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Purification and Characterization of Six Cytochrome P-450 Isozymes from Human Liver Microsomes†

Philip P. Wang, Philippe Beaune, Laurence S. Kaminsky, Ghazi A. Dannan, Fred F. Kadlubar, Dominique Larrey, and F. Peter Guengerich*

ABSTRACT: Six cytochrome P-450 (P-450) isozymes were purified to electrophoretic homogeneity from the livers of four human organ donors, with three of these isozymes purified from a single individual. Differences were noted between all six P-450s for some or all of the parameters determined by the techniques of sodium dodecyl sulfate–polyacrylamide gel electrophoresis, peptide mapping, spectral analysis of ferrous–carbon monoxide complexes, double-diffusion immunoprecipitin analysis or crossed immunoelectrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis/peroxidase-coupled staining) with rabbit antisera raised to five of the P-450s, or catalytic activity toward *d*-benzphetamine, benzo[*a*]pyrene, acetanilide, debrisoquine, (*R*)- and (*S*)-warfarin, and 1-naphthylamine. While NADPH-fortified human liver microsomal preparations showed catalytic activity toward trichloroethylene, 7-ethoxycoumarin, 2-naphthylamine, and 2-aminofluorene in addition to the other substrates mentioned, none of the P-450s which we purified from these microsomes catalyzed the oxidation of these compounds in reconstituted enzyme systems containing purified rat liver NADPH–P-450 reductase. Antibodies raised against one of the purified P-450s inhibited *d*-benzphetamine *N*-demethylase activity in microsomal incubations but did not inhibit the metabolism of 7-ethoxycoumarin, acetanilide, benzo[*a*]pyrene, or debrisoquine. The data provide a strong biochemical basis for the view that distinct isozymes of P-450 exist in humans and that these isozymes differ in catalytic activity toward drugs and carcinogens.

Interindividual variations in the metabolism of foreign compounds such as therapeutic drugs have been known for many years. Both beneficial and harmful effects to patients can result from unusually slow or rapid metabolism, depending upon the situation under consideration (Conney, 1982; Idle & Smith, 1979; Penno et al., 1981; Ritchie et al., 1980; Sjöqvist & von Bahr, 1973). Such variation appears to have a strong genetic component (Dayer et al., 1982; Nebert & Felton, 1976; Penno et al., 1981; Sloan et al., 1981; Vesell, 1977), although environmental modifications of metabolizing capability (Alvares et al., 1979; Conney, 1982; Nebert et al., 1969) and intraindividual tissue variation (Autrup et al., 1982) must also be taken into consideration. The variation in metabolism is exemplified by the case of debrisoquine, where approximately 10% of the population metabolizes the drug relatively slowly (Kahn et al., 1982; Mahgoub et al., 1977; Ritchie et al., 1980; Sloan et al., 1981). A similar fraction of the human population metabolizes a number of other drugs at unusually low rates, including phenformin, guanoxan, phenacetin, sparteine, metaprolol, bufaralol, perhexiline, mucodyne, and mephenytoin (Kahn et al., 1982, and references therein). Variations in

† From the Department of Biochemistry and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232 (P.P.W., P.B., G.A.D., D.L., and F.P.G.), Center for Laboratories and Research, New York Department of Health, Albany, New York 12201 (L.S.K.), and Division of Carcinogenesis, National Center for Toxicological Research, Food and Drug Administration, Environmental Protection Agency, Jefferson, Arkansas 72079 (F.F.K.).
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metabolism that lead to varying effects of drugs might well be expected to be associated with differences in the susceptibility of different individuals to the effects of chemical carcinogens (Conney, 1982; Nebert, 1981). Considerable intraindividual variation is also found in the ability of derived human samples to metabolize and activate carcinogens in vitro (Conney, 1982; Prough et al., 1979; Sabadie et al., 1980).

While variations in metabolism are often attributed to qualitative and quantitative changes in the isozyme composition of cytochrome P-450 (P-450)[1] and other enzymes which metabolize xenobiotics, little biochemical evidence is available to support this hypothesis and to probe its molecular basis. In the last decade, a wide variety of techniques have been utilized to demonstrate convincingly that P-450 exists in multiple forms in experimental animals and that such polymorphism, if present in humans, may be important to overall health [for reviews see Guengerich (1979) and Lu & West (1980)]. Because of the potential similarity of P-450 isozymes, highly purified proteins are needed as probes to utilize biochemical techniques in the study of multiplicity and regulation. Work with human systems has lagged behind experimental animal models due to technical considerations, unavailability of samples, and the inability to modulate human systems because of ethical reasons. Human liver P-450 has been partially purified in several laboratories (Beaune et al., 1979; Erickson & Bösterling, 1981; Kamataki et al., 1979; Kitada & Kamataki, 1979) and has been purified to electrophoretic homogeneity in this laboratory (Wang et al., 1980). To date the purification of several distinct P-450s from human sources has not been accomplished to verify the hypothesis that multiple forms of P-450 exist in humans. Although partial cross-reactivity of antibodies raised to P-450s isolated from experimental animals has been of some use in characterizing human P-450s (Fujino et al., 1982; Guengerich et al., 1981a; Robie-Suh et al., 1980; Wang et al., 1980), ultimately techniques must be developed for separation of purification of individual human P-450s in order to investigate these enzymes properly.

We report here the purification of six different P-450s from human liver to homogeneity and some of the electrophoretic, spectral, catalytic, and immunochemical properties of these purified isozymes.

Experimental Procedures

Assays. P-450 concentrations were determined from ferrous–CO vs. ferrous difference spectra by using the procedure of Omura & Sato (1964). Protein concentrations were estimated as described by Lowry et al. (1951). The procedure for the estimation of activities of P-450s toward the substrates *d*-benzphetamine (N-demethylation), benzo[*a*]pyrene (total phenolic products), 7-ethoxycoumarin (O-deethylation), 7-ethoxyresorufin (O-deethylation), and acetanilide (para-hydroxylation) are described elsewhere (Guengerich et al., 1982b). Regioselective hydroxylation of (*R*)- and (*S*)-warfarin was measured as described elsewhere (Kaminsky et al., 1980). Metabolism of trichloroethylene to chloral was measured as described previously (Miller & Guengerich, 1982). N-Oxidation and ring hydroxylation of ^{3}H-labeled 2-aminofluorene, 1-naphthylamine, and 2-naphthylamine were measured by high-performance liquid chromatography (Frederick et al., 1981, 1982) and liquid scintillation spectrometry. Hydroxylation of debrisoquine to its 4-hydroxy derivative was measured by gas chromatography/mass spectrometry as described by Kahn et al. (1982). $NaDodSO_4$–polyacrylamide gel electrophoresis and immunoelectrophoresis were carried out as described elsewhere (Guengerich et al., 1982a,b). Two-dimensional combined isoelectric focusing–$NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by using the general procedure of O'Farrell (1975) as modified by Vlasuk & Walz (1980). Peptide mapping involved the procedure of Cleveland et al. (1977) as modified elsewhere (Guengerich, 1978).

