Purification and Characterization of the Rat Liver Microsomal Cytochrome P-450 Involved in the 4-Hydroxylation of Debrisoquine, a Prototype for Genetic Variation in Oxidative Drug Metabolism†

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ABSTRACT: Genetic polymorphism in oxidative drug metabolism is perhaps best exemplified in the case of debrisoquine 4-hydroxylase activity, where the incidence of deficient metabolism ranges from 1% to 30% in various populations and this defect is also linked to an impaired ability to metabolize a number of other drugs effectively. Sprague-Dawley (SD) rats possess this activity, but females of the DA strain do not, although total cytochrome P-450 (P-450) levels are similar. We have purified, by using debrisoquine 4-hydroxylase activity as an assay, a minor P-450 to electrophoretic homogeneity from male SD rats and designate this as P-450$_{UT-H}$. P-450$_{UT-H}$ differs from eight other purified rat liver P-450s as judged by peptide mapping and immunochemical analysis and thus appears to be isozymic with these other P-450s. P-450$_{UT-H}$ exhibited considerably more debrisoquine 4-hydroxylase activity than any of the other purified P-450s and, on a total P-450 basis, more than total microsomal P-450. Antibodies raised against P-450$_{UT-H}$ specifically recognized P-450$_{UT-H}$ and inhibited more than 90% of the debrisoquine hydroxylase activity present in SD rat liver microsomes. The level of P-450$_{UT-H}$ in SD rat liver microsomes accounted for <10% of the total P-450, as judged by immunochemical quantitation. These assays also indicated that the level of P-450$_{UT-H}$ in female DA rat liver microsomes is only about 5% of that in male or female SD rat liver microsomes, consonant with the view that deficiency of this form of P-450 is responsible for the defective debrisoquine 4-hydroxylase activity in the former animals. The Soret spectrum of P-450$_{UT-H}$ was perturbed by debrisoquine in a manner indicative of binding, and P-450$_{UT-H}$ showed varying levels of activity, in addition to debrisoquine 4-hydroxylase, toward the P-450 substrates phenacetin, propranolol, encainide, 7-ethoxycoumarin, 7-ethoxyresorufin, aniline, 4-nitroanisole, *d*-benzphetamine, ethylmorphine, aminopyrine, dimethylnitrosamine, benzo[*a*]pyrene, (*R*)- and (*S*)-warfarin, androst-4-ene-3,17-dione, and testosterone. Activities were not stimulated by the presence of cytochrome b_5. The results clearly demonstrate the role of a specific P-450 in a model for genetic polymorphism of oxidative drug metabolism, and the biochemical characterization of enzymes provides a rationale for further studies of the basis of such polymorphism.

Several genetic polymorphisms in oxidative drug metabolism within various human populations have been recognized recently through clinical and epidemiological studies (Eichelbaum, 1982; Kalow, 1982). Debrisoquine [3,4-dihydro-2-(1*H*)-isoquinolinecarboxiimidamide] is the first drug for which monogenic control of its oxidative metabolism was established in large population studies (Mahgoub et al., 1977; Tucker et al., 1977). This antihypertensive agent is extensively metabolized in normal individuals to 4-hydroxydebrisoquine, a major urinary metabolite. However, approximately 1–10% of Caucasians have been identified as poor metabolizers of debrisoquine (Eichelbaum, 1982; Kalow, 1982). The frequency of poor metabolizers of debrisoquine in various racial populations ranges from 1 to 30% (Islam et al., 1980; Kalow et al., 1980). Familial studies suggest that the formation of 4-hydroxydebrisoquine in humans is under the control of a single autosomal gene and that impaired hydroxylation is due to a recessive allele at that locus. The impaired metabolic oxidation of at least 14 other drugs with diverse structures and pharmacological actions has been associated with the phenotype of poor debrisoquine metabolism (Lennard et al., 1983): these include sparteine, guanoxan, perhexiline, phenformin, phenacetin, nortriptyline, encainide, and bufuralol.

The biochemical basis for the genetic polymorphism of debrisoquine metabolism has not been established. Oxidative metabolism of most drugs and other xenobiotics proceeds via a microsomal mixed function oxidase system in mammalian liver. Liver microsomal cytochrome P-450 (P-450),[1] which serves as the terminal oxidase for this drug-metabolizing system, exists in multiple forms in both rodent and human livers (Guengerich, 1979; Wang et al., 1983). Poor hydroxylation of debrisoquine among certain individuals is probably due to a missing, aberrant, or low level of a particular P-450 which is responsible for debrisoquine hydroxylation in normal individuals (Boobis et al., 1983). Decreased capacity to hydroxylate debrisoquine is not due to an overall impairment in the oxidative metabolism of xenobiotics, as poor hydroxylators of debrisoquine metabolize antipyrine and other sub-

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[1] Abbreviations: P-450, liver microsomal cytochrome P-450; NaDodSO$_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G; SD, Sprague-Dawley; TLC, thin-layer chromatography. The rationale for the nomenclature used in the designation of individual forms of P-450 is described elsewhere, and comparisons are made to preparations isolated in other laboratories (Guengerich et al., 1982a); in this paper the forms that were previously designated P-450$_{PB/PCN-E}$ and P-450$_{\beta NF/ISF-G}$ are termed P-450$_{PCN-E}$ and P-450$_{ISF-G}$ for convenience.

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strates in a normal manner (Eichelbaum et al., 1983). Further, debrisoquine 4-hydroxylase activity in liver microsomes from poor metabolizers is much lower than that observed in microsomes from extensive metabolizers (Davies et al., 1981). In vitro enzyme activity in normal individuals is NADPH dependent and inhibited by carbon monoxide (Kahn et al., 1982a), suggesting P-450-dependent hydroxylation. However, poor and extensive metabolizers of debrisoquine have comparable levels of total spectrally determined P-450, NADPH–P-450 reductase, and cytochrome b_5 and similar activities toward several typical P-450 substrates (Meier et al., 1982).

An animal model for poor debrisoquine metabolism has recently been identified. Female DA rats have a very low capacity to hydroxylate debrisoquine in vivo and in vitro as compared to male DA rats and several other strains of rats (Al-Dabbagh et al., 1981; Kahn et al., 1982b, 1983). Spectral heme perturbation studies have shown that debrisoquine (as well as sparteine, phenformin, and guanoxan) elicits a so-called "type I" binding spectrum with microsomes from Lewis, SD, and Fischer rats, all of which are capable of hydroxylating debrisoquine in vivo, but not with microsomes from female DA rats (Kupfer et al., 1982). These data suggest that impaired debrisoquine metabolism in this strain is due to decreased amounts of a functional P-450 responsible for debrisoquine hydroxylation in the other strains of rats. Thus, the rat may be a useful model for debrisoquine hydroxylation in humans.

