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Immunological Comparison of Rat, Rabbit, and Human Microsomal Cytochromes P-450†

F. Peter Guengerich,* Philip Wang, Patricia S. Mason, and Margaret B. Mitchell

ABSTRACT: Antibodies were raised in rabbits to electrophoretically homogeneous cytochromes P-450 isolated from rat and human liver microsomes. These antibodies were used to compare various forms of rat, rabbit, and human cytochromes P-450 present in microsomes and in purified preparations by using double-diffusion analysis, immunoelectrophoresis, quantitative microcomplement fixation, competitive radioimmune assay, and inhibition of enzyme activity toward *d*-benzphetamine and benzo[*a*]pyrene. The results indicate that (1) at least some forms of cytochrome P-450 from the three species share certain common immunological determinants, (2) there are immunological differences between cytochromes P-450 isolated from the three species, (3) some immunological differences exist between cytochromes P-450 isolated from rats of different strains, (4) immunologically distinguishable forms of cytochrome P-450 exist within individual human liver samples, and (5) human liver samples obtained from different individuals contain immunologically different forms of cytochrome P-450. Quantitative microcomplement fixation techniques were used to assign immunological distances to different forms of rat, rabbit, and human liver microsomal cytochrome P-450. Cross-reactivity was observed in all systems tested, and the extent of immunological similarity was dependent upon the particular assay used.

Cytochrome P-450[1] serves a pivotal role as the terminal oxidase in a microsomal mixed-function oxidase system that catalyzes the metabolism of a great variety of xenobiotics as well as endogeneous compounds (Coon et al., 1977; Gillette et al., 1974). The hepatic enzyme has been purified to apparent homogeneity as judged by various criteria from rats (Guengerich, 1978; Ryan et al., 1979; West et al., 1979), rabbits (Coon et al., 1978; Imai & Sato, 1974; Johnson & Muller-Eberhard, 1977; Kawalek et al., 1975; Philpot & Arinc, 1976), and mice (Huang et al., 1976). A large body of evidence has been accumulated which suggests that multiple forms of P-450 exist in experimental animals and that some are inducible [for reviews, see Guengerich (1979) and Lu (1979)]. Different animal species appear to contain different subsets of P-450s. This multiplicity appears to be important in the regulation of the types of P-450-mediated reactions carried out in a given individual (Coon et al., 1977).

Wide variation of carcinogen-metabolizing activities attributed to P-450 has been observed in human samples (Harris et al., 1979; Sabadie et al., 1980). Extension of the knowledge of P-450s in humans had been hampered by technical diffi-

† From the Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received May 2, 1980; revised manuscript received September 2, 1980.* This work was supported in part by Grants ES 01590 and ES 00267 and Contract NCI NO 1 CP 85672 from the U.S. Public Health Service.

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[1] Abbreviations used: P-450, liver microsomal cytochrome P-450; C′, complement; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; RIA, competitive radioimmune assay; IgG, immunoglobulin G fraction; PB, phenobarbital; 3MC, 3-methylcholanthrene; BNF, β-naphthoflavone (5,6-benzoflavone); $NaDodSO_4$, sodium dodecyl sulfate; M_r, apparent monomeric molecular weight as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis.

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Table I: Human Liver Autopsy Samples

patient	age (years)	sex	cause of death	time between death and freezing of sample (h)	microsomal P-450 [nmol (mg of protein)$^{-1}$]
1	50	male	seizure disorder	4	0.086
2	54	male	cardiac arrest	10	0.069
3	70	male	myocardial infarction	3	0.13
4	4 days	female	cardiac arrest	12	0.36
5	69	female	primary breast and ovarian carcinoma	5	0.067
6	32	female	gunshot wound	12	0.18
7	52	female	subarachnoid hematoma	5	0.078
8	31	female	cerebral edema	4	0.35
9	14	male	cardiac arrest	3	0.21
10	64	female	myocardial infarction	6	0.066
11	52	female	pulmonary carcinoma	7	nd[a]
12	47	female	accidental blow to head	8	nd
13	64	male	subarachnoid hemorrhage and adenocarcinoma of lung	9	nd
14	27	male	gunshot wound	5	0.29
15	37	male	gunshot wound	4	0.12
16	20	male	stab wound	9	0.29

[a] nd, not determined.

culties in working with human samples. Kaschnitz & Coon (1975) have solubilized, partially separated, and reconstituted this mixed-function oxidase from human liver, and Kamataki et al. (1979) have made considerable progress in the purification of the human system. This laboratory has utilized technical advances in purification technology to isolate human liver P-450s that are apparently homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis, have high specific contents of P-450, and are free of certain suspected contaminating enzyme activities (Wang et al., 1980). In this report, we describe a series of studies utilizing rabbit antibodies raised to highly purified rat and human liver P-450s in a battery of immunological assays for the purpose of examining the extents of similarity of rat, human, and rabbit P-450s. In this and the following paper, we examine variation with regard to P-450 as well as NADPH–P-450 reductase (Guengerich et al., 1981).

Experimental Procedures

Preparation of Microsomes and P-450s. Male rats of Sprague-Dawley descent, weighing 125–150 g unless noted differently, were obtained from Harlan Industries, Indianapolis, IN. Treatment with PB consisted of the addition of 0.1% (w/v) PB to the drinking water for 6 days prior to sacrifice. BNF and 3MC treatments consisted of an intraperitoneal injection of 40 and 25 mg kg^{-1}, respectively, in 0.5 mL of corn oil once each day for 3 days prior to sacrifice. Male rats of Long–Evans origin, weighing 50–60 g, were obtained from Blue Spruce Farms, Altamont, NY. Treatment with 3MC was as in the case of the Sprague-Dawley rats, but PB treatment consisted of four daily intraperitoneal injections of 80 mg of PB kg^{-1} in 0.9% NaCl.

A list of the human liver autopsy samples used in this work is given in Table I.

Male New Zealand white rabbits (2–2.5 kg) were obtained from Hilltop Farms, Columbia, TN. The rabbits were treated with PB as in the case of the Sprague-Dawley rats. BNF treatment consisted of a single intraperitoneal injection of 80 mg kg^{-1} in 20 mL of corn oil 40 h prior to sacrifice.

Microsomes were prepared from rat liver (van der Hoeven & Coon, 1974), rabbit liver (van der Hoeven & Coon, 1974), and human liver and lung (Wang et al., 1980) as described elsewhere and stored in 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA, 20% (v/v) glycerol, and 0.4 mM phenylmethanesulfonyl fluoride at −70 °C.