Enzymes. (*1*) *General.* The gels and buffers were prepared by using materials described previously (Guengerich et al., 1982b). Purification procedures were carried out at 0–4 °C with the exception of DEAE-cellulose chromatography, which was carried out at room temperature.

(*2*) *Preparation and Solubilization of Human Liver Microsomes.* Human liver microsomes were prepared and solubilized as described (Wang et al., 1980). Liver samples from organ donors, who met accidental deaths, were obtained within 60 min of clinical death (patient 21S, 65-year-old male; patient 25, 21-year-old male; patient 26, 24-year-old male; patient 27, 38-year-old female).

In contrast to previous work with human liver microsomes prepared from autopsy samples (Wang et al., 1980), we found that 75–90% of the P-450 could be solubilized at a sodium cholate to protein ratio of 3 to 1 [i.e., 0.6% (w/v) cholate concentration]. The remaining P-450 could be solubilized when the ratio of cholate to protein was raised to 6 to 1. Only the 0.6% sodium cholate fractions were used in subsequent work.

The cholate-solubilized microsomal supernatant remaining after centrifugation for 60 min at 10^5g (1000–1200 nmol of P-450) was divided and applied to two *n*-octylamino-Sepharose 4B columns (2.6 × 50 cm), and the columns were washed and eluted with the buffer containing 0.06% Renex 690 described elsewhere (Wang et al., 1980). A second P-450 fraction was recovered when the Renex concentration in the elution buffer was raised to 0.3% (w/v). The combined recovery at this step was greater than 90%. The fractions eluted with the 0.06% Renex 690 buffer were pooled and concentrated by ultrafiltration (Amicon PM-30 membrane system, Amicon, Lexington, MA) to <70 mL total volume.

(*3*) *DEAE-cellulose Chromatography.* The concentrated fraction from the previous step was dialyzed against 20 volumes of 5 mM potassium phosphate buffer (pH 7.7) containing 20% (v/v) glycerol, 0.1 mM EDTA, 0.1% (w/v) Renex 690, and 0.2% (w/v) sodium cholate. The buffer was changed 4 times over a 48-h period. A series of Whatman DE-51 (1.6 × 7 cm), DE-52 (1.6 × 15 cm), and DE-53 (1.6 × 25 cm) columns (Whatman Separation Products, Clifton, NJ) were connected in tandem (Guengerich et al., 1982b). The columns were equilibrated with 1000 mL of the dialysis buffer. The dialyzed fraction was applied to the system, and the columns were washed with 300 mL of dialysis buffer and then with 500 mL of the same buffer in which the Renex 690 and sodium cholate concentrations were raised to 0.2% and 0.5%, respectively. A major portion of the P-450 was not bound to the columns and was collected in the void volume. The bound P-450 was eluted from these columns by using a 600-mL linear gradient of 0–250 mM NaCl in the latter buffer described above. Electrophoretically homogeneous cytochrome P-450 fractions were obtained with some microsomal preparations at NaCl concentrations of 45–60 mM ($P\text{-}450_3$ from patient

[1] Abbreviations: P-450, microsomal cytochrome P-450; P-420, cytochrome P-420 (representing denatured forms of P-450); IgG, immunoglobulin G; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate. The individual P-450 isozymes are arbitrarily denoted with Arabic numerals as indicated. The nomenclature used with the rat P-450 isozymes is described elsewhere (Guengerich et al., 1982b).

27 and P-450$_4$ and P-450$_7$ from patient 25).

(*4*) *CM-cellulose Chromatography.* The void volume fraction from the DEAE-cellulose step was dialyzed against 20 volumes of 5 mM potassium phosphate buffer (pH 6.5) containing 20% glycerol, 0.1 mM EDTA, and 0.2% Renex 690 (w/v). The buffer was changed twice over a period of 24 h. A Whatman CM-52 column (1.6 × 30 cm) was equilibrated with the dialysis buffer (250 mL). The dialyzed fraction was applied and washed into the column with 250 mL of the equilibration buffer. P-450 was eluted from the column with a 500-mL linear NaCl gradient (0–150 mM) in the same buffer. Electrophoretically homogeneous P-450 was eluted from this column at a NaCl concentration of 65 mM (P-450$_2$ from patient 21S and P-450$_5$ from patient 25). The fraction isolated from patient 26 was not homogeneous and was further purified by using hydroxylapatite chromatography, with a stepwise gradient of 5, 20, 40, 80, 150, and 300 mM potassium phosphate buffers (pH 7.25) containing 20% glycerol, 0.1 mM EDTA, and 0.2% Renex 690. Electrophoretically homogeneous P-450 was eluted in the 300 mM phosphate fraction (P-450$_8$ from patient 26).

In the case of the sample derived from patient 25, approximately 50% of the P-450 that was applied to the CM-52 column was eluted at a NaCl concentration of 110 mM (P-450$_6$). Further chromatography of this fraction, which was not electrophoretically homogeneous, on hydroxylapatite and DE-53 did not result in further purification.

P-450$_1$ was purified from patient 8 (Guengerich et al., 1981a) by using a procedure described elsewhere (Wang et al., 1980). The fraction was eluted from a hydroxylapatite column in the 300 mM phosphate fraction.

Rat liver NADPH–P-450 reductase was purified to apparent homogeneity as described previously (Guengerich et al., 1982b).

(*5*) *Antibodies.* Antisera were raised in female New Zealand White rabbits by using the following injection schedule: 50 μg of P-450 in an equal volume of Freund's complete adjuvant, injected intradermally along the back; repetition of the first step after 1 week; intramuscular injection of 10 μg of P-450 after 4 more weeks; first bleeding after 1 more week (Kaminsky et al., 1982).

Results

Purification of P-450s. The procedure used for purification involved solubilization of microsomes with sodium cholate and chromatography by using columns of *n*-octylamino-Sepharose 4B, DEAE-cellulose, and (in some cases) CM-cellulose and hydroxylapatite. The work is based on previous schemes used for the purification of rat and human liver P-450s (Guengerich et al., 1982b; Wang et al., 1980) and differs from earlier work with human samples in the order and number of steps used (Wang et al., 1980). In this work, only liver samples obtained within a short period of time after clinical death were used, and patients were exposed to a minimum of therapeutic agents.

The yields (based on total microsomal P-450) and specific contents (in parentheses) of these purified P-450s were the following: P-450$_2$, 4% (7.8 nmol of P-450/mg of protein); P-450$_3$, 0.9% (10.6 nmol of P-450/mg of protein); P-450$_4$, 0.6% (8.9 nmol of P-450/mg of protein); P-450$_5$, 0.7% (11.5 nmol of P-450/mg of protein); P-450$_6$, 1.3% (10.3 nmol of P-450/mg of protein); P-450$_7$, 0.15% (11.3 nmol of P-450/mg of protein); P-450$_8$, 1.5% (12.8 nmol of P-450/mg of protein).

