Evidence has been presented in the preceding paper (Guengerich, F. P., Wang, P., Mitchell, M. B., and Mason, P. S. (1979) J. Biol. Chem. 254, 12248-12254) that multiple forms of liver microsomal epoxide hydratase can be isolated from either rats or humans. An antibody preparation was raised to the "A" fraction purified from phenobarbital-treated rats as described in that report and used to examine the similarities and differences between various epoxide hydratase preparations.

The antibody preparation did not inhibit epoxide hydratase activity but could precipitate >90% of the styrene-7,8-oxide hydratase activity from human liver or untreated rat liver, kidney, lung, or testis microsomes or from liver microsomes prepared from rats treated with phenobarbital, 3-methylcholanthrene, or trans-stilbene oxide. Analysis of several of the immunoprecipitates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that all of the immunoprecipitated epoxide hydratases examined had similar apparent molecular weights. Immunodiffusion techniques were used to demonstrate the presence of an immunologically distinct form of epoxide hydratase in livers of untreated and 3-methylcholanthrene-treated rats which was not detected in microsomes prepared from phenobarbital- or trans-stilbene oxide-treated rats. This epoxide hydratase fraction contained a significant portion of the microsomal epoxide hydratase activity, depending upon the substrate examined, and could be isolated using hydroxyapatite chromatography.

Complement fixation methods were used to demonstrate differences among several of the purified epoxide hydratase fractions that were not revealed by other immunological techniques. Microsomal preparations derived from five different humans differed in their reactivity with the antibody preparation, as judged by complement fixation, when reactivity was plotted either versus microsomal protein or versus styrene-7,8-oxide hydratase activity.

In the accompanying paper methods were presented for the purification of various forms of rat and human liver microsomal epoxide hydratase (3). This report presents a variety of immunological experiments that compare the various purified enzyme preparations and the enzymes in extrahaepatic and human liver microsomal preparations. In addition, immunological techniques were used to examine the location of the enzyme in microsomal membranes.

**Experimental Procedures**

**Epoxide Hydratase Preparations**—The preparations described in the preceding paper (3) were used in this work. Protein and activity determinations were made as described in that report.

**Preparation of Antibodies**—The epoxide hydratase A fraction was purified from phenobarbital-treated rats as described (3). Female New Zealand white rabbits were immunized using a schedule described elsewhere (4, 5). Sera from three rabbits were pooled and IgG fractions were prepared as described (6), with the exception that antiserum were heated 20 min at 66°C prior to ammonium sulfate fractionation.

**Preparation of Microsomes**—Rat extrahaepatic and rabbit liver microsomes were prepared as described elsewhere (7). The specific styrene-7,8-oxide hydratase activities found for the various microsomal preparations were as follows (nanomoles per min per mg of protein, mean of three assays ± S.D.): untreated rat kidney, 0.74 (± 0.15); untreated rat lung, 0.49 (± 0.05); untreated rat testis, 0.98 (± 0.11); and 3-methylcholanthrene-treated rabbit liver, 5.9 (± 0.2).

**Results**

**Immunoprecipitation Studies**—Preliminary studies indicated that the immunoglobulin complex of epoxide hydratase remains catalytically active, as reported by another laboratory (9). Immunoprecipitation techniques were first used to examine the similarity of the epoxide hydratases of various microsomal systems with the enzyme preparation used to raise antibodies. The results indicate that the antibody preparation precipitated ≥90% of the styrene-7,8-oxide hydratase activity in microsomes prepared from untreated rat liver, human liver (Fig. 1), phenobarbital-, 3-methylcholanthrene-, or trans-stilbene oxide-treated rat liver, or lungs, kidneys, or testes of untreated rats (data not shown).

**Rat Liver Microsomes**—The abbreviations used arc. SDS, sodium dodecyl sulfate; C', complement; IgG, immunoglobulin G; BP, benzo(a)pyrene.

