Characterization of Three Highly Purified Cytochromes P-450 from Hepatic Microsomes of Adult Male Rats*

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Three hepatic microsomal cytochromes P-450 (P-450f, P-450g, and P-450h) have been purified to electrophoretic homogeneity from both untreated and ethanol-treated adult male rats. By all criteria examined, the hemoproteins isolated from untreated rats are indistinguishable from the corresponding enzymes purified from rats administered ethanol. Highly purified cytochromes P-450f, P-450g, and P-450h are characterized by minimum M, of 51,000, 50,000, and 51,000, respectively, and unique coordinates in two-dimensional isoelectric focusing-sodium dodecyl sulfate-polyacrylamide gels. The CO-reduced spectral maxima of cytochromes P-450f and P-450g are at 447-448 nm, and the peak of cytochrome P-450h is at 451 nm. Cytochrome P-450h is a versatile catalyst exhibiting high activity toward benzphetamine, hexobarbital, and estradiol-17β and moderate activity toward benzo[a]pyrene and zoxazolamine. In contrast, cytochromes P-450f and P-450h catalyze the hydroxylation of testosterone with different regio- and stereospecificities and overall rates. Both cytochromes P-450f and P-450h catalyze the hydroxylation of testosterone at the 16α-position; however, cytochrome P-450h also oxidizes the steroid at the 2α- and 17β-position (androstenedione formation). Testosterone is oxidatively metabolized at the 6β-, 15α- and an unknown position by cytochrome P-450g.

Peptide maps, generated by proteolytic or chemical digestion of the hemoproteins, indicate that cytochromes P-450f, P-450g, and P-450h differ structurally from each other and five previously characterized rat hepatic microsomal cytochromes P-450 (P-450a, P-450b, P-450c, P-450d, and P-450e). Cytochromes P-450f, P-450g, and P-450h do not react with antibodies directed against these inducible hemoproteins by Ouchterlony immunodiffusion in the presence of detergent; however, in the absence of detergent, cytochrome P-450f cross-reacts weakly with anti-P-450b. Results of this study indicate that rat hepatic microsomal cytochromes P-450 are composed of at least four hemoproteins with CO-reduced absorbance maxima between 447-448 nm. Furthermore, a minimum of four microsomal cytochromes P-450 are now known to 16α-hydroxylate testosterone.

Hepatic endoplasmic reticulum is composed, in part, of a complex population of various cytochromes P-450 which, in conjunction with NADPH-cytochrome c reductase and phospholipid, participate in the metabolism of a multitude of structurally dissimilar endogenous and exogenous substrates (1, 2). The cytochrome P-450 composition of microsomes has long been known to be regulated by factors such as the species, age, and sex of an animal and is subject to induction by numerous compounds. Several different rat hepatic microsomal cytochromes P-450 have been purified and characterized (3-12), including five isozymes (cytochromes P-450a, P-450b, P-450c, P-450d, and P-450e) previously reported by this laboratory (4, 6, 12). Cytochromes P-450b, P-450c, and P-450d refer to the major rat liver hemoproteins induced by phenobarbital, 3-methylcholanthrene, and asafoetide, respectively (4, 6). Cytochrome P-450a is modestly inducible by both phenobarbital and 3-methylcholanthrene, whereas cytochrome P-450e levels appear to be under coordinate regulation with cytochrome P-450b (13, 14). All five of these hemoproteins are inducible in rat liver by the polychlorinated biphenyl mixture Aroclor 1254 (14).

Chronic ethanol treatment of rats has been demonstrated to result in a marked proliferation of hepatic smooth endoplasmic reticulum (15) accompanied by an induction in cytochrome P-450 content associated with a Soret maximum at 451 nm in the CO-reduced difference spectrum as well as an enhancement of microsomal hydroxylation of aniline and oxidation of ethanol (16-22). The microsomal pathway of ethanol oxidation was first reported by Lieber and De Carli, Teschke et al., and Ohnishi and Lieber (16, 19, 21) to be independent of alcohol dehydrogenase and catalase. The SDS2-polyacrylamide gel electrophoretic pattern of microsomal proteins from ethanol-treated rats is distinct from the profiles observed after induction by several other compounds (21, 23). These observations have led to the proposal that ethanol treatment of rats results in the induction of a unique form(s) of cytochrome P-450 unlike those hemoproteins induced by phenobarbital or 3-methylcholanthrene (17, 21, 23).

*These hemoproteins have been designated in a nondescriptive manner as they are purified because of the lack of a single sufficiently specific criterion to differentiate them. The term isozyme has been reserved for those rat hepatic cytochromes P-450 that have been established to have different primary amino acid sequences.

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Ethanol-inducible rat hepatic microsomal cytochrome P-450 has been reported to exhibit high affinity for cyanide (17) and tetrahydrofuran (24) and to generate a reverse type I spectral change upon addition of dimethyl sulfoxide (22). Tschekke et al. and Ohnishi and Lieber (19, 21) reconstituted a cytochrome P-450 fraction with NADPH-cytochrome c reductase and phospholipid that was capable of oxidizing ethanol and other alcohols. Villeneuve et al. (20) reported the partial purification of ethanol-inducible cytochrome P-450 from rat hepatic microsomes with enhanced catalytic activity toward the hydroxylation of aniline. To date, however, a unique cytochrome P-450 isoform selectively induced by ethanol in rat liver has not been purified to homogeneity. In contrast, Koop et al. (25) have purified ethanol-inducible cytochrome P-450LM3a from rabbit hepatic microsomes and showed that this isoform to be clearly distinct from several other rabbit liver cytochromes P-450 by various parameters. Cytochrome P-450LM3a exhibits high catalytic activity toward the hydroxylation of aniline and the oxidation of alcohols to aldehydes (26).

