificity, minimal molecular weight in SDS-PAGE,[3] and response to activators and inhibitors of catalytic activity (6). However, the enzymes from rat and human liver have been shown to be immunochemically distinct when tested with antibody prepared against the purified rat liver enzyme (6, 7).

Epoxide hydrolase catalytic activity can be markedly inhibited or activated *in vitro* by a number of small molecular weight modulators (7–12). It is presently unknown whether the variation in liver microsomal epoxide hydrolase catalytic activity among different human subjects (13–15) is a result of endogenous modulators of catalysis or the result of different amounts of the enzyme

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[1] Hoffmann-La Roche Inc.

[2] Karolinska Institute.

[3] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G.

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in different human subjects. In the present study, antibody was prepared in sheep against purified human liver microsomal epoxide hydrolase, and this antibody was used to immunoquantitate the absolute level of epoxide hydrolase protein in 11 human liver microsomal samples. These studies show that interindividual variation in human hepatic microsomal epoxide hydrolase in the 11 subjects studied is due to differences in the amount of epoxide hydrolase protein and is not a result of modifiers of the catalytic activity of the enzyme.

MATERIALS AND METHODS

Purification of liver microsomal epoxide hydrolase. Liver microsomes were prepared from three human liver samples obtained at autopsy (4–6 hr after death). Epoxide hydrolase was purified by a modification of the method of Lu *et al.* (6). After hydroxylapatite chromatography (6), the enzyme was dialyzed against 5 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.2% Lubrol-PX, 0.5% sodium cholate, and 0.1 mM EDTA. The sample was applied at room temperature to a Whatman DE-52 column (2 × 90 cm) previously equilibrated with 900 ml of the dialysis buffer. After sample application, the column was washed with 150 ml of the equilibration buffer followed by a linear gradient of NaCl (0–0.25 M) in 500 ml of the same buffer. The column fractions were analyzed by SDS-PAGE for epoxide hydrolase. Epoxide hydrolase was the first protein to elute from the column, and only fractions free of contaminating proteins were pooled. The sample (80 ml) was treated with 20 g of Amberlite XAD-2 beads (Rohm and Haas) for 2 hr at 4° to remove detergents. The beads were removed, and the epoxide hydrolase was dialyzed overnight against 50 mM potassium phosphate buffer (pH 7.4) and concentrated by ultrafiltration. Rat liver epoxide hydrolase was purified to apparent electrophoretic homogeneity from isosafrole- and phenobarbital-treated rats as previously described (16, 17).

Assay methods. Protein concentration was determined by the method of Lowry *et al.* (18) with crystalline bovine serum albumin as standard. SDS-PAGE of purified human liver epoxide hydrolase was performed by the method of Laemmli (19) in gels consisting of 7.5% acrylamide which were 0.75 mm thick and 10 cm long. Following electrophoresis, proteins were fixed in 25% isopropyl alcohol-10% acetic acid, stained in the same solution containing 0.05% Coomassie Brilliant Blue R, and destained in 10% isopropanol-10% acetic acid (20).

The catalytic activity of purified and microsomal epoxide hydrolase toward several substrates was measured as previously described (6, 21). Activity was determined under conditions in which the rate of product formation was constant with respect to protein concentration and time of incubation.

Immunization and immunochemical procedures. One female sheep was immunized with 400 μg of pure human epoxide hydrolase in Freund's complete adjuvant at more than 30 intradermal sites on a shaved area of the flanks. Ten weeks later the animal was boosted intravenously with 70 μg of the enzyme in phosphate-buffered saline [10 mM sodium phosphate buffer (pH 7.4), 137 mM NaCl, 2.6 mM KCl, and 0.2 mM EDTA], and blood samples were obtained 2 weeks later. The IgG fraction of the immune sera was isolated as previously described (22). Specifically purified antibody was prepared in the following manner. The IgG fraction was passed through a column of partially purified human epoxide hydrolase (70–80% pure) covalently bound to Sepharose 4B. The IgG which bound to the column was eluted with 2 M KSCN, dialyzed against phosphate-buffered saline, and concentrated by ultrafiltration with an Amicon XM-50 membrane. This specifically purified antibody was used for Ouchterlony double-diffusion analysis. Ouchterlony plates were made as described (23). Radial immunodiffusion was performed as detailed (23), except that the agarose gel containing the antibody was cast on GelBond film (FMC Corporation, Rockland, Me.) instead of glass plates. Liver microsomes were solubilized for immunodiffusion analysis with a buffer containing 1% sodium cholate, 0.2% Emulgen 911, 50 mM potassium phosphate buffer (pH 7.4), 20% glycerol, 0.2 mM EDTA, 1.0 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and aprotinin (40 μg/ml) (Mobay Chemical Corporation, New York, N. Y.).

