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Neonatal Imprinting and Hepatic Cytochrome *P*-450

I. Comparison of Testosterone Hydroxylation in a Reconstituted System between Neonatally Imprinted and Nonimprinted Rats

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SUMMARY

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Rat hepatic microsomal cytochrome *P*-450s were resolved by DEAE-cellulose column chromatography. A comparison was made between the elution profiles of the cytochrome *P*-450 from the neonatally imprinted (adult male and adult male castrated at 4 weeks of age) and nonimprinted (adult female and adult male castrated at birth) rats. Four peaks of cytochrome *P*-450 (designated peaks I, II, III, and IV) were eluted by a linear salt gradient from 0 to 0.25 M NaCl. No consistent qualitative difference was found in the elution profiles of cytochrome *P*-450 from the solubilized microsomes of these rats. However, further resolution of the catalytic activities of the various peaks of cytochrome *P*-450 in a reconstituted system revealed a form or forms of cytochrome *P*-450 in the peak II fraction that can be imprinted by gonadal hormones during the neonatal period. Only the form of cytochrome *P*-450 isolated from the neonatally imprinted animals was capable of hydroxylating testosterone at the 16α position to a significant degree similar to that reported for the intact microsomes. Phenobarbital treatment enhanced the total as well as the various peaks of cytochrome *P*-450 content in the hepatic microsomes of both adult male and female rats. Cytochrome *P*-450 content in peak III/IV, however, was differentially induced by the phenobarbital treatment. This differentially induced form or forms of cytochrome *P*-450 hydroxylated testosterone in a reconstituted system at the 16α but not at the 7α or 6β positions.

INTRODUCTION

Sex-dependent differences in steroid and drug metabolism in rats have been well documented (1–4). Some aspects of these differences are attributed to androgen imprinting during the neonatal period (5–8). For example, the activities of several steroid-metabolizing enzymes (5–7), the development and the kinetics of some microsomal drug-oxidizing enzyme systems (8), and the relative proportions of certain forms of cytochrome *P*-450 (7) are determined neonatally by androgen imprinting. These sex-dependent differences persist for life even if the adult male host has been deprived of androgenic steroids via castration for a prolonged period of time. Although the exact site(s) of androgen imprinting on the drug-metabolizing enzyme systems has not yet been determined, alterations of the forms of cytochrome *P*-450 have been suggested (7–10). Levin *et al.* (7) showed that the proportion of cytochrome *P*-450 with certain characteristic fast/slow turnover rates is determined during the neonatal period. The typical male characteristic was found in animals castrated at 4 weeks of age, whereas adult male rats castrated at birth resembled that of the females. Similar requirements of neonatal androgen imprinting and the acquisition of 16α-hydroxylase activity in the hepatic microsomes toward testosterone (7), dehydroepiandrosterone (11), and 5α-androstane-3,17-dione (6) were demonstrated in the adult rats. More recently, Kamataki *et al.* (10) observed sex differences in the O-dealkylation of 7-ethoxycoumarin in rat microsomes, and that these differences persist upon the addition of exogenous NADPH–cytochrome *c* reductase or cumene hydroperoxide. These data suggest that the sex-dependent differences in 7-ethoxycoumarin metabolism may reside in different forms of cytochrome *P*-450.

We have previously reported the existence of a sex-dependent and neonatally imprinted form(s) of hepatic microsomal cytochrome *P*-450 (12). This form of cytochrome *P*-450 was obtained by DEAE-cellulose column chromatography. In a reconstituted system, this form of

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cytochrome *P*-450 was capable of hydroxylating testosterone at the 16α position. Furthermore, this enzyme activity was confined primarily to the liver microsomes of the neonatally imprinted rats.