Several other fractions were obtained that contained more than one polypeptide as judged by electrophoretic techniques, and most of the data related to these preparations are not presented. The NaDodSO$_4$–polyacrylamide gel electrophoretograms of the six P-450s purified to homogeneity in this study (P-450$_2$, P-450$_3$, P-450$_4$, P-450$_5$, P-450$_7$, and P-450$_8$), one P-450 purified in a previous study (P-450$_1$; Guengerich et al., 1981a; Wang et al., 1980), and one P-450 fraction which contained three polypeptides (P-450$_6$) are shown in Figure 1. The apparent monomeric molecular weights of these isozymes are as follows: P-450$_1$, 51 400; P-450$_2$, 53 200; P-450$_3$, 54 300; P-450$_4$, 54 300; P-450$_5$, 53 700; P-450$_7$, 53 200; P-450$_8$, 49 600, as estimated by comparisons with proteins of accepted molecular weight. P-450s are known to aggregate in solution in the absence of strong detergents (Guengerich & Holladay, 1979), but we have not yet examined the hydrodynamic properties of these preparations in detail.

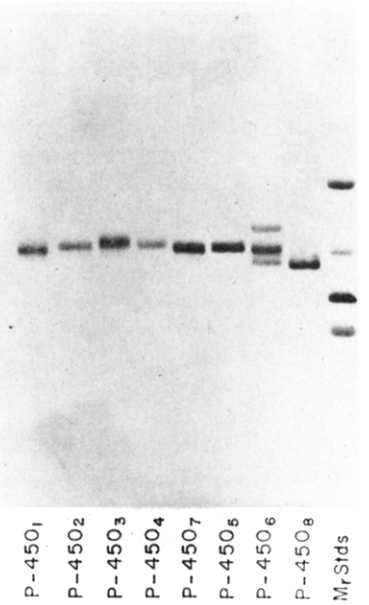

FIGURE 1: Electrophoresis of purified human P-450 preparations. Polyacrylamide gel electrophoresis was carried out in the presence of NaDodSO$_4$ as described by Laemmli (1970) by using a 7.5% (w/v) acrylamide concentration. The anode was at the bottom of the figure. The gel was stained by using the procedure of Wray et al. (1981). About 0.5 μg of P-450 protein was included in each well, and well 9 contained a mixture of standard proteins, including (1 μg each) bovine serum albumin (accepted M_r 68 000), *Eschericia coli* L-glutamate dehydrogenase (M_r 53 000), equine liver alcohol dehydrogenase (M_r 43 000), and rabbit muscle aldolase (M_r 40 000).

Some of the P-450 preparations were subjected to two-dimensional combined isoelectric focusing–NaDodSO$_4$–polyacrylamide gel electrophoresis. While several of the human and rat liver P-450s (Guengerich et al., 1982b) streaked in the isoelectric focusing dimension, as opposed to giving a single spot or family of spots, some of the P-450s were well focused. Figure 2 shows such an electrophoretogram of P-450$_2$. P-450$_4$ also electrophoresed as a single spot under these conditions. The other human P-450s tended to streak in the isoelectric focusing dimension. Similar results have been noted previously for purified P-450s (Guengerich, 1979; Vlasuk & Walz, 1980), and the reasons are not clear.

The purified human liver P-450s were partially digested with *Staphylococcus aureus* V8 protease or α-chymotrypsin, and the resulting peptides were electrophoresed in polyacrylamide gels in the presence of NaDodSO$_4$. Both maps indicated that P-450$_2$, P-450$_5$, and P-450$_7$ have some similarity but shared no bands with the other P-450s examined.

Spectral Properties of P-450s. All of the isolated P-450s appeared to exist predominantly in the low-spin form, as judged by the appearance of the Soret spectra (Ullrich, 1979). Examination of the Soret wavelength maxima observed in the

Table I: Metabolism of Various Compounds by Human Liver Microsomes and Purified P-450s

substrate	rate: purified P-450[a] [nmol of product min^{-1} (nmol of P-450)$^{-1}$]						rate: human liver microsomes [nmol of product min^{-1} (nmol of P-450)$^{-1}$]			
	P-450$_2$	P-450$_3$	P-450$_4$	P-450$_5$	P-450$_7$	P-450$_8$	21S	25	26	27
d-benzphetamine	0.3	11	33	3.2	33	9.1	10	11	12	12
benzo[*a*]pyrene	0.09	0.75	0.24	0.19	0.41	0.17	0.25	0.21	0.23	0.67
acetanilide	0.09	0.50	0.03	0.005	0.01	0.005	0.35	0.28	0.61	0.29
trichloroethylene	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	2.2	0.41	0.56	0.43
7-ethoxycoumarin	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.43	0.61	1.04	0.40
debrisoquine	<0.005	<0.005	<0.005	0.038	<0.005	<0.005	0.063	0.22	0.33	0.052
1-naphthylamine[b]										
N-hydroxy	<0.05	*c*	<0.05			<0.05	<0.04	<0.02	<0.04	<0.03
2-hydroxy	<0.05		0.16			0.13	0.51	0.65	0.67	0.28
4-hydroxy	<0.05		<0.05			<0.05	0.14	0.39	0.28	0.05
2-naphthylamine[c]										
N-hydroxy	<0.05		<0.05			<0.05	<0.04	0.05	0.12	0.14
1-hydroxy	<0.05		<0.05			<0.05	<0.04	0.22	0.29	0.18
3-hydroxy	<0.05		<0.05			<0.05	<0.04	0.05	0.05	0.07
6-hydroxy	<0.05		<0.05			<0.05	0.14	0.10	0.09	0.04
2-aminofluorene										
N-hydroxy	<0.05		<0.05			<0.05	<0.04	0.28	0.56	0.11

[a] For reference, P-450$_2$ was purified from patient 21S, P-450$_3$ was purified from patient 27, P-450$_8$ was purified from patient 26, and P-450$_4$, P-450$_5$, and P-450$_7$ were purified from patient 25. [b] The lower limits of detection vary in the microsomal assays because a fixed amount of protein was used in all assays. [c] A blank space indicates that a determination was not made.

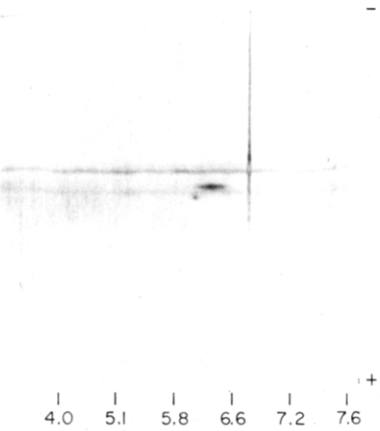

FIGURE 2: Two-dimensional combined isoelectric focusing–NaDodSO$_4$–polyacrylamide gel electrophoresis of P-450$_2$. The electrophoresis was carried out as described under Experimental Procedures. One microgram of protein was electrophoresed, and the gel was stained as described by Wray et al. (1980). The pH values are indicated for the isoelectric focusing dimension, and the cathode for the NaDodSO$_4$–polyacrylamide gel electrophoresis dimension was at the bottom of the figure. The two horizontal lines are artifacts due to 2-mercaptoethanol, and the vertical line is an artifact of overlaying the gel.

