Immunological Comparison of Rat, Rabbit, and Human Liver NADPH–Cytochrome P-450 Reductases†

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

ABSTRACT: NADPH–cytochrome P-450 reductase (EC 1.6.2.4) preparations were purified to electrophoretic homogeneity from rat, rabbit, and human liver microsomes. These preparations had apparent monomer molecular weights (M_r's) of 72 000–74 000 and were catalytically active in reducing rat and rabbit liver cytochromes P-450 as well as cytochrome *c*. A form of the human liver reductase devoid of a peptide of about M_r 6000 was isolated in the absence of protease inhibitors; this enzyme catalyzed the reduction of cytochrome *c* but not cytochromes P-450. Rabbits were immunized with purified rat liver NADPH–cytochrome P-450 reductase and the resulting antibody preparation was used to examine the species specificity of the enzyme. Immunological differences among the three species were detected by using double-diffusion analysis, quantitative microcomplement fixation, and inhibition of enzyme activity. Microcomplement fixation techniques indicated immunological differences in both rat and human reductase preparations due to removal of a peptide of M_r 6000–8000; these differences were not detected by using double-diffusion analysis. The antibody inhibited rat liver microsomal *d*-benzphetamine *N*-demethylase activity to the same extent as NADPH–cytochrome *c* reductase activity, suggesting that the level of reductase controls the rate of this cytochrome P-450-mediated activity. On the other hand, the antibody was much less effective in inhibiting rat liver benzo[*a*]pyrene hydroxylase activity. The antibody exerted different effects in inhibiting *d*-benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase activities as compared to NADPH–cytochrome *c* reductase activity in human liver microsomes.

NADPH–cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4) supplies reducing equivalents to P-450,[1] the terminal oxidase of the microsomal mixed-function oxidase system. The enzyme has been purified to apparent homogeneity from rat liver (Strobel & Dignam, 1978; Vermilion & Coon, 1978; Yasukochi & Masters, 1976), rabbit liver (French & Coon, 1979) and rabbit lung (Guengerich, 1977a; Serabjit-Singh et al., 1979) and shown to contain one molecule each of FAD *and* FMN per subunit in every case, as originally demonstrated by Iyanagi & Mason (1973) to be the situation in the rabbit enzyme isolated by protease digestion. Studies with both rat and rabbit liver systems suggest that an equimolar complex of P-450 and the reductase has optimal functional activity in reconstituted monooxygenase systems (French et al., 1980; Miwa et al., 1978). The in vivo situation may be more complex since the ratio of total P-450 to NADPH–P-450 reductase has been estimated to be as high as 20:1 (Estabrook et al., 1976). The rat or rabbit enzyme is readily cleaved with proteases to yield a 66 000–68 000-dalton portion, which retains activity toward cytochrome *c* and other artificial electron donors, and a low M_r hydrophobic peptide, which appears to be located at the *N* terminus of the enzyme (Black et al., 1979). The presence of the hydrophobic peptide seems to be necessary for binding of the reductase and efficient electron transfer to P-450 (Black et al., 1979; Coon et al., 1976; Vermilion & Coon, 1978).

Studies with the human liver enzyme have been limited. The human reductase has been partially purified both by this laboratory (Wang et al., 1980) and by Kamataki et al. (1979). Both preparations have been reported to be functionally active in the reconstitution of P-450-dependent activities, being as effective as the rat liver enzyme when added at optimal concentrations. The latter group has also reported that the human liver enzyme contains both FAD and FMN (Kamataki et al., 1979). We have now purified human liver NADPH–P-450 reductase to apparent homogeneity as judged by NaDodSO_4–polyacrylamide gel electrophoresis. Different preparations containing the enzyme isolated in varying M_r were compared to the rat and rabbit reductases by using activity toward P-450 and a battery of immunological tests to examine the similarity of the various NADPH–P-450 reductases.

Experimental Procedures

Preparation of Microsomes and NADPH–P-450 Reductase. Liver microsomes were prepared from male rats of Sprague-Dawley descent (125–150 g; Harlan Industries, Indianapolis, IN) which had been induced with PB as described elsewhere (Guengerich et al., 1981). When noted, rats were treated instead with Aroclor 1254 by administering a single intraperitoneal dose of 300 mg kg^{-1} in corn oil 3 days prior to sacrifice (Ryan et al., 1977). Liver microsomes were prepared from PB-treated rabbits as described (Guengerich et al., 1981) Human liver microsomes were prepared from various individuals as described in the preceding paper in this issue (Guengerich et al., 1981).

NADPH–P-450 reductase was purified from rat and rabbit liver microsomes by using a modification of the method of Yasukochi & Masters (1976) described elsewhere (Guengerich, 1978a; Guengerich & Martin, 1980). In this work, the

† 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 00267 and ES 01590 and Contract NCI NO 1 CP 85672 from the U.S. Public Health Service.

* Address correspondence to this author. He is a recipient of Research Career Development Award ES 00041 from the U.S. Public Health Service.

[1] Abbreviations used: C′, complement; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid; IgG, immunoglobulin G fraction of antisera; PB, phenobarbital; NaDodSO_4, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; P-450, liver microsomal cytochrome P-450; M_r, apparent monomeric molecular weight as determined by NaDodSO_4–polyacrylamide gel electrophoresis.

0006-2960/81/0420-2379$01.25/0

buffer used to elute the reductase from the 2′,5′-ADP–agarose column contained 0.4 mM PMSF but was devoid of FMN.