MATERIALS AND METHODS

Adult Wistar rats and pregnant females were purchased from either Canadian Breeding Farm (St. Constant, Quebec) or Microbiological Associates (Bethesda, Md.) and maintained on cottonwood shavings and Purina Laboratory Chow *ad libitum*. In some experiments, adult male and female rats were fed with phenobarbital (1 mg/ml) in their drinking water for a period of 10 days. Where indicated, male rats were castrated either at birth or at the age of 4 weeks according to procedures previously described (8, 9). The nonandrogenized and androgenized male rats represent rats castrated at birth and treated at days 2 and 4 with either 50 μl of corn oil or testosterone propionate (500 μg/day, 10 mg/ml), respectively. Animals were killed by cervical dislocation between the ages of 3 to 4 months and the liver microsomes were isolated by the procedure described previously (8). The microsomal drug or steroid-metabolizing enzyme activities in the androgenized male rats in general resemble those of the male rats castrated at either 3 or 4 weeks of age (7–9). ^{3}H-Labeled cytochrome *P*-450 was obtained from male rats that had been injected with δ-[3,5-^{3}H]aminolevulinic acid (505 mCi/mmol) 1 h prior to sacrifice according to the procedures described previously (9). In brief, animals were injected with δ-aminolevulinic acid (1.87 μmol/kg) that was dissolved in 0.9% NaCl through the jugular vein under ether anesthesia.

Solubilization of hepatic microsomes and fractionation of cytochrome P-450 by DEAE-cellulose column chromatography. Freshly prepared microsomes were solubilized in 10 mM sodium phosphate buffer (pH 7.4) containing 0.5% (w/v) cholic acid, 0.2% (w/v) Emulgen 911 (KAO Atlas, Tokyo), 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol (buffer A) at a ratio of 1 ml/g of liver. The microsomal suspension was allowed to stand at room temperature for 3 to 7 h. This procedure was found to solubilize greater than 90% of the microsomal cytochrome *P*-450 and resulted in no demonstrable proteolytic hydrolysis of the *P*-450 during solubilization (13).

All column chromatography was conducted at room temperature according to the procedures described by Warner *et al.* (13). This procedure has been reported to provide a high yield and better resolution of the cytochrome *P*-450 (13, 25). The solubilized microsomal cytochrome *P*-450 (40–70 ml or 400–700 nmol) was applied to a DEAE-cellulose column (Whatman DE-52, 3 × 30 cm) that had been previously equilibrated with buffer A. The column was then washed with 300 to 400 ml of buffer A; the cytochrome *P*-450 eluted by buffer A was designated peak I. The bulk of *P*-450 that remained on the column was then eluted by 500 ml of buffer A containing a linear NaCl gradient from 0 to 0.25 M (250 ml of buffer A in each reservoir). These eluted fractions were designated peaks II, III, and IV. Since peaks III and IV represent only a small fraction of the total cytochrome *P*-450, these two peaks were designated peak III/IV and were combined for enzymatic activity analysis. Total recovery of *P*-450 by this procedure was greater than 70% of that of the *P*-450 originally present in the solubilized microsomes. The isolated cytochrome *P*-450 peaks were concentrated by membrane ultrafiltration (Amicon) and stored immediately at −20°C. Under these storage conditions, peaks II and III/IV were stable for at least 4 months as judged by their characteristic CO-difference spectra and their catalytic activities against testosterone and benzphetamine in a reconstituted system (see below). Under these same storage conditions, however, peak I was unstable and decomposed to form cytochrome *P*-420 within a few weeks.