Using techniques developed previously by this laboratory for the purification and immunoquantitation of eight isozymes of rat liver P-450, we have purified an additional isozyme of P-450 to electrophoretic homogeneity from livers of untreated SD rats which is responsible for the 4-hydroxylation of debrisoquine. Immunochemical techniques were used to demonstrate that the biochemical basis of poor debrisoquine hydroxylation in the female DA rat is the decreased level of this P-450 enzyme.

Experimental Procedures

Enzymes. SD rats weighing 200–250 g and obtained from Harlan Industries (Indianapolis, IN) were used unless indicated otherwise. Microsomes from untreated male SD rats were solubilized with sodium cholate, and *n*-octylamino-Sepharose 4B chromatography was carried out as described in detail elsewhere (Guengerich & Martin, 1980; Guengerich et al., 1982b). Fractions were assayed for debrisoquine 4-hydroxylase activity in the presence of NADPH–P-450 reductase and L-α-dilauroyl-*sn*-glycero-3-phosphocholine (Guengerich et al., 1982b). The bulk of the debrisoquine 4-hydroxylase activity was eluted in the 0.5% Lubrol PX fraction. A 50% (w/v) solution of poly(ethylene glycol) 6000 was added to this fraction to give a final concentration of 18% (w/v). The turbid solution was stirred at 4 °C for 20 min and centrifuged for 20 min at $(2 \times 10^4)g$ at 4 °C. The clear supernatant was discarded, and the pellet was gently homogenized in a minimal volume of buffer A [5 mM potassium phosphate (pH 7.4) containing 0.1 mM EDTA, 20% (v/v) glycerol, 0.2% (w/v) Emulgen 911, and 0.5% (w/v) sodium cholate]; this solution was dialyzed for 2 h vs. 20 volumes of buffer A at 4 °C. The dialyzed sample was applied to a 1.2 × 15 cm column of Whatman DE-52 (DEAE-cellulose) which had been equilibrated with buffer A. The column (run at 4 °C) was washed with 100 mL of buffer A and then with 100 mL of buffer A containing 10 mM KCl. A 250-mL linear gradient of buffer A (plus 10 mM KCl) to buffer A containing 10 mM KCl and 200 mM NaCl was applied to the column. Four major hemoprotein peaks (A_{405}) were eluted. The second (buffer A wash) and third (eluted with buffer A plus 10 mM KCl) peaks contained most of the P-450 and a major peptide with an apparent monomeric molecular weight of 52000. Of the four, only the third fraction was found to catalyze debrisoquine 4-hydroxylation. This fraction was applied to a 1.0 × 8 cm column of hydroxylapatite (at 4 °C). The column was washed successively with 90-mL portions of 10, 20, 40, 90, and 180 mM potassium phosphate (pH 7.25) buffers containing 0.1 mM EDTA, 20% (v/v) glycerol, and 0.3% (w/v) Lubrol PX. The only P-450 fraction was eluted with the 90 mM phosphate buffer. This material was dialyzed vs. 10 mM Tris–acetate buffer (pH 7.4) containing 1 mM EDTA and 20% (v/v) glycerol, treated with Bio-Beads SM-2 (Guengerich et al., 1982b), and stored at −20 °C.

NADPH–P-450 reductase, epoxide hydrolase, cytochrome b_5, and P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, P-450$_{PCN-E}$, P-450$_{UT-F}$, and P-450$_{ISF-G}$ were purified as described previously (Guengerich et al., 1982b).

Antisera were raised and treated essentially as described elsewhere (Kaminsky et al., 1981).

Assays. Spectral measurements of P-450 utilized the method of Omura & Sato (1964). Protein concentrations were estimated by using the procedure of Lowry et al. (1951), with correction for detergent levels, or by quantitative amino acid analysis in the case of highly purified fractions (AAA Laboratories, Seattle, WA).

The methods for measuring the catalytic activity toward aniline, *d*-benzphetamine, ethylmorphine, aminopyrine, *N*,*N*-dimethylnitrosamine, and benzo[*a*]pyrene are described elsewhere (Guengerich et al., 1982b). 7-Ethoxyresorufin *O*-deethylase, 7-ethoxycoumarin *O*-deethylase, 4-nitroanisole *O*-demethylase, NADPH oxidase, and NADPH–cytochrome *c* reductase activities were monitored by using continuous assays as described in detail elsewhere (Prough et al., 1978; Guengerich et al., 1982b). All assays were carried out in 0.1 M Tris-HCl buffer (pH 7.6). The products of (*R*)- and (*S*)-warfarin were measured by Dr. L. S. Kaminsky, New York State Department of Health, Albany, NY (Kaminsky et al., 1983). Testosterone and androst-4-ene-3,17-dione hydroxylation products were measured by Dr. D. J. Waxman (Waxman et al., 1983).

Fractions were assayed for debrisoquine 4-hydroxylase activity [after 12-h dialysis and removal of ≥90% of the nonionic detergent with Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, CA) (Guengerich et al., 1982b), assayed as described elsewhere (Garewal, 1973; Goldstein & Blecker, 1975)]. The substrate concentration was 1.0 mM, the P-450 concentration was 50 nM, and the NADPH–P-450 reductase concentration was 100 nM. The incubation time was 15 min at 37 °C. 4-Hydroxydebrisoquine was measured by combined gas–liquid chromatography/chemical ionization–negative ion mass spectrometry using the deuterated analogue as an internal standard (Kahn et al., 1982a). [*ring*-^{3}H]Phenacetin (20 μM, 7.2 mCi/mmol) (added in acetone) was incubated with the enzyme system at 37 °C for 10 min (in a final volume of 0.15 mL). Incubations were analyzed for 4-hydroxyacetanilide essentially as described elsewhere (Guengerich & Martin, 1980) with the addition of 1% (v/v) concentrated NH_4OH to the chromatography solvent system. Propranolol was used at a substrate concentration of 20 μM, the incubation time was 10 min (37 °C), and the products were separated by high-performance liquid chromatography (Nation et al., 1978). Encainide was used at a substrate concentration of 0.2 mM, the incubation time was 10 min, and the products were separated by high-performance liquid chromatography (Wang et al., 1984).

Chemicals. [*ring*-^{3}H]Phenacetin was prepared by ethylation of [*ring*-^{3}H]acetanilide (Garland et al., 1976), and its chemical and radiochemical purity was demonstrated by TLC (vide supra). Debrisoquine, propranolol, and encainide and their metabolites were generously provided by Hoffmann-La Roche (Nutley, NJ), ICI Ltd. (Macclesfield, U.K.), and Mead Johnson Pharmaceuticals (Evansville, IN), respectively. Other chemicals and reagents were as described in previous reports (Guengerich, 1977; Guengerich & Martin, 1980; Guengerich et al., 1982a,b).