Rat liver P-450s were purified by using modifications of methods previously described (Guengerich, 1977, 1978; Guengerich & Martin, 1980). Briefly, microsomes were solubilized with cholate and chromatographed on *n*-octylamino-Sepharose 4B columns as described (Guengerich, 1977, 1978), except that the nonionic detergent Emulgen 913 was replaced with Lubrol PX. The fractions containing P-450 were pooled, concentrated by ultrafiltration, dialyzed, and chromatographed on 2.5 × 40 cm columns of DEAE-cellulose. The buffer system contained 10 mM potassium phosphate (pH 7.7), 0.1 mM EDTA, 20% (v/v) glycerol, 0.1% (w/v) Lubrol PX, and 0.2% (w/v) sodium cholate; a linear 0–0.25 M NaCl gradient was used for elution. The major P-450 fraction from each set of rats was the last peak eluted in each case; the NaCl concentration was 60 mM in the case of the PB-treated rat P-450 and 80 mM in the case of BNF- or 3MC-treated rat P-450 at the height of the peak. Each column was monitored by using A_{417}:A_{280} ratios and $NaDodSO_4$–polyacrylamide gel electrophoresis. Pooled fractions were lowered in detergent concentration by treatment with calcium phosphate gel (van der Hoeven & Coon, 1974), concentrated by ultrafiltration, dialyzed vs. 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% glycerol, and stored at −20 °C. Preparations routinely contained 14–18 nmol of P-450 (mg of protein)$^{-1}$, were apparently homogeneous as judged by $NaDodSO_4$–polyacrylamide gel electrophoresis, and had A_{417}:A_{280} ratios of 1.6 in the case of PB-treated rat P-450 and 1.3 in the case of BNF- or 3MC-treated rat P-450.

Rabbit P-450 LM-2 was purified by using modifications of published procedures. PB-treated rabbit liver microsomes were solubilized and chromatographed on *n*-octylamino-Sepharose 4B as described elsewhere (Imai & Sato, 1974). The resulting P-450 fraction was chromatographed on columns of DEAE-cellulose and hydroxylapatite (van der Hoeven & Coon, 1974; Coon et al., 1978) and treated with calcium phosphate gel (van der Hoeven & Coon, 1974). The specific content of the preparation was 18.0 nmol (mg of protein)$^{-1}$. Rabbit P-450 LM-4 was isolated from BNF-treated rabbit liver microsomes by using cholate extraction, chromatography on columns of *n*-octylamino-Sepharose 4B (Hashimoto & Imai, 1976), DEAE-cellulose (Coon et al., 1978), and hydroxylapatite (Coon et al., 1978; Guengerich, 1977), and treatment with calcium phosphate gel (van der Hoeven & Coon, 1974). The

specific content of the preparation was 16.1 nmol (mg of protein)$^{-1}$. Both the rabbit P-450 LM-2 and LM-4 preparations were apparently homogeneous as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis.

Human liver P-450 preparations were obtained by using procedures described elsewhere (Wang et al., 1980). The procedure involves stepwise cholate extraction of microsomes; each sample is denoted I or II, depending on whether the 0–0.6% (I) or 0.6–1.5% (II) cholate fraction was used. ("HL" and the first number denote "human liver" and the patient number.) The phosphate concentration (mM) at which the P-450 was subsequently eluted from a hydroxylapatite (HA) column is also denoted. The following preparations were electrophoretically homogeneous as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis: HL6$_{II}$-HA$_{300}$ [M_r 55 000, 13.1 nmol of P-450 (mg of protein)$^{-1}$]; HL7$_I$-HA$_{300}$ [M_r 53 000, 10.5 nmol of P-450 (mg of protein)$^{-1}$]; HL7$_{II}$-HA$_{300}$ [M_r 55 000, 8.7 nmol of P-450 (mg of protein)$^{-1}$]; HL8$_{II}$-HA$_{150}$ [M_r 55 000, 12.7 nmol of P-450 (mg of protein)$^{-1}$]; and HL15$_{II}$-HA$_{300}$ [M_r 54 000; 9.9 nmol of P-450 (mg of protein)$^{-1}$]. Other preparations used include the following: HL6$_I$-HA$_{80}$ (0.41 nmol of P-450 (mg of protein)$^{-1}$]; HL8$_I$-HA$_{80}$ [1.4 nmol of P-450 (mg of protein)$^{-1}$]; HL8$_I$-HA$_{150}$ [3.1 nmol of P-450 (mg of protein)$^{-1}$]; and HL8$_{II}$-HA$_{150}$ [7.2 nmol of P-450 (mg of protein)$^{-1}$].

Antibodies. Antibodies were isolated from adult female New Zealand White rabbits immunized with PB- or BNF-treated rat P-450 (Guengerich, 1978) or human P-450 (HL6$_{II}$-HA$_{300}$) (Thomas et al., 1976a) by using injection and bleeding schedules described in the references listed. In each case, antisera were pooled from three different rabbits.

Antisera were collected by allowing the blood to clot for 1 h at 23 °C and then 16 h at 4 °C and centrifuging (for 10 min at 2000*g* and 4 °C). The supernatants were heated at 56 °C for 20 min and centrifuged for 10 min at 10^4g. Such antisera were treated with ammonium sulfate and DEAE-cellulose as described elsewhere (Dean & Coon, 1977) to obtain IgG fractions which were concentrated by ultrafiltration and stored at −20 °C. The IgG could be concentrated for several of the studies and gave less interference than crude antisera in the double-diffusion precipitin analysis and enzyme activity inhibition assays. Titers were determined by mixing a fixed quantity of human liver microsomes (solubilized in 1% Lubrol PX) with varying amounts of the antibody. In a typical preparation, a maximum of 5.6 μg of immunoprecipitated protein was formed from 4 mg of liver (patient 16) microsomes and 1.0 mg of the antibody IgG fraction (at this antibody concentration, optimum precipitation occurred).

Double-Diffusion Analysis. The gels contained 1 M sodium glycine (pH 7.4), 0.09 M NaCl, 0.02% (w/v) NaN_3, 0.9% (w/v) agarose, and 0.2% (w/v) Lubrol PX (Thomas et al., 1976a). The gel was ~2 mm thick; wells were 4 mm in diameter, and the distance between centers of adjacent wells was usually 8 mm. Each was filled with 12 μL of antibody or antigen. Microsomal preparations were solubilized by the addition of Lubrol PX and sodium cholate to final concentrations of 2% and 1.5% (w/v) and centrifuged for 60 min at 48000*g* at 4 °C. Protein pellets were discarded. Photographs were taken over indirect light by using either Kodak Pan X-100 or 5069 high contrast copy film. Some of the gels were stained for heme with 3,3′,5,5′-tetramethylbenzidine essentially as described elsewhere (Thomas et al., 1976b) and photographed over a light box with a white background, using the same film. In these gels, Lubrol PX was removed by extensive washing, as the detergent was found to inhibit staining.