1 The abbreviations used are: SDS, sodium dodecyl sulfate; C', complement; IgG, immunoglobulin G; BP, benzo(a)pyrene.
Rat and Human Epoxide Hydratase

Fig. 1. Immunoprecipitation of styrene-7,8-oxide hydratase activity from Lubrol PX-solubilized microsomes with IgG raised to epoxide hydratase. Microsomes (10 mg ml\(^{-1}\)) were solubilized by the addition of Lubrol PX to 1% (w/v) in 0.2 M phosphate buffer (pH 7.25) and centrifuged for 1 h at 105,000 \(\times\) g. Varying amounts of IgG were added to aliquots of each microsomal supernatant and the mixtures were kept at 4°C for 16 to 20 h. After subsequent centrifugation at 20,000 \(\times\) g for 20 min, aliquots of each supernatant were assayed for styrene-7,8-oxide hydratase activity (8) in triplicate. Results are expressed as means ± S.D. for each microsomal sample using epoxide hydratase IgG (M) or preimmune IgG (O--O).

One unit of epoxide hydratase is defined as that amount of enzyme converting 1 nmol of styrene-7,8-oxide to styrene glycol min\(^{-1}\) at 37°C. A, untreated rat liver; B, human liver (No. 1).

microsomes (Fig. 3A). These diffusion studies were carried out at 23°C; single lines were observed in all cases when diffusion took place at 4°C.

Both of the double diffusion precipitin lines derived from untreated or 3-methylcholanthrene-treated rat liver microsomes contained epoxide hydratase activity. Samples were allowed to migrate for 24 h at 23°C and washed by gentle rocking with eight changes of 50 mM potassium phosphate buffer (pH 8.0) containing 300 mM NaCl over another 24 h; both bands were excised and assayed for activity, as binding of IgG did not inhibit activity. In the case of untreated rat liver microsomes, the inner precipitin line contained 3% of the recovered styrene-7,8-oxide hydratase activity and 48% of the recovered BP-4,5-oxide hydratase activity. With 3-methylcholanthrene-treated rat liver microsomes, the inner precipitin line contained 50% of the recovered styrene-7,8-oxide hydratase activity and 49% of the recovered BP-4,5-oxide hydratase activity.

A single precipitin line was also observed when liver microsomes prepared from trans-stilbene oxide-treated rats were tested against the antibody preparation. (Fig. 3B). Rat kidney microsomes yielded a very faint precipitin line.

Fig. 3C indicates that the epoxide hydratase A\(_2\) fractions prepared from untreated and phenobarbital-, 3-methylcholanthrene-, and trans-stilbene oxide-treated rat livers shared a line of common identity and that the A\(_1\) fraction prepared from untreated rats was immunochemically distinct. The B fractions prepared from rats treated in various ways appeared to be immunochemically identical with each other and to the A\(_2\) fractions but distinct from the untreated rat A\(_1\) fraction (Fig. 3D). The A\(_1\) fraction appeared to contain two compo-
Solubilized by the addition of Lubrol PX to containing 0.2% Emulgen 913 as described elsewhere (4, 12). Microsomal preparations were solubilized by the addition of Lubrol PX to containing 0.2% Emulgen 913 as described elsewhere (4, 12).

In Fig. 3E, the epoxide hydrolases of human liver microsomes derived from four different patients were found to be immunologically similar but distinct from rat liver epoxide hydrolases. Subsequent analysis of various purified human liver epoxide hydrolase preparations indicated that the DE preparations of various individual patients were immunologically similar (Fig. 3F). Moreover, the Fractions HA, and DE200 were similar to the DE preparations (Fig. 3G).

C' Fixation Studies using Microsomes—C' fixation was used to examine differences in microsomal preparations. A preliminary experiment indicated that microsomes prepared from three different humans produced >90% C' fixation and that the human liver microsomes were less effective in C' fixation than were rat liver microsomes (Fig. 5). A purified human liver epoxide hydrolase preparation was more effective in C' fixation than the microsomal preparation from which it was derived, and both preparations produced the same maximum level of C' fixation (Fig. 6).