Results

Purification of P-450$_{UT\text{-}H}$. Chromatographic techniques were used to purify the P-450 responsible for the 4-hydroxylation of debrisoquine from the livers of untreated male SD rats, and enzyme fractions were monitored for debrisoquine 4-hydroxylase activity (Table I). Hydrophobic affinity chromatography of cholate-solubilized microsomes on an *n*-octylamino-Sepharose 4B column yielded the majority of debrisoquine 4-hydroxylase activity in fractions eluted with a buffer containing 0.5% Lubrol PX; very little enzyme activity appeared in the earlier (0.06%) Lubrol fraction (Guengerich et al., 1982b). The pooled fractions were purified further by chromatography on a Whatman DE-52 column. Debrisoquine 4-hydroxylase activity was found only in the third of four hemoprotein peaks eluted from the column. Fractions from this peak contained a single major polypeptide of apparent monomeric M_r 52 000 as judged by NaDodSO$_4$–polyacrylamide gel electrophoresis. Since these fractions still contained a minor polypeptide contaminant of apparent M_r 30 000, active fractions from the DE-52 column were pooled and applied to a hydroxylapatite column for further purification. However, debrisoquine 4-hydroxylase activity was not enriched further (and actually decreased) by this chromatographic step. Only one hemoprotein peak was eluted from the hydroxylapatite column (90 mM potassium phosphate buffer), but this fraction had lower debrisoquine 4-hydroxylase activity than the pooled DE-52 fraction applied to the column. The minor low molecular weight polypeptide contained in the DE-52 fraction was diminished but not completely removed by this chromatography step as judged by NaDodSO$_4$–polyacrylamide electrophoresis.

Nearly homogeneous fractions from either the DE-52 or the hydroxylapatite column chromatography steps which contained the protein band of apparent M_r 52 000 and also had debrisoquine 4-hydroxylase activity were designated as P-450$_{UT\text{-}H}$. These fractions were used for subsequent electrophoretic, catalytic, and immunochemical studies. The overall yield of P-450$_{UT\text{-}H}$ after the DE-52 chromatography step, on the basis of the total P-450 present in untreated rat liver microsomes, was 1.7%; the overall apparent yield of debrisoquine 4-hydroxylase activity was 11%. A 65-fold purification of debrisoquine 4-hydroxylase activity (on the basis of protein content from microsomes) was achieved in electrophoretically homogeneous preparations. The calculated yields were lower after the hydroxylapatite chromatography step, presumably because of inactivation. (These values provide a rough comparison with microsomes but are not completely accurate because of the addition of optimal levels of reductase to the purified fractions.) The specific contents of P-450$_{UT\text{-}H}$ in the DE-52 and hydroxylapatite-purified fractions were 11.0 and 14.7 nmol/mg of protein, respectively, on the basis of amino acid analysis for determination of protein content. On the basis of subsequent immunochemical estimates of the level of P-450$_{UT\text{-}H}$ in SD rat liver microsomes (vide infra), P-450$_{UT\text{-}H}$ was purified about 140-fold in this procedure.

Table I: Purification of P-450$_{UT\text{-}H}$ from SD Rat Liver Microsomes

step	protein (mg)	P-450 (nmol)	specific content (nmol of P-450/mg of protein)	specific debrisoquine 4-hydroxylase activity [nmol min^{-1} (mg of protein)$^{-1}$]	total debrisoquine 4-hydroxylase activity (nmol/min)	yield (%) on the basis of spectrally determined P-450	yield (%) on the basis of debrisoquine 4-hydroxylase activity
microsomes	4220	4430	1.05	0.48	2030	(100)	(100)
n-octylamino-Sepharose 4B (0.5% Lubrol)	413	690	1.67	1.56	64.3	16	32
DE-52 (buffer A + 10 mM KCl)	7.0	77	11.0	31.4	220	1.7	11
hydroxylapatite (90 mM phosphate)	1.2	17.2	14.7	4.22	5.1	0.4	0.2

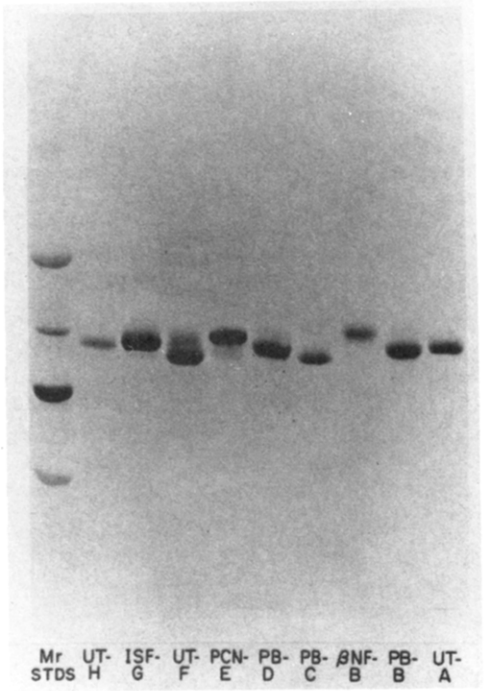

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of rat P-450s. Electrophoresis [7.5% (w/v) acrylamide] was carried out with 1–2 μg of each of the indicated P-450 preparations (Laemmli, 1970). The molecular weight standards ("M_r STDS") included bovine serum albumin (accepted monomeric M_r 68 000), *Escherichia coli* L-glutamate dehydrogenase (M_r 53 000), equine liver alcohol dehydrogenase (M_r 43 000), and carbonic anhydrase (M_r 29 000). The anode was at the bottom of the figure, and the gel was stained with alkaline $AgNO_3$ (Wray et al., 1981).

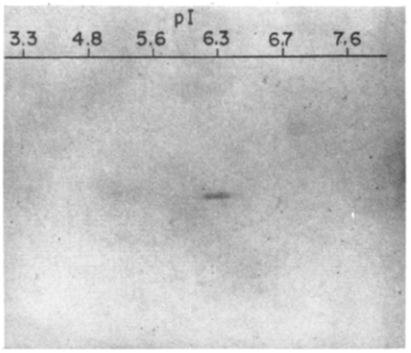

FIGURE 2: Two-dimensional combined isoelectric focusing $NaDodSO_4$–polyacrylamide gel electrophoresis of P-450_{UT-H}. Electrophoresis was carried out by using 5 μg of P-450_{UT-H} (O'Farrell, 1975; Vlasuk & Walz, 1980; Hashimoto et al., 1983). The anode for the isoelectric focusing dimension was to the left of the figure. In the $NaDodSO_4$–polyacrylamide gel electrophoresis dimension, the acrylamide concentration was 7.5% (w/v), and the anode was at the bottom of the figure.

Electrophoretic Characterization. $NaDodSO_4$–polyacrylamide gel electrophoresis of purified P-450_{UT-H} along with samples of the eight different isozymes of P-450 previously isolated from rat liver, as shown in Figure 1, indicated that P-450_{UT-H} is most similar in electrophoretic mobility to P-450_{ISF-G}. By comparison to known molecular weight standards, the molecular weight of P-450_{UT-H} is estimated to be 52 000.