Purification of NADPH–cytochrome c reductase activity. Adult rats were treated for 10 days with phenobarbital (1 mg/ml) in their drinking water. Liver microsomes were isolated and solubilized as described above. NADPH–cytochrome *c* reductase was purified from the solubilized microsomes by the procedures described by Yasukochi and Masters (14) with the following modifications. Solubilized microsomes were mixed with a slurry of Whatman DEAE-cellulose at a ratio of approximately 1 g of liver microsome/equivalent g of DE-52. DE-52 was washed with distilled water and equilibrated previously with buffer A. This step and all of the subsequent steps were performed at 2°C. Over 90% of the reductase activity was bound to the DEAE-cellulose after 15 min of mixing. The gel slurry was then centrifuged at 9000*g* for 5 min, the supernatant was decanted, and the pellets were washed three times with buffer A (4 ml/g DE-52). This was followed by three washes of the pellets with 0.2 M NaCl in buffer A (4 ml/g DE-52) to remove the cytochrome *P*-450. The crude NADPH–cytochrome *c* reductase was then recovered from the gel by three washes with 0.3 M NaCl in buffer A (4 ml/g DE-52). This extract was then filtered through Whatman No. 2 paper and concentrated by membrane ultrafiltration (Amicon) to approximately 30 ml. The crude enzyme preparation was dialyzed overnight against 2 liters of a buffer containing 10 mM sodium phosphate (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol (buffer W). The dialyzed sample was then allowed to pass through a 2′,5′-ADP-Sepharose 4B column (0.9 × 12 cm, Pharmacia, Piscataway, N. J.), preequilibrated with buffer W at a flow rate of approximately 0.25 ml/min. The column was then washed with 2 column volumes of buffer W and the purified NADPH–cytochrome *c* reductase was eluted with 15 ml of buffer W containing 1 mM 2′-AMP (Sigma, St. Louis, Mo.). This purified reductase was then dialyzed overnight against 2 liters of buffer W. Total recovery of reductase activity was between 55 and 65%. The purified NADPH–cytochrome *c* reductase has a specific activity of 20,700 units/mg of protein. One unit of enzyme activity is defined as 1 nmol of cytochrome *c* reduced per min according to the method of Phillips and Langdon (15). The purified reductase exhibited one major band on sodium dodecyl sulfate gel electrophoresis when separated according to the method of Laemmli (16).

Removal of detergent from cytochrome P-450 fractions. Since detergents are known to affect the catalytic activities of cytochrome *P*-450 in a reconstituted system, different cytochrome *P*-450 fractions (peaks II and III/

IV) were thawed and subjected to the following treatment prior to use for reconstitution studies. Cholic acid in the *P*-450 samples was removed either by dialysis against buffer W or by repeated washing with buffer W through an ultrafiltration apparatus (Amicon). The thoroughness of cholic acid removal was monitored by the addition of trace amounts of [^{3}H]cholic acid (New England Nuclear, Boston, Mass.) to representative samples. Emulgen 911 was removed by three or four washes with Bio-Beads (SM-2, Bio-Rad, Richmond, Calif.). Since both protein and Emulgen 911 absorbed at the wavelength of 278 nm, the residual Emulgen 911 concentrations were estimated by correcting the contribution of light absorbed by the protein at this wavelength. Light absorption of heme in the purified rat hemoprotein preparation (kindly provided by Dr. A. Lu) at this wavelength accounts for less than 5% that of the respective protein. Residual Emulgen 911 concentrations in samples were then estimated from a standard curve containing Emulgen 911 (0–1%, w/v) in buffer W and were found to be <0.01%. Protein concentrations were determined by the method of Lowry *et al.* (17) using bovine serum albumin as the reference standard.

Reconstitution of testosterone hydroxylase activities. Testosterone hydroxylase activities were reconstituted in the presence of purified NADPH–cytochrome *c* reductase (100 units), cytochrome *P*-450 (0.2 nmol), dilauryl phosphatidyl choline (15 μg, Sedary Research Laboratory, London, Ontario), [1,2-^{14}C]testosterone (160 nmol, 46 mCi/mmol, New England Nuclear, Boston, Mass.), $MgCl_2$ (10 μmol), and NADPH (1 μmol) in a final volume of 1 ml according to the general procedures described by Lu and Levin (18). Testosterone hydroxylase activities were determined as described previously (19) utilizing the method developed by Conney and Schneidman (20). In brief, the incubation was allowed to proceed for 15 min at 35°C in the presence of atmospheric air. The reaction was terminated by the addition of 5 ml of methylene chloride. Testosterone and its metabolites were extracted into the organic phase by constant shaking at room temperature for 30 min. The organic phase was removed, dried under N_2, and redissolved in a small aliquot of ethyl acetate containing 20 μg each of the three testosterone standards (7α, 16α, and 6β). This sample was then separated by either paper chromatography with the solvent system of 2,2,4-trimethylpentane:toluene:methanol:H_2O (3:5:4:1) or thin-layer chromatography (plastic plates coated with silica gel G and fluorescence indicators, Eastman Kodak, Rochester, N. Y.) with the solvent system of chloroform:ethyl acetate:ethanol (4:1:0.4). In some experiments, 2β-hydroxytestosterone (gift of Prof. D. N. Kirk, British Medical Research Council) was also used as the reference standard. The R_f values for 16α-, 2β-, 7α-, and 6β-hydroxytestosterone in the thin-layer chromatographic system were 0.32, 0.40, 0.45, and 0.61, respectively. The identity of these metabolites on the thin-layer chromatography was established by derivatization and recrystallization (21). The R_f values for the reference standards in the paper chromatographic system could not be assessed because the separation requires the solvent system to overrun the paper chromatograms, which usually take 65 h at 8°C (19). The locations of reference standards were detected by an ultraviolet light (254 nm), cut, and counted by a liquid scintillation spectrophotometer (Beckman LS-150) in 6 ml of PCS (Amersham/Searle, Des Plains, Ill.).