NADPH–P-450 reductase was isolated from human liver microsomes, using the same general techniques as described for other species, during the isolation of P-450 and epoxide hydrolase (Wang et al., 1980; Guengerich et al., 1979a,b). The fractions from the *n*-octylamino-Sepharose 4B column which contained NADPH–cytochrome *c* reductase activity were pooled and applied to a 2.5 × 50 cm column of DEAE-cellulose previously equilibrated with 10 mM potassium phosphate buffer (pH 7.4) which contained 0.1 mM EDTA, 20% (v/v) glycerol, 0.1% (w/v) sodium deoxycholate, 0.35% (w/v) sodium cholate, and 0.4 mM PMSF. The column was washed with 500 mL of the same buffer. The reductase was eluted by using a 1000-mL linear gradient of 0–0.4 M NaCl in the equilibration buffer. Fractions containing NADPH–cytochrome *c* reductase activity were pooled and applied to a 5-mL column of 2′,5′-ADP–agarose. The column was washed with Lubrol PX and deoxycholate based buffers and eluted with 2′-AMP essentially as described elsewhere (Guengerich, 1978; Guengerich & Martin, 1980) except that 0.1 mM PMSF was added to all buffers. The reductase fraction was dialyzed twice for 24 h vs. 50 volumes of 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol and stored at −70 °C in small aliquots after the addition of 0.4 mM PMSF. When necessary, the enzyme was concentrated by using an Amicon PM-30 ultrafiltration cell.

Bromelain-treated Sprague-Dawley rat liver NADPH–cytochrome *c* reductase (Pederson et al., 1973) was a generous gift of Dr. S. D. Aust, Michigan State University, East Lansing, MI.

Antibodies. Antibodies were raised in female New Zealand White rabbits immunized with rat liver NADPH–P-450 reductase by using an injection and bleeding schedule described elsewhere (Thomas et al., 1976). Antisera were pooled from three different rabbits. IgG preparations were isolated as described elsewhere (Guengerich et al., 1981; Dean & Coon, 1977). A typical precipitation titer was 0.6 μg of rat liver reductase per mg of IgG fraction, as determined by the ability of the antibody to precipitate NADPH–cytochrome *c* reductase activity from Lubrol PX solubilized microsomes. When rat liver microsomes were electrophoresed in $NaDodSO_4$–polyacrylamide gel and the resolved proteins were transferred to nitrocellulose, the only band detected by antibody–second antibody–antiperoxidase–peroxidase staining (Guengerich et al., 1981) corresponded to the antigen.

Immunological Studies. Double-diffusion analyses, micro-C′ fixation, and enzyme inhibition studies were carried out by using the techniques described in the preceding paper (Guengerich et al., 1981). When noted, double-diffusion plates were stained for NADPH–nitrotetrazolium blue reductase activity as described elsewhere (Guengerich, 1977a) and photographed against a white background.

Assays. Protein concentrations were estimated as described by Lowry et al. (1951). NADPH–P-450 reductase was monitored during purification by using NADPH–cytochrome *c* reductase activity; 1 unit is defined as 1 μmol of cytochrome *c* reduced min^{-1} at 30 °C in 0.3 M potassium phosphate buffer (pH 7.7). $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). *d*-Benzphetamine *N*-demethylase (Guengerich & Holladay, 1979) and benzo[*a*]pyrene hydroxylase activities (Nebert & Gelboin, 1968) were determined as described elsewhere.

Materials. 2′,5′-ADP–agarose and 2′-AMP were purchased from P-L Biochemicals. DEAE-cellulose (DEAE-Sephacel) was purchased from Pharmacia. PMSF, obtained from Sigma, was dissolved in 1-propanol, stored at -20 °C, and added to buffers immediately before use.

Results

Purification of Human Liver NADPH–Cytochrome P-450 Reductase. NADPH–P-450 reductase was purified from human liver microsomes by using a combination of procedures previously described for the purification of the enzyme from other sources, i.e., solubilization with cholate (Imai, 1976), *n*-octylamino-Sepharose 4B chromatography (Guengerich, 1978b; Imai, 1976), DEAE-cellulose chromatography (Strobel & Dignam, 1978; Vermilion & Coon, 1978; Yasukochi & Masters, 1976), and 2′,5′-ADP–agarose chromatography (Yasukochi & Masters, 1976). This procedure was utilized to purify the enzyme from the same microsomal preparations used for the isolation of P-450 (Wang et al., 1980) and epoxide hydrolase (Guengerich et al., 1979a,b). The yields, based upon NADPH–cytochrome *c* reductase activity, of the preparations purified from patients 8A (first portion of the liver of patient 8), 8B (second portion of the liver of patient 8), 14, and 15 were 43%, 37%, 63%, and 75% respectively.

Subunit M_r's of Rat, Rabbit, and Human NADPH–Cytochrome P-450 Reductase Preparations. Subunit M_r's were estimated by using $NaDodSO_4$–polyacrylamide gel electrophoresis and standard proteins with defined M_r's. Knapp et al. (1977) have presented evidence that the M_r estimate obtained by this technique is nearly identical with that obtained by sedimentation equilibrium measurements made in the presence of 6 M guanidine hydrochloride. The M_r of the rat liver monomer was estimated at 74000 (Figure 1), while the rabbit liver monomer had an apparent M_r of 72000. The difference was confirmed by electrophoresing the samples together (a broad band was observed) and supports the difference of about 2000 daltons reported by French & Coon (1979).

Proteolysis was a particular problem with the human liver reductase. As shown in Figure 1, the preparation derived from patient 8A consisted of a single polypeptide of 66000 daltons. The preparation derived from patient 14 contained a considerable portion of this same polypeptide in addition to two polypeptides of subunit M_r 70000 and 72000. In the purification of reductase from patient 8B, PMSF was added to all buffers immediately prior to use, and chromatography steps were carried out without intermittent storage of fractions. The apparent subunit of that preparation was 72000, identical with that of the rabbit liver prepartion but not with that of the rat liver preparation. Since both M_r 72000 and M_r 66000 preparations were derived from patient 8, we conclude that the apparent proteolysis is an artifact of the isolation procedure; i.e., the M_r 66000 species is derived from the M_r 72000 species. The procedure was repeated to obtain reductase from patient 15 with a M_r of 72000.