Sources of human liver tissue. Eleven human liver microsomal preparations were obtained from a "liver bank" (24). The tissues had been obtained from cadaveric (cerebral infarction) kidney transplant donors shortly after circulatory arrest. Liver samples had been characterized by light and electron microscopy, and the levels of several drug-metabolizing enzymes were measured for specimens numbered 1–13 (24), whereas livers numbered 15–20 have been added to the "liver bank" since that report.

RESULTS AND DISCUSSION

Electrophoretic, catalytic, and structural properties. The high purity of human liver microsomal epoxide hydrolase is shown by SDS-PAGE, in which increasing amounts of the enzyme were electrophoresed (Fig. 1). Only a single protein-staining band is observed at 1 μg of protein (*Well D*) electrophoresed in a slab gel 0.75 mm thick. Human liver microsomes (*Well A*) have a clearly discernible band with the same mobility as purified epoxide hydrolase. The intensity of the protein-staining band in microsomes may not accurately reflect the actual amount of epoxide hydrolase present, since it is not known whether any other microsomal proteins also migrate to this position in the gel. Our previous preparations of purified human liver epoxide hydrolase (6) contained small amounts of contaminating proteins, which precluded their use for preparation of antibodies and structural comparisons with the rat liver enzyme. The additional purification step used in the present study (see Materials and Methods) effectively removed these contaminants, and the enzyme had slightly higher catalytic activity for the hydration of alkene and arene oxides than the previous preparations. The specific activities of human liver epoxide hydrolase for the metabolism of octene 1,2-oxide, styrene 7,8-oxide, and phenanthrene 9,10-oxide were 2430, 787, and 2030 nmoles of diol formed per minute per milligram of protein, respectively. Finally, the purified human enzyme used in these studies has yielded

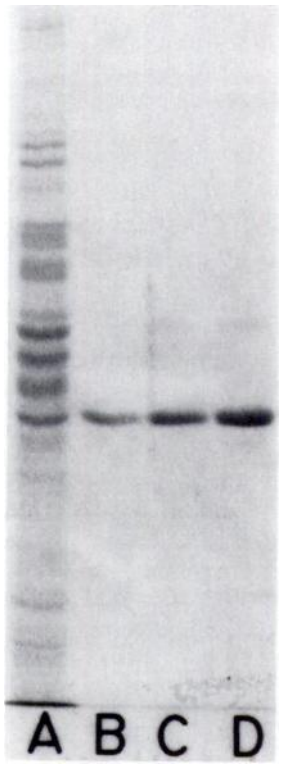


FIG. 1. *SDS-PAGE of purified human liver microsomal epoxide hydrolase and human liver microsomes*

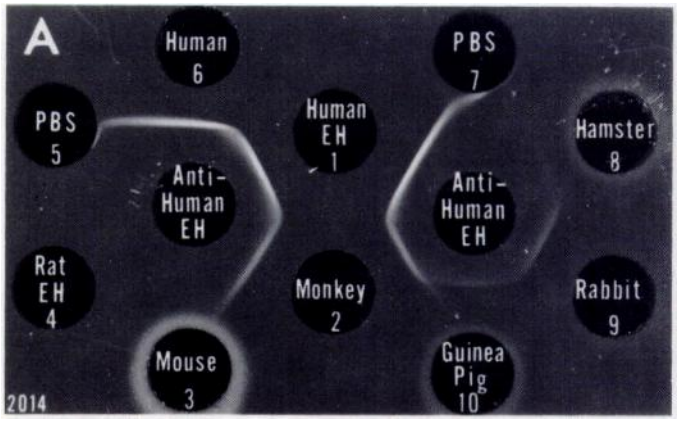
Well A contained 5 μg of human liver microsomal protein. *Wells B, C,* and *D* contained 0.25, 0.5, and 1 μg of purified microsomal epoxide hydrolase from human liver.

a single NH_2-terminal sequence when subjected to automated Edman degradation (25).