Micro-C′ Fixation. The basic methodology is described elsewhere (Guengerich et al., 1979; Levine & Van Vanukis, 1967; Prager & Wilson, 1971a,b; Sarich & Wilson, 1966; Wang et al., 1980). For each set of experiments, the appropriate control incubations devoid of IgG, antigen, or C′ and containing the immunogen were carried out. All assays were carried out in duplicate, with results expressed as means. Data were plotted as maximum micro-C′ fixation (observed at a fixed antibody concentration by using variable levels of antigen) vs. log IgG concentration (Sarich & Wilson, 1966). The lines were nearly parallel in all cases, and the points at which 50% micro-C′ fixation occurred were used in calculation of each immunological distance between two given antigens, which is defined as 100 × log of the difference in IgG concentration at which 50% micro-C′ fixation was found (Prager & Wilson, 1971a,b).

RIA. Antigens were labeled with ^{125}I in the following manner. Twenty-five micrograms of antigen was incubated with an equimolar amount of Na^{125}I (containing 1–2 mCi of radioactivity) and 20 μL of immobilized lactoperoxidase/glucose oxidase beads. Reaction was initiated by the addition of 20 μL of a 1% (w/v) D-glucose solution which had been allowed to stand at 4 °C for 16 h to mutarotate. After standing for 60 min at 23 °C, the incubation was centrifuged for 10 min at 3000*g*. The supernatant was applied to a 2 × 40 cm column of Sephadex G-25 which had been previously equilibrated with 50 mM potassium phosphate buffer containing 0.1 mM EDTA, 0.1 M KCl, 0.02% (w/v) NaN_3, and 0.05% (w/v) bovine serum albumin. The column was developed at 23 °C by using the equilibration buffer. The radioactive fractions eluted in the void volume were pooled and dialyzed for 16 h vs. 40 volumes of 5 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol. The dialyzed PB-treated rat P-450 was applied to a 2 × 6 cm column of DEAE-cellulose previously equilibrated with 10 mM potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 0.1% (w/v) Lubrol PX, 0.2% (w/v) sodium cholate, and 0.02% (w/v) ovalbumin. The column was washed with 200 mL of the equilibration buffer. The ^{125}I-labeled P-450 was eluted by applying a 300-mL linear gradient of 0 to 0.25 M NaCl in the equilibration buffer to the column. The fractions (~5 mL each) were assayed for ^{125}I by using a γ counter. From each tube in the region of the major peak, an aliquot containing 10^3 cpm was mixed with 1 μg of IgG raised to the antigen in 0.5 mL of a diluent consisting of 50 mM potassium phosphate buffer (pH 7.4) containing 0.02% (w/v) NaN_3 and 0.1% (w/v) bovine serum albumin. These tubes stood for 1 h at 23 °C and then at 4 °C for 24 h. To each tube was added 200 μL of immobilized goat antirabbit IgG (second IgG); these tubes were incubated at 37 °C with shaking for 2 h and then centrifuged for 10 min at 10^4g. The pellets were mixed with 0.5 mL of the above diluent and recentrifuged; tubes were decanted to recover the pellets. ^{125}I in the pellets was quantitated by using a γ counter, and those fractions from the DEAE-cellulose column containing significant levels of precipitable ^{125}I were pooled for use in the RIA. The specific activity was on the order of 10 μCi/μg of P-450.

Aliquots of ^{125}I-labeled P-450 containing 2000 cpm were mixed with varying levels of IgG, plus sufficient preimmune IgG to bring the total amount of IgG to 5 μg, in 0.5 mL of the diluent described above. Tubes were allowed to stand 1 h at 23 °C and then 48 h at 4 °C. Immobilized goat antirabbit IgG (0.2 mL) was added to each tube. Tubes were incubated at 37 °C for 2 h with shaking and centrifuged and washed

twice as described above. From plots of precipitated ^{125}I vs. IgG concentration, a level of IgG was chosen for the RIA that was sufficient to precipitate 25–55% of the ^{125}I.

RIAs were carried out by using 2000 cpm of ^{125}I-labeled P-450, IgG, varying levels of each antigen tested, and sufficient diluent to bring the total volume to 0.5 mL. Tubes were allowed to stand 1 h at 23 °C and then 48 h at 4 °C. Immobilized goat antirabbit IgG (0.2 mL) was added to each tube, and the tubes were incubated at 37 °C for 2 h with shaking. Pellets recovered by centrifugation were washed twice with diluent as described above and counted 10 min in a γ counter. Results are expressed as percent ^{125}I precipitated vs. micrograms of each antigen added. The data presented in each figure were obtained in a single experiment with the same ^{125}I-labeled P-450 and IgG preparations, and each point represents the mean of three determinations.

Inhibition Studies. Microsomes were mixed with varying levels of a given antibody (IgG fraction) in 50 mM potassium phosphate buffer (pH 7.7) and allowed to stand for 20 min at 23 °C. Cofactors and substrate were then added, and incubations proceeded for 10 min at 37 °C under the conditions described for each assay. Parallel experiments were carried out with rabbit preimmune antibody (IgG fraction) in every case. Each data point represents the mean of three individual determinations.

Other Assays. Protein was estimated as described by Lowry et al. (1951). P-450 was estimated in human microsomes by using reduced-CO vs. oxidized-CO difference spectroscopy as described by Matsubara et al. (1976); in other cases, reduced-CO vs. reduced difference spectroscopy was used (Omura & Sato, 1964). A Cary 219 spectrophotometer was used in the automatic base-line correction mode for these measurements. *d*-Benzphetamine *N*-demthylase activity was estimated by using *d*-[*N-methyl*-^{14}C]benzphetamine as described elsewhere (Guengerich & Holladay, 1979), except that the buffer used was 50 mM potassium *N'*-(2-hydroxyethyl)-piperazine-*N*-2-ethanesulfonate (pH 7.7) and the temperature was 37 °C. Benzo[*a*]pyrene hydroxylase activity was determined fluorimetrically as described by Nebert & Gelboin (1968). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970).