Proteolytic digestion of rat liver NADPH–P-450 reductase is known to destroy catalytic activity toward P-450 (but not cytochrome *c*) (Vermilion & Coon, 1978; Welton et al., 1973), presumably by removing a peptide necessary for binding to P-450 (Black et al., 1979; Coon et al., 1976). The rat, rabbit, and human preparations used in this work were examined for NADPH–cytochrome *c* reductase activity and NADPH–P-450 reductase activity, as estimated by the rate of NADPH oxidation in the presence of an excess of either highly purified rat or rabbit liver P-450 and *d*-benzphetamine (Table I). As expected, the low M_r forms of the enzyme contained very little activity toward either P-450. We have previously reported that a partially purified human liver NADPH–P-450 reductase

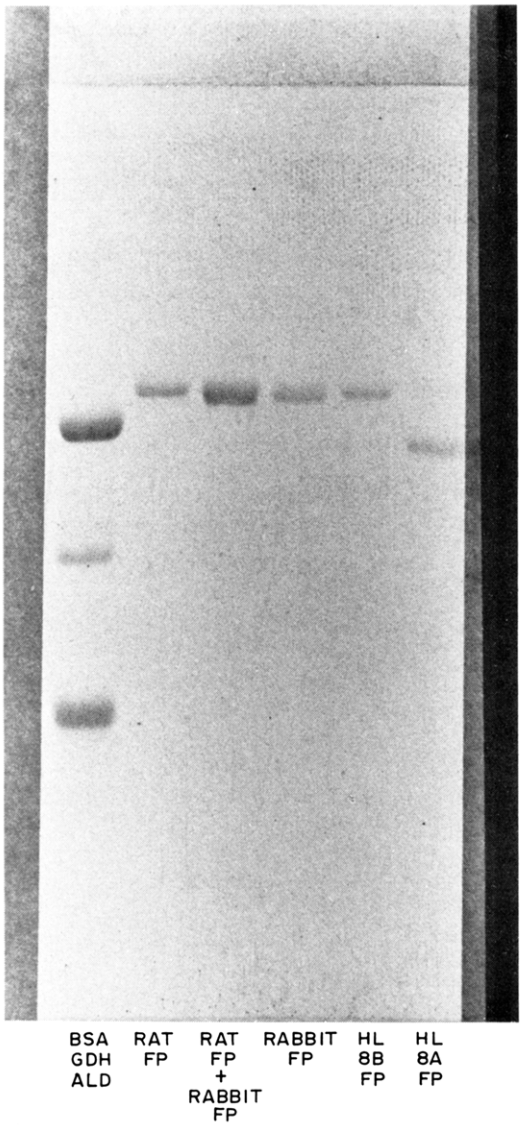

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of NADPH–P-450 reductase preparations. Electrophoresis was carried out according to Laemmli (1970) in a 0.075 × 10 × 20 cm Hoefer slab gel electrophoretic apparatus using a 6% acrylamide gel. The anode was at the bottom of the gel. Staining was carried out as described by Fairbanks et al. (1971) except that staining and destaining times were 4 h for each step. Samples analyzed included the following (1) mixture of 1 μg each of bovine serum albumin (accepted monomer M_r 68 000), *Escherichia coli* L-glutamate dehydrogenase (M_r 53 000), and rabbit muscle aldolase (M_r 40000); (2) PB-treated rat liver NADPH–P-450 reductase, 0.2 μg; (3) mixture of PB-treated rat liver and PB-treated rabbit liver NADPH–P-450 reductases, 0.2 μg each; (4) PB-treated rabbit liver NADPH–P-450 reductase, 0.2 μg; (5) human (patient 8B) liver NADPH–P-450 reductase, 0.3 μg; and (6) human (patient 8A) liver NADPH–P-450 reductase, 0.3 μg.