Analysis of P-450_{UT-H} by two-dimensional electrophoresis in a combined isoelectric focusing/$NaDodSO_4$–polyacrylamide gel system is shown in Figure 2. Only one major polypeptide spot was observed.

Peptide mapping of P-450_{UT-H} was carried out by using *Staphylococcus aureus* V8 protease digestion and $NaDodSO_4$–polyacrylamide gel electrophoresis (Cleveland et al., 1977). P-450_{UT-H} was compared with the eight other rat liver P-450 isozymes treated in a similar manner (Figure 3) and was judged to be a distinct polypeptide.

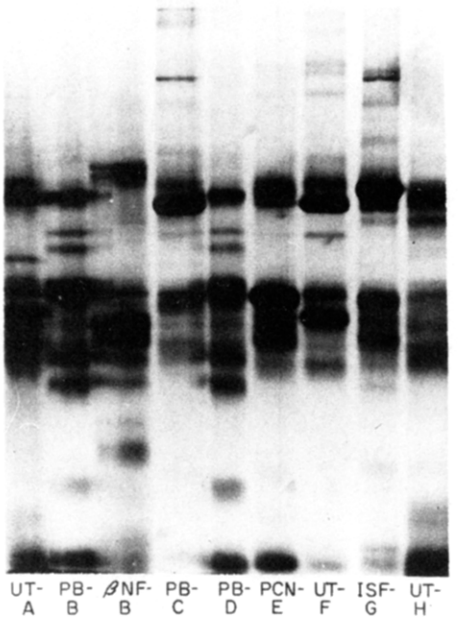

FIGURE 3: Peptide mapping of rat P-450s. Five micrograms of each of the indicated P-450s was treated with *S. aureus* V8 protease, and the peptides were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis [12% (w/v) acrylamide] (Cleveland et al., 1977). The anode was at the bottom of the figure.

Spectral Properties. Debrisoquine was previously shown to elicit a so-called type I spectral change in liver microsomes from SD, Lewis, and Fischer rats but not microsomes from female DA rats (Kupfer et al., 1982). This pattern of spectral change with debrisoquine (characterized by an absorption peak at 385–390 nm and a trough at 420 nm in the difference spectrum) was confirmed in our studies with liver microsomes from untreated male SD rats (Figure 4A) and untreated female DA rats (Figure 4B). With purified P-450_{UT-H}, type I binding spectra with debrisoquine were observed (Figure 4C). The magnitude of the spectral change due to debrisoquine binding to this isozyme was low, but binding was concentration dependent with respect to debrisoquine. The weaker perturbation of purified P-450s by substrates is common and not associated with decreased metabolism (Guengerich, 1983). The subsequent addition of *d*-benzphetamine, a known type I substrate of P-450, produced an enhanced spectral change with P-450_{UT-H} (Figure 4C).

For purified P-450_{UT-H}, the Soret wavelength maximum of the ferrous–carbonyl complex vs. the ferrous difference spectrum was 449.2 nm, as determined by using first-derivative spectroscopy. In the oxidized state, P-450_{UT-H} had a Soret maxima at 417 nm (ϵ = 131 mM^{-1} cm^{-1}), indicative of low-spin iron (Guengerich, 1983). The α and β bands were found at 526 and 569 nm. Purified preparations of P-450_{UT-H} contained no detectable cytochrome P-420 (i.e., <5% of P-450).

Catalytic Activities. None of the other eight P-450 isozymes had more than 5% of the debrisoquine 4-hydroxylase activity displayed by P-450_{UT-H} when all were examined.

In addition to debrisoquine 4-hydroxylase, the catalytic activities toward a variety of substrates were examined by using purified P-450_{UT-H}, male SD microsomes, and female DA microsomes. These comparative enzyme activities are presented in Table II. Relative to the enzyme activities found in microsomes from male SD rats, purified P-450_{UT-H} was

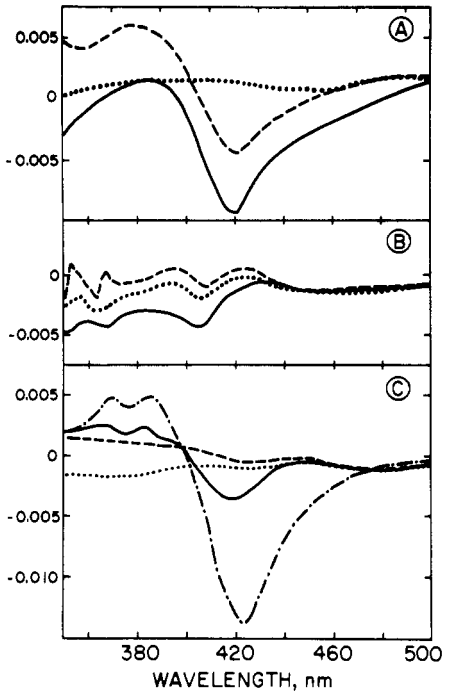

FIGURE 4: Spectral examination of binding of debrisoquine to P-450s. In all experiments, a sample of rat liver microsomes or P-450$_{UT-H}$ (in 0.1 M potassium phosphate buffer, pH 7.4) was divided into two cuvettes, and a base line was recorded (···) in a Cary 219 spectrophotometer (25 °C). Aliquots of a 36 mM aqueous solution of debrisoquine hemisulfate were added to give final concentrations of 17 μM (---) and then 185 μM (—). An equivalent amount of water was added to the reference cuvette in each case. Absorbance is shown on the ordinate at the left. (A) Male SD rat liver microsomes, 2 μM P-450. (B) Female DA rat liver microsomes, 2 μM P-450. (C) Purified P-450$_{UT-H}$, 1.4 μM. The final trace (C) shows the difference spectrum obtained with the subsequent addition of 0.2 mM *d*-benzphetamine hydrochloride (-·-).

particularly active (2-fold or greater enrichment of activity) in catalyzing several enzyme activities. One of these, propranolol *N*-deisopropylase, was also present in female DA rat liver microsomes at a level less than 10% of that observed in microsomes prepared from male SD rats. Other substrate oxidations which both were efficiently catalyzed by P-450$_{UT-H}$ and were present at reduced levels in the microsomes from female DA rats were propranolol 4-hydroxylase, encainide *O*-demethylase, 7-ethoxycoumarin *O*-deethylase, *d*-benzphetamine *N*-demethylase, and aminopyrine *N*-demethylase. P-450$_{UT-H}$ was also active in the N,O-demethylation of encainide, the 4-hydroxylation of aniline, and the N-demethylation of *N,N*-dimethylnitrosamine. For these latter substrate oxidations, however, there were only small differences in the catalytic activities observed in microsomes from male SD rats and female DA rats.

Addition of equimolar amounts of cytochrome b_5 to the reconstituted systems with the purified P-450$_{UT-H}$ either had no effect or decreased catalytic activity (Table II). In a number of other experiments not shown, the addition of cytochrome b_5 never enhanced the debrisoquine 4-hydroxylase activity of P-450$_{UT-H}$ or any of the other purified rat liver P-420s.