This report describes the characterization of three hepatic microsomal cytochromes P-450 (P-450f, P-450g, and P-450h) that have been highly purified with equivalent recoveries from untreated and ethanol-treated male rats. By several criteria, the hemoproteins isolated from untreated rats are identical with the corresponding cytochromes P-450 purified from hepatic microsomes of rats maintained on a diet containing ethanol. The purification studies suggest but do not provide definitive evidence that ethanol treatment of rats does not result in dramatic changes in the levels of cytochromes P-450f, P-450g, or P-450h although the induction by ethanol of another form(s) of cytochrome P-450 is indicated. Results of additional experiments included in the current investigation suggest sex differences in microsomal levels of cytochromes P-450f and P-450h and provide a protocol for the isolation of a female-specific cytochrome P-450 (P-450f). Several approaches have been used to establish that cytochromes P-450f, P-450g, and P-450h are different hemoproteins. Furthermore, these enzymes are distinct from five inducible cytochrome P-450 isozymes previously purified in this laboratory (4, 6, 12) based on spectral properties, minimum \( M_m \), peptide fragments generated by proteolytic or chemical digestion, immunochemical characteristics, and substrate specificities. A comparison of the properties of cytochromes P-450f, P-450g, and P-450h with other purified rat hepatic isozymes (3–12) indicates that cytochromes P-450f and P-450g have not been previously purified.

**EXPERIMENTAL PROCEDURES**

**Purification of Microsomal Enzymes**

Cytochromes P-450f, P-450g, and P-450h—Fifteen to twenty-five Sprague-Dawley male rats (120–150 g) from Charles River Breeding Laboratories, Wilmington, MA were maintained on a nutritionally adequate liquid diet containing ethanol for approximately five weeks as previously described by Lieber and De Carli (27). Male and female

[Diagram of purification scheme]

**Fig. 1.** Purification scheme for the isolation of cytochromes P-450f, P-450g, and P-450h from rat hepatic microsomes.
Sprague-Dawley rats of equivalent weights fed standard rat chow were also utilized for purification studies. The rats were killed by decapitation, and hepatic microsomes were prepared as reported (28).

The specific content of several microsomal preparations from ethanol-fed rats ranged from 1.22-1.55 nmol of cytochrome P-450/mg of protein. Microsomes prepared from the livers of untreated rats had a specific content of approximately 0.7 nmol of cytochrome P-450/mg of protein.

An outline of the purification of cytochromes P-450f, P-450g, and P-450h is shown in Fig. 1. A microsomal preparation containing 3-4 g of protein (4-6 ml of cytochrome P-450) was diluted (1:1) in a single 0.005 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 911 (Kao-Atlas Ltd., Japan), and 0.5% sodium cholate (Calbiochem-Behring). After homogenization, the preparation was centrifuged for 1 hour at 100,000 × g to remove insoluble material. The supernatant was divided in half, and each portion was applied to a Whatman DE52 column (2.4 × 60 cm) that had been equilibrated at room temperature with 770 ml of 0.005 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 911, and 0.5% sodium cholate. After sample application, each column was washed with 80 ml of equilibration buffer, and protein was subsequently eluted with a linear gradient of 0-0.25 M NaCl in 600 ml of equilibration buffer. The flow rate of the columns was maintained at 0.8 ml/min. Five peaks containing A450 were eluted from each column. The second peak was used for the further purification of cytochromes P-450f and P-450h, whereas the third peak containing 1% Emulgen 911 eluted 16-22% of the cytochrome P-450 applied to the columns is recovered in the second peak, and 4-7% is eluted in the third peak. The appropriate fractions were pooled based on their SDS-polyacrylamide gel electrophoretic profiles.

The fractions containing cytochromes P-450f and P-450h (second peak) were pooled and dialyzed overnight at 4 °C against 6 liters of 0.01 M Tris HCl (pH 7.7) containing 30% glycerol, 0.1 mM EDTA, and 0.1 mM DTT. The sample was brought to room temperature, and a final concentration of 0.25% sodium cholate was added. This preparation was applied to a Whatman DE53 column (2.4 × 60 cm) that had been equilibrated at room temperature with 800 ml of 0.01 M Tris-HCl (pH 7.7) containing 30% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.2% Emulgen 911, and 0.5% sodium cholate and washed (1.5 ml/min) with the same buffer until the first peak of A450 eluted. Cytochromes P-450f and P-450h co-elute with the equilibration buffer, and pooling of the fractions was based on SDS-polyacrylamide gel electrophoresis. The partially purified preparation of cytochromes P-450f and P-450h was subsequently dialyzed for 5 hours at room temperature against 6 liters of 0.01 M potassium phosphate buffer (pH 6.5) containing 20% glycerol, 1.0 mM EDTA, and 0.1 mM DTT and applied to a CM-Sepharose (CL-6B) column (2.2 × 25 cm) equilibrated with 400 ml of the same buffer mixture containing 0.1% Emulgen 911. After sample application, the column was washed with 70 ml of equilibration buffer and eluted (1.2 ml/min) with a gradient of 0-0.25 M NaCl in 500 ml of the same buffer mixture. The chromatographic step effects a separation of the two hemoproteins; cytochrome P-450h elutes as a sharp peak earlier in gradient elution than the broad peak containing cytochrome P-450f. The appropriate fractions were selected based on SDS-polyacrylamide gel electrophoretic patterns.