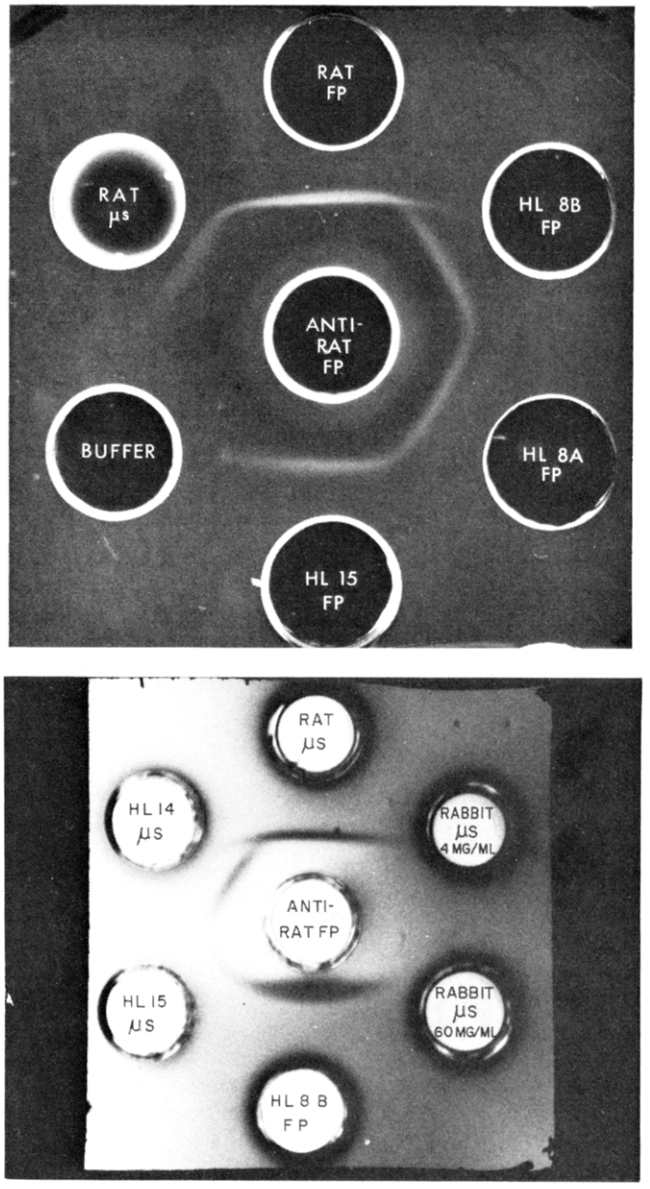

FIGURE 2: Double-diffusion analysis of rat, rabbit, and human liver NADPH–P-450 preparations using rabbit anti-rat NADPH–P-450 reductase. The center wall contained the IgG fraction at 29 mg mL^{-1}. In part A, peripheral wells contained (clockwise from top) the following: (well 1) rat liver reductase (FP), 0.20 mg mL^{-1}; (well 2) human liver (patient 8B) reductase (M_r 72 000), 0.29 mg mL^{-1}; (well 3) human liver reductase (patient 8A, M_r 66 000), 0.3 mg mL^{-1}; (well 4) human liver reductase (patient 15, M_r 72 000), 0.2 mg mL^{-1}; (well 5) 10 mM Tris–acetate buffer (pH 7.4) containing 20% glycerol and 1 mM EDTA; (well 6) PB-treated rat liver microsomes (μS), 40 mg mL^{-1}. Plate A was photographed directly. In part B, the peripheral wells contained the following preparations (clockwise from top): (well 1) PB-treated rat liver microsomes (μS), 10 mg mL^{-1}; (wells 2 and 3) PB-treated rabbit liver microsomes at 4 and 60 mg mL^{-1}, respectively; (well 4) purified human NADPH–P-450 reductase (patient 8B, M_r 72 000), 0.2 mg mL^{-1}; (well 5) human liver microsomes (patient 15), 30 mg mL^{-1}; (well 6) human liver microsomes (patient 14), 30 mg mL^{-1}. Plate B was stained for NADPH–nitrotetrazolium blue reductase activity. In both parts, microsomes were solubilized as described elsewhere for these studies (Guengerich et al., 1981).

preparation which had an apparent monomer M_r of ~2000 daltons less than the rat liver enzyme was as active (when added at saturating concentrations) in the reconstitution of human liver P-450-dependent *d*-benzphetamine N-demethylation and 7-ethoxycoumarin O-deethylation as the rat liver enzyme (Wang et al., 1980).

Double-Diffusion Precipitin Analysis. Diffusion of the rat liver reductase against antibody raised to that enzyme produced a single precipitin line (Figure 2A). A precipitin line could not be detected when purified rabbit liver NADPH–P-450 reductase was tested against the same antibody. Human liver reductase preparations of M_r 72 000 and 66 000 yielded precipitin lines that formed a pattern of fusion with each other but not with the precipitin line formed with the rat liver reductase. The immunoprecipitates contain NADPH–nitrotetrazolium blue reductase activity, and we found that double-diffusion plates could be stained for this activity. The resulting patterns were identical with those obtained by direct observation of the protein lines; no line could be observed for the wells containing the rabbit liver reductase. The data of Figure 2 indicate that in both rats and humans precipitin lines formed a pattern of fusion for microsomes and the purified

Table I: NADPH-P-450 Reductase Activity of Purified Rat, Rabbit, and Human Preparations[a]

reductase preparation	M_r	cytochrome *c* reduction [μmol min^{-1} (mg of reductase)$^{-1}$]	NADPH oxidation in the presence of excess rat P-450 and benzphetamine [μmol min^{-1} (mg of reductase)$^{-1}$]	NADPH oxidation in the presence of excess rabbit P-450 and benzphetamine [μmol min^{-1} (mg of reductase)$^{-1}$]
rat	74 000	65	10.8	7.8
bromelain-solubilized rat	66 000	57	0.14	0.28
rabbit	72 000	58	6.45	6.55
human (patient 8A)	66 000	37	0.18	0.65
human (patient 8B)	72 000	43	3.58	2.85
human (patient 14)	72 000/ 70 000/ 66 000	41	1.96	1.52

[a] Assays were carried out at 37 °C by using 0.5–5 μg of NADPH-P-450 reductase, 30 pmol of PB-treated rat liver P-450 fraction B (Guengerich, 1977b, 1978b) or PB-treated rabbit liver P-450 LM-2 (Guengerich, 1977b), 4 μg of L-α-dilauroylglyceryl-3-phosphorylcholine, and 20 μg of sodium deoxycholate in 0.20 mL of buffer containing 50 mM potassium *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonate (pH 7.7), 15 mM $MgCl_2$, and 1 mM *d*-benzphetamine. After equilibration for 3 min in a Cary 219 spectrophotometer, the reactions were initiated by the addition of NADPH to 0.15 mM and monitored by recording absorbance changes at 340 nm. Results are presented as means of duplicate experiments.

Table II: Comparison of Liver Microsomal NADPH-P-450 Preparations by Micro-C′ Fixation Using Antibody Raised to Rat Liver NADPH-P-450 Reductase[a]

NADPH-P-450 reductase preparation	immunological distance						
	rat_1[b]	rabbit	rat_2[b]	bromelain-solubilized rat	human 8A (M_r 66 000)	human 14 (M_r 66 000, 70 000, 72 000)	human 8B (M_r 72 000)
rat_1[b]		0	4	13	21	24	80
rabbit			4	13	21	24	80
rat_2[b]				9	17	20	76
bromelain-solubilized rat					8	11	71
human 8A (M_r 66 000)						3	59
human 14 (M_r 66 000, 70 000, 72 000)							56
human 8B (M_r 72 000)							

[a] The procedure was carried out with the appropriate controls, including the immunogen, as described elsewhere (Sarich & Wilson, 1966), with the maximum micro-C′ fixation observed with varying antigen concentration plotted vs. antibody concentration. At least three points in the range of 30–80% micro-C′ fixation were used in the calculations. The points at which 50% micro-C′ fixation was observed were used to determine immunological distances, as defined by Prager & Wilson (1971). For the rat or rabbit NADPH-P-450 reductases, 50% micro-C′ fixation was obtained by using 3.5 μg of the IgG fraction derived from the antiserum. [b] Duplicate preparations.

reductases, both intact and protease cleaved.