Utilizing the above separating procedures, an excellent correlation was found between the paper and thin-layer chromatographic systems with regard to separation of major hydroxylated testosterone metabolites (16α, 7α, and 6β). Although 2β-hydroxytestosterone was not detected on paper chromatograms, it appeared only as a minor product in the present thin-layer chromatographic system. In this regard, the reconstituted enzyme activity of individual cytochrome *P*-450 fractions differed from that of the total extracted *P*-450 as reported recently by Shiverick and Neims (21).

Cytochrome *P*-450 was measured according to the method of Omura and Sato (22). Total protein content in cytochrome *P*-450 samples was determined by the method of Lowry *et al.* (17) with bovine serum albumin as the reference standard. Proteins were routinely precipitated in chilled 0.8 N perchloric acid and redissolved in 1 N NaOH before analyses.

RESULTS

Solubilized microsomal cytochrome *P*-450s from adult male and female rats were separated on DEAE-cellulose columns. Four *P*-450 fractions (peaks I, II, III, and IV) were eluted from the column at NaCl concentrations of 0, 80, 130, and 160 mM, respectively (Fig. 1). Comparing the elution profiles of these microsomal cytochrome *P*-450 preparations reveals no consistent, qualitative sex-dependent differences. Although small sex-dependent differences were observed in the peak III/IV region, further studies using ^{3}H-labeled cytochrome *P*-450 (peak III) isolated from the male rats pretreated with δ-[^{3}H] aminolevulinic acid, combined with the unlabeled cytochrome *P*-450 from peak III of the female rats, failed to show separation between the ^{3}H-labeled and the unlabeled cytochrome *P*-450 when eluted from a DEAE-

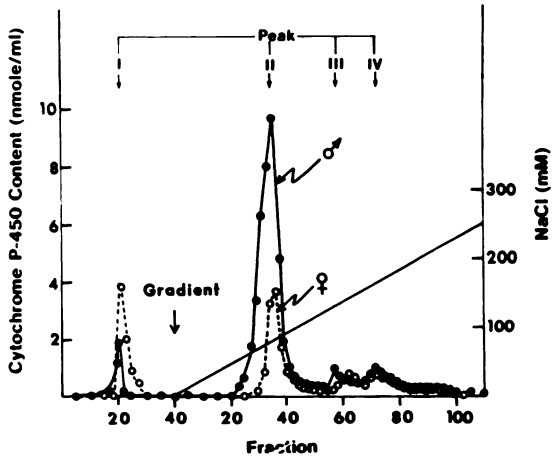

FIG. 1. *DEAE-cellulose elution profiles of the solubilized microsomes isolated from the untreated adult male and female rats*

Hepatic microsomal cytochrome *P*-450 from adult male or female rats solubilized and eluted from DEAE-cellulose column according to Materials and Methods. Four peaks of cytochrome *P*-450, designated peaks I, II, III, and IV, were eluted from the column at NaCl concentrations of 0, 80, 130, and 160 mM, respectively.

cellulose column with a linear NaCl gradient of 50 to 200 mM. These data suggest that these hemoproteins were not distinguishable by column elutions.

The elution profiles of microsomal cytochrome *P*-450 from the androgenized, nonandrogenized, and adult male rats castrated at 4 weeks (28 days) of age are depicted in Fig. 2. In general, the eluting patterns of these preparations were similar to those of the adult male and female rats (see Fig. 1).