The regioselective hydroxylation of the R and S enantiomers of warfarin was examined with purified P-450$_{UT-H}$. With both enantiomers, the primary site of hydroxylation was at the 4-position: 0.77 and 0.92 nmol of this product were formed per min per nmol of P-450$_{UT-H}$ for (*R*)- and (*S*)-warfarin, respectively. Traces of dehydrowarfarin and 6- and 7-hydroxywarfarin were formed from both enantiomers, but at rates <15% of 4′-hydroxylation in all cases.

Table II: Catalytic Activities of Rat Liver Microsomes and Purified P-450$_{UT-H}$

	nmol of product min^{-1} (nmol of P-450)$^{-1}$		
	rat liver microsomes		
assay[a]	SD males	DA females	P-450$_{UT-H}$ (+b_5)[b]
NADPH–cytochrome *c* reductase	150	117	
NADPH oxidase	10.2	12.3	53 (36)
debrisoquine 4-hydroxylase	0.60	0.044	3.5
phenacetin *O*-deethylase	0.10	0.15	0.048 (0.013)
propranolol			
4-hydrolase	1.2	0.11	2.2 (2.7)
N-deisopropylase	3.6	0.27	7.3 (6.1)
glycol formation	<0.04	<0.04	<0.04 (<0.04)
encainide			
N-demethylase	2.7	1.4	3.0
O-demethylase	2.0	0.6	14.2
N,O-demethylase	0.6	0.7	1.3
7-ethoxyresorufin *O*-deethylase	0.054	0.022	<0.005 (<0.005)
7-ethoxycourmarin *O*-deethylase	1.7	0.5	5.2 (0.8)
aniline 4-hydroxylase	0.52	0.42	2.4 (0.6)
4-nitroanisole *O*-demethylase	1.1	0.5	<0.1 (<0.1)
d-benzphetamine *N*-demethylase	10	3.1	19 (3)
ethylmorphine *N*-demethylase	16	4	2.8 (<0.1)
aminopyrine *N*-demethylase	12	4	33 (0.8)
N,N-dimethylnitrosamine *N*-demethylase			
1 mM	0.78	0.86	0.94
100 mM	2.0	1.5	6.0
benzo[*a*]pyrene hydroxylase	0.07	0.03	0.02

[a] See Experimental Procedures for details of assays. All incubations were carried out in duplicate, and values are presented as means. [b] When indicated, cytochrome b_5 was added at a concentration equivalent to that of P-450$_{UT-H}$, and the rates are expressed in parentheses.

The ability of purified P-450$_{UT-H}$ to form various metabolites of androst-4-ene-3,17-dione was also examined. The rate of total metabolism was rather low [<0.5 nmol of total products formed min^{-1} (nmol of P-450)$^{-1}$], with the 7α-, 6β-, 16α-, and 16β-alcohols accounting for most of the products [≤0.1 nmol of each of these products formed min^{-1} (nmol of P-450)$^{-1}$]. Further, only negligible metabolism of testosterone was observed.

Immunochemical Comparison of P-450$_{UT-H}$ with Other P-450 Isozymes. Antisera raised to purified P-450$_{UT-H}$ in rabbits were examined for reactivity toward P-450$_{UT-H}$ and other purified P-450 isozymes, as well as toward other microsomal proteins, by using double-diffusion immunoprecipitin analysis and crossed immunoelectrophoresis. Additionally, antibodies raised to other P-450 isozymes and microsomal proteins were examined for their cross-reactivity with P-450U$_{T-H}$ by immunodiffusion analysis.

In double-diffusion immunoprecipitin experiments, anti-P-450$_{UT-H}$ recognized P-450$_{UT-H}$ but not P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PCN-E}$, P-450$_{UT-F}$, or P-450$_{ISF-G}$ (Figure 5A). Antibodies to P-450$_{UT-H}$ also produced a precipitin line with microsomes from male SD microsomes, forming a pattern of fusion with the precipitin line observed between anti-P-450$_{UT-H}$ and P-450$_{UT-H}$. A weaker precipitin

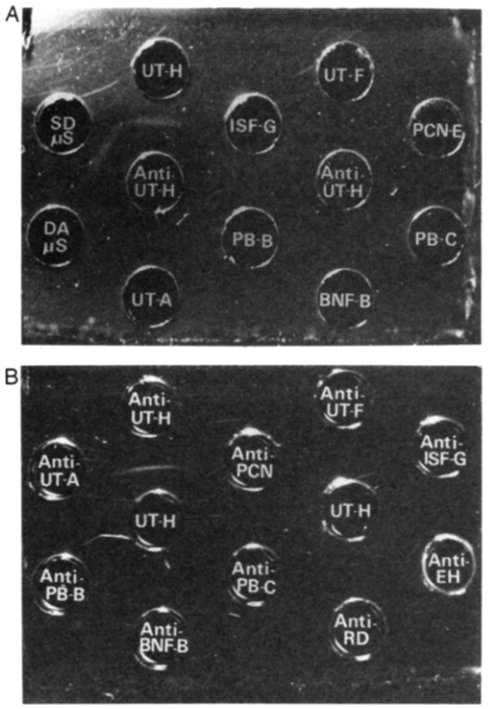

FIGURE 5: Double-immunodiffusion analysis of P-450 immunochemical reactions. (A) Antisera to P-450$_{UT-H}$ were placed in the central wells, and the peripheral wells contained 2–5 μM concentrations of each of the indicated purified P-450s or, alternatively, male (♂) SD or female (♀) DA rat liver microsomes (5 mg of protein/mL) which were solubilized with 1% (w/v) Lubrol PX (Guengerich et al., 1981). (B) P-450$_{UT-H}$ (0.7 μM) was placed in the central wells, and the peripheral wells contained antisera raised to the indicated P-450s. The gels included 0.2% (w/v) Lubrol PX and were photographed over indirect light.