Residual contaminants in the cytochrome P-450h preparation were subsequently removed by chromatography on a Whatman phosphocellulose column at room temperature. The sample was dialyzed overnight at 4 °C against 5 liters of 0.05 M potassium phosphate buffer (pH 7.7) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT and applied to a phosphocellulose column (2.2 × 6 cm) equilibrated with 150 ml of 0.05 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.05% Emulgen 911. Purified cytochrome P-450h was recovered from the column by stepwise elution with the equilibration buffer and 0.075 M potassium phosphate buffer (pH 7.4) containing the same components. The eluting fractions were analyzed by SDS-polyacrylamide gel electrophoresis prior to pooling the samples.

Detergent was removed from the final purified cytochrome P-450h preparation by phosphatase gel (Bio-Bad) fractionation. The hemoprotein was dialyzed overnight at 4 °C against 6 liters of 0.025 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT. The gel was added to the sample at a ratio of 20 mg/Ag约为。After washing the gel with 25 ml of 0.025 M potassium phosphate buffer (pH 7.4), cytochrome P-450h was eluted with 25 ml of 0.4 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, dialyzed overnight against 3 liters of 0.05 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT, and concentrated by ultrafiltration through an Amicon PM-30 membrane.

The cytochrome P-450f fraction that eluted from the CM-Sepharose column contained the hemoprotein and one contaminant which, fortuitously, was recognized by the antibody used in the purification of cytochrome P-450d (6). Therefore, an immunofinity column (2.2 × 8 cm) containing antibody covalently bound to Sepharose 4B, prepared as previously described (6), was used to remove the remaining contaminating protein. The column was equilibrated with 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.2 mM EDTA, 0.2 mM KC1, 0.2% Emulgen 911, and 0.5% sodium cholate. The following components (final concentrations) were added to the sample prior to application to the column; 0.2 M solid KC1, 0.05 M potassium phosphate buffer (pH 7.4), 0.1% Emulgen 911, and 0.5% sodium cholate. The sample was applied to the column, eluted with the column equilibration buffer at a flow rate of 0.8 ml/min, and judged to be homogeneous by SDS-polyacrylamide gel electrophoresis. The purified cytochrome P-450f preparation was dialyzed, absorbed by calcium phosphate gel to remove detergent, and concentrated by ultrafiltration by the same protocol used for cytochrome P-450h.

Whatman DE53 and CM-Sepharose column chromatography were also used for the further purification of cytochrome P-450g recovered in the third peak of A450 eluting from the Whatman DE52 column. This preparation was dialyzed at room temperature for 4 hours against 6 liters of 0.01 M phosphate buffer (pH 7.4) containing 20% glycerol, 1.0 mM EDTA, 0.1 mM DTT, and 0.1 mM NaCl, and sodium cholate (final concentration 0.25%) was added to the sample. The fraction was applied to a Whatman DE53 column equilibrated at room temperature with 625 ml of 0.01 M Tris-HCl (pH 7.7) containing 30% glycerol, 1.0 mM EDTA, 0.1 mM DTT, 0.2% Emulgen 911, and 0.5% sodium cholate. After washing the column with 70 ml of equilibration buffer, the hemoprotein was eluted as a sharp peak at a linear gradient of 0-0.5 M NaCl in 600 ml of equilibration buffer at a flow rate of 0.8 ml/min. The appropriate fractions were pooled based on SDS-polyacrylamide gel electrophoretic profiles. The partially purified cytochrome P-450g preparation was then dialyzed overnight at 4 °C against 6 liters of 0.005 M potassium phosphate buffer (pH 6.5) containing 20% glycerol and 1.0 mM EDTA, and applied to a CM-Sepharose column (2.2 × 10 cm) equilibrated with 200 ml of the same buffer mixture also containing 0.1 mM DTT and 0.1% Emulgen 911. Purified cytochrome P-450g was recovered from the column (1.0-1.2 ml/min) by stepwise elution by increasing ionic strength of phosphate buffers (0.006, 0.030, and 0.075 M) at pH 6.5 containing 20% glycerol, 1.0 mM EDTA, 0.1 mM DTT, and 0.1% Emulgen 911. Fractions eluting with 0.075 M buffer containing purified cytochrome P-450g were pooled after analysis by SDS-polyacrylamide gel electrophoresis. This purified hemoprotein preparation was dialyzed, treated with calcium phosphate gel to remove detergent, and concentrated by ultrafiltration, as detailed in the final step for the purification of cytochrome P-450h.

The specific contents of several purified cytochromes P-450 (P-450f, P-450g, and P-450h) from either untreated or ethanol-treated rats ranged from 12-17 nmol of cytochrome P-450/mg of protein, and each preparation was apparently homogeneous based on SDS-polyacrylamide gel electrophoresis and two-dimensional isoelectric focusing-SDS-gel electrophoresis. The recovery of each hemoprotein ranged from 0.6-1.1% of total microsomal cytochrome P-450 whether purified from untreated or ethanol-treated rats. The purified enzymes contained 0.02-0.03 mg of Emulgen 911/nmol of cytochrome P-450 based on a purification performed in the presence of [3H]Emulgen 911. Cytochrome P-450f, P-450g, and P-450h were stable for several months when frozen at -90 °C.