The failure to demonstrate certain consistent sex-dependent and neonatally imprinted differences in the cytochrome *P*-450 elution profiles prompted us to search for the possible enzymatic differences in the eluted cytochrome *P*-450 fractions from these rats. Since it was well documented that the 16α-hydroxylase activity in the hepatic microsomes is imprinted by testicular androgens during the neonatal period (6, 7, 11), this enzyme was therefore chosen as a marker for assessing the differences between neonatally imprinted and nonimprinted rats. We have established optimal assay conditions with respect to cytochrome *P*-450 concentrations for several testosterone hydroxylase activities (16α, 7α, and 6β). Figure 3 shows that when peak II isolated from untreated adult male rats was used in the reconstituted system, the hydroxylation of testosterone at the 16α, 7α, or 6β positions was time, *P*-450, and reductase dependent. The increasing concentrations of cytochrome *P*-450 resulting in a slight inhibition of 16α- and 7α-hydroxylase activities may be due to an altered molar ratio of lipid to total contents of cytochrome *P*-450 and reductase in the reconstituted assay system (23). However, the possibility of the presence of trace amounts of detergent in samples resulting in inhibition cannot be excluded. The absolute amount of 6β-hydroxytestosterone formed seemed to vary between experiments, whereas the amount of 16α- and 7α-hydroxytestosterone formed remained rather constant. This is particularly obvious when comparing the

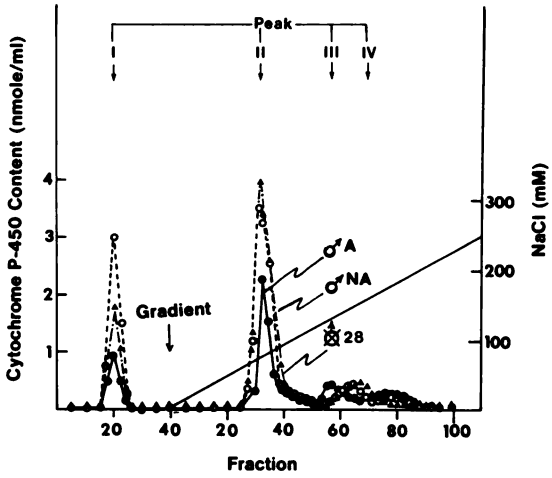

FIG. 2. *DEAE-cellulose elution profiles of the solubilized microsomes isolated from androgenized, nonandrogenized, and adult male rats castrated at 4 weeks of age*

Hepatic microsomal cytochrome *P*-450 isolated from adult male rats neonatally androgenized (♂ A), non-androgenized (♂ NA), or castrated at 4 weeks (⊠ 28) were separated by DEAE-cellulose column chromatography according to Materials and Methods. Note that the elution profiles of these groups of rats are similar to those in Fig. 1.

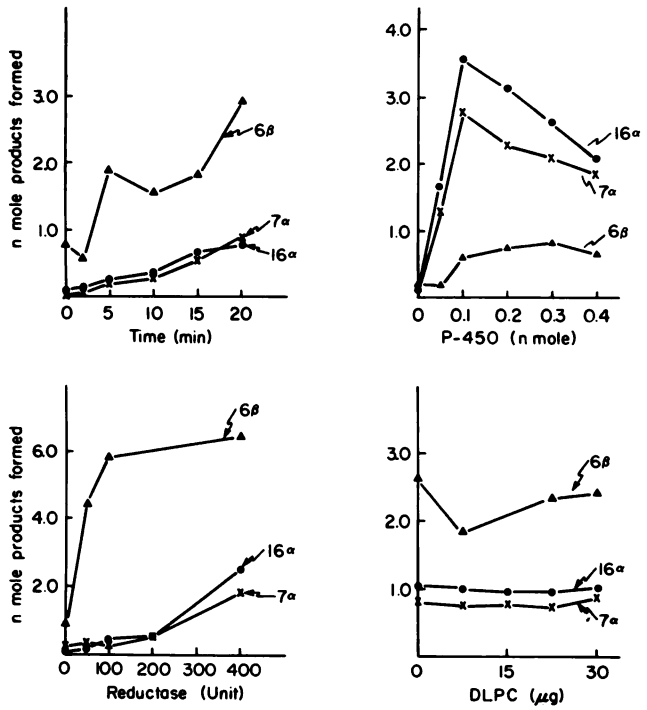

FIG. 3. *Effects of time or concentrations of P-450, reductase, or lipid on testosterone hydroxylase activities in a reconstituted system*