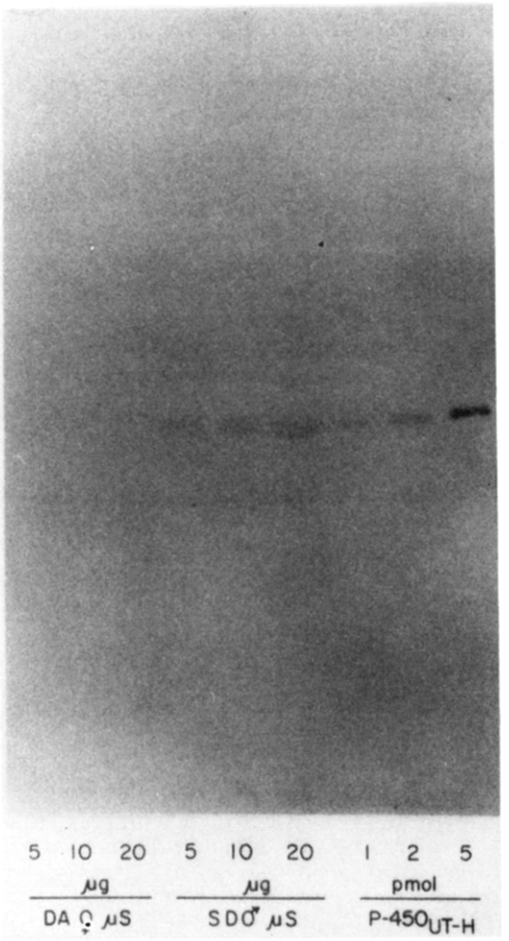

FIGURE 6: Immunoelectrophoresis using anti-P-450$_{UT-H}$ with liver microsomes and P-450$_{UT-H}$. Various amounts of P-450$_{UT-H}$ and microsomal protein from female DA (donated by Dr. A. Küpfer, Bern, Switzerland, and shipped at −70 °C) and male SD rats were electrophoresed in the indicated lanes (Laemmli, 1970). The gel contained 7.5% (w/v) acrylamide, and the nitrocellulose sheet, after the protein transfer (Guengerich et al., 1982a,b), was stained by using a 1/50 dilution of rabbit antisera raised to P-450$_{UT-H}$. The anode was at the bottom of the figure.

line was also detected between microsomes from female DA rats and anti-P-450$_{UT-H}$ under these conditions.

In other immunodiffusion experiments, P-450$_{UT-H}$ was not recognized by any of the antibodies raised to several other isozymes of P-450 and to other purified microsomal enzymes (Figure 5B), including P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PCN-E}$, P-450$_{UT-F}$, P-450$_{ISF-G}$, epoxide hydrolase, and NADPH–P-450 reductase.

The specificity of anti-P-450$_{UT-H}$ toward its homologous antigen was also demonstrated by crossed immunoelectrophoresis (Guengerich et al., 1982a,b). Samples of microsomes or purified microsomal enzymes were electrophoresed in $NaDodSO_4$–polyacrylamide gels, and separated protein bands were transferred to nitrocellulose sheets. The sheets were then treated sequentially with rabbit anti-P-450$_{UT-H}$ and a protein A/horseradish peroxidase complex; protein bands recognized by anti-P-450$_{UT-H}$ were visualized by staining the treated nitrocellulose sheets with 3,3′-diaminobenzidine/H_2O_2. With this technique, anti-P-450$_{UT-H}$ was found to recognize only P-450$_{UT-H}$ among the purified microsomal enzymes examined (Figure 6); no immunoreaction was observed with epoxide hydrolase, NADPH–P-450 reductase, P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, P-450$_{PCN-E}$, P-450$_{UT-F}$, or P-450$_{ISF-G}$. With liver microsomes from male SD rats, anti-P-450$_{UT-H}$ recognized a single protein band of the same apparent molecular weight as purified P-450$_{UT-H}$. However, only a very weak band was recognized by anti-P-450$_{UT-H}$ in liver microsomes from female DA rats.

Having established the specificity of anti-P-450$_{UT-H}$ for P-450$_{UT-H}$, we used crossed immunoelectrophoresis to quantitate the levels of P-450$_{UT-H}$ in microsomes from male SD rats and female DA rats (Figure 6). By comparison of the immunoreactivity of anti-P-450$_{UT-H}$ with P-450$_{UT-H}$ in a standard curve, the level of P-450$_{UT-H}$ in these particular male SD microsomes was 0.065 nmol/mg of microsomal protein. In contrast, microsomes from female DA rats had less than 5% of this amount of P-450$_{UT-H}$ (i.e., 0.003 nmol/mg of protein). Relative to the total spectral P-450 content of these microsomes, P-450$_{UT-H}$ constitutes about 6% and 0.3% of the total P-450 in microsomes from male SD rats and female DA rats, respectively.

Immunoinhibition of Debrisoquine 4-Hydroxylase Activity. The high level of debrisoquine 4-hydroxylation catalyzed by purified P-450$_{UT-H}$ and the extremely low levels of this isozyme in female DA rats which hydroxylate debrisoquine poorly in vivo and in vitro suggest that P-450$_{UT-H}$ is indeed responsible for the majority of debrisoquine 4-hydroxylation in male SD rats. To determine the contribution of P-450$_{UT-H}$ to this microsomal enzyme activity, an IgG fraction prepared from rabbit antisera raised against P-450$_{UT-H}$ was examined for its ability to inhibit debrisoquine 4-hydroxylase catalyzed by purified P-450$_{UT-H}$ and by microsomes prepared from SD rats (Figure 7). In reconstituted systems containing purified P-450$_{UT-H}$, anti-P-450$_{UT-H}$ was able to inhibit debrisoquine 4-hydroxylase activity by >99%. In microsomes from SD rats, anti-P-450 inhibited debrisoquine 4-hydroxylase activity by 90%. Rabbit preimmune IgG fractions had no effect on en-

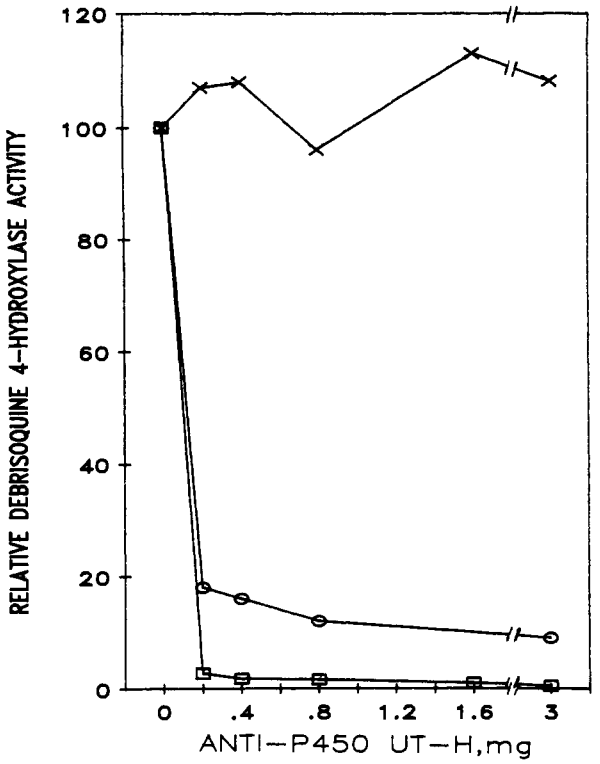

FIGURE 7: Inhibition of debrisoquine 4-hydroxylase activity by anti-P-450$_{UT-H}$. The indicated amounts of the IgG fraction of rabbit anti-P-450$_{UT-H}$ were added to standard incubations containing 50 pmol of P-450$_{UT-H}$ (□), or liver microsomal protein from male SD rats (○) equivalent to 50 pmol of P-450 and debrisoquine 4-hydroxylase activity was measured. The effect of a preimmune IgG fraction on the activity of the rat liver microsomal preparation is also shown (×). Antibodies were incubated with P-450s for 30 min at 23 °C prior to enzyme assay. Activities in the absence of the IgG fractions (100%) for incubations with microsomes and P-450$_{UT-H}$ were 0.342 and 3.01 nmol of 4-hydroxydebrisoquine min^{-1} (nmol of P-450)$^{-1}$, respectively.