Previous comparisons of the rat and human liver microsomal enzymes revealed marked similarities between the two enzymes (6). Both enzymes have a minimal molecular weight of 49,000 in SDS-PAGE. The substrate specificities of both epoxide hydrolases are very broad; furthermore, a comparison of the metabolic rates of nine alkene and arene oxides revealed only minor quantitative differences between the purified rat and human enzyme (6). Human and rat liver microsomal preparations have also been compared by using the hydration rate of 11 alkene and arene oxides, and only modest differences between the human and rat subcellular fractions were found (13). The selective effects of activators and inhibitors on catalytic activity were also strikingly similar for both enzymes, and an active site-directed inhibitor (2-bromo-4′-nitroacetophenone) of the rat enzyme (26) also inhibits metabolism by the human enzyme (6). Furthermore, there is 68% homology in the first 19 NH_2-terminal amino acid residues in human and rat epoxide hydrolase (25).

Immunochemical properties. Despite the many similarities between human and rat liver epoxide hydrolase, these enzymes differ markedly in immunological properties. Antibody against rat epoxide hydrolase prepared in sheep, donkey, goat, or rabbit shows very weak (rabbit antibody) or no cross-reaction (sheep, donkey, and goat antibodies) with the human enzyme in Ouchterlony double-diffusion analysis (6). Since antibodies are directed against several distinct and spatially distant surface antigenic determinants (27, 28), the scarcity of common antigenic determinants between the human and rat enzymes implies the absence of large regions of surface homology. Extensive immunological studies with globular proteins of known sequence have indicated that cross-reactivity between proteins requiring a multivalent antigenic interaction does not usually disappear until there is approximately a 40% difference in the amino acid sequence of the proteins (29–31).

Figure 2 shows the reactivity of specifically purified



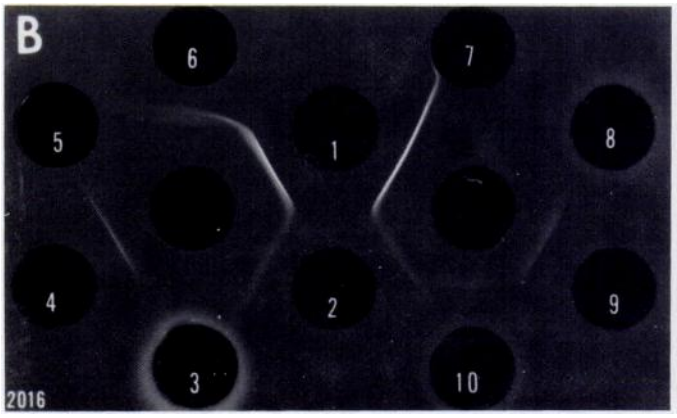


FIG. 2. *Ouchterlony immunodiffusion plates comparing the reactivity of epoxide hydrolase from humans and other species with the antibody made against the human enzyme*

The plates shown in A and B are identical except for the inclusion in A of the detergent Emulgen 911 at 0.2% final concentration. The central two wells contain specifically purified sheep IgG (2 mg/ml) directed against human microsomal epoxide hydrolase. *Wells 1* and *4* contain pure microsomal epoxide hydrolase from human liver (0.24 mg/ml) and rat liver (0.25 mg/ml), respectively. *Wells 5* and *7* contain phosphate-buffered saline. The remaining wells contain detergent-solubilized microsomes at the final protein concentrations given: rhesus monkey (8.7 mg/ml) in *Well 2*; C57BL/6J × DBA/2J F_1 mouse (9.0 mg/ml) in *Well 3*; human (11 mg/ml) in *Well 6*; Syrian golden hamster (8.6 mg/ml) in *Well 8*; New Zealand White rabbit (10 mg/ml) in *Well 9*; and Hartley guinea pig (6.1 mg/ml) in *Well 10*. All wells were 4 mm in diameter.