Other Methods

Protein was determined by the method of Lowry et al. (31) with crystalline bovine serum albumin as standard. Cytochrome P-450 content was calculated according to the method of Omura and Sato (32) in the CO-reduced difference spectrum based on an extinction coefficient of 9,000 M−1 cm−1 at 415 nm.
coefficient of 91 mM$^{-1}$ cm$^{-1}$. The binding of ethylisocyanide (2.0 mM final concentration) and metyrapone (100 mM final concentration) to ferrous cytochrome P-450 was measured as previously reported (33, 34). The hemoprotein concentrations and buffer components used for spectral determinations are detailed in the appropriate figure legends. SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (35) in a separating gel (7.5% acrylamide) 0.75 mm thick and 10 cm long. The gels were stained with Coomassie blue R-250 and destained as described (6). Cytochromes P-450f, P-450g, and P-450h (2.5 μg each) were subjected to two-dimensional isoelectric focusing-SDS-gel electrophoresis by the protocol of Vlasuk and Walz (36). Limited proteolytic digestion of the eight purified hemoproteins in the presence of SDS was conducted as described by Cleveland et al. (37). The cytochromes P-450 were incubated with the protease for 10 min at 37 °C at protein ratios described in the figure legend. Cyanogen bromide cleavage of the hemoproteins in gel slices was accomplished using the method of Nikodem and Fresco (38). Both the cleavage products and proteolytic digests of the cytochromes P-450 were subjected to SDS-polyacrylamide gel electrophoresis (35) in gels containing 12.5% acrylamide that were 1.5 mm thick and 10 cm long.

Antibodies to purified cytochromes P-450f, P-450g, P-450h, and P-450d were produced, purified, and appropriately absorbed as previously described (39-41). Ouchterlony double diffusion plates were prepared, with and without 0.2% Emulgen 911, as described (40).

Catalytic activity of cytochromes P-450f, P-450g, and P-450h was assayed under conditions in which metabolism was proportional to hemoprotein concentration and time of incubation. In each experiment, other cytochromes P-450 with known activity were included for reference. Saturating amounts of NADPH-cytochrome c reductase and optimal dilaurylphosphatidylcholine were used in all experiments. Dilauroylphosphatidylcholine was prepared in 0.02 M Tris-HCl (pH 8.0) containing 1 mM EDTA and sonicated immediately before use. The following references contain the methods for assays of the various substrates: [N-methyl-$^3$H]benzphetamine (42), [2,4,6-$^3$H]hexobarbital (43, 44), benzo[a]pyrene (45), [4,6-$^3$H]oxygenolamine (46), [2-$^3$H]estradiol-17β (47), 7-ethoxyresorufin (48), p-nitroanisole (49), and testosterone (50). Metabolites of testosterone were analyzed by reverse phase high pressure liquid chromatography (51, 52).

RESULTS AND DISCUSSION

Purification of Cytochromes P-450—Cytochromes P-450f, P-450g, and P-450h were purified from hepatic microsomes of either untreated or ethanol-treated male rats by the protocol outlined in Fig. 1. As detailed under "Experimental Procedures," the first three steps for the isolation of the hemoproteins involved Whatman DE52, DE53, and CM-Sepharose column chromatography at room temperature. In the presence of Emulgen 911 and sodium cholate, cytochromes P-450f and P-450h co-eluted from both anionic exchangers (DE52 and DE53), and higher salt concentrations were required for the elution of cytochrome P-450g. CM-Sepharose chromatography in the presence of Emulgen 911 yielded a separation of cytochromes P-450f and P-450h. Trace contaminants remaining in the cytochrome P-450h preparation were removed by phosphocellulose chromatography in the presence of a nonionic detergent. Fortuitously, antibody used previously in the purification of cytochrome P-450d (6) recognized the contaminant remaining in the cytochrome P-450f preparation and, therefore, immunoabsorption was used as the final step for the purification of cytochrome P-450f. Cytochrome P-450g was essentially electrophoretically homogeneous after CM-Sepharose chromatography. The specific contents of the three purified cytochromes P-450 from either untreated or ethanol-treated rats ranged from 12-17 nmol/mg of protein. Therefore, by the procedure schematically shown in Fig. 1, three cytochromes P-450 can be simultaneously purified from microsomes of adult male rats. Cytochromes P-450f, P-450g, and P-450h were highly purified with equivalent recoveries (0.6-1.1% of total microsomal cytochrome P-450) from hepatic microsomes of untreated or ethanol-treated male rats. This result suggested that microsomal levels of these hemoproteins are not dramatically altered by ethanol treatment of male rats. Purification studies, however, do not provide a direct assessment of relative levels of these hemoproteins in microsomes.

Properties of Cytochromes P-450f, P-450g, and P-450h—Fig. 2A shows the SDS-polyacrylamide gel electrophoretic mobilities of highly purified cytochromes P-450f, P-450g, and P-450h isolated from male rats treated with ethanol. At 0.5-1.0 μg of protein in 0.75-mm thick gels, essentially one protein-staining band was observed in each purified cytochrome P-450 preparation. The electrophoretic mobilities of cytochromes P-450f, P-450g, and P-450h isolated from untreated male rats are directly compared to the corresponding hemoproteins from animals administered ethanol in Fig. 2B. The M, of each of the purified enzymes from untreated rats is identical to the corresponding hemoprotein purified from ethanol-treated rats. The minimum M, of these hemoproteins in the SDS-gel system of Laemmli (35) was calculated to be 51,000 (P-450f), 50,000 (P-450g), and 51,000 (P-450h). Cytochromes P-450f and P-450h have identical electrophoretic mobility in this one-dimensional gel system and are inseparable when mixed together, even at low protein concentrations. The co-migration of cytochromes P-450f and P-450h is reminiscent of the identical electrophoretic mobilities of rat hepatic cytochromes P-450b and P-450d (6).