Materials. *n*-Octylamino-Sepharose 4B and hydroxylapatite were prepared as described elsewhere (Guengerich, 1977). Immobilized lactoperoxidase/glucose oxidase (Immunoperoxidase), immobilized goat antirabbit IgG (Immunobeads), and agarose (low m_r) were purchased from Bio-Rad. Sephadex G-25 and DEAE-cellulose (DEAE-Sephacel) were purchased from Pharmacia. Na^{125}I (2.03 Ci/μmol) was purchased from Amersham-Searle. Lubrol PX was obtained through Sigma.

Results

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. An electrophoretogram of the human immunogen (Wang et al., 1980: Figure 2, tracks 4–6 and 10) and typical electrophoretograms to the rat immunogens (Guengerich, 1978) been published elsewhere. We have used a technique in which proteins are electrophoresed from polyacrylamide onto nitrocellulose filters (Towbin et al., 1979) and treated sequentially with rabbit antibody, goat antirabbit IgG, rabbit anti-horseradish peroxidase, horseradish peroxidase, 3,3'-diaminobenzidine, and H_2O_2 (Glass et al., 1981) to detect proteins which react with an antibody. Such a procedure was carried out with the antibodies used here and the microsomal preparations from which the immunogens were derived. In every case, the antibody recognized only one band, corresponding to the immunogen, in such a complex mixture of proteins, as shown for rat and several human microsomal preparations in Figure 1.

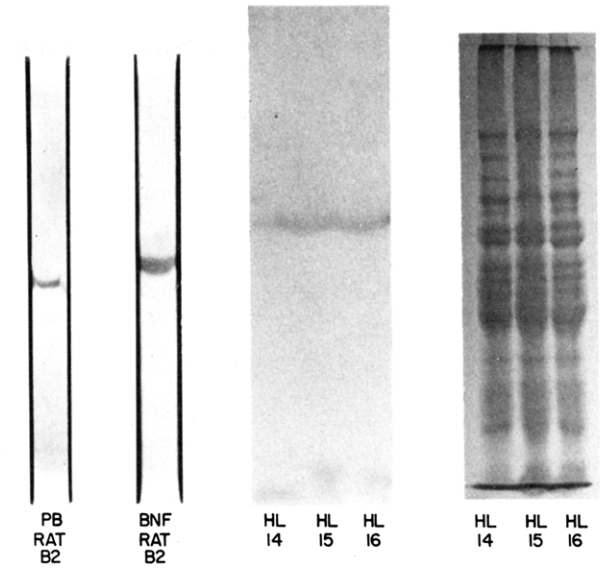

FIGURE 1: $NaDodSO_4$ immunoelectrophoresis of rat and human liver microsomes. Approximately 15 μg of PB- or BNF-treated rat liver microsomes or 100 μg of human liver microsomes was subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis by using 0.4 × 10 cm sample tracks of a slab gel which was 7.5% (w/v) in acrylamide (Laemmli, 1970). The gel thickness was 0.075 cm in the case of the rat liver microsomes and 0.30 cm in the case of the human liver microsomes. The tracking dye was Pyronin Y. The proteins in each gel track were transferred to sheets of nitrocellulose as described elsewhere (Towbin et al., 1979). Some of the human liver gels were stained with Coomassie brilliant blue before transfer (far right). Staining the residual gel after transfer and Amido Black staining of the nitrocellulose sheet indicated that microsomal proteins were all transferred. The other microsomal electrophoretograms were treated sequentially with the appropriate rabbit antibody (anti-PB rat B_2 for PB-treated rat liver microsomes, anti-BNF rat B_2 for BNF-treated rat liver microsomes, and anti-HL for human liver microsomes), goat anti-rabbit IgG, rabbit anti-horseradish peroxidase/horseradish peroxidase complex, 3,3'-diaminobenzidine, and H_2O_2 with intermittent washings with a buffer containing albumin and calf serum (Glass et al., 1981). Photographs were taken of the benzidine-stained nitrocellulose gels over a fluorescent light box. The two rat liver electrophoretograms and the three human liver electrophoretograms on the left were stained by using the immunological technique. In all cases, the anode was at the bottom of the gel, and a faint band corresponding to the tracking dye can be seen.

Double-Diffusion Precipitin Analysis. When the antibody raised to purified human liver P-450 was tested against either human preparation $HL6_{II}$-HA_{300} or $HL7_{II}$-HA_{300}, a single precipitin line was formed (Figure 2A). The precipitin lines for the two antigens formed a pattern of fusion.[2] Other human P-450 preparations derived from patient 8 also yielded single precipitin lines. These lines did not form a pattern of fusion with the immonogen, but the line met the immunogen line and did not cross it. Thus, these preparations derived from patient 8 appear to contain some, but not all, of the determinants of the immunogen. The precipitin lines formed by the human P-450 preparations yielded positive stains when tested for heme (Figure 2B), consistent with the view that the antibody is directed toward P-450. When the antibody preparation raised to human P-450 (50 mg of IgG mL^{-1}) was tested against purified human epoxide hydrolase (Guengerich et al., 1979; 0.05–0.8 mg mL^{-1}) or human NADPH–P-450 reductase (Guengerich et al., 1981; 0.01–0.16 mg mL^{-1}), no visible

[2] Such an observation is termed a "pattern of fusion" as opposed to a "line of identity", as suggested by Crowle (1973).