Solubilized microsomes were also examined by using the double-diffusion technique (Figure 2B). A pattern of fusion was observed for the microsomes derived from different humans, and this line formed a spur with the line produced by solubilized rat liver microsomes. Because of the low concentration of reductase in human liver microsomes, these plates required NADPH–nitrotetrazolium blue reductase activity staining for visualization of the precipitin lines. Again, no lines were visible for the wells containing the rabbit liver enzyme.

Micro-C′ Fixation. A number of rat, rabbit, and human liver microsomal NADPH–P-450 reductase preparations were examined by using quantitative micro-C′ fixation (Prager & Wilson, 1971) (Table II). Two individual preparations of the rat liver enzyme were separated by an immunological distance of 4, which is regarded as a measure of variation between apparently identical samples. The rabbit liver enzyme was not distinguished from the corresponding rat liver preparations by this criterion. On the other hand, the bromelain-treated rat liver enzyme yielded a line 21 distance units removed from the intact rat liver reductases. The low M_r preparation derived from patient 8 yielded a line 67 distance units removed from the prepartion (derived from the same patient) which had a M_r identical with that of the rabbit liver enzyme (i.e., 72 000). The preparation isolated from patient 14, which contained both high and low M_r forms of the reductase, yielded a distance between the two human antigens, being much closer to the low M_r form.

While the rat and rabbit liver enzymes appeared identical, the data indicated that the concentration of antigen required for maximum micro-C′ fixation with the rabbit enzyme was 250-fold greater than with the rat enzyme; i.e., with an IgG concentration of 4 μg mL^{-1}, 0.02 μg of the rat liver reductase (either of two preparations) yielded optimal micro-C′ fixation (60–65%), but 5 μg of rabbit reductase was required for optimal micro-C′ fixation (70%). This difference is certainly greater then expected for any small differences in specific activity (Table I), and we conclude that micro-C′ fixation indicates dissimilarity of the rat and rabbit reductases as well as the human reductases.

Activity Inhibition Studies. Immunological differences between purified rat and rabbit liver NADPH–P-450 reductases were shown by their inhibition with antibody raised to the rat liver enzyme (Figure 3); i.e., the concentration of antibody required to produce a given degree of inhibition of NADPH–cytochrome *c* reductase activity was roughly an order of magnitude higher for the rabbit liver reductase than for the rat liver enzyme. Other experiments were carried out

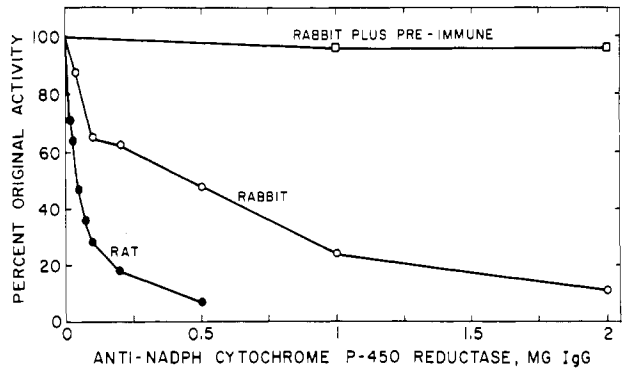

FIGURE 3: Inhibition of NADPH–cytochrome *c* reductase activity of purified rat and rabbit liver NADPH–P-450 reductases by antibody raised against the rat liver enzyme. Reductase (0.2 μg of either preparation) was incubated with varying amounts of antibody for 20 min at 23 °C. Cytochrome *c* and potassium phosphate buffer (pH 7.7) were added to each tube to final concentrations of 40 μM and 0.3 M, respectively, in a total volume of 1.0 mL. After incubation for 3 min at 30 °C, NADPH (0.1 mM) was added to each cuvette, and A_{550} was recorded as a function of time. Data points represent means of duplicate determinations; the initial ΔA_{550} min^{-1} was 0.14 in both cases. Points are shown for rat (●) and rabbit (○) liver reductases with antireductase IgG and for rabbit liver reductase with preimmune IgG (□).

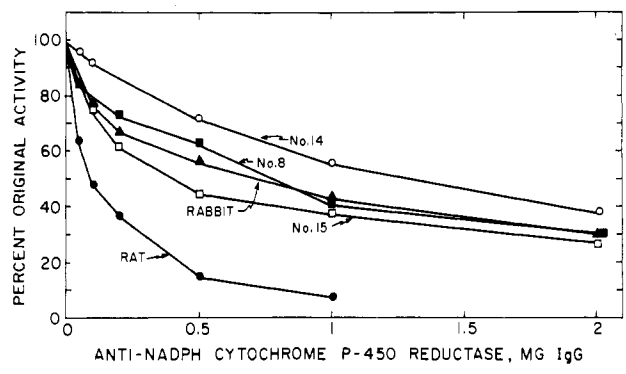

FIGURE 4: Inhibition of NADPH–cytochrome *c* reductase in microsomes derived from rats, rabbits, and humans by antibody raised to rat liver NADPH–P-450 reductase. Amounts of microsomal preparations catalyzing the reduction of 7 nmol of cytochrome *c* min^{-1} were incubated with varying amounts of the antibody for 20 min at 23 °C. Cytochrome *c* and potassium phosphate buffer (pH 7.7) were added to each tube to final concentrations of 40 μM and 0.3 M, respectively, in a total volume of 1.0 mL. NADPH (0.1 mM) was added to each cuvette, and A_{550} was recorded as a function of time. The points shown are means of duplicate determinations with microsomes prepared from Aroclor 1254-treated rats (●), PB-treated rabbits (▲), human patient 8 (■), human patient 14 (○), and human patient 15 (□). In control experiments, the inhibition observed with 1 mg of preimmune IgG was no more than 14% in any case.

with microsomal preparations (Figure 4). These studies also indicate that the rat liver enzyme is considerably more sensitive to inhibition by the antibody than are the NADPH–P-450 reductases of rabbits and humans. The inhibition patterns for the various human preparations were somewhat different.