In Fig. 3, a mixture of the three hemoproteins isolated from ethanol-treated male rats was analyzed by two-dimensional isoelectric focusing-SDS-polyacrylamide gel electrophoresis in the system of Vlasuk and Walz (36) which was designed to optimize the resolution of microsomal proteins. To date, this technique has been shown to be capable of resolving all known rat liver cytochromes P-450 including very closely related...
strain variants (13, 53). As illustrated in Fig. 3, cytochromes P-450f, P-450g, and P-450h are each characterized by unique coordinates which differ from one another and other purified cytochromes P-450 (13, 53). Although cytochromes P-450f and P-450h cannot be separated in the SDS-gel system of Laemmli (35), these two hemoproteins differ markedly in isoelectric points. Cytochrome P-450h tends to streak in the two-dimensional gel system, as do two other rat liver cytochromes P-450f, P-450g, and P-450h, as shown in Fig. 4, however, no protein-staining band corresponding in mobility to cytochrome P-450g was observed in the third peak of the Whatman DE52 elution profiles of microsomal hemoproteins from females that were obtained with male rats with one difference. An additional small peak of \( A_{417} \) eluted intermediate to the regions corresponding to the second and third peaks. When analyzed by SDS-polyacrylamide gel electrophoresis, similar proteins appeared to be present in the second peak of \( A_{417} \) from female and male rats. As shown in Fig. 4, however, no protein-staining band corresponding to cytochrome P-450g was observed in the third peak of \( A_{417} \) eluting from the DE52 column containing solubilized microsomes from female rats. Despite the lack of detectable cytochrome P-450g in the fractions from female rats, many of the same other microsomal proteins were observed when compared to the electrophoretic profile of the corresponding region from male rats. All of the column fractions from the DE52 column were monitored electrophoretically to ensure that cytochrome P-450g had not eluted at a different salt concentration. Therefore, hepatic microsomes from untreated female rats do not appear to contain detectable amounts of cytochrome P-450g.

In contrast, cytochrome P-450f was highly purified with an equivalent recovery from hepatic microsomes of female rats by the described protocol. As illustrated in Fig. 4, electrophoretically homogeneous cytochrome P-450f was obtained; the hemoprotein had the same electrophoretic mobility as cytochrome P-450f isolated from male rats. All of the properties of the enzyme from females were identical to the characteristics of cytochrome P-450f from male rats. Only trace amounts of cytochrome P-450h were isolated from female rats; furthermore, the preparation obtained was significantly contaminated with a protein of slightly smaller minimum Mr (Fig. 4). Therefore, levels of cytochrome P-450h in hepatic microsomes may also be dependent upon the sex of the animal.

As shown in Fig. 4, the contaminant present in the cytochrome P-450h preparation from female rats had the same electrophoretic mobility as cytochrome P-450i, a female-specific hemoprotein. Cytochrome P-450i was purified from the additional peak of \( A_{417} \) which eluted from DE52 column intermediate to the regions corresponding to cytochromes P-450f/P-450h and P-450g. A protein corresponding to cytochrome P-450i was never observed in DE52 elution profiles of microsomal proteins from untreated or ethanol-treated male rats. This female-specific hemoprotein was purified to apparent homogeneity by the protocol described for cytochrome P-450g and was electrophoretically distinct from cytochromes P-450f, P-450g, and P-450h (Fig. 4). Although cytochrome P-450i has not been completely characterized, the CO-reduced difference spectral maximum is at 449 nm, and the minimum Mr has been calculated to be 50,500.

The CO-reduced difference spectra of purified cytochromes P-450f, P-450g, and P-450i, as shown in Fig. 5, illustrate that...
Multiple Forms of Rat Liver Microsomal Cytochrome P-450

**FIG. 5. CO-reduced difference spectra of purified cytochrome P-450f, P-450g, and P-450h.** Each hemoprotein was diluted to 1 nmol/ml in 0.1 M potassium phosphate buffer (pH 7.4) containing 20% glycerol, 0.1 mM EDTA, 0.2% Emulgen 911, and 0.5% sodium cholate. The spectrum of each hemoprotein was generated according to the method of Omura and Sato (32).