Human liver microsomes prepared from five different patients were compared for C' fixation activity in parallel assays. Considerable variation was found in the concentrations of antigen required to produce 50% C' fixation when the antigen.
Fig. 4. C’ fixation by various purified rat and human liver epoxide hydratase fractions. The procedure was carried out according to the method of Levine and Van Vanukis (13) using 2 μg of epoxide hydratase IgG and the indicated epoxide hydratase preparations. Preparations examined included: A, untreated (UT) rat liver A₁ (●—●); untreated rat liver A₂ (△—△); untreated rat liver B (■—■); and phenobarbital rat liver A (●—●); B, human [No. 1] liver DE (●—●); human [No. 3] liver DE (○—○); human [No. 1] liver HA₅ (△—△); and human [No. 1] liver HA₆ (□—□).

Fig. 5. C’ fixation by rat and human liver microsomes as a function of antibody concentration. All assays were carried out using 2 μg of phenobarbital-treated rat liver (●—●) or human liver microsomal protein and varying levels of IgG prepared from antisera raised to phenobarbital-treated rat liver epoxide hydratase A. The human liver microsomes were prepared from Patient 2 (○—○), Patient 3 (△—△), Patient 4 (H), Patient 5 (□—□), and Patient 6 (□—□). C’ fixation is plotted versus protein concentration in A and versus styrene-7,8-oxide hydratase activity in B.

Fig. 6. C’ fixation by human liver microsomes and purified human liver epoxide hydratase. C’ fixation experiments were carried out as in Fig. 4 in the presence of 4 × 10⁻⁷ % (w/v) Lubrol PX using either human liver microsomes derived from Patient 6 (○—○) or the DE epoxide hydratase fraction (●—●) purified from those microsomes.

Fig. 7. C’ fixation by microsomes prepared from different humans. The basic procedure was carried out according to the method of Levine and Van Vanukis (13) using 5 μg of IgG raised to phenobarbital rat liver epoxide hydratase A₂ and the indicated preparations: Patient 2 (○—○), Patient 3 (△—△), Patient 4 (H), Patient 5 (□—□), and Patient 6 (○—○). C’ fixation is plotted versus protein concentration in A and versus styrene-7,8-oxide hydratase activity in B.

Fig. 8. Effect of detergent solubilization upon C’ fixation of rat liver microsomes in the presence of anti-epoxide hydratase. A, C’ fixation assays were carried out according to the method of Levine and Van Vanukis (13) using 5 μg of IgG raised to phenobarbital rat liver epoxide hydratase A₂. Varying amounts of phenobarbital rat liver epoxide hydratase A₂ were added in the presence (■—■) or absence (●—●) of 1.25 × 10⁻⁷ % (w/v) Lubrol PX. B, C’ fixation assays were carried out as above with phenobarbital-treated rat liver microsomes in the absence of Lubrol PX (●—●). In the other part of the experiment, the same phenobarbital rat liver microsomes (10 mg ml⁻¹) were solubilized with 1% (w/v) Lubrol PX and aliquots were used in the C’ fixation assays (○—○); Lubrol PX was added to give an equal concentration of 1.25 × 10⁻⁷ % in all incubations.
C’ Fixation as a Probe of Epoxide Hydratase Accessibility—Epoxide hydratase has been postulated to be buried deep in microsomal membranes (14). On the other hand, immunological studies suggest that cytochrome P-450 and NADPH-cytochrome P-450 reductase are either fixed near the outer membrane edge or migrate and have access to the outside of the membrane (6, 7, 15, 16). Such a hypothesis concerning compartmentalization of epoxide hydratase and cytochrome P-450 and its reductase is of potential significance in understanding pathways of bioactivation and detoxification of xenobiotics.