The sensitivity of microsomal mixed-function oxidase activities to antibody raised to rat liver NADPH–P-450 reductase was also examined. In these studies, microsomes were derived from rats treated with the polychlorinated biphenyl mixture Aroclor 1254, which appears to induce P-450s involved both in *d*-benzphetamine N-demethylation and in benzo[*a*]pyrene hydroxylation (Alvares et al., 1973; Ryan et al., 1977). The pattern of inhibition for *d*-benzphetamine N-demethylation was virtually identical with that observed for inhibition of NADPH–cytochrome *c* reductase activity (Figure 5). On the other hand, benzo[*a*]pyrene hydroxylase activity was significantly less sensitive to inhibition by the antibody.

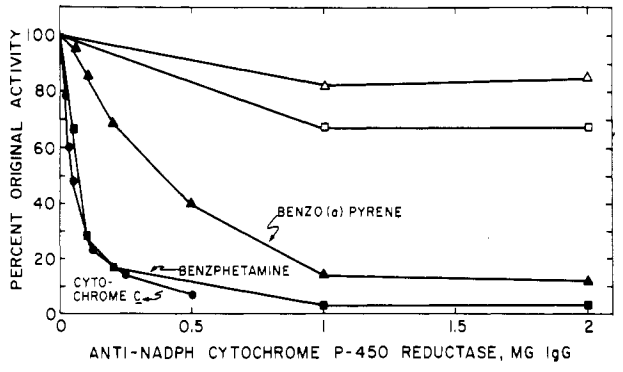

FIGURE 5: Inhibition of NADPH–cytochrome *c* reductase, *d*-benzphetamine *N*-demethylase, and benzo[*a*]pyrene hydroxylase activities in liver microsomes prepared from Aroclor 1254-treated rats by antibody raised to rat liver NADPH–P-450 reductase. In each case, 40 μg of microsomal protein was incubated with an indicated amount of antibody for 20 min at 23 °C. Incubates were chilled on ice, and remaining cofactors were added prior to assay for each activity. Data points represent means of duplicate determinations for NADPH–cytochrome *c* reductase (●) and *d*-benzphetamine *N*-demethylase (■) activities and means of triplicate determinations for benzo[*a*]pyrene hydroxylase (▲) activity. Points are also shown for control experiments with preimmune IgG for *d*-benzphetamine *N*-demethylase (□) and benzo[*a*]pyrene hydroxylase activities (△). The initial specific activities were 371 nmol of cytochrome *c* reduced, 17.7 nmol of HCHO formed (from *d*-benzphetamine), and 1.44 nmol of 3-hydroxybenzo[*a*]pyrene equivalent formed min^{-1} (mg of protein)$^{-1}$.

The data presented in Figure 6A indicate that *d*-benzphetamine *N*-demethylase and benzo[*a*]pyrene hydroxylase activities of human liver microsomes were inhibited somewhat less than NADPH–cytochrome *c* reductase activity by the antibody. On the other hand, similar studies with the microsomes derived from another individual showed that benzo[*a*]pyrene hydroxylase activity was inhibited to the same extent as NADPH–cytochrome *c* reductase activity but *d*-benzphetamine *N*-demethylase activity was not significantly inhibited (Figure 6B).

Discussion

Human liver NADPH–P-450 reductase was purified to electrophoretic homogeneity in a catalytically active form by using modifications of methods described for other animal species. This appears to be the first such purified human preparation reported. The purified enzyme has an apparent monomer M_r identical with that of the rabbit enzyme and slightly less than that of the rat liver enzyme (Figure 1). A small M_r peptide appears to be essential for reductase activity toward cytochrome P-450 but not cytochrome *c*, as reported previously for the rat (Welton et al., 1973) and rabbit (Black et al., 1979) liver enzymes. This difference in activity has been attributed to a role of the hydrophobic peptide in binding to cytochrome P-450 (Black et al., 1979; Coon et al., 1976; Gum & Strobel, 1979). The apparently intact human liver reductase was about one-third to one-half as active in catalyzing the reduction of either rat or rabbit liver microsomal cytochrome P-450, as were the rat and rabbit liver reductases (Table I). The immunological differences between the high and low monomer M_r forms of the enzyme may be due to either (1) the presence of antigenic sites in the small hydrophobic peptide or (2) changes in the antigenicity of the remainder of the enzyme that occur after the release of the small hydrophobic peptide.

A number of studies indicate that NADPH–P-450 reductases isolated from different species are highly similar in their functional properties (e.g., French & Coon, 1979). Masters et al. (1973) reported that an antibody raised to protease-treated porcine liver NADPH–P-450 reductase (i.e., the major