The unique substrate specificities of cytochromes P-450f, P-450g, and P-450h are apparent from the results presented in Table II. The turnover numbers listed in the table were obtained with the hemoproteins purified from untreated male rats, whereas the values in parentheses were determined using cytochromes P-450f, P-450g, and P-450h from untreated male rats. Cytochrome P-450f is ineffective at catalyzing the metabolism of benzphetamine, 7-ethoxycoumarin, and hexobarbital (31), although rates of metabolism of certain substrates (e.g. benzphetamine, 7-ethoxytocumarin, and hexobarbital) are slightly higher when catalyzed by cytochrome P-450f compared to cytochrome P-450g. Cytochrome P-450h is an efficient catalyst of the N-demethylation of benzphetamine as well as the hydroxylation of hexobarbital, benzo[a]pyrene, zoxazolamine, estradiol-17β, 7-ethoxytocumarin, or p-nitroanisole. These substrates are also poorly metabolized by cytochrome P-450g, although rates of metabolism of certain substrates (e.g. benzphetamine, 7-ethoxytocumarin, and hexobarbital) are slightly higher when catalyzed by cytochrome P-450g compared to cytochrome P-450f. Cytochrome P-450h is ineffective at catalyzing the metabolism of benzphetamine, hexobarbital, benzo[a]pyrene, zoxazolamine, estradiol-17β, 7-ethoxytocumarin, or p-nitroanisole. These substrates are also poorly metabolized by cytochrome P-450g, although rates of metabolism of certain substrates (e.g. benzphetamine, 7-ethoxytocumarin, and hexobarbital) are slightly higher when catalyzed by cytochrome P-450g compared to cytochrome P-450f. Cytochrome P-450h is an efficient catalyst of the N-demethylation of benzphetamine as well as the hydroxylation of hexobarbital, benzo[a]pyrene, zoxazolamine, and estradiol-17β. Despite this catalytic ver-
Catalytic activity is expressed as nanomoles of product formed/min/nmol of cytochrome P-450 determined under conditions in which metabolism was proportional to hemoprotein concentration and time of incubation. Saturating NADPH-cytochrome c reductase and optimal dialuroylphosphatidylcholine concentrations were used in all experiments. The turnover numbers listed in the table were obtained with the hemoproteins from ethanol-treated rats, whereas the values in parentheses were determined using cytochromes P-450f, P-450g, and P-450h from untreated animals. ND, not detected.

![Table II](https://www.jbc.org/content/124/5/1245)

| Substrate      | Reaction         | P450f | P450g | P450h |
|----------------|------------------|-------|-------|-------|
| Benzphetamine  | N-demethylation  | 1.3   | 1.2   | 52.1  |
| Hexobarbital   | 3-hydroxylation  | <0.5  | 0.7   | 22.6  |
| Benzo[a]pyrene | 3- and 9-hydroxylation | <0.1 | 0.1   | 1.8   |
| Zoxazolamine   | 6-hydroxylation  | 0.9   | 0.2   | 7.4   |
| Estradiol-17β  | 2-hydroxylation  | 0.5   | 0.6   | 8.0   |
| 7-Ethoxycoumarin | O-dealkylation   | 0.2   | 1.1   | 0.9   |
| p-Nitroanisole | N-demethylation  | <0.5  | <0.5  | 1.5   |
| Testosterone   | 2α-OH            | ≤0.15 | ≤0.15 | ND    |
|                | 6β-OH            | ≤0.07 | 2.3   | ND    |
|                | 15α-OH           | ND    | 0.5   | ND    |
|                | 16α-OH           | 0.8   | 2.2   | 7.9   |
|                | Δ5-3OH oxidation (androstenedione) | ND | ND | 2.8 |
| Unknown metabolite | ND | 0.6 | ND | ND |

satellit, 7-ethoxycoumarin and p-nitroanisole are poorly metabolized by cytochrome P-450h. The addition of purified cytochrome b5, at a ratio of 0.8 nmol/nmol of cytochrome P-450, had no effect on the metabolism of p-nitroanisole catalyzed by cytochrome P-450f, P-450g, or P-450h (data not shown). Cytochromes P-450f, P-450g, and P-450h metabolize testosterone with markedly different effec
tivities. Cytochrome P-450f catalyzes the oxidation of testos
terone almost entirely at the 16α-position but at a rate only 10% of that catalyzed by cytochrome P-450h. Three metabolites, 6β-hydroxytestosterone, 15α-hydroxytestosterone, and an unknown product, are formed when cytochrome P-450g catalyzes the metabolism of the steroid. Cytochrome P-450h catalyzes the oxidation of testosterone at the 16α-position but at a rate only 10% of that catalyzed by cytochrome P-450h.
shown that microsomes from untreated rats contain very low levels of cytochromes P-450a, P-450b, P-450c, P-450d, and P-450e (54). As judged by the intensity of corresponding protein-staining bands from ethanol-treated rats, amounts of these inducible hemoproteins do not appear to be markedly altered upon ethanol treatment. Subtle differences in the levels of of cytochromes P-450f, P-450g, and P-450h may, of course, exist.

The microsomal electrophoretic patterns shown in Fig. 7 indicate that ethanol treatment of rats results in the appearance of a protein-staining band (indicated by the arrow) with a mobility intermediate to cytochromes P-450b/P-450d and P-450f/P-450h. The minimum $M_c$ of this protein-staining band is approximately 51,500. Potentially, the induction of a cytochrome(s) P-450 by ethanol may be reflected in the appearance of the 51,500 protein-staining band, since no other unique characteristics of the electrophoretic pattern of microsomes from ethanol-treated rats compared to untreated animals were observed (Fig. 7). Unfortunately, we have not been able to purify this protein to homogeneity, although preliminary results indicate that it is a form of cytochrome P-450. The enhancement of this protein after ethanol treatment was found to be somewhat variable among several different microsomal preparations (Fig. 7, wells 2 and 3). Interestingly, Morgan et al. (22) have proposed a high rate of turnover for ethanol-inducible rat hepatic cytochrome P-450 because the ethanol effect on microsomal aniline hydroxylation disappeared 24 h after the discontinuation of treatment. Morgan et al. (26) and Koop (55) have reported that ethanol has a variable effect on the specific content of cytochrome P-450 and the rate of aniline hydroxylation in rabbit liver microsomes.