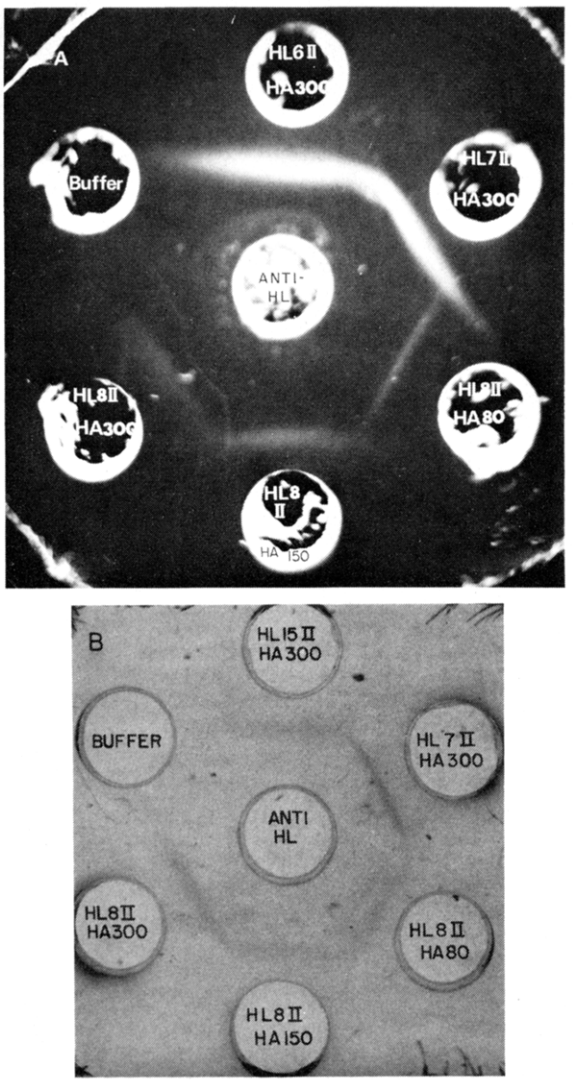

FIGURE 2: Double-diffusion precipitin analysis of purified and partially purified human liver P-450s using antibody raised to human liver P-450. The antibody (anti-HL) was placed in the center well at a concentration of 50 mg of IgG mL^{-1}. Antigens were added to the peripheral wells at the following concentrations. (Plate A) $HL6_{II}$-HA_{300}, 0.31 mg mL^{-1}; $HL7_{II}$-HA_{300}, 0.25 mg mL^{-1}; $HL8_{II}$-HA_{80}, 1.31 mg mL^{-1}; $HL8_{II}$-HA_{150}, 70 μg mL^{-1}; $HL8_{II}$-HA_{300}, 29 μg mL^{-1}. (Plate B) $HL15_{II}$-HA_{300}, 0.30 mg mL^{-1}; $HL7_{II}$-HA_{300}, 0.25 mg mL^{-1}; $HL8_{II}$-HA_{80}, 7.8 mg mL^{-1}; $HL8_{II}$-HA_{150}, 0.42 mg mL^{-1}; $HL8_{II}$-HA_{300}, 0.18 mg mL^{-1}. The buffer was 10 mM Tris–acetate (pH 7.4) containing 1 mM EDTA and 20% glycerol. Plate A was photographed directly; plate B was photographed after staining for heme, using a Vivitar 25A red filter.

precipitin lines were observed under the assay conditions. Further evidence for the specificity of this antibody was obtained by the finding that concentrations of as much as 10 mg mL^{-1} did not inhibit NADPH–cytochrome *c* reductase activity of human liver microsomes.

A single precipitin line was also observed when purified human P-450 preparations $HL6_{II}$-HA_{300} and $HL7_{II}$-HA_{300} were tested against the antibody raised to human P-450 in Figure 3. PB-treated rat P-450 B_2 also yielded a single line when tested against antibody raised to that immunogen. When solubilized human liver microsomes were tested against anti-human P-450, multiple lines were observed. All liver preparations contained an outer line which formed a pattern of fusion with the immunogen, although this line was weak in the case of patient 6. The inner lines all met that line when allowed to diffuse for longer periods of time and did not cross it in any case. A single line was observed for the reaction of

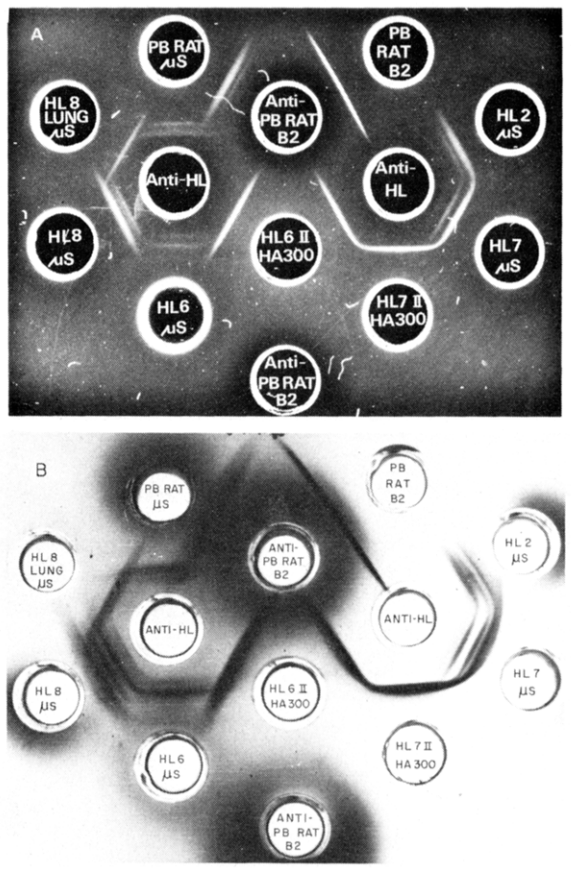

FIGURE 3: Double-diffusion precipitin analysis using antibodies raised to rat and human P-450s. Antibodies raised to PB-treated rat (anti-PB rat B_2) and human (anti-HL) P-450s were present at concentrations of 50 mg of IgG mL^{-1}. The purified antigens PB rat B_2, $HL6_{II}$-HA_{300}, and $HL7_{II}$-HA_{300} were added at concentrations of 0.30, 0.30, and 0.25 mg mL^{-1}, respectively. PB-treated rat microsomes (PB rat μS) and human lung microsomes (μS) were added at concentrations of 5 and 12.6 mg mL^{-1}, respectively. Human liver microsomes (HL μS) were added at protein concentrations varying from 6.5 to 10.5 mg mL^{-1}. Plate A was photographed directly, and plate B was photographed after further diffusion, washing, and staining with Naphthol Blue Black (Thomas et al., 1976a).

human lung microsomes—this line met the outer human liver microsomal line, forming a spur, and did not cross it. No line between PB-treated rat P-450 B_2 and anti-human P-450 was observed, nor was a line observed for the diffusion of anti-PB-treated rat P-450 B_2 against purified PB-treated rat P-450 B_2 in these tests. However, multiple lines were observed for the reaction of PB-treated rat liver microsomes with both antibodies. Similar results were obtained when PB-treated P-450 B_2 and its antibody were replaced with BNF-treated rat P-450 B_2 and its antibody, i.e., a single line with the immunogen and no lines between the anti-rat antibody and human microsomes. The precipitin lines formed by the rat P-450 immunogens and their antibodies also give positive heme stains (Guengerich, 1978).