antibody to human epoxide hydrolase with the antigen, purified rat liver epoxide hydrolase, and solubilized microsomes from various species by Ouchterlony double-diffusion analysis in the presence (Fig. 2A) and absence (Fig. 2B) of Emulgen 911. Several features of these double-diffusion plates deserve comment. First, the specificity of antibody to human epoxide hydrolase prepared in sheep provides additional evidence for the homogeneity of the purified human enzyme. As shown in Fig. 2A and B, a single precipitin band is formed between the antibody (*center well*) and detergent-solubilized human liver microsomes (*Well 6*). To eliminate the possibility that an additional immunogenic protein was obscured by the reaction of epoxide hydrolase with its antibody, double-diffusion analysis was also performed with both antibody and antigen in excess; in each case, only one immunoprecipitin band was observed with human liver microsomes (results not shown). The purity of the human epoxide hydrolase is further indicated by the lack of spurs at the point of fusion of the two immunoprecipitin bands produced from the reaction of the antibody with liver microsomes (*Well 6*) and the antigen of immunization (*Well 1*). Antibody to human epoxide hydrolase reacts with the purified antigen (*Well 1*), forming a sharp immunoprecipitin band independent of detergent inclusion in the plate; however, a more distinct reaction is observed between human microsomal epoxide hydrolase (*Well 6*) and the antibody in the presence of Emulgen 911 (Fig. 2A) than without detergent (Fig. 2B). Anti-human epoxide hydrolase, like the antibody directed against the purified rat enzyme (7), is a noninhibitory antibody. Immunoprecipitated human liver epoxide hydrolase retains full catalytic activity for the hydration of styrene 7,8-oxide, octene 1,2-oxide, and phenanthrene 9,10-oxide (data not shown).

Second, antibody against human epoxide hydrolase cross-reacts with detergent-solubilized liver microsomes from a variety of other species, as shown in Fig. 2. Of the species tested (hamster, rabbit, guinea pig, mouse, rat, and monkey), solubilized microsomes from rhesus monkey cross-react with the antibody to the greatest extent and show immunochemical identity with human epoxide hydrolase. Rabbit and guinea pig microsomes also react with the antibody to human epoxide hydrolase, showing a reaction of immunochemical identity to each other but only partial identity with human (and monkey) epoxide hydrolase. Spurring is observed at the junction of the immunoprecipitin bands of monkey (*Well 2*) and guinea pig (*Well 10*) solubilized microsomes, although the spur is weak. The partial identity of rabbit and guinea pig microsomal epoxide hydrolase with the human enzyme, as indicated by the spurring, can be accentuated by placing human epoxide hydrolase next to solubilized rabbit or guinea pig microsomes in a double-diffusion plate (results not shown). The cross-reactivity of epoxide hydrolase in hamster liver microsomes (*Well 8*) with antibody to the human enzyme is extremely weak and often difficult to discern, but the immunoprecipitin band formed appears to fuse with the band from rabbit liver microsomes. Immunoprecipitin bands are observed between hamster, rabbit, guinea pig, and monkey microsomes and antibody to human epoxide hydrolase when detergent is present or absent from the Ouchterlony plate. In contrast, however, solubilized mouse liver microsomes (*Well 3*) do not react with specifically purified antibody to human epoxide hydrolase under the conditions in Fig. 2A or B.

Third, the presence of Emulgen 911 in the double-diffusion plate dramatically influences the reactivity of purified rat liver epoxide hydrolase with the antibody to the human enzyme. Interestingly, only when detergent is omitted from the agarose (Fig. 2B) does purified rat epoxide hydrolase react reasonably well with the antibody. If Emulgen 911 is incorporated into the agarose matrix (Fig. 2A), no immunoprecipitin band formation is observed between the purified rat enzyme and the antibody to human epoxide hydrolase. However, without detergent in the agarose, detergent-solubilized rat liver microsomes show no reaction (results not shown), and detergent-solubilized microsomes from human and monkey react with a weaker precipitin band than they do when detergent is present. Clearly, detergent has two effects: inhibition of the weaker cross-reaction of rat epoxide hydrolase and enhancement of the diffusion and consequently the formation of the precipitin band of detergent-solubilized microsomal samples.

The relative reactivities of hepatic epoxide hydrolase from different species with antibody to human epoxide hydrolase can be ranked as follows: human ≅ monkey > guinea pig ≅ rabbit > hamster > rat ≥ mouse. From our previous data (7) and additional data on the monkey (not shown), the order of reactivity of these proteins with anti-rat epoxide hydrolase can be described as: rat ≅ mouse > hamster > guinea pig > rabbit > monkey ≅ human. It is interesting that the order of reactivities toward the human epoxide hydrolase antibody is close to being the inverse of the order toward the antibody to the rat enzyme.

Correlation of microsomal catalytic activity with absolute levels of epoxide hydrolase. The catalytic activities of rat and human liver microsomal epoxide hydrolase toward 11 substrates have previously been compared (13). A markedly similar, but not identical, substrate specificity profile was found for the human and rat enzymes. However, there was substantial interindividual variation in the rates of hydration of these substrates by microsomes from nine human subjects. The significance of the individual differences in metabolic rate is unclear, since there are many known modulators of the catalytic activity of epoxide hydrolase (7–12). Furthermore, lipid and detergent are known to have a marked effect on the kinetics of hydration of several arene oxides by microsomes and purified rat liver epoxide hydrolase (32, 33).