An experiment was set up to examine the accessibility of microsomal epoxide hydratase to antibodies. Preliminary experiments established that a 1% (w/v) concentration of the nonionic detergent Lubrol PX would completely release epoxide hydratase activity from membranes (3) and that, after dilution to a final concentration of 1.25 x 10⁻⁴%, Lubrol PX had only a slight inhibitory effect on the C’ fixation ability of a purified epoxide hydratase preparation (Fig. 8). Rat liver microsomes were found to fix C’ well without solubilization, and the effect of treatment with Lubrol was essentially identical with that observed in the case of the purified enzyme. Thus, epoxide hydratase does not appear to be so deeply imbedded in membranes that the enzyme is rendered inaccessible to antibodies.

DISCUSSION

The data suggest that the various rat and human epoxide hydratases are rather immunochemically similar enzymes, as the antibody preparation used here precipitated the enzyme nearly quantitatively from a variety of sources. However, immunological techniques were useful in demonstrating differences among several individual forms of the enzyme that were not always readily apparent using other techniques (3). For instance, the A2 and B fractions isolated from untreated rat liver were distinguished by C’ fixation, as were several human liver epoxide hydratase fractions (Fig. 4). The untreated rat liver epoxide hydratase A1 and the various human liver hydratases differed considerably from the remainder of the rat liver enzymes.

The C’ fixation data suggest that individual variations occur among humans with regard to the types of liver microsomal epoxide hydratase, and such a view is supported by the isolation of individual forms of the enzyme (3). However, the possible lability of autopsy sample material may be a problem, and further studies will be required to elucidate the role of multiplicity of epoxide hydratase in humans.

The isolation of multiple forms of epoxide hydratase is probably not a result of proteolytic artifacts. Similar chromatographic (3) and immunological results were obtained regardless of whether 0.1 mm (freshly dissolved) phenylmethylisulfonyl fluoride was added to buffers. Since all human and rat epoxide hydratase preparations had very similar apparent molecular weights, any extensive proteolysis could not have occurred. Finally, immunological double diffusion results (Fig. 3) were a function of inducing rats (with phenobarbital or trans-stilbene oxide), which would not be expected to increase contamination of microsomes with proteolytic enzymes.

The immunological results support the conclusions reached in the accompanying paper (3); i.e. that the untreated rat A1, phenobarbital-treated rat A and the various human liver epoxide hydratase preparations are distinct enzymes and that the other rat A2 and rat B fractions are distinct classes of enzymes, which may or may not vary slightly after treatment of rats with 3-methylcholanthrene or trans-stilbene oxide. Clearly, the most distinct form of rat liver epoxide hydratase is Fraction A, prepared from untreated rat liver. Double diffusion analysis against the antibody separated two distinct lines having epoxide hydratase activity, and the enzyme fractions giving rise to each of these two lines could be separated by hydroxyapatite chromatography (3). The isolated fractions differed in substrate specificity (3). Although the recovery of Fraction A was low, assays of the double diffusion immunoprecipitates indicated that this fraction can account for 3 to 50% of the total microsomal epoxide hydratase activity. In general, the various human and rat liver epoxide hydratases are similar enzymes with regard to a variety of criteria but apparent differences exist in several cases. The levels of individual forms can be affected by induction and repression by exogenous chemicals. Thus, epoxide hydratase is similar to microsomal cytochrome P-450 (4, 17) and cystolic glutathione-S-transferase (18), two other xenobiotic-metabolizing systems, in this regard. The cross-reactivity of the rat and human enzymes and the availability of highly purified human liver epoxide hydratase preparations may be useful in using immunological techniques to define the role of epoxide hydratase in humans.

Acknowledgments—We wish to thank Doctors Robert Briggs and Lubomir S. Hnilica for their help in the complement fixation work and Dr. John Edland for help in the procurement of autopsy samples.

REFERENCES

Rat and human microsomal epoxide hydratase. Immunological characterization of various forms of the enzyme.
F P Guengerich, P Wang, P S Mason and M B Mitchell