Since the antibodies yielded single, heme-stained precipitin lines when tested against their immunogens, all precipitin lines were continuous with the line formed with the immunogen (i.e., no lines formed by the reaction of different human liver antigens with an antisera crossed the immunogen line), and each antibody reacted specifically with a single band obtained by $NaDodSO_4$–polyacrylamide gel electrophoresis of microsomes (Figure 1), we feel that each of the antibodies recognizes only P-450s. Thus, the multiple lines observed with microsomal samples are the result of precipitation of P-450s not identical with the immunogen. That is, these other P-450s share only

Table II: Comparison of Various Rat Liver P-450 Preparations and Human Liver Microsomes by Micro-C′ Fixation with Antibody Raised to Sprague-Dawley PB-Treated Rat P-450[a]

P-450 preparation	immunological distance						
	Sprague-Dawley PB rat$_1$[b]	Sprague-Dawley PB rat$_2$[b]	Long-Evans PB rat	Long-Evans 3MC rat	Sprague-Dawley BNF rat	Sprague-Dawley 3MC rat	human (no. 8) liver microsomes
Sprague-Dawley PB rat$_1$[b]		7	12	37	47	48	100
Sprague-Dawley PB rat$_2$[b]			5	30	40	41	93
Long-Evans PB rat				25	35	36	88
Long-Evans 3MC rat					10	11	63
Sprague-Dawley BNF rat						1	53
Sprague-Dawley 3MC rat							52
human (no. 8) liver microsomes							

[a] Experiments were carried out as described under Experimental Procedures. Fifty percent C′ fixation was obtained with Sprague-Dawley rat P-450$_{(1)}$ by using 1.2 μg of IgG fraction isolated from the antiserum. [b] Duplicate preparations.

some of the determinants of the immunogen. This view is supported by the pattern of Figure 2A, in which different human P-450 fractions yielded precipitin lines that were continuous with but did not form a pattern of fusion with the immunogen line. Species specificity exists, although some P-450s of rat and human liver share immunological determinants. Tissue specificity also exists within an individual human. Qualitative differences exist among liver samples of different humans. Evidence that these differences were not due to artifacts associated with storage of human autopsy samples was obtained in the following experiment. An autopsy liver sample was obtained from patient 9 3 h after death. Microsomes were prepared and frozen immediately and after the liver was kept at 23 °C for an additional 3 and 21 h. All three solubilized liver microsomal preparations yielded three precipitin lines when tested against antihuman P-450, and these lines all formed patterns of fusion. This pattern of fusion was lost after an additional 24 h of storage of the liver at 23 °C, although none of the precipitin lines crossed those obtained with the other samples. All of the other human autopsy samples were obtained and frozen within considerably less time than 24 h (Table I).

Micro-C′ Fixation. This technique was utilized because of its sensitivity and selectivity, particularly with regard to antibody populations that are not involved in precipitation. We have previously used modifications of this technique to demonstrate that human and rat liver P-450s share certain immunological determinants (Wang et al., 1980). The use of the quantitative method allowed the assignment of immunological distances to related antigens. Antibodies raised to the major P-450s isolated from PB- and BNF-treated rats were used to study the similarity of rat liver P-450s, and the antibody raised to human liver P-450 was used to explore differences among human P-450s.

The major P-450s isolated from PB- and 3MC-treated Sprague-Dawley rats have previously been shown to be immunologically distinct but to share some elements of similiarity, as judged by double-diffusion analysis and by immunoprecipitation using fluorescein isothiocyanate labeled P-450s (Guengerich, 1978). These P-450s can also be distinguished by using inhibition of enzyme activity (Guengerich & Mason, 1979; Kaminsky et al., 1980) and immunohistochemical localization (Baron et al., 1978) techniques. We have recently isolated what appears to be the major P-450 present in liver microsomes of BNF-treated Sprague-Dawley rats and find this to be indistinguishable from the enzyme isolated from such 3MC-treated rats as judged by chromatographic properties, monomer M_r, spectral properties, activity toward substrates, and double-diffusion immunological analysis (Guengerich & Martin, 1980). Some evidence had been presented that strain differences exist in P-450-containing mixed-function oxidases (Cram et al., 1965; Creel et al., 1976; Dent et al., 1980; Gold & Widnell, 1975). Several rat liver P-450 preparations were tested by using micro-C′ fixation with antibody raised to P-450 isolated from PB-treated Sprague-Dawley rats (Table II). Duplicate preparations of the immunogen yielded an immunological distance of 7, which can be used as a measure of variation among replicate preparations. The major P-450 isolated from PB-treated male Long–Evans rats by the same procedure yielded a value slightly different but not really distinguishable from the first two preparations. The P-450s isolated from BNF- and 3MC-treated Sprague-Dawley rats yielded nearly superimposable lines separated by a distance of 1. The P-450 isolated from 3MC-treated Long–Evans rats, using the same procedure, yielded a line shifted 10–11 distance units to the left to the Sprague-Dawley preparations. This line is slightly out of the limits of identity to the 3MC- and BNF-treated Sprague-Dawley preparation, but the distance is not really large enough to conclusively define a difference. Human microsomes contain some cross-reacting determinants, as previously reported (Wang et al., 1980), but the differences between the various rat and human preparations are greater than the differences between the different rat preparations. If human microsomes contain several forms of P-450, this assay will only select for the most immunologically related form (or forms) in each case. Quantitative micro-C′ fixation is not dependent upon the purity or concentration of an antigen in a crude mixture unless inhibitory cross-reactive antigens are present (Sarich & Wilson, 1966). Even if inhibitory cross-reactive antigens are present, differences in immunological distance reflect immunological differences between partially related antigens.

In a complementary experiment using antibody raised to the major P-450 isolated from BNF-treated Sprague-Dawley rats, two duplicate preparations of the homologous antigen were separated by an immunological distance of 6 (Figure 4). The P-450 isolated from 3MC-treated Long–Evans rats was not distinguished from these preparations using this assay. Two preparations of PB-treated Sprague-Dawley rat P-450 gave superimposable lines 100 distance units to the right of the homologous antigens. The PB-treated Long–Evans rat preparation clearly gave a line distinct from the other preparations. Thus, the Long–Evans rat P-450 induced by PB is clearly immunologically distinct from the corresponding Sprague-Dawley rat P-450. This difference is attributed to the strain and not age, as P-450 preparations derived from 60-, 125-, and 250-g Sprague-Dawley rats differed by immunological distances of no more than 8 units when examined with

Table III: Comparison of Various Human, Rat, and Rabbit P-450 Preparations by Micro-C′ Fixation by Using Antibody Raised to Human Liver P-450[a]

	immunological distance									
preparation	8_{II}-HA_{300}	7_I-HA_{300}	8_I-HA_{150}	7_{II}-HA_{300}	6_{II}-HA_{300}	8_{II}-HA_{150}	6_I-HA_{80}	8_I-HA_{80}	rabbit LM-4	BNF rat	rabbit LM-2
8_{II}-HA_{300}		5	10	17	22	32	53	81	107	108	152
7_I-HA_{300}			5	12	17	27	48	76	102	103	147
8_I-HA_{150}				7	12	22	43	71	97	98	142
7_{II}-HA_{300}					5	15	36	64	90	91	135
6_{II}-HA_{300}						10	31	59	85	86	130
8_{II}-HA_{150}							21	49	75	76	120
6_I-HA_{80}								28	54	55	99
8_I-HA_{80}									26	27	71
rabbit LM-4										1	45
BNF rat											46
rabbit LM-2											

[a] Experiments were carried out as described under Experimental Procedures. Fifty percent C′ fixation was obtained with 2.4 μg of the IgG fraction of the antiserum in the assays with 8_{II}-HA_{300}. Unless otherwise noted, preparations were derived from humans. (See Experimental Procedures for explanation of terms.)