The catalytic activity of the cytochrome *P*-450 in the peak II fraction isolated from adult untreated male rats was reconstituted with NADPH–cytochrome *c* reductase and dilauryl phosphatidyl choline using testosterone as the reacting substrate. The standard assay conditions consist of cytochrome *P*-450 (0.2 nmol), NADPH–cytochrome *c* reductase(100 units), dilauryl phosphatidyl choline (15 μg), [1,2-^{14}C]-testosterone (160 nmol), $MgCl_2$ (10 μmol), and NADPH (1 μmol) in a final volume of 1.0 ml. The reaction is initiated by the addition of NADPH at 35°C and is allowed to proceed for 15 min. At the end of incubation, the reaction is terminated by the addition of 5 ml of methylene chloride to the reaction mixture at 2°C. The effects of time, reductase, and lipid concentrations on the reconstituted enzyme activities are derived from one experiment, whereas the effects of cytochrome *P*-450 concentration are derived from a separate experiment. The formation of 16α-, 7α-, and 6β-hydroxytestosterone was determined according to Materials and Methods. The identity of these steroid metabolites was further confirmed by paper chromatography, derivatization, and recrystallization (see Materials and Methods and Ref. 21).

hydroxylation reaction between varied *P*-450 concentrations or varied time or reductase concentrations (Fig. 3). Low concentrations of dilauryl phosphatidyl choline had little effect on the overall hydroxylation reaction, but high concentrations of this lipid inhibited the hydroxylation reaction. The lack of effect of lipid in the present reconstituted system may be due to the presence of trace amounts of detergent in samples. Subsequently, all reconstituted reactions were performed under conditions of 0.2 nmol cytochrome *P*-450, 100 units NADPH–cytochrome *c* reductase, 15 μg dilauryl phosphatidyl choline, and the reaction was allowed to proceed at 35°C for 15 min. Table 1 shows a comparison of testosterone hydroxylase activities from various cytochrome *P*-450 fractions (peaks II and III/IV) isolated from neonatally imprinted (adult male and male castrated at 4 weeks of age) and nonimprinted (adult female and male castrated at birth) rats. Peaks II and III/IV were both capable of hydrox-

TABLE 1

Reconstituted testosterone hydroxylase activities in two cytochrome P-450 fractions, peaks II and III/IV, isolated from neonatally imprinted (adult male and adult male castrated at 4 weeks) and nonimprinted (adult female and adult male castrated at birth) rats

Conditions[a]	Testosterone hydroxylase activities[b]					
	16α		7α		6β	
	II	III/IV	II	III/IV	II	III/IV
Adult male (5)	2.15 ± 0.59	0.11	0.91 ± 0.14	0.04	3.18 ± 0.76	Nil
Adult female (4)	0.13 ± 0.02	Nil	0.29 ± 0.07	Nil	0.60 ± 0.05	Nil
Adult male castrated at birth (4)	0.12 ± 0.03	0.03 ± 0.006	0.76 ± 0.42	0.02 ± 0.001	1.00 ± 0.33	3.28 ± 1.25
Adult male castrated at 4 weeks (5)	2.02 ± 0.87	0.16 ± 0.06	1.31 ± 0.21	0.08 ± 0.03	3.44 ± 0.37	0.50 ± 0.15

[a] Number in parentheses indicates the total number of experiments from which cytochrome *P*-450 fractions (peaks II and III/IV) were derived. Each experiment usually involves the isolation of cytochrome *P*-450 fractions from 3 to 5 rats. The average specific content of cytochrome *P*-450 was 2.3 ± 0.3 (SE) nmol/mg for peak II and was not determined for peak III/IV. (Using this same isolation procedure, the reported specific content for peaks III and IV were 3.1 and 1.9, respectively; see Ref. 13.)