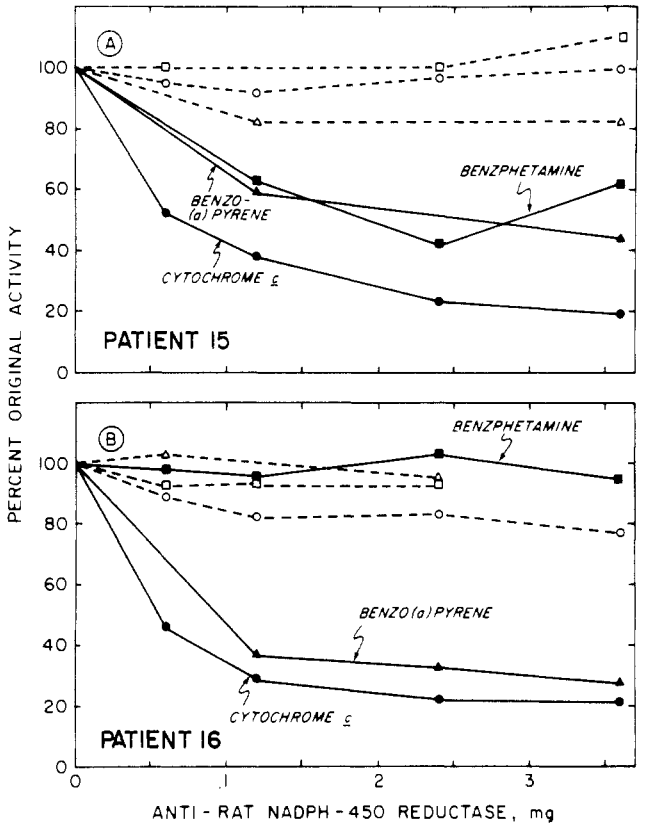

FIGURE 6: Inhibition of NADPH–cytochrome *c* reductase *d*-benzphetamine *N*-demethylase, and benzo[*a*]pyrene hydroxylase in human liver microsomes. Assays were carried out as described under Figure 5 with the exception that 500 μg of microsomal protein [from patient 15 (A) or patient 16 (B)] was used in each determination. The data points represent NADPH–cytochrome *c* reductase (●) [preimmune IgG (○)], *d*-benzphetamine *N*-demethylase (■) [preimmune IgG (□)], and benzo[*a*]pyrene hydroxylase (▲) [preimmune IgG (△)] activities. The respective uninhibited specific activities were 14 nmol of cytochrome *c* reduced, 0.23 nmol of HCHO formed (from *d*-benzphetamine), and 0.23 nmol of 3-hydroxybenzo[*a*]pyrene equivalent formed min^{-1} (mg of protein)$^{-1}$ in (A). The corresponding values in part B were 22 nmol of cytochrome *c* reduced, 0.41 nmol of HCHO formed, and 0.12 nmol of 3-hydroxybenzo[*a*]pyrene equivalent formed min^{-1} (mg of protein)$^{-1}$.

fraction of about M_r 66000) was able to inhibit NADPH–cytochrome *c* reductase activity to varying degrees in microsomes isolated from different species. On the other hand, French & Coon (1979) concluded that NADPH–P-450 reductase was not immunologically species specific. The species specificity of NADPH–cytochrome P-450 reductase was clearly indicated in these studies by double-diffusion analysis (Figure 2). The rabbit enzyme did not produce a precipitin line while the human enzyme produced a line that did not form a pattern of fusion with that observed with the rat liver enzyme. The simplest explanation of the data is that the human reductase contains some, but not all, of the determinants of the rat enzyme. The precipitin patterns of the rat and human reductases were not affected by the excision of the M_r 6000–8000 hydrophobic peptide. Solubilized microsomes derived from different humans produced a pattern of fusion. We have not yet utilized immunological techniques to determine if extensive human interindividual variation exists with regard to this enzyme.

The difference in rat and rabbit NADPH–P-450 reductases was also confirmed in experiments in which the NADPH–cytochrome *c* reductase activity in either purified or microsomal preparations was titrated with antibody raised to the rat liver enzyme (Figures 3 and 4). Human and rat liver reductases also clearly differ (Figure 4). Some variation was observed between microsomal preparations derived from different humans. At the present time, it is unclear whether the immunological differences reflect differences in primary sequence or postranslational modifications. We favor the hypothesis that differences in primary structure are responsible since some differences in the peptide maps of the rat and rabbit liver enzymes have been found (Guengerich, 1978c). If the estimate of Ibrahimi et al. (1979) of 2.5 immunological distance units per amino acid substitution is appropriate here, then the human and rat liver enzymes would differ by approximately 30 residues.

The quantitative micro-C′ fixation data indicated that rabbit and rat NADPH–P-450 reductases were identical (Table II) although 250 times more rabbit antigen was needed to observe this response than in the case of the rat antigen. Prager et al. (1978) have analyzed a similar result obtained with lysozymes and attributed the finding to contamination of individual preparations in the laboratory. This is a possibility here, although the enzymatic activity of the rat antigen was only inhibited 10 times more effectively than was the activity of the rabbit antigen (Figures 3 and 4). Moreover, similar inhibition results were obtained with microsomal preparations, and the possibility of 10% cross-contamination is extremely unlikely. If the micro-C′ fixation results are due to artifacts of contamination, then the inhibitory and C′ fixing antibody populations must be quite unrelated. Prager et al. (1978) have offered other possibilities to explain such a micro-C′ fixation observation. Both the micro-C′ fixation and enzyme inhibition data as well as the double-diffusion data definitely support the hypothesis that the rat and rabbit enzymes can be distinguished immunologically. The fact that the antibodies were raised in rabbits may tend to make the rabbit enzyme seem less related to the rat enzyme than the human enzyme, if rabbits actually screen against determinants present in their own proteins (Reichlin, 1975)

d-Benzphetamine *N*-demethylase activity was inhibited by the antibody to the same extent as NADPH–cytochrome *c* reductase activity in liver microsomes prepared from Aroclor 1254 treated rats (Figure 5). On the other hand, the antibody was much less effective in inhibiting benzo[*a*]pyrene hydroxylase activity in the same preparation.[2] These observations are consistent with the hypothesis that NADPH–P-450 reductase is the rate-limiting component in the mixed-function oxidation of *d*-benzphetamine but not benzo[*a*]pyrene under these conditions. In human liver microsomes, the situation is more complex. Neither *d*-benzphetamine *N*-demethylase nor benzo[*a*]pyrene hydroxylase activity was inhibited by the antibody to the extent that NADPH–cytochrome *c* reductase activity was in microsomes derived from one patient (Figure 6A). In microsomes derived from another patient, benzo[*a*]pyrene hydroxylase was inhibited to the same extent as NADPH–cytochrome *c* reductase activity, but *d*-benzphetamine *N*-demethylase was insensitive to the antibody (Figure 6B). The differences in the ratios of the three activities in the various situations are not instructive as to why the reductase appears to be limiting in some cases but not others. The data indicate that in rats and humans the role of NADPH–P-450 reductase, depending upon the substrate and individual in question, may vary with regard to being a limiting factor in catalysis of P-450-mediated reactions. Alternatively, the results may also be consistent with the view that different P-450s

[2] Glazer et al. (1971) previously reported the inhibition of benzo[*a*]-pyrene hydroxylase activity of rat liver microsomes by an antibody preparation raised to protease-solubilized NADPH–P-450 reductase (i.e., NADPH–cytochrome *c* reductase).

bind to the reductase at different sites, which are not blocked by the antibodies in the same manner.

