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Neonatal Imprinting and Hepatic Cytochrome P-450

Immunochemical Evidence for the Presence of a Sex-Dependent and Neonatally Imprinted Form(s) of Hepatic Cytochrome P-450

HAIYEN CHAO[1] AND LELAND W. K. CHUNG[1,2]

Pharmacology/School of Pharmacy, University of Colorado, Boulder, Colorado 80309, and Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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SUMMARY

Some aspects of the sexual differences in hepatic microsomal drug and steroid metabolism in adult rats are imprinted by adrogenic steroids during the neonatal period. This study provides immunochemical evidence to support further the concept that neonatal androgen imprints or programs a form(s) of hepatic cytochrome P-450, which in turn regulates the major imprintable differences of drug and steroid metabolism in adult life. This concept is based on the following experimental results. Cytochrome P-450 partially purified from the neonatally androgen-imprinted animal expresses imprintable enzyme activity (testosterone 16α-hydroxylase) in a reconstituted assay system; the expression of non-imprintable enzyme activities, such as testosterone 7α-hydroxylase, testosterone 6β-hydroxylase, and benzphetamine *N*-demethylase, failed to show a similar correlation with imprinting state of the animal. Immunoglobulin G (IgG), isolated from rabbits immunized with partially purified cytochrome P-450 from liver microsomes of adult male rats, inhibited the activity of neonatally androgen-imprintable testosterone 16α-hydroxylase in both intact microsomes and the reconstituted system. Immunoadsorption data suggest that the IgG fraction contained a specific class of immunoglobulin molecules that react only with cytochrome P-450 isolated from the neonatally androgen-imprinted animal. Polyacrylamide gel electrophoretic patterns of partially purified cytochrome P-450 from neonatally androgen-imprinted and non-imprinted rats revealed that the imprintable form of cytochrome P-450 has an apparent molecular weight of 50,000. The neonatally imprintable form of hepatic cytochrome P-450 appeared to have a rather slow rate of turnover. No significant *in vivo* incorporation of ^{14}C-labeled-δ-aminolevulinic acid into this hemoprotein was observed during a 48-hr period.

INTRODUCTION

Hepatic monooxygenases that metabolize a large number of drugs and steroids in rats show sexual dimorphism during postnatal development (1–4). Some aspects of these differences are determined by circulating gonadal hormones. Castration of the male or testosterone administration to the female during adulthood eliminates these sexual differences in metabolism. However, other aspects of these differences are determined by androgen imprinting during the neonatal period (5–8). In adulthood, expression of these neonatally androgen-imprinted or programmed differences of metabolism is insensitive to depletion or replacement therapy of postpubertal sex steroids (5–8). We have been interested in the molecular basis of neonatal imprinting, particularly the possibility that hepatic cytochrome P-450[3] may be the primary target for androgen imprinting during the neonatal period (previous inquiries into this phenomenon, however, were limited by technical considerations). Recent methodologies which allow effective isolation and purification of hepatic cytochrome P-450 from untreated rats (9–11) and rabbits (12, 13), in combination with the reconstituted assay system and immunochemical approaches, have extended our capability of identifying the representative androgen-imprinted form(s) of cytochrome P-450 and its associated enzyme activities.

The presence of multiple forms of cytochrome P-450 in hepatic microsomes has been demonstrated by genetic (14, 15), inductive (16, 17), developmental (4, 7, 18, 19),

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[1] University of Colorado, Boulder.

[2] University of Colorado Health Sciences Center, Denver.

[3] Cytochrome P-450 within the context of this paper describes a heterogeneous group of hemoproteins that exhibits a characteristic CO-difference spectrum with λ_{max} at 450 nm.