[b] Testosterone hydroxylase activities were measured by the procedures described in Materials and Methods. Enzyme activities were expressed as nmol products formed/0.2 nmol cytochrome *P*-450/15 min. Values represent average ± SEM or the average of two determinations. Nil indicates nondetectable enzyme activities.

ylating testosterone at the 16α position, with peak II hydroxylating testosterone at a rate 4- to 20-fold higher than that of peak III/IV. The 16α-hydroxylase activity in peak II from the imprinted rats was consistently higher (17-fold) than that from the nonimprinted animals (Table 1). 7α-Hydroxylase activity, however, failed to show similar imprinting-related differences, whereas 6β-hydroxylase activity exhibited differences to a lesser degree in this regard. These data correspond well with data obtained from the intact microsomes (7). The large difference between neonatally imprinted and nonimprinted rats with respect to 16α-hydroxylase activity was not accountable by the possible sex difference of NADPH–cytochrome *c* reductase since similar results were obtained when NADPH–cytochrome *c* reductase isolated from either male or female rats was used.

Phenobarbital (PB) treatment increased total hepatic cytochrome *P*-450 content approximately two- to three-fold in both male and female rats. This is reflected in the overall increases of all fractions in cytochrome *P*-450 content (Figs. 1 and 4). The peak III/IV region was differentially induced by PB treatment from less than 5% in the untreated adult to about 25% in the PB-treated animals. PB treatment, however, did not alter the elution profiles of various cytochrome *P*-450 fractions from a DEAE-cellulose column. This differential induction of cytochrome *P*-450 content in this peak area by phenobarbital, 3-methylcholanthrene, or β-naphthoflavone pretreatment in adult or weanling *male* rats has recently been reported by Warner and Neims (24) and Robbins and Mannering (25). The percentage of increase in peak III/IV reported by these investigators differed from the present report. This may be attributed to differences in the experimental conditions such as the age of the rats and the dose of inducers administered.

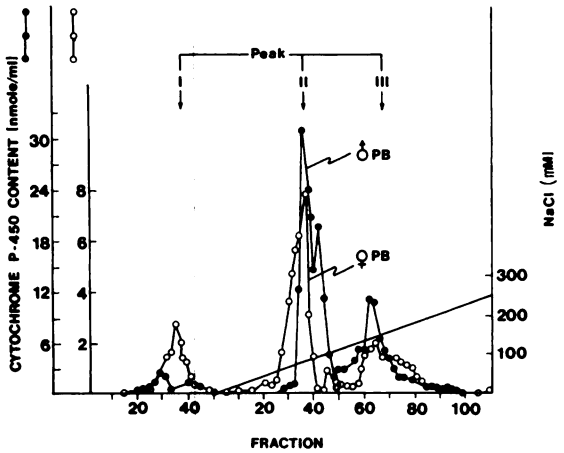

FIG. 4. *DEAE-cellulose elution profiles of the solubilized microsomes isolated from phenobarbital-treated male or female rats*

Adult male or female rats were treated with phenobarbital for 10 days. Liver microsomes were isolated, solubilized, and separated by DEAE-cellulose column chromatography according to Materials and Methods. Note that the cytochrome *P*-450 in the peak III/IV region was differentially induced by phenobarbital treatment as compared to the untreated rats (see Fig. 1).

This PB-induced cytochrome *P*-450 (peak III/IV) was subjected to similar reconstitution analysis as that described in Fig. 4. This form of cytochrome *P*-450 appeared to hydroxylate only testosterone at the 16α position in a time-, *P*-450-, and reductase-dependent manner (Fig. 5). Under these assay conditions, an insignificant amount of 7α- or 6β-hydroxytestosterone was formed. PB treatment, however, did not alter the relative catalytic efficiency of this form of cytochrome *P*-450 with respect to 16α-hydroxytestosterone formation (see Table 1).