Ouchterlony immunodiffusion analyses, as shown in Fig. 8, were used to evaluate possible reactivities of cytochromes P-450f, P-450g, and P-450h with antibodies prepared against cytochromes P-450a, P-450b, P-450c, and P-450d (39–41). The double diffusion plates shown in A, B, and C contained the same components except for the omission of Emulgen 911 from the agarose matrix in the plate shown in C. In A, a mixture of antibodies to cytochromes P-450a and P-450b (center well) was tested against the homologous antigens, cytochrome P-450e, which has been established to be immunochemically identical to cytochrome P-450b (12, 53) and cytochromes P-450f, P-450g, and P-450h in the peripheral wells. There is no detectable reaction of cytochrome P-450f, P-450g, or P-450h with either antibody in this Ouchterlony
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A mixture of antibodies to cytochromes P-450c and P-450d was applied to the center well of the double diffusion plate shown in B; only the homologous antigens reacted with these antibodies. Interestingly, although cytochromes P-450f and P-450g have Soret peaks between 447-448 nm in the CO-reduced difference spectra and exhibit similar ethylisocyanide difference spectra, these hemoproteins are not recognized by antibodies to cytochromes P-450c and P-450d which also exhibit similar absorbance maxima. Based on immunodiffusion analysis in the presence of detergent (Fig. 8, A and B) cytochromes P-450f, P-450g, and P-450h do not react with antibodies against cytochromes P-450a, P-450b, P-450c, or P-450d.

Recently, Thomas et al. (54, 57) have reported that the presence of detergent can partially inhibit immunoprecipitation between an antibody and a weakly cross-reactive heterologous antigen. Therefore cytochromes P-450f, P-450g, and P-450h were tested against the various antibodies in Ouchterlony plates prepared without Emulgen 911. None of the hemoproteins reacted with antibodies to cytochromes P-450a, P-450c, or P-450d in the absence of detergent (data not shown). Neither cytochrome P-450g nor P-450h react with antibody to cytochrome P-450b in immunodiffusion plates prepared without detergent. As shown in Fig. 8C, however, a very weak immunoprecipitin band is observed when cytochrome P-450f is tested against antibody to cytochrome P-450b in the absence of detergent. The immunoprecipitin band observed shows apparent partial identity with that formed by the homologous antigen, which suggests that cytochromes P-450f and P-450b may share some minor antigenic sites. In additional experiments (data not shown), various cytochrome P-450f preparations from both untreated and ethanol-treated rats were applied to adjacent peripheral wells of an immunodiffusion plate lacking Emulgen 911 in which antibody to cytochrome P-450b was added to the central well. The faint immunoprecipitin bands which were produced by the cross-reaction fused in a line of identity indicating that the same antigenic sites were present in all the preparations of cytochrome P-450f. Antibody to cytochrome P-450b was also capable of inhibiting the 16α-hydroxylation of testosterone catalyzed by cytochrome P-450f (data not shown). The presence of cytochrome P-450f, however, did not influence immunoenquantitation of cytochrome P-450b determined by radial immunodiffusion. The results presented in Fig. 8 indicate that cytochromes P-450f, P-450g, and P-450h differ in immunological properties from five inducible rat liver cytochrome P-450 isozymes (P-450a, P-450b, P-450c, P-450d, and P-450e).

The same immunodiffusion experiments were performed with the hemoproteins purified from untreated rats and identical results were obtained (data not shown).

SDS-polyacrylamide gel profiles of the peptides of the eight hemoproteins generated by proteolytic or chemical digestion are shown in Fig. 9. The constituent peptides of each cytochrome P-450 (labeled α-h) were analyzed electrophoretically.

\[\text{Preliminary results have indicated that cytochromes P-450f, P-450g, and P-450h do not react with several monoclonal antibodies to cytochrome P-450c (56).}\]

\[\text{mixture of antibodies to cytochromes P-450a (50 mg/ml) and P-450b (20 mg/ml) was applied to the center well; the center well in B contains antibodies to cytochromes P-450c (10 mg/ml) and P-450d (3 mg/ml); and antibody to P-450b alone (20 mg/ml) was applied to C. In A and B, all of the cytochromes P-450, designated by their subscripts, were present at 5 \(\mu\)M except cytochrome P-450a which was applied at 2.5 \(\mu\)M. In C, in the absence of detergent cytochromes P-450b and P-450f were present at 15 and 5 \(\mu\)M, respectively. Wells containing phosphate-buffered saline are indicated by PBS.}\]
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FIG. 9. Peptide fragments of eight purified cytochromes P-450 generated by proteolytic or chemical digestion. Cytochromes P-450f, P-450g, and P-450h were isolated by the method detailed under "Experimental Procedures." In A and B, each hemoprotein was subjected to limited proteolytic digestion under identical conditions according to the method of Cleveland et al. (37), and 2.8 µg of each cytochrome P-450 were applied to the gels (12.5% acrylamide) in wells labeled according to the cytochrome. A shows the chymotryptic digests at a ratio of 0.05 mg of chymotrypsin/mg of hemoprotein. and chymotrypsin itself was applied to the first well. B illustrates the peptides of the eight cytochromes P-450 generated by S. aureus V8 protease at a ratio of 0.15 mg/mg of hemoprotein. The protease was applied to the last well (V8 prot.). In C, each cytochrome P-450 (10 µg) as labeled was digested in a gel slice by cyanogen bromide, and the peptide fragments were electrophoresed into a second gel (12.5% acrylamide) as described by Nikodem and Fresco (38).