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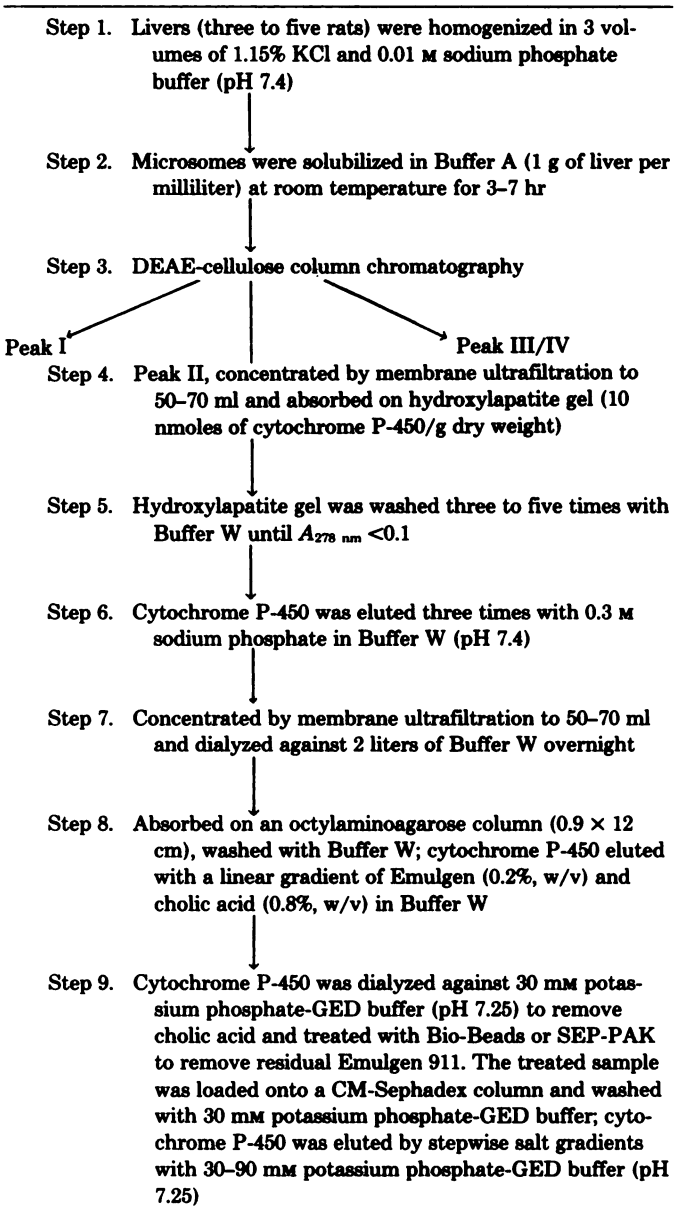
and inhibitory (20, 21) studies. Vigorous biochemical and immunochemical means have documented differences between various inducible forms of cytochrome P-450. Immunochemical data further suggest the lack of homology between the inducible forms and the bulk of the constitutive forms of hepatic cytochrome P-450 (22). Although sexual differences in hepatic drug and steroid metabolism in rats are well known, the presence of a sex-dependent form of hepatic cytochrome P-450 has not been documented. Levin *et al.* (7) reported sexual differences in the turnover of cytochrome P-450 and that such suggested differences are determined by gonadal hormone imprinting during the neonatal period.

By taking advantage of the sex-dependent and neonatally imprinted differences in testosterone hydroxylase activity from intact microsomes, we have identified by reconstitution experiments a form of hepatic cytochrome P-450 which showed sex-dependent differences and imprinting characteristics in untreated adult male rats (18). In this communication, we report procedures to purify partially this form of cytochrome P-450 from liver microsomes of neonatally androgen-imprinted rats (adult male and adult male castrated at 28 days). Immunochemical data of this partially purified cytochrome P-450 and studies of the reconstituted enzyme activities are consistent with the concept of the existence of a sex-dependent and neonatally imprinted form of cytochrome P-450 in hepatic microsomes.

MATERIALS AND METHODS

Animals. Adult Wistar rats (200–300 g) were purchased from Microbiological Associates (Bethesda, Md.). Animals were maintained on Purina laboratory chow and water *ad libitum*. Newborn litters were derived from random matings of one male and two females per cage. Rats routinely were weaned at 21–23 days. Male rats were surgically castrated under hypothermic conditions within 16 hr after birth or under diethyl ether anesthesia at 4 weeks of age (8).