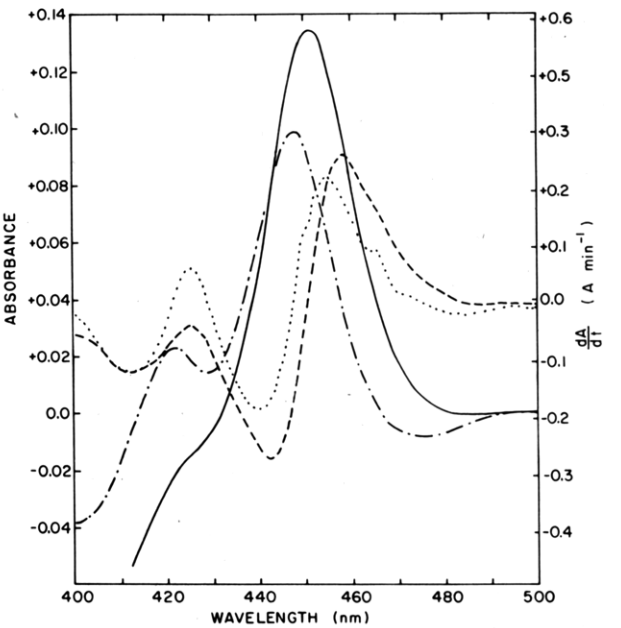

FIGURE 3: Ferrous–CO vs. ferrous difference spectra of purified P-450s. The buffer included 0.1 M potassium phosphate (pH 7.25), 1 mM EDTA, 20% (v/v) glycerol, and 0.5% (w/v) Renex 690. The sample cuvette contained excess $Na_2S_2O_4$ and CO, and the reference cuvette contained only excess $Na_2S_2O_4$. The resulting difference spectra of P-450$_1$ (—) and P-450$_2$ (–·–) are shown. The first derivative spectra (recorded using a Cary 219 derivative accessory with a scan rate of 1 nm s^{-1} and full scale setting of 1 A min^{-1} are also shown for P-450$_1$ (- - -) and P-450$_2$ (···).

ferrous–CO vs. ferrous difference spectra indicated that P-450$_2$ showed a wavelength maximum at 447 nm (Figure 3), confirmed by first derivative spectroscopy. All of the other P-450s showed wavelength maxima in the region of 450–451 nm.

The spectra indicated the presence of P-420 in some of the purified preparations (e.g., see Figure 3). However, in no case did the amount P-420 account for more than one-third of the total heme. No evidence for the presence of cytochrome b_5 (which is tightly bound to *n*-octylamino-Sepharose and DEAE-cellulose) was found in other spectral experiments.

Catalytic Activities. The electrophoretically homogeneous P-450 preparations were examined for mixed-function oxidase activity toward several typical P-450 substrates in reconstituted enzyme systems containing purified rat liver P-450 reductase and L-α-dilauroylglyceryl-3-phosphocholine (Table I). Previous studies indicated that, although rat and human liver NADPH–P-450 reductases can be distinguished immunochemically (Guengerich et al., 1981b), the reductases from the two sources can be used interchangeably in the reconstitution of P-450-dependent monooxygenase activity (Wang et al., 1980). The results are expressed as turnover numbers (based on limiting P-450) under conditions where initial reaction velocities were being measured, and comparison is made with the microsomal preparations from which these P-450s were derived.

d-Benzphetamine N-demethylation was most efficiently catalyzed by P-450$_4$ and P-450$_7$. Benzo[*a*]pyrene hydroxylation (conversion to total phenolic products) was catalyzed

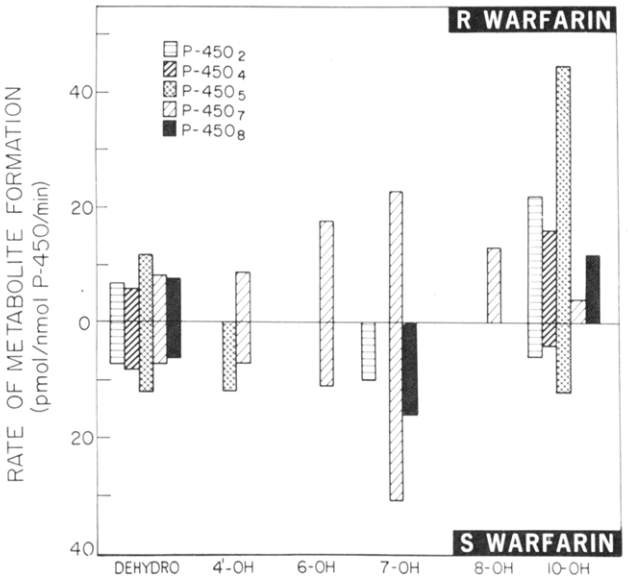

FIGURE 4: Rates of formation of dehydrowarfarin (dehydro), 4′-hydroxywarfarin (4′-OH), 6-hydroxywarfarin (6-OH), 7-hydroxywarfarin (7-OH), 8-hydroxywarfarin (8-OH), and 10-hydroxywarfarin (10-OH) from (*R*)- and (*S*)-warfarin catalyzed by highly purified P-450 isozymes from human livers. The reconstituted system used was based on that optimized with rat P-450 isozymes (Guengerich et al., 1982b; Kaminsky et al., 1980). Reactions were performed at 37 °C for 20 min. The P-450 concentration was 1 μM. The metabolites were analyzed by a high-performance liquid chromatography method (Kaminsky et al., 1982).

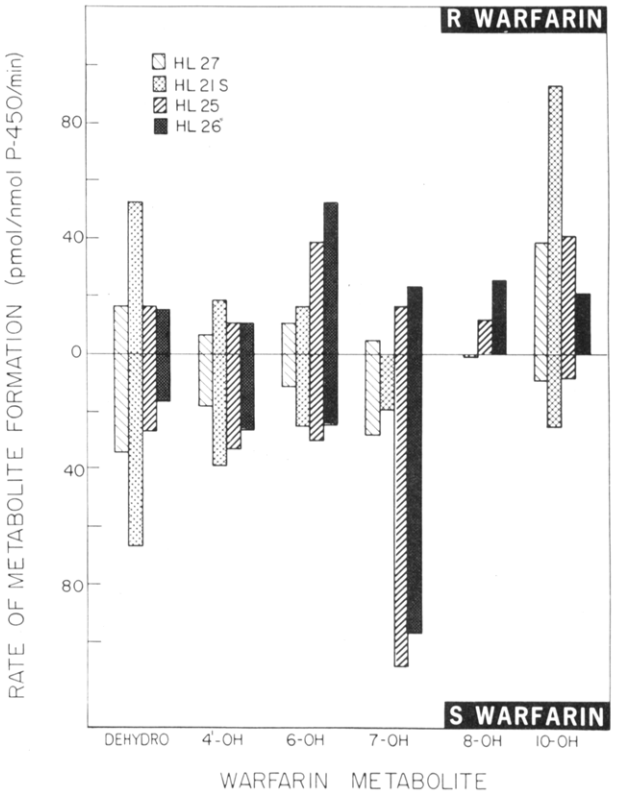

FIGURE 5: Rates of formation of the metabolites of (*R*)- and (*S*)-warfarin by human liver microsomal preparations. Reactions were performed for 10 min by previously reported methods (Kaminsky et al., 1982). Other details were as in Figure 4.

most efficiently by P-450$_3$. P-450$_3$ was also the enzyme most active in catalyzing acetanilide hydroxylation. In several of the above cases the rate of metabolism in the reconstituted system was greater than the rate observed in the microsomes from which the P-450 was derived, when activities are expressed on a P-450 basis.