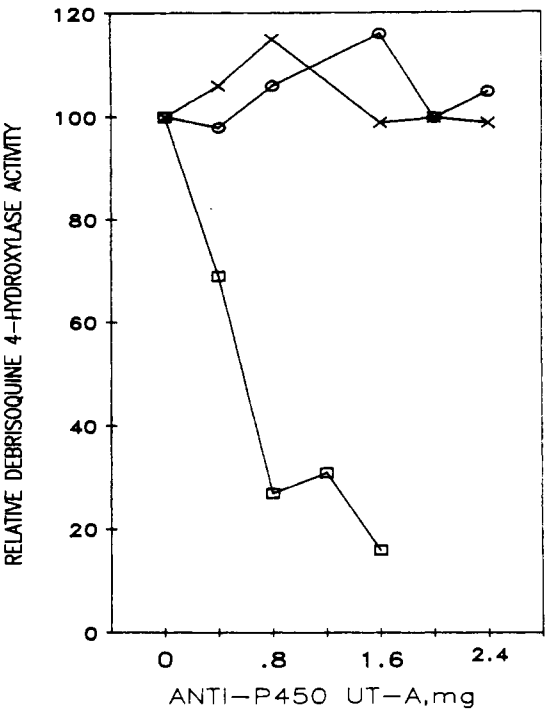

FIGURE 8: Inhibition of debrisoquine 4-hydroxylase activity by anti-P-450$_{UT-A}$. The indicated amounts of the IgG fraction of rabbit anti-P-450$_{UT-A}$ were added to standard incubations containing 50 pmol of P-450$_{UT-A}$ (□), or liver microsomal protein from male SD rats equivalent to 50 pmol of P-450 (○) and debrisoquine 4-hydroxylase activity was measured. The effect of a preimmune IgG fraction on the activity of the microsomal preparation is also shown (×). Antibodies were incubated with P-450s for 30 min at 23 °C prior to enzyme assay. Activities in the absence of the IgG fractions (100%) for incubations with microsomes and P-450$_{UT-A}$ were 0.328 and 0.088 nmol of 4-hydroxydebrisoquine min^{-1} (nmol of P-450)$^{-1}$, respectively.

zyme activity catalyzed by purified P-450$_{UT-H}$ or liver microsomes. Thus, these immunoinhibition experiments indicated that P-450$_{UT-H}$ is responsible for nearly all of the debrisoquine 4-hydroxylase activity found in liver microsomes from SD rats.

Among the eight other isozymes of P-450 examined, P-450$_{UT-A}$ was the only one present at an appreciable level in untreated SD rats that had appreciable catalytic activity toward debrisoquine (ca. 5%). Immunoinhibition experiments were also conducted with an IgG fraction prepared from rabbit antisera raised against P-450$_{UT-A}$, reconstituted systems containing P-450$_{UT-A}$, and microsomes isolated from male SD rats. As shown in Figure 8, anti-P-450$_{UT-A}$ inhibited debrisoquine 4-hydroxylase activity catalyzed by purified P-450$_{UT-A}$. However, anti-P-450$_{UT-A}$ had no effect on this enzyme activity catalyzed by liver microsomes from SD rats. We conclude that P-450$_{UT-A}$, which has a low level of debrisoquine 4-hydroxylase activity in reconstituted systems, does not significantly contribute to this enzyme activity observed in liver microsomes prepared from SD rats.

Inducibility and Developmental Pattern of P-450$_{UT-H}$. To examine the inducibility of P-450$_{UT-H}$ and its associated debrisoquine 4-hydroxylase activity in male SD rats, the levels of P-450$_{UT-H}$ and enzyme activity were determined in liver microsomes from animals pretreated with a group of chemicals known to induce other P-450s (Guengerich et al., 1982b), including phenobarbital, β-naphthoflavone, pregnenolone-16α-carbonitrile, isosafrole, and Aroclor 1254. In these studies, pretreatment of rats with any of these compounds did not increase the hepatic microsomal content of P-450$_{UT-H}$ or the levels of debrisoquine 4-hydroxylase activity. There were no significant differences in the level of P-450$_{UT-H}$ or in debrisoquine 4-hydroxylase activity in microsomes prepared from female and male SD rats.

With antibodies prepared against seven other isozymes of P-450 isolated from livers of male SD rats, levels of each homologous P-450 were immunoquantitated in the liver microsomes from female DA rats and compared to the levels observed in microsomes from male and female SD rats (Table III). Each of these other P-450 isozymes was present in the female DA rat at levels comparable to those in female and male SD rats (P-450$_{UT-A}$ and P-450$_{PCN-E}$ were sex specific in the SD rats).

Finally, the developmental pattern of P-450$_{UT-H}$ was examined in male and female SD rats (Figure 9). In both sexes, levels of P-450$_{UT-H}$ and debrisoquine 4-hydroxylase activity increased up to 8 weeks. In females, both the level of enzyme and the activity then declined. In the males, the enzyme level fell somewhat after 8 weeks while activity increased slightly.

Discussion

Using specific catalytic activity as the basis for microsomal P-450 purification, we purified to electrophoretic homogeneity a minor form of hepatic P-450, designated as P-450$_{UT-H}$, from livers of male SD rats which appears to be responsible for nearly all of the debrisoquine 4-hydroxylase activity observed in microsomes. To the best of our knowledge, no corresponding P-450 preparation has been isolated before. None of the other eight isozymes of P-450 isolated previously from rat liver by this laboratory was particularly active in debrisoquine hydroxylation. Antibodies raised against P-450$_{UT-H}$ inhibited debrisoquine 4-hydroxylase activity both in reconstituted systems with purified P-450$_{UT-H}$ and in microsomes from

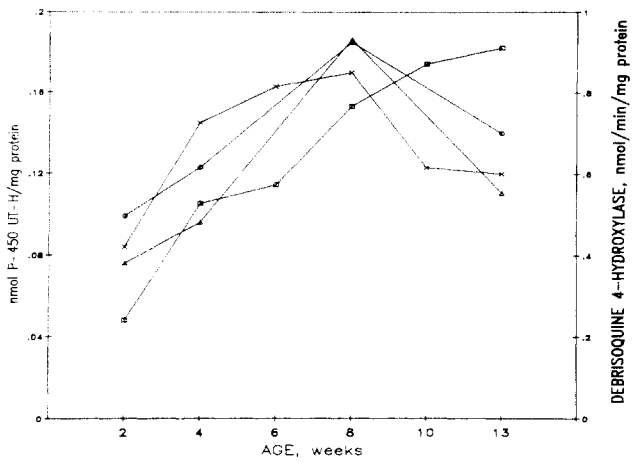

FIGURE 9: Developmental pattern of P-450$_{UT-H}$ and debrisoquine 4-hydroxylase activity. The levels of P-450$_{UT-H}$ in microsomes from male SD (×) and female SD (○) rats of various ages were measured by immunoelectrophoresis as described under Figure 6. Debrisoquine 4-hydroxylase activty of these microsomes from male (□) and female (△) rats was also determined. The rats used in this study were obtained from Charles River Laboratories (Wilmington, MA) and donated by Dr. D. J. Waxman, Harvard University.