Partial purification of a sex-dependent and neonatally imprinted form of hepatic cytochrome P-450 from adult male rats. Unless otherwise indicated, all isolation procedures were conducted at 2°. The individual purification steps are depicted in Scheme 1. Detailed procedures from Step 1 to Step 7 have been described (8). Briefly stated, isolated liver microsomes were resuspended (1 g/ml) in Buffer A [10 mM sodium phosphate, 0.5% (w/v) cholic acid, 0.2% (w/v) Emulgen 911 (Kao Atlas, Tokyo, Japan) 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol, pH 7.4] by gentle homogenization in a Potter-Elvehjem homogenizer fitted with a Teflon pestle and solubilized at room temperature for 3–7 hr. The solubilized microsomes were fractionated by DEAE-cellulose column chromatography (DE-52, Whatman, England) and adsorbed by hydroxylapatitie gel (Bio-Rad, Richmond, Calif.). The gel was washed three to five times with Buffer W [10 mM sodium phosphate, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% (v/v) glycerol, pH 7.4], and cytochrome P-450 was eluted from the gel with Buffer W containing 0.3 M sodium phosphate. The eluted P-450 fraction was concentrated and dialyzed overnight against Buffer W. The cytochrome P-450 was subsequently fractionated by an affinity hydrophobic column, octylaminoagarose (P-L Biochemicals, Milwaukee, Wis.), according to procedures described by Imai and Sato (ref. 23, step 8). The column (0.9 × 12 cm) was pre-equilibrated with Buffer W, and the sample was loaded at a flow rate of 0.5 ml/min. The column was washed with 25 ml of Buffer W. Cytochrome P-450 was eluted from the column with a linear gradient of 200 ml each of Buffer W and Buffer W containing 0.2% (w/v) Emulgen 911 and 0.8% (w/v) decolorized and recrystallized cholic acid (ref. 23, step 8). For further purification, the cytochrome P-450 was eluted by stepwise gradients (30, 50, 70, and 90 mM potassium phosphate-GED buffer,[4] pH 7.25) from a CM-



SCHEME 1. *Partial purification of hepatic cytochromes P-450 from rat microsomes*

[4] The abbreviations used are: GED, 20% (v/v) glycerol, 0.2% (w/v) Emulgen 911, and 0.2 mM dithiothreitol; SDS, sodium dodecyl sulfate; ^{14}C-δ-ALA, δ-[4-^{14}C]aminolevulinic acid; IgG, immunoglobulin G.

Sephadex (Pharmacia, Piscataway, N.J.) column (ref. 11, step 9). Potassium phosphate-GED buffer contained varying concentrations of potassium phosphate which was dissolved in 20% (v/v) glycerol, 0.2% (w/v) Emulgen 911, and 0.2 mM dithiothreitol. The partially purified cytochrome P-450 was dialyzed overnight against 50–100 volumes of Buffer W to remove cholic acid. The residual Emulgen 911 was removed by three to four washes with Bio-Beads (SM-2, Bio-Rad). In some experiments, Emulgen was removed directly by passing the sample through a hydrophobic C-18 cartridge (SEP-PAK, Waters Associates, Milford, Mass.). The cholic acid and Emulgen-free cytochrome P-450 was used for reconstitution studies, polyacrylamide gel electrophoretic analyses, and immunochemical studies.

Reconstitution of testosterone hydroxylase and benzphetamine N-demethylase activities. Unless otherwise indicated, testosterone hydroxylase by the addition of the following components (18): partially purified cytochrome P-450 (0.1 nmole); affinity column-purified NADPH-cytochrome *c* reductase (50 units), purified by procedures described by Yasukochi and Masters (24); dilauroylphosphatidylcholine (7.5 μg, Sedary Research Laboratory, London, Ont.); $MgCl_2$ (5 μmoles); NADPH (0.5 μmole); and substrates (1,2-[^{14}C]testosterone, 80 nmoles, 46 mCi/mmole, or *N*-[^{14}C]benzphetamine, 264 nmoles, 0.14 mCi/mmole), in a final volume of 0.5 ml according to the general procedures reported by Lu and Levin (25). Testosterone hydroxylase activities were assayed by the method described (26). Benzphetamine *N*-demethylase activity was determined by procedures described by Thomas *et al.* (27) with slight modification (28).

Polyacrylamide gel electrophoresis. The crude DEAE-eluate (Peak II) and the partially purified rat cytochrome P-450 from adult males, adult females, adult males castrated at birth, and adult males castrated at 28 days were subjected to SDS-polyacrylamide slab-gel electrophoresis according to the procedures of Laemmli (29). The final concentrations of acrylamide and SDS were 7.5% (w/v) and 0.2% (w/v), respectively. The protein markers used were bovine serum