Although human liver microsomes catalyzed the O-deethylation of 7-ethoxycoumarin and the oxidation of trichloroethylene to chloral, none of the purified human P-450s carried out these reactions. Debrisoquine was metabolized by all of the microsomal preparations, but only one of the purified P-450s hydroxylated this substrate, and the rate was low in comparison to the microsomes. Two of the three purified P-450s tested hydroxylated 1-naphthylamine at the 2-position, but none formed the *N*- or 4-hydroxy metabolites, the latter of which was also formed in microsomal incubations. Microsomal preparations catalyzed the N-, 1-, 3-, and 6-hydroxylation of 2-naphthylamine and the N-hydroxylation of 2-aminofluorene; however, none of the purified P-450s catalyzed these oxidations.[2] In other incubations carried out with purified rat liver P-450s in similar reconstituted systems, the rates of trichloroethylene oxidation (by P-450$_{PB-B}$), 7-ethoxycoumarin O-deethylation (by P-450$_{\beta NF-B}$), and debrisoquine 4-hydroxylation (by P-450$_{UT-A}$) were 8.4, 39, and 0.30 nmol of product (nmol of P-450)$^{-1}$ min^{-1}, respectively.

[2] The N-hydroxylation of primary aromatic amines can also be catalyzed by microsomal flavin-containing monooxygenase (EC 1.14.13.8; *N*,*N*-dimethylaniline N-oxidizing) (Frederick et al., 1982). In order to examine the possibility that this enzyme was contributing to the hydroxylation rates observed in microsomes, all assays involving the microsomes from patient 25 were repeated in the presence of 0.5 mM 2-[(2,4-dichloro-6-phenyl)phenoxy]ethylamine. The presence of this inhibitor reduced the formation of all of the metabolites of 1- and 2-naphthylamine and 2-aminofluorene to levels below the limits of detection, indicating that essentially all of the metabolism of these amines could be attributed to P-450s or that the flavin-containing monooxygenase (if present) was inactive in this particular preparation.

None of the human liver microsomes or purified P-450s examined here were found to catalyze the O-deethylation of 7-ethoxyresorufin.

The metabolism of warfarin, by yielding regio- and stereoselective data, provides a powerful comparison of the substrate specificities of P-450 isozymes. The rates of metabolite formation from each of the warfarin enantiomers are shown, catalyzed by five of the purified isozymes (Figure 4) and by four source microsomal preparations (Figure 5). All five isozymes tested (P-450$_2$, P-450$_4$, P-450$_5$, P-450$_7$, and P-450$_8$) yielded approximately equivalent rates of (*R*)- and (*S*)-dehydrowarfarin formation, and while there were other overlapping metabolites, certain distinct patterns were observed.

P-450$_2$, which was isolated from microsomal preparation 21S, yielded primarily (*R*)-10-hydroxywarfarin, which was also the major metabolite from the source microsomal preparation. This isozyme also yielded (*S*)-10- and (*S*)-7-hydroxywarfarin. While the (*R*)-10-hydroxylase activity in the microsomal preparation (based on total P-450) was 4.3-fold greater than that of the P-450$_2$ reconstituted system, the *R*/*S* ratio in both systems was the same (3.7).

P-450$_7$, which was isolated from microsomal preparation 25, was primarily an (*S*)-7-hydroxylase of warfarin, as was the microsomal preparation. P-450$_4$ also yielded (*R*)- and (*S*)-4′-, (*R*)- and (*S*)-6-, (*R*)-7-, (*R*)-8-, and (*R*)-10-hydroxylase activity. The (*S*)-7-hydroxylase activity of the microsomal preparation was 3.5-fold greater than that of the P-450$_7$ reconstituted system.

P-450$_5$, which was also isolated from microsomal preparation 25, was primarily an (*R*)-10-hydroxylase of warfarin. It also catalyzed the formation of (*S*)-10- and (*S*)-4′-hydroxywarfarin. The ratio of (*R*)- to (*S*)-10-hydroxywarfarin formation rates for P-450$_5$ was the same as that for P-450$_2$, but