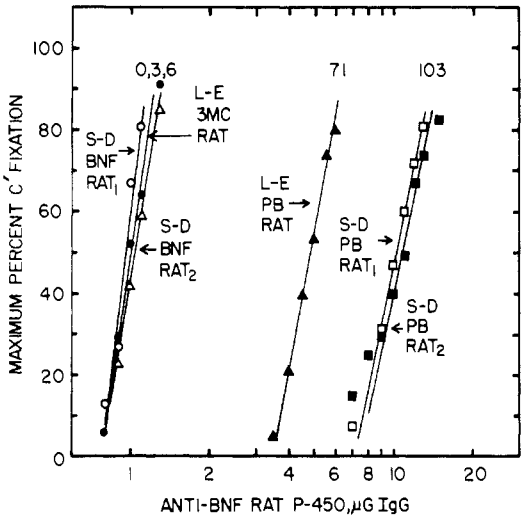

FIGURE 4: Comparison of various rat liver P-450s by quantitative micro-C′ fixation by using antibody raised to Sprague-Dawley BNF-treated rat P-450. The procedure was carried out as described by using the following antigens: Sprague-Dawley (SD) BNF-treated rat P-450 (○ and △), Long–Evans (LE) 3MC-treated rat P-450 (●), Long–Evans PB-treated rat P-450 (▲), and Sprague-Dawley PB-treated rat P-450 (□ and ■). Immunological distances are given above each line relative to Sprague-Dawley BNF-treated rat P-450 preparation 1.

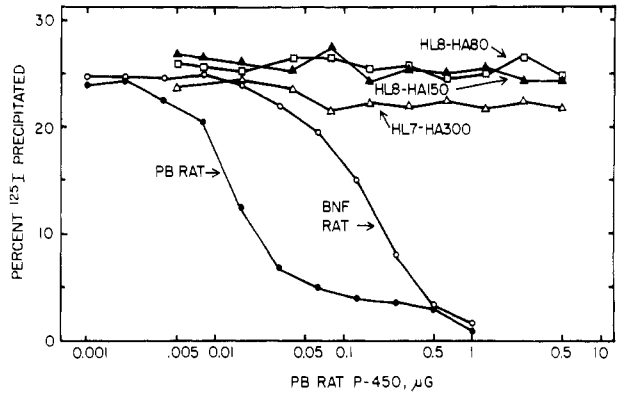

FIGURE 5: Comparison of rat and human liver (HL) P-450 preparations by RIA by using antibody raised against PB-treated P-450 and that antigen labeled with ^{125}I. The procedure was carried out as described by using the following P-450 preparations: PB-treated rat (●), BNF-treated rat (○), HL7-HA_{300} (△), HL8-HA_{150} (▲), and HL8-HA_{80} (□).

the antibody raised to BNF-treated rat P-450.

Antibody raised to human liver P-450 was used to explore the extents of immunological differences among various P-450 fractions isolated from three different humans (Table III). Some weak similarity of human P-450 to P-450s isolated from rats and rabbits was found. The data may tend to overestimate the differences between the rabbit P-450s and the other P-450s; the antibodies were raised in rabbits, and there is evidence that animals may screen their own proteins when forming antibodies (Reichlin, 1975). The available fractions isolated from patient 6 differed in immunological distance by as much as 31 units, and the fractions tested from patient 8 differed by as much as 81 units. Of course, all of the P-450s have probably not been recovered from the microsomes, and further variations in dissimilarity may exist. Some of the preparations tested, particularly the HA_{80} fractions, are not homogeneous, and if more than one cross-reacting P-450 is present, only the most related form or forms would be detected in these experiments.

The contribution of antigenic changes accompanying storage of human liver autopsy samples to the observed differences among fractions was considered. For assessment of the degree of such changes, a liver sample, obtained 3 h after death, was kept at 23 °C, and microsomes were prepared and frozen after varying lengths of time. The apparent immunological distance observed after an additional 3 h at 23 °C was 9, and the distance after 21 h at 23 °C was 17. Storage does appear to introduce some differences in immunological reactivity. However, distances as great as 81 were observed between different P-450 fractions obtained from a single patient, no. 8, from which the liver sample was obtained 4 h after death. The control experiment presented here must be considered a rough estimate of the contribution of storage to observed immunological differences, as any changes occurring prior to procurement of samples cannot be ascertained and this experimental design focuses only on the most antigenic P-450s in the microsomes. However, the variation of immunological data both between individuals and within an individual would appear to be greater than that attributable to storage changes.

RIA. RIA was also used to examine similarity of P-450 preparations. When ^{125}I-labeled PB-treated rat P-450 and the antibody raised to the (unlabeled) enzyme were utilized, the data presented in Figure 5 were obtained. BNF-treated rat P-450 shared some antigenic sites with PB-treated rat P-450 but was only about one-tenth as effective in the RIA as the immunogen. None of three different human liver P-450 preparations examined were effective in displacing the radioactive antigen, even at concentrations 500 times that of the immunogen.