DISCUSSION

Some aspects of the hepatic microsomal metabolism of drugs and steroids in the male rats are known to be imprinted or programmed by gonadal hormones during the neonatal period (5–8). Hepatic cytochrome *P*-450 is also known to exist in many forms and appears to determine substrate specificities in a reconstituted system. This study focused on resolving the possible differences of cytochrome *P*-450 as a result of sexual differentiation of hepatic microsomal enzyme systems during the neonatal period. Although no qualitative difference in the column elution profiles of cytochrome *P*-450 can be found between the neonatally imprinted and nonimprinted rats, reconstitution of the resolved forms of cytochrome *P*-450 reveals that there is a form(s) of cytochrome *P*-450 in the peak II fraction that may be imprinted by the gonadal

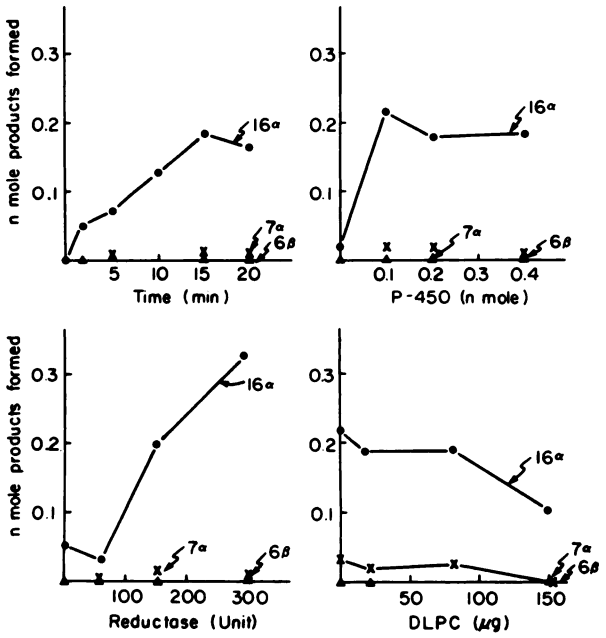

FIG. 5. *Testosterone hydroxylase activities (16α, 6β, and 7α) in the cytochrome P-450 fraction isolated from peak III/IV of phenobarbital-treated male rats*

Effects of time and concentrations of *P*-450, reductase, or lipid.

hormones during the neonatal period. This imprinted form of cytochrome *P*-450 is responsible for the hydroxylation of testosterone at the 16α position later in adult life. Multiplicity of cytochrome *P*-450 for testosterone hydroxylation has been demonstrated previously by several laboratories (7, 21, 26). Recently, Kremers *et al.* (27) demonstrated that multiplicity even exists within steroid-16α-hydroxylase systems when testosterone, progesterone, dehydroepiandrosterone, or pregnenolone is used as substrate. In the present study, we have observed that both peak II and peak III/IV are capable of hydroxylating testosterone at the 16α position, albeit at vastly different turnover rates. Interestingly, this enzyme activity in both peak II and peak III/IV followed closely the imprinting states of the animals, i.e., higher activity in the neonatally imprinted than the nonimprinted animals. Whether this neonatally imprinted form of hepatic cytochrome *P*-450 in peak II or III/IV represents one or several molecular species awaits further study.

In the present study, it is possible that the 16α-hydroxylase activity observed in peak III/IV may be due to contamination from peak II during the elution procedure. However, this possibility seems unlikely since peaks II and III/IV can be rechromatographed identically on a DEAE-cellulose column (13). Furthermore, peak III/IV, but not peak II, is differentially induced by PB in both imprinted and nonimprinted adult rats.

The use of reconstituted systems for studying the developmental aspects of microsomal testosterone hydroxylation *in vivo* is particularly significant since it has been demonstrated that the temporal relationship of 16α- and 7α-hydroxylase activities in the immature and adult microsomal suspensions can be replicated in a reconstituted system (4, 21). In the present study, we have demonstrated in a reconstituted system that the imprinted testosterone 16α-hydroxylase activity in the hepatic microsomes of the male rats resides primarily in the peak II fraction. The pattern of imprinting of this enzyme activity is in agreement with the results previously reported by Levin *et al.* on the neonatal imprinting of the 16α- and 6β-hydroxylase activities in the intact hepatic microsomes (7). To further evaluate the forms of cytochrome *P*-450 in this peak, we have attempted to purify this form of *P*-450, characterized its reconstituted enzyme activities, and identified these hemoproteins by polyacrylamide gel electrophoresis. Results of these studies will be reported elsewhere (28).

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