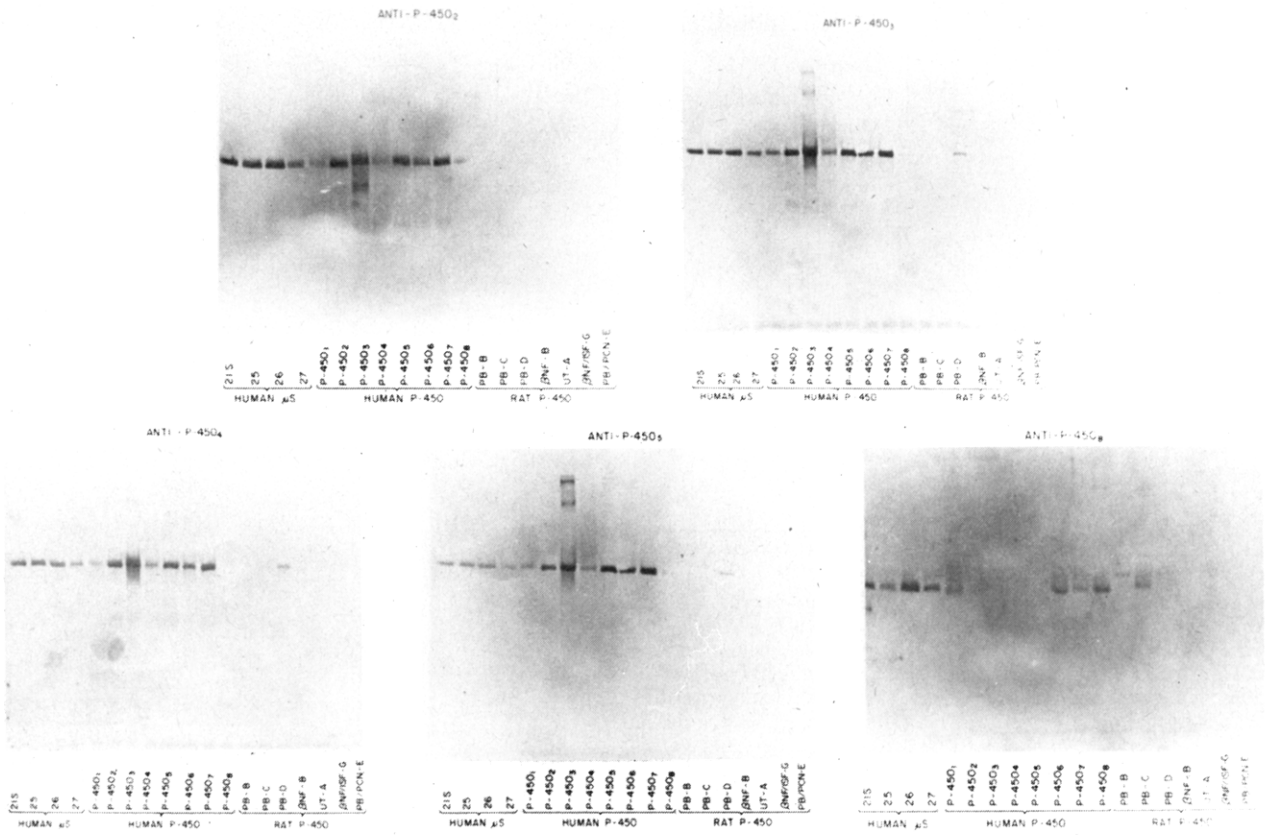

FIGURE 6: Immunochemical staining of $NaDodSO_4$–polyacrylamide gel electrophoretograms after transfer to nitrocellulose. Two picomoles of each of the purified proteins or 4 μg of each of the indicated preparations of liver microsomes was electrophoresed in each slab gel. The resolved proteins were electrophoretically transferred to nitrocellulose sheets which were stained by using the indicated antisera (at dilutions of 1/100) (Glass et al., 1981; Guengerich et al., 1982b; Towbin et al., 1979). Sheets were stained by using (top left) anti-P-450_2, (top right) anti-P-450_3, bottom left) anti-P-450_4, (bottom middle) anti-P-450_5, and (bottom right) anti-P-450_8.

other metabolites catalyzed by these isozymes differed. The rates of (*R*)-10-hydroxylase activities by P-450_5 and microsomal preparation 25 were similar.

P-450_4, which was also isolated from microsomal preparation 25, was also primarily an (*R*)-10-hydroxylase of warfarin with some (*S*)-10-hydroxylase activity (ratio of $R/S = 4.0$). It differed from the other human 10-hydroxylases of warfarin in yielding no other metabolites apart from dehydrowarfarin.

P-450_8, which was isolated from microsomal preparation 26, was primarily an (*S*)-7-hydroxylase of warfarin as was its source microsomal preparation. The microsomal preparation also yielded some (*R*)-10-hydroxylase activity. The ratio of the microsomal to the reconstituted (*S*)-7-hydroxylase activity was 6.1.

In general microsomes from patients 25 and 26 yielded quantitatively and qualitatively similar warfarin metabolite profiles, while microsomes from patient 21S yielded a markedly different profile, suggesting that P-450 isozyme compositions of humans can differ qualitatively and quantitatively.

Immunochemical Studies. Antisera were raised to the purified human P-450s in rabbits and utilized to examine the immunochemical relatedness of the purified P-450s by using several techniques.

One technique which was used involved the electrophoresis of the P-450s by using $NaDodSO_4$–polyacrylamide gels, transfer of the electrophoresed proteins to sheets of nitrocellulose paper, and subsequent staining of the P-450s by using antisera with visualization involving a peroxidase-coupled method (Guengerich et al., 1982a) (Figure 6). Anti-P-450_2 recognized P-450_2, P-450_3, P-450_4, P-450_5, P-450_6, and P-450_7, but none of the eight rat liver P-450s examined showed extensive cross-reactivity. Anti-P-450_3, anti-P-450_4, and anti-P-450_5 all recognized P-450_1, P-450_2, P-450_3, P-450_4, P-450_5, P-450_6, P-450_7, and rat liver P-450_{PB-D}. Anti-P-450_4 showed a weak recognition of rat P-$450_{\beta NF/ISF-G}$. Cross-reactivity of all these P-450s with P-450_8 was very weak. Anti-P-450_8 strongly recognized P-450_8, showed slight recognition of P-450_6 and P-450_7, and recognized rat liver P-450_{PB-B}, P-450_{PB-C}, and P-450_{PB-D}. When the antisera were tested against electrophoresed human liver microsomes (Figure 6), and antisera all recognized apparent single bands corresponding to the antigen in molecular weight, except in the case of anti-P-450_8, in which a slight reaction with P-450s having molecular weights in the region of other P-450s was noted. In other experiments which are not presented, none of the antisera recognized purified human liver microsomal epoxide hydrolase.

Human liver microsomes were also electrophoresed in two dimensions (isoelectric focusing followed by electrophoresis in the presence of $NaDodSO_4$), and the resolved proteins were electrophoretically transferred to sheets of nitrocellulose. Staining of such sheets with antibodies raised to two of the purified human P-450s is shown in Figure 7. In both cases only a single major spot was visualized, consistent with a high degree of purity of the P-450s and specificity of the antibodies.

The antisera were also characterized by using double-diffusion immunoprrecipitin analysis (Figure 8). The antisera were tested against all of the P-450s with the exception of P-450_1 and P-450_6. Anti-P-450_2 precipitated P-450_2 and P-450_5, and the precipitin lines formed a pattern of fusion; P-450_3 was also weakly precipitated. Anti-P-450_3 formed a strong precipitin line with P-450_3 and weak lines (which formed an apparent pattern of fusion) with P-450_2 and P-450_4. Both anti-P-450_4 and anti-P-450_5 showed the same patterns: the precipitin lines formed with P-450_2, P-450_4, and P-450_5

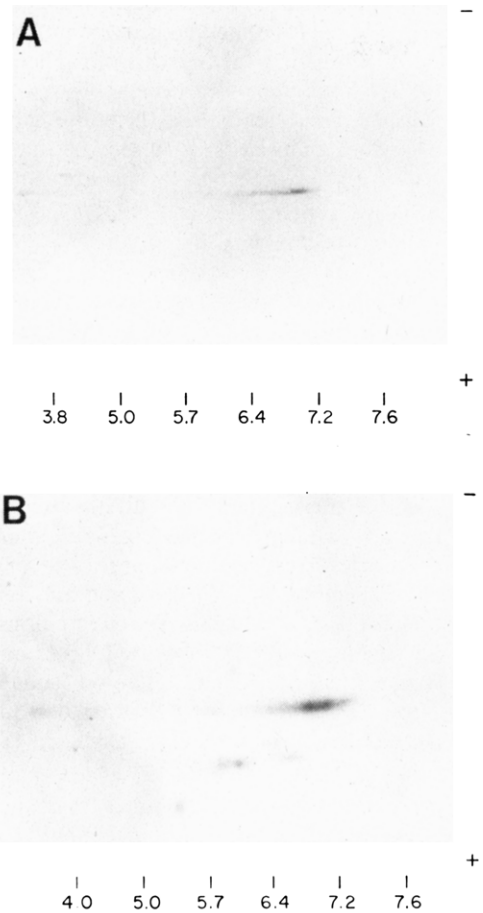

FIGURE 7: Immunochemical staining of a two-dimensional electrophoretogram of human liver microsomes after transfer to nitrocellulose. Fifty micrograms of microsomal protein (from patient 25) were separated by combined isoelectric focusing and $NaDodSO_4$–polyacrylamide gel electrophoresis as in Figure 2. The resolved proteins were electrophoretically transferred to nitrocellulose sheets which were stained with antisera (1/75 dilution) directed to $P\text{-}450_2$ (part A) or $P\text{-}450_8$ (part B).