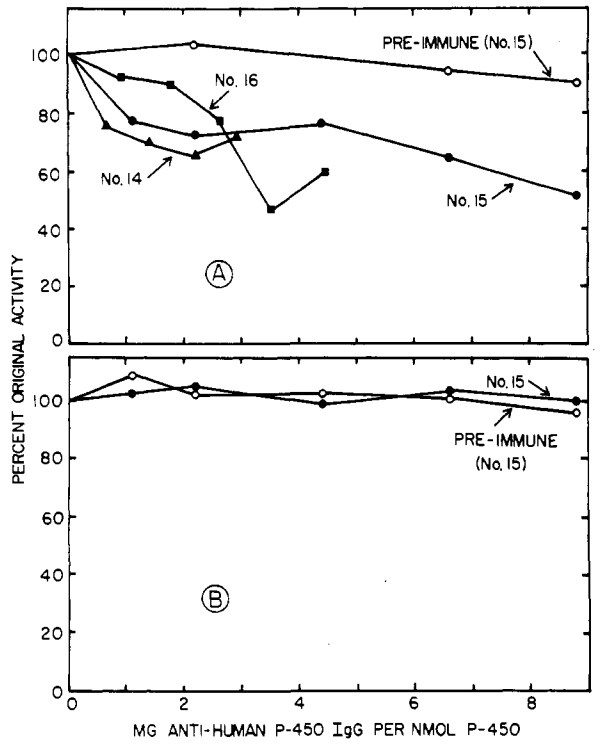

FIGURE 6: Inhibition of mixed-function oxidase activities in human liver microsomes with antibody raised to purified human liver P-450. Microsomes from various individuals were preincubated for 20 min with varying amounts of antibody, and *d*-benzphetamine *N*-demethylase (part A, upper frame) and benzo[*a*]pyrene hydroxylase (part B, lower frame) activities were determined. The uninhibited rates of *d*-benzphetamine *N*-demthylase activity were 0.45 nmol min^{-1} $nmol^{-1}$ P-450 for patient 14 (▲), 2.5 nmol min^{-1} $nmol^{-1}$ P-450 for patient 15 (●), and 0.89 nmol min^{-1} $nmol^{-1}$ P-450 for patient 16 (■). The rate of benzo[*a*]pyrene hydroxylation for patient 15 (●) was 0.22 nmol min^{-1} $nmol^{-1}$ P-450.

Inhibition of Enzyme Activity. Human P-450s prepared by using the procedure we have previously described are capable of metabolizing *d*-benzphetamine (Wang et al., 1980) but metabolize benzo[*a*]pyrene at slow rates in the presence of NADPH–P-450 reductase of either rat or human origin. The antibody raised to human P-450 was able to inhibit approximately one-half of the *d*-benzphetamine *N*-demethylase activity of human liver microsomes (Figure 6A).

The benzo[*a*]pyrene hydroxylase activity of a microsomal preparation was not inhibited by the antibody under the same conditions in which *d*-benzphetamine *N*-demethylase activity was partially inhibited (Figure 6B).

Discussion

Human liver microsomal P-450 was purified to apparent homogeneity (Wang et al., 1980) and used to raise antibodies in rabbits. Differences in various P-450 fractions of individuals were observed in double-diffusion precipitin analysis, as judged by spurs and multiple lines for the various fractions. The precipitin line patterns suggest that forms of P-450 in the lung differ from those of the liver in a single patient. The activity inhibition curves (Figure 6) correlate well with the observed substrate specificity of human P-450s; i.e., these P-450 preparations show a reasonable amount of activity toward *d*-benzphetamine (Wang et al., 1980) but are essentially inactive toward benzo[*a*]pyrene. These results are consistent with the view that different forms of P-450 preferentially metabolize *d*-benzphetamine and benzo[*a*]pyrene within a given human liver.

Quantitative micro-C′ fixation data indicated that the available P-450 preparations derived from three individual humans could be resolved into at least five families of lines (i.e., immunological distances of 0–5, 10–22, 32, 53, and 81) (Table III). Again, a distance of 10 is estimated as a maximum due to variation between assays and changes in antigenicity accompanying storage under these conditions.

Evidence for strain differences in rat P-450s was also obtained by using micro-C′ fixation (Figure 4). These differences were observed when rats were treated identically and identical purification techniques were employed. Thus, the major P-450s present in PB-treated Long–Evans and Sprague-Dawley rats are not identical. Differences between the major 3MC- (or BNF-) induced forms of P-450 in these two strains of rats could not be conclusively demonstrated by using this technique (Figure 4, Table II). On the other hand, the major form of P-450 present in PB-treated Sprague-Dawley rats did not change with age (i.e., weight range of 60–250 g) as judged by micro-C′ fixation. 3MC and BNF appear to induce the same major form of P-450 in Sprague-Dawley rats, as judged by quantitative micro-C′ fixation (Figure 4, Table II) as well as a variety of other criteria[3] (Guengerich & Martin, 1980). RIA results (Figure 5) also substantiated other data (Guengerich, 1978) in that the major forms of Sprague-Dawley rat liver P-450 induced by PB and BNF are immunologically distinct but share some antigenic sites.

Thus, a variety of immunological techniques have been used to compare rat, rabbit, and human P-450s. The data suggest that immunological differences exist among and within humans with regard to P-450. Immunological differences of P-450s in rats and rabbits are associated with variations in amino acid composition (Coon & Dean, 1977; Guengerich, 1978; Ryan et al., 1979). To date, forms of P-450 differing in post-translational modification have not been identified, although such differences could give rise to immunological variation. Ibrahimi et al. (1979) have suggested that in bird lysozymes an immunological distance of 2.5 is related to each amino acid substitution. Amino acid composition data for P-450s (Wang et al., 1980) indicate that there may be as many as 55 amino acid substitutions between the major forms of rat liver P-450 induced by PB and 3MC. This can be compared with an immunological distance of 50–100 between the proteins. Human liver P-450 $HL6_{11}$-HA_{300} differs from 3MC- (or BNF-) treated rat liver P-450 by as many as 101 residues (Wang et al., 1980); the immunological distance between these protein is 86. Thus, one can roughly estimate that each amino acid substitution corresponds to 1–2 distance units if the contribution of heme and any carbohydrates to antigenicity is not considered.

It is tempting to speculate on the number of different P-450s that may exist in the total human population, which is subject to genetic differences and to a tremendous variety of xenobiotics potentially capable of P-450 induction. In this regard, Nebert (1979) has postulated that hundreds or even thousands of individual forms of P-450 exist, being induced by individual xenobiotics. In this work, the micro-C′ fixation data obtained with three individuals fell into about five families of lines (Table III), although we do not claim to have examined all possible forms of P-450. The limited number of samples used here and the caveats presented about the use of autopsy samples argue against strong conclusions concerning the extent of multiplicity of P-450 in humans. The differences in the histories of various patients, along with the small number of samples used and the limited information about the patients,

[3] E. Gozukara, H. V. Gelboin, and F. P. Guengerich, unpublished results.

suggest that attempts to correlate the isolation of specific forms of P-450 with various aspects of patient history are also premature. Moreover, it is possible that some of the immunological differences may not reflect major changes in P-450 function. However, extension of some of the immunological techniques presented here may permit examination of larger numbers of patients, using more readily available tissues, to better correlate differences in P-450s with exposure to xenobiotics, differences in metabolism, and the like. To date, few proteins, particularly those which can exist in multiple forms, have been examined for variation in human populations.

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