In summary, the results provide evidence for the species specificity of NADPH–P-450 reductase, at least with regard to rats, rabbits, and humans. However, the meaning of these differences is not clear, as the enzymes isolated from the various sources were all capable of reducing either cytochrome *c* or cytochrome P-450 at fairly similar rates (Table I). On the other hand, differences in the ability of antibody to inhibit *d*-benzphetamine *N*-demethylase activity in rat and human microsomes suggest differences in regulation of overall catalysis, which may be a reflection of properties of the reductase.

Acknowledgments

We thank Drs. R. C. Briggs and L. S. Hnilica for their help in carrying out the microcomplement fixation assays and in photographing the double-diffusion analyses. We also thank Drs. F. Chytil and R. C. Briggs for their advice and for constructively reviewing the manuscript. Drs. J. Edland and M. Arnott were of help in providing human liver autopsy samples. Dr. S. D. Aust kindly furnished bromelain-solubilized NADPH–cytochrome *c* reductase.

References

Alvares, A. P., Bickers, D. R., & Kappas, A. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1321–1325.

Black, S. D., French, J. S., Williams, C. H., Jr., & Coon, M. J. (1979) *Biochem. Biophys. Res. Commun. 91*, 1528–1535.

Coon, M. J., Haugen, D. A., Guengerich, F. P., Vermilion, J. L., & Dean, W. L. (1976) in *The Structural Basis of Membranes Function* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 409–427, Academic Press, New York.

Dean, W. L., & Coon, M. J. (1977) *J. Biol. Chem. 252*, 3255–3261.

Estabrook, R. W., Werringloer, J., Masters, B. S. S., Jonen, H., Matsubara, T., Ebel, R., O'Keefe, D., & Peterson, J. A. (1976) in *The Structural Basis of Membrane Function* (Hatefi, Y., & Djavadi-Ohaniance, L., Eds.) pp 429–445, Academic Press, New York.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

French, J. S., & Coon, M. J. (1979) *Arch. Biochem. Biophys. 195*, 565–577.

French, J. S., Guengerich, F. P., & Coon, M. J. (1980) *J. Biol. Chem. 255*, 4112–4119.

Glazer, R. I., Schenkman, J. B., & Sartorelli, A. G. (1971) *Mol. Pharmacol. 7*, 683–688.

Guengerich, F. P. (1977a) *Mol. Pharmacol. 13*, 911–923.

Guengerich, F. P. (1977b) *J. Biol. Chem. 252*, 3970–3979.

Guengerich, F. P. (1978a) *Biochemistry 17*, 3633–3639.

Guengerich, F. P. (1978b) *J. Biol. Chem. 253*, 7931–7939.

Guengerich, F. P. (1978c) *Biochem. Biophys. Res. Commun. 82*, 820–827.

Guengerich, F. P., & Holladay, L. A. (1979) *Biochemistry 18*, 5442–5449.

Guengerich, F. P., Wang, P., Mitchell, M. B., & Mason, P. S. (1979a) *J. Biol. Chem. 254*, 12248–12254.

Guengerich, F. P., Wang, P., Mason, P. S., & Mitchell, M. B. (1979b) *J. Biol. Chem. 254*, 12255–12259.

Guengerich, F. P., Wang, P., Mason, P. S., & Mitchell, M. B. (1981) *Biochemistry* (preceding paper in this issue).

Gum, J. R., & Strobel, H. W. (1979) *J. Biol. Chem. 254*, 4177–4185.

Ibrahimi, I. M., Prager, E. M., White, T. J., & Wilson, A. C. (1979) *Biochemistry 18*, 2736–2744.

Imai, Y. (1976) *J. Biochem. (Tokyo) 80*, 264–276.

Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308.

Kamataki, T., Sugiura, M., Yamazoe, Y., & Kato, R. (1979) *Biochem. Pharmacol. 28*, 1993–2000.

Knapp, J. A., Dignam, J. D., & Strobel, H. W. (1977) *J. Biol. Chem. 252*, 437–443.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Masters, B. S. S., Nelson, E. B., Schacter, B. A., Baron, J., & Isaacson, E. L. (1973) *Drug Metab. Dispos. 1*, 121–128.

Miwa, G. T., West, S. B., & Lu, A. Y. H. (1978) *J. Biol. Chem. 253*, 1921–1929.

Nebert, D. W., & Gelboin, H. V. (1968) *J. Biol. Chem. 243*, 6242–6249.

Pederson, T. C., Buege, J. A., & Aust, S. D. (1973) *J. Biol. Chem. 248*, 7134–7141.

Prager, E. M., & Wilson, A. C. (1971) *J. Biol. Chem. 246*, 5978–5989.

Prager, E. M., Wilson, A. C., Pĕrin, J.-P., & Jollĕs, P. (1978) *Immunochemistry 15*, 577–583.

Ryan, D. E., Thomas, P. E., & Levin, W. (1977) *Mol. Pharmacol. 13*, 521–532.

Sarich, V. M., & Wilson, A. C. (1966) *Science (Washington, D.C.) 154*, 1563–1566.

Serabjit-Singh, C. J., Wolf, C. R., & Philpot, R. M. (1979) *J. Biol. Chem. 254*, 9901–9907.

Strobel, H. W., & Dignam, J. D. (1978) *Methods Enzymol. 52*, 89–96.

Thomas, P. E., Lu, A. Y. H., Ryan, D., West, S. B., Kawalek, J., & Leven, W. (1976) *Mol. Pharmacol. 12*, 746–758.

Vermilion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 2694–2704.

Wang, P., Mason, P. S., & Guengerich, F. P. (1980) *Arch. Biochem. Biophys. 199*, 206–219.

Welton, A. F., Pederson, T. C., Buege, J. A., & Aust, S. D. (1973) *Biochem. Biophys. Res. Commun. 54*, 161–167.

Yasukochi, Y., & Masters, B. S. S. (1976) *J. Biol. Chem. 251*, 5337–5344.