untreated male SD rats. By immunoquantitation techniques, we demonstrated that liver microsomes from female DA rats, which are genetically deficient in the capacity to 4-hydroxylate debrisoquine, have extremely low levels of P-450$_{UT-H}$ as compared to the levels of P-450$_{UT-H}$ in microsomes from male and female SD rats. In vitro, debrisoquine 4-hydroxylase activity and immunochemically quantitated levels of P-450$_{UT-H}$ were both present in female DA rat liver microsomes at levels about 5% of those in male or female SD rat liver microsomes. Thus, we have shown that the biochemical basis for impaired hydroxylation of debrisoquine in female DA rats is a decreased level of a specific isozyme of P-450 that is responsible for this drug oxidation in the SD rat. This is the first study to document the association of a genetic impairment of oxidative drug metabolism in rats with the relative lack of a specific isozyme of hepatic P-450 by using such a biochemical approach.

The female DA rat has been suggested as an animal model for individuals of the phenotype for poor debrisoquine hydroxylation among human populations (Al-Dabbagh et al., 1981). From the work presented in this paper, the female DA rat does appear to lack the P-450 isozyme responsible for debrisoquine hydroxylation in normal rats (e.g., SD). Furthermore, P-450$_{UT-H}$ from normal rats is also very efficient in the O-demethylation of encainide. Impairment of this drug oxidation has been associated with the phenotype of poor debrisoquine hydroxylation in humans (Wang et al., 1984). The 4-hydroxylation of propranolol in humans also appears to be under the same genetic control as debrisoquine hydroxylation (Raghuran et al., 1984), and in our studies, the level of propranolol 4-hydroxylase in microsomes from DA rats was <10% of the level in microsomes from SD rats (Table II). Purified P-450$_{UT-H}$ also catalyzed this propranolol oxidation to a greater extent than did microsomes from the SD rat.

Other observations in our investigation, however, indicate that the female DA rat is not a complete model for impaired debrisoquine hydroxylation in humans. First, the rat P-450 isozyme which hydroxylates debrisoquine was not very efficient in phenacetin *O*-deethylation, and microsomes from both the DA rat and the SD rat *O*-deethylated phenacetin at comparable levels (Table II). In another recent study, no differences were observed in microsomal phenacetin *O*-deethylase activity

Table III: P-450 Levels and Debrisoquine 4-Hydroxylase Activity as a Function of Strain and Sex[a]

source of microsomes	debrisoquine 4-hydroxylase activity [nmol min^{-1} (mg of protein)$^{-1}$]	nmol of P-450/mg of protein							
		UT-H	UT-A	βNF-B	PB-B/D	PB-C	PCN-E	UT-F	ISF-G
DA female, untreated	0.023	0.003	0.008	<0.05	0.04	0.24	<0.01	0.11	0.021
SD female, untreated	0.41	0.11	<0.005	<0.05	0.01	0.13	0.02	0.10	0.009
SD male, untreated	0.47	0.11	0.16	<0.05	0.06	0.39	0.22	0.08	0.039

[a] Specific contents of each P-450 isozyme were measured by immunoelectrophoresis (Guengerich et al., 1982a,b). Anti-P-450$_{PB-B}$ recognizes both P-450$_{PB-B}$ and P-450$_{PB-D}$, and the two enzymes were not separated in this study (Guengerich et al., 1982b).

among DA, Lewis, and Fischer rats (Kahn et al., 1983). Clinical pharmacokinetic investigations and studies with human liver microsomes have indicated that the O-deethylation of phenacetin is impaired among poor debrisoquine hydroxylators (Davies et al., 1981; Devonshire et al., 1983). Presumably, the same isozyme of P-450 in human liver catalyzes the oxidation of both phenacetin and debrisoquine. In rats, however, it appears that different P-450 isozymes catalyze the O-deethylation of phenacetin and the 4-hydroxylation of debrisoquine. Further, no sex difference has been reported for debrisoquine hydroxylation in humans. This pattern is in contrast to DA rats, where females are deficient in the enzyme ($P\text{-}450_{UT\text{-}H}$). Our current view is that female DA rats contain the gene coding for $P\text{-}450_{UT\text{-}H}$ but that its expression is impaired by estrogenic steroids or other factors. Further experimentation will be required to verify this hypothesis.

Though the rat may not be a complete model for the genetic polymorphism of debrisoquine hydroxylation in humans, the approach used to purify $P\text{-}450_{UT\text{-}H}$ from rat liver could be used to isolate the analogous isozyme of P-450 from human liver and initiate biochemical studies. Among the six isozymes of human liver purified to date in this laboratory (Wang et al., 1983), none was particularly active in debrisoquine hydroxylation. The P-450 responsible for debrisoquine hydroxylation may be a relatively minor form in human liver, as is the case with $P\text{-}450_{UT\text{-}H}$ in the rat liver. The isolation of the debrisoquine-hydroxylating P-450 and other P-450 isozymes from human liver should be facilitated by using specific catalytic activities as a monitor of progress during purification.

Acknowledgments

We thank Dr. L. S. Kaminsky for the warfarin hydroxylation assays, Dr. D. J. Waxman for the testosterone and androstenedione hydroxylation assays, Dr. J. P. Shea for the synthesis of [*ring*-^{3}H]phenacetin, M. V. Martin, A. McNaron, C. B. McAllister, R. L. Koshakji, and H. T. Wolfenden for technical assistance, Dr. R. A. Branch for helpful discussions, and Dr. A. Küpfer for the female DA rat liver samples.

Registry No. P-450, 9035-51-2; debrisoquine 4-hydroxylase, 83061-52-3; debrisoquine, 1131-64-2; phenacetin, 62-44-2; propranolol, 525-66-6; encainide, 66778-36-7; 7-ethoxycoumarin, 31005-02-4; 7-ethoxyresorufin, 5725-91-7; aniline, 62-53-3; 4-nitroanisole, 100-17-4; *d*-benzphetamine, 156-08-1; ethylmorphine, 76-58-4; aminopyrine, 58-15-1; dimethylnitrosamine, 62-75-9; benzo[*a*]pyrene, 50-32-8; (*R*)-warfarin, 5543-58-8; (*S*)-warfarin, 5543-57-7; androst-4-ene-3,17-dione, 63-05-8; testosterone, 58-22-0.

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