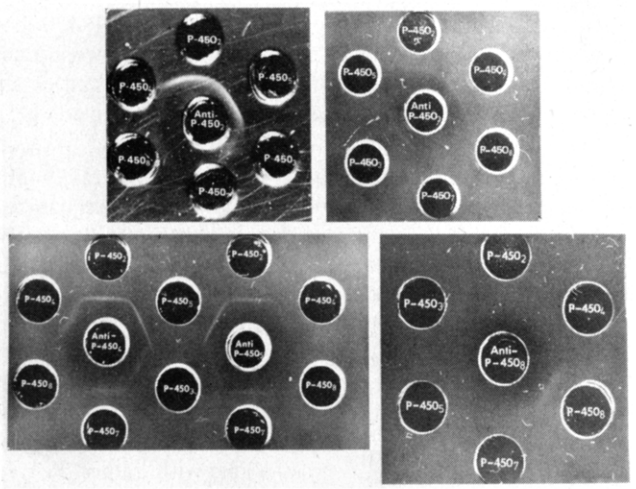

FIGURE 8: Double-diffusion immunoprecipitin analysis of purified human liver P-450s using antisera raised to the purified enzymes. In each case undiluted antisera were placed in the center well, and the purified P-450s were placed in the peripheral wells (at concentrations of 1–2 μM). The plates were developed for 8 h at room temperature and photographed over indirect light.

formed a pattern of fusion, and the line formed with $P\text{-}450_3$ formed a spur with the line precipitated by using $P\text{-}450_7$ and also the line formed with the other P-450s. Anti-$P\text{-}450_8$ only recognized $P\text{-}450_8$ in this assay, giving a weak precipitin line.

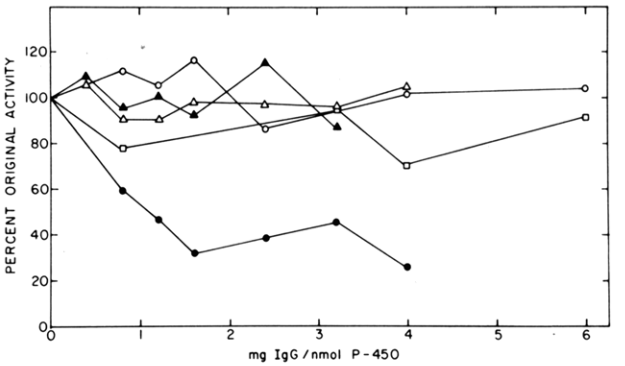

FIGURE 9: Inhibition of mixed-function oxidase activities in human liver microsomes (prepared from patient 25) with anti-$P450_3$. Microsomes (100 pmol of P-450; 0.23 mg of protein) were incubated with the indicated amount of the IgG fraction of anti-$P\text{-}450_3$ for 20 min at 23 °C, and *d*-benzphetamine *N*-demethylase (●), benzo[*a*]-pyrene hydroxylase (○), 7-ethoxycoumarin *O*-deethylase (△), acetanilide hydroxylase (▲), and debrisoquine 4-hydroxylase (□) activities were determined as described under Experimental Procedures. Representative uninhibited initial enzyme velocities are presented in Table I. All points shown represent means of duplicate experiments. Preimmune IgG, added at the same concentrations as anti-$P\text{-}450_3$ IgG, had no significant effect on any of the activities.

The IgG fraction prepared from anti-$P\text{-}450_3$ was tested for its ability to inhibit mixed-function oxidase activities in the microsomes derived from patient 25. Although purified $P\text{-}450_3$ was approximately as effective a catalyst for oxidation of *d*-benzphetamine, benzo[*a*]pyrene, and acetanilide as the P-450 (based on the total P-450 pool) in the microsomes (Table I), the antibody preparation inhibited *d*-benzphetamine N-demethylation to the extent of 75% but did not affect benzo[*a*]pyrene or acetanilide hydroxylation (Figure 9). The microsomes from patient 25 catalyzed the oxidation of debrisoquine and 7-ethoxycoumarin, and purified $P\text{-}450_3$ did not; anti-$P\text{-}450_3$ had no effect on either of these activities.

The apparent concentrations of $P\text{-}450_8$ were estimated in liver microsomes derived from several patients by using immunochemical staining. As in the case of Figure 6, major bands were stained corresponding in molecular weight to the antigen. The concentrations are presented in Table II to show the variation among patients. Since anti-$P\text{-}450_8$ did not show extensive cross-reaction with other P-450s and, when cross-reaction was observed, the molecular weight were sufficiently separated (Figure 6), staining of this band was taken as a measure of $P\text{-}450_8$. Single preparations of human kidney, lung, and placental microsomes from other patients were also examined with the antisera by using this immunochemical assay, but cross-reactive P-450s were not detected at a limit of 2 pmol (mg of protein)$^{-1}$ when either anti-$P\text{-}450_5$ or anti-$P\text{-}450_8$ was used.

Discussion

In this report the purification of six human liver P-450s to homogeneity is described. On the basis of the spectral, electrophoretic, proteolytic, catalytic, and immunochemical data presented, these P-450s, although similar, are indeed distinct proteins. Since all samples were obtained from organ donors shortly after death and patients were not exposed to drastic drug regimens, we feel that the proteins described here are representative of human P-450 enzymes. This is the first instance in which a group of electrophoretically homogeneous P-450s have been isolated from humans and definitively shown to differ by using the criteria examined, particularly catalytic activity. Although the multiplicity of human P-450s might be inferred from animal models and clinical observations, we

Table II: Immunochemical Estimation of P-450 Isozymes in Liver Microsomes of Individual Human Liver Microsomal Samples

	nmol of P-450 (mg of microsomal protein)$^{-1}$		
	immunochemical determination	spectral determination	
patient	$P\text{-}450_8$	total P-450	total P-450 plus P-420
21S	0.04	0.36	0.50
25	0.19	0.92	1.95
26	0.06	0.38	0.76
27	0.15	0.44	0.99
38	0.17	0.49	1.86
39	0.13	0.24	0.76
41	0.21	0.75	1.37
47	0.19	0.53	1.46
50	0.24	0.41	0.83
51	0.75	0.78	1.43
55	0.30	0.36	0.83
56	0.37	0.53	0.94
63	0.70	0.39	1.50
66	0.66	0.32	0.70
69	0.67	0.51	0.98
70	1.33	0.61	1.18
72	1.18	0.52	1.10
73	0.87	0.39	1.32
75	1.11	0.67	1.12
81	0.72	0.64	1.06
83	1.00	0.55	1.40
84	1.25	0.48	1.23
mean ± SD (n = 22)	0.56 ± 0.42	0.51 ± 0.16	1.15 ± 0.37

feel that this work represents an important biochemical basis for the hypothesis. Yields of homogeneous P-450s have been low thus far, but the work does demonstrate the usefulness of hydrophobic and ion-exchange chromatography in isolations; further technical improvements are currently under investigation.