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by each of these four hemoproteins is taken into account, it is evident that there is no detectable cross-contamination of cytochromes P-450b, P-450e, P-450f, and P-450h.

The correlation of forms of rat liver microsomal cytochromes P-450 isolated in different laboratories has been complicated by the lack of a uniform nomenclature system and different approaches to the characterization of these hemoproteins. We surveyed the literature to determine if cytochromes P-450f, P-450g, P-450h, and P-450i correspond to hemoproteins purified earlier by others.

Kamataki et al. (10) have partially purified both a male- and a female-specific cytochrome P-450 from hepatic microsomes of untreated adult Sprague-Dawley rats. Most likely, cytochrome P-450h corresponds to the male-specific form, and cytochrome P-450i is the female-specific form. This identification is based on the similar chromatographic behavior of the proteins, Soret maxima in the CO-reduced difference spectrum, and minimum Mr. Although the substrate specificity of cytochrome P-450i has not been studied in our laboratory, both cytochrome P-450h and the male-specific form have high catalytic activity toward benzphetamine. Kamataki et al. (10) calculated, based on radial immunodiffusion analysis, that the male-specific cytochrome P-450 constituted 16% of total microsomal cytochrome P-450 in the livers of male rats. Results of their immunquantitation (10) indicated an absence of the female cytochrome P-450 in males as well as the lack of the male hemoprotein in females. In our purification studies, no cytochrome P-450i was detected in microsomal preparations from males, and only trace amounts, if any, of cytochrome P-450h was detected in females.

The purification of two forms of rat hepatic microsomal cytochrome P-450 from untreated male rats has been reported by Agosin et al. (3) and Cheng and Schenkenman (7). Based on a composite of the properties of cytochrome P-450 (fraction A) and P-450.8 (fraction B) described by Agosin et al. (3), neither of those hemoproteins correspond to cytochromes P-450f, P-450g, or P-450h. Cytochrome P-450 RLM4 described by Cheng and Schenkenman (7), however, is probably the same hemoprotein as cytochrome P-450h based on minimum Mr, spectral characteristics, and catalytic activity. One difference between these hemoproteins regards the metabolic attack of testosterone. Whereas 2α-hydroxytestosterone was identified as a major metabolite of testosterone catalyzed by cytochrome P-450h (Table II) based on cochromatography with authentic standard, Cheng and Schenkenman (7) reported that 2β-hydroxytestosterone was a major product of testosterone metabolism by cytochrome P-450 RLM4. The method of Shiverick and Neins (58) was used by those investigators for product identification, and van der Hoeven (51) has suggested a possible misassignment of the 2α-product as 2β-hydroxytestosterone by that procedure. Although certain characteristics of cytochrome P-450 RLM4 are similar to cytochrome P-450f, the profile of testosterone metabolites indicates that the two enzyme preparations are different. A significant amount of 7α-hydroxytestosterone is observed when cytochrome P-450 RLM4 catalyzes the metabolism of testosterone (7), but none of this product is observed when catalysis is supported by cytochrome P-450 (Table II).

Furthermore, the CO-reduced difference spectral peak of cytochrome P-450 RLM4 is at 449 nm (7) whereas the absorption maximum of cytochrome P-450f is at 447.5 nm (in contrast, the CO-reduced difference spectral peaks of cytochrome P-450 RLM4 and P-450h are at the same wavelength).

Cytochrome P-450 UT-A purified by Guengerich et al. (9), which has high catalytic activity for benzphetamine and benzo[a]pyrene, metabolizes testosterone at the 2α-, 16α-, and 17β positions, and has low catalytic activity for 7-ethoxycoumarin and p-nitroanisole, corresponds to cytochrome P-450 based on substrate specificity as well as Soret maximum in the CO-reduced difference spectrum (451 nm) and chromatographic behavior. Whereas cytochrome P-450 UT-A was reported (9) to have a minimum Mr, slightly higher than cytochrome P-450 PB-B (P-450h), the minimum molecular weight of cytochrome P-450h is significantly smaller than cytochrome P-450 (Fig. 7). The reason for this discrepancy is unknown.

Based on a variety of criteria, cytochromes P-450f, P-450g, and P-450h do not correspond to rat microsomal cytochrome P-450 inducible by pregnenolone 16α-carbonitrile (5), β-naphthoflavone (11), or clofibrate (8). For example, the three hemoproteins differ chromatographically, spectrally, electrophoretically, and catalytically from the cytochrome P-450 induced by treatment of rats with pregnenolone 16α-carbonitrile (5). Cytochromes P-450f, P-450g, and P-450h have different spectral and catalytic properties from the five cytochromes P-450 purified from β-naphthoflavone-treated rats by Lau and Strobel (11) as well as the hemoprotein induced by clofibrate described by Gibson et al. (8).

In conclusion, cytochrome P-450h apparently corresponds to the male-specific hemoprotein (10), cytochrome P-450 RLM4, and cytochrome P-450 UT-A (9), and cytochrome P-450i is the female-specific form of cytochrome P-450 (10). Based on our survey of the literature, however, neither cytochrome P-450f nor P-450h have previously been purified to electrophoretic homogeneity from rat liver microsomes. The purification procedure reported in this study offers the advantage of the simultaneous purification of three cytochromes P-450 from male rats and permits the purification of an additional form of cytochrome P-450 from female rats.

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