To determine whether microsomal epoxide hydrolase catalytic activity is a reliable index of the amount of enzyme protein in human liver, epoxide hydrolase in 11 human liver microsomal samples was measured by the rate of octene oxide hydration (13, 21) and by a radial immunodiffusion assay (23, 34). The radial immunodiffusion assay measures the absolute amount of epoxide hydrolase irrespective of the catalytic activity of the enzyme. All human liver samples were obtained from a human "liver bank" (24) where tissue was received from kidney transplant donors shortly after circulatory arrest. Since these liver tissues were obtained under relatively controlled conditions and were well-characterized mor-

TABLE 1

Comparisons of epoxide hydrolase catalytic activity with the amount of epoxide hydrolase protein in microsomal preparations from 11 human livers

Liver numbers correspond to those in the human liver bank (24). Liver no. 12 had abnormal morphology (24). The amount of epoxide hydrolase was determined by radial immunodiffusion with antibody prepared against the human liver enzyme as described under Materials and Methods. Values represent the means ± standard error of duplicate determinations performed at three protein concentrations on each liver sample. Specific activities (column 4) were calculated by dividing the rate of octene oxide hydration in liver microsomes (column 2) by the amount of epoxide hydrolase protein in 1 mg of microsomal protein (column 3).

Liver no.	Octene 1,2-oxide hydration	Epoxide hydrolase protein	Specific activity
	nmoles glycol/min/mg microsomal protein	*mg/100 mg microsomal protein*	*nmoles octene glycol/min/mg epoxide hydrolase*
15	42.3 ± 1.6	1.40 ± 0.03	3020
12	47.0 ± 0.8	1.81 ± 0.04	2600
10	63.9 ± 3.8	2.35 ± 0.06	2720
17	64.5 ± 2.6	2.60 ± 0.06	2480
19	67.8 ± 2.8	2.49 ± 0.04	2720
6	92.7 ± 1.5	3.35 ± 0.09	2770
13	96.4 ± 7.4	3.52 ± 0.10	2740
16	96.6 ± 2.3	3.88 ± 0.06	2490
18	105.3 ± 3.7	4.21 ± 0.15	2500
11	105.6 ± 1.8	4.15 ± 0.10	2540
20	123.7 ± 3.6	4.78 ± 0.13	2590

phologically and biochemically, they serve as an ideal source for this comparison. As shown in Table 1, the values for the rate of octene oxide hydration varied 2.9-fold among the different liver specimens, which correlated well with the observed 3.4-fold variation in absolute amount of epoxide hydrolase protein as measured by a radial immunodiffusion assay. Figure 3 illustrates the excellent correlation (r = 0.99) between epoxide hydrolase catalytic activity and the amount of epoxide hydrolase protein for the 11 human subjects.

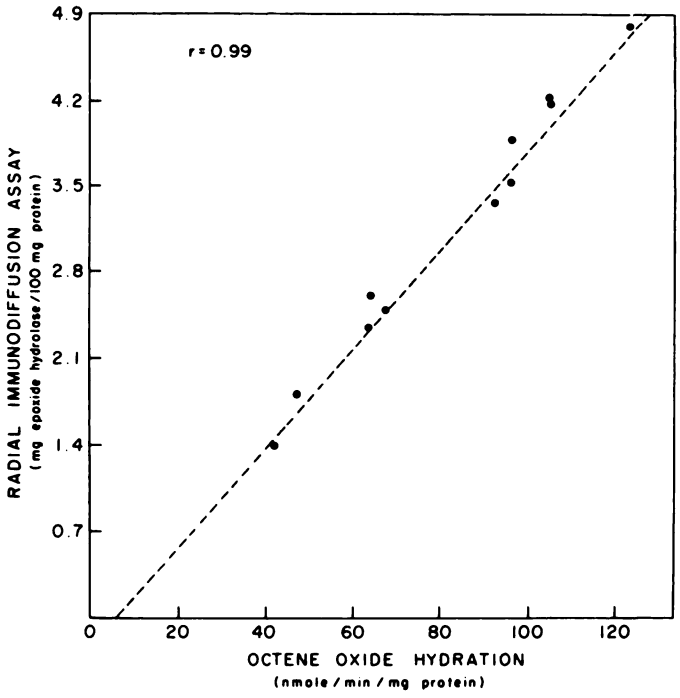


FIG. 3. *Correlation of epoxide hydrolase protein and octene 1,2-oxide hydration by liver microsomes from eleven human subjects*

It is somewhat surprising that the amount of epoxide hydrolase protein determined immunochemically corresponds so well to the catalytic activity of the enzyme in human liver, since Guengerich *et al.* (14) have proposed the existence of multiple forms of this enzyme, each having broad and overlapping substrate specificity and showing immunochemical relatedness. Since the basis of the two assays for epoxide hydrolase used in the present study are unrelated, such an excellent correlation would be unexpected if there were multiple forms of immunologically and catalytically distinct epoxide hydrolase. A comparison between octene oxide hydration and immunoquantitation among liver microsomal samples from rats treated with a variety of inducers has yielded a similar correlation (23, 34). Guengerich *et al.* (35) have reported no correlation between styrene oxide hydration and immunochemically assayed epoxide hydrolase (measured by complement fixation) when liver microsomes from five human subjects or various purified preparations from human liver microsomes were examined. However, Guengerich *et al.* (35) assayed human liver autopsy samples which had not been characterized and used antibody against rat liver epoxide hydrolase, which cross-reacts weakly with human liver epoxide hydrolase. These investigators recognized that their use of uncharacterized livers obtained at autopsy could present problems in their studies (35). The choice of octene oxide in this study or styrene oxide by Guengerich *et al.* (35) to measure catalytic activity should not have resulted in the discrepancy, since these two catalytic activities have previously been shown (13) to correlate well with one another (r = 0.98) in nine human liver microsomal samples. Moreover, the hydration of nine other arene and alkene oxides also correlated well with each other and with octene oxide and styrene oxide hydration (r = 0.87 to 0.99 for all substrate pairs) in these human livers (13). Although the possibility of the presence of multiple forms of human liver microsomal epoxide hydrolase cannot be eliminated, we can conclude that, if multiple forms of the enzyme exist, they are under similar regulatory control in different individuals.

In additional experiments, we have eliminated any possibility of a significant contribution of soluble epoxide hydrolase (36) toward the metabolism of octene oxide catalyzed by human liver microsomes. Octene oxide hydration by 100,000 × g supernatant of human liver occurs at less than 10% of the rate (nanomoles per minute per milligram of protein) of hydration by microsomes at pH 8.2 (data not shown). Since the microsomes used in the present study were resuspended in 0.15 M Tris-HCl (pH 7.6) and subjected to a second centrifugation at 100,000 × g (24), the contribution of soluble epoxide hydrolase to the catalytic activity of the microsomes was negligible.

An important consideration for all studies with purified enzymes is the possibility of inactivation of the enzyme during purification. This is an especially important consideration with microsomal epoxide hydrolase, which is

an integral membrane enzyme and must be solubilized and purified from its native lipophilic milieu in the presence of detergents. We can address this problem by calculating the catalytic activity per milligram of epoxide hydrolase in each human liver microsomal sample, using the radial immunodiffusion assay to determine the amount of epoxide hydrolase. Catalytic activities per milligram of epoxide hydrolase appear in the fourth column of Table 1. These individual determinations of epoxide hydrolase specific activity (2440–3020 nmoles of octene oxide hydrolyzed per minute per milligram of epoxide hydrolase protein) compare favorably with the specific activity of pure human epoxide hydrolase (2430 nmoles/min/mg). From these results we can conclude that the human microsomal epoxide hydrolase which was purified and used as an antigen is not significantly inactivated by the detergent-solubilization and purification procedure.

In conclusion, antibody to pure human liver microsomal epoxide hydrolase has been used to show substantial immunochemical differences between hepatic epoxide hydrolases isolated from humans and rats. Immunochemical differences are in contrast to the many similarities in chemical and catalytic properties of these two enzymes. We have also shown that the interindividual variation in human epoxide hydrolase catalytic activity in 11 subjects correlates well with the amount of immunochemically assayed enzyme and is, therefore, not an indirect effect of endogenous modifiers on the catalytic activity of the enzyme.

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Send reprint requests to: Dr. Paul E. Thomas, Building 86, Hoffmann-La Roche Inc., 340 Kingsland Street, Nutley, N. J. 07110.