Of interest is the observation that one of the purified P-450s ($P\text{-}450_2$) yielded a ferrous–CO complex which had an absorption maximum at 447 nm (Figure 3), in either the difference or absolute (not shown) spectra. None of the other partially purified or homogeneous P-450 fractions to date have shown corresponding absorption maxima outside of the range of 450–452 nm. However, $P\text{-}450_2$ showed only low catalytic activity toward any of the substrates examined (Table I), including some of those which are rapidly metabolized by the rat liver isozymes which show the same blue-shifted ferrous–CO absorption maximum at 447 nm (i.e., $P\text{-}450_{\beta NF\text{-}B}$ and $P\text{-}450_{\beta NF/ISF\text{-}G}$). Immunochemical reactivity with those two forms of rat liver P-450 was not detected (Figure 4).

Some of the catalytic activities exhibited by the purified human P-450s in reconstituted systems were as high as or higher (on a P-450 basis) than the activities in the microsomes from which the preparations were derived (Table I). However, some other activities, which were demonstrated in microsomes, were not recovered in any of the P-450 fractions obtained to date. At the current time, we feel that these activities are expressed by other P-450s which have not been recovered in homogeneous form yet, as opposed to the possibility that the P-450s are denatured during purification or that reconstitution conditions are not adequate. The results of the anitbody inhibition studies (Figure 9) were unexpected. We suspected that anti-$P\text{-}450_3$, which reacts with several P-450s (Figure 6), would be strongly inhibitory against all activities examined in the microsomes. However, the antibody inhibited *d*-benzphetamine *N*-demethylase activity very well but did not inhibit the oxidation of the other four substrates, some of which (e.g., benzo[*a*]pyrene and acetanilide) were readily metabolized by this isozyme. The catalytic and immunochemical studies to date suggest that none of the P-450s isolated to date are responsible for the bulk of the metabolism of debrisoquine, an important prototype for genetic polymorphism in humans (Idle & Smith, 1979). Variations in the metabolism of warfarin among humans (Figures 4 and 5) may be of use in predicting the P-450 isozyme composition and metabolism of other compounds after more basic information is obtained.

The quantitative immunochemical data support the view that wide variations exist in the composition of P-450 isozymes in humans, as judged by the data presented for 22 organ donors in Table II. The data are limited by caveats about cross-reactivity, but the values presented can, at the very least, be considered to represent an estimate of a P-450 (strictly speaking, apo- *and* holo-P-450) family having similar molecular weights and immunochemical determinants. In many cases the data indicate that $P\text{-}450_8$ accounts for a considerable portion of the total P-450 pool. The level of immunochemically estimated P-450 is greater than the total amount estimated spectrally (even with allowance for P-420) in some cases. Similar discrepancies have been noted previously in rat liver quantitations (Guengerich et al., 1982b) and may reflect the existence of pools of apo-P-450. In previous studies we demonstrated that the level of a P-450 varied 8-fold among 10 patients (Guengerich et al., 1982a). Here we found that the level of $P\text{-}450_8$ varied 33-fold among 22 organ donors. These variations are considerably greater, as judged by the range and SD, than observed for total P-450.

In other experiments with 14 of the human liver microsomal preparations, we found a significant correlation ($p < 0.05$) between immunochemically determined levels of $P\text{-}450_8$ and benzo[*a*]pyrene 3-hydroxylase ($r^2 = 0.64$), *p*-nitroanisole *O*-demethylase ($r^2 = 0.63$), and 7-ethoxycoumarin *O*-deethylase ($r^2 = 0.56$). No correlation was seen with aldrin epoxidase, *d*-benzphetamine *N*-demethylase, aniline 4-hydroxylase, pregnenolone 16α-hydroxylase, or testosterone 6β-hydroxylase. These correlations are not completely consistent with the observed substrate specificity of purified $P\text{-}450_8$. The variation may be due to changes in the activity of $P\text{-}450_8$ during purification or to the presence of P-450s in microsomes which tend to be expressed along with $P\text{-}450_8$ but are lost during purification. Nevertheless, the work does demonstrate that certain P-450-related activities can be associated with distinct forms of P-450 in human liver.

Also of interest is the observation that of the five antisera prepared against human P-450s, three recognized rat P-450s as well as human P-450s (Figure 4). Anti-$P\text{-}450_8$ recognized $P\text{-}450_{PB\text{-}B}$, $P\text{-}450_{PB\text{-}C}$, and $P\text{-}450_{PB\text{-}D}$. Anti-$P\text{-}450_3$, anti-$P\text{-}450_4$, and anti-$P\text{-}450_5$ recognized only $P\text{-}450_{PB\text{-}D}$. These results were surprising, since anti-$P\text{-}450_{PB\text{-}B}$ does not recognize $P\text{-}450_{PB\text{-}C}$ and anti-$P\text{-}450_{PB\text{-}B}$ recognizes both $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{PB\text{-}D}$. These results do, in general, support previous observations that rat liver $P\text{-}450_{PB\text{-}B}$ cross-reacts with human liver P-450s, as judged by antibody inhibition and microcomplement fixation results (Guengerich et al., 1981a; Wang et al., 1980). Rat $P\text{-}450_{\beta NF\text{-}B}$ cross-reacts with human P-450s in extrahepatic tissues (Fujino et al., 1982; Robie-Suh et al., 1980) but does not cross-react extensively with human liver P-450s (Fujino et al., 1982), as borne out by these results (Figure 6). The cross-reactivity among the human P-450s is a problem in utilizing immunochemical methods for characterization of P-450s in human populations, and techniques such as cross-adsorption and, more appropriately, monoclonal antibodies (Fujino et al., 1982; Park et al., 1982) will be needed to resolve the isozymes in such assays. On the other hand, the specificity of these antisera for P-450s and the recognition

of a number of the isozymes may be useful in other immunochemical studies such as localizing major portions of P-450s in immunohistochemical localization studies (Baron et al., 1982) or exploring the degree of relatedness of different P-450s using techniques such as quantitative microcomplement fixation (Guengerich et al., 1981a).

Registry No. P-450, 9035-51-2; *d*-benzphetamine, 156-08-1; benzo[*a*]pyrene, 50-32-8; acetanilide, 103-84-4; trichloroethylene, 79-01-6; 7-ethoxycoumarin, 31005-02-4; debrisoquine, 1131-64-2; *N*-hydroxy-1-naphthylamine, 607-30-7; 2-hydroxy-1-naphthylamine, 2834-92-6; 4-hydroxy-1-naphthylamine, 2834-90-4; *N*-hydroxy-2-naphthylamine, 613-47-8; 1-hydroxy-2-naphthylamine, 606-41-7; 3-hydroxy-2-naphthylamine, 5417-63-0; 6-hydroxy-2-naphthylamine, 4363-04-6; *N*-hydroxy-2-aminofluorene, 53-94-1; (*R*)-warfarin, 5543-58-8; (*S*)-warfarin, 5543-57-7; dehydrowarfarin, 67588-18-5; 4′-hydroxy-(*R*)-warfarin, 63740-78-3; 6-hydroxy-(*R*)-warfarin, 63740-75-0; 7-hydroxy-(*R*)-warfarin, 63740-76-1; 8-hydroxy-(*R*)-warfarin, 63740-77-2; 4′-hydroxy-(*S*)-warfarin, 68407-05-6; 6-hydroxy-(*S*)-warfarin, 63740-80-7; 7-hydroxy-(*S*)-warfarin, 63740-81-8; 10-hydroxywarfarin, 83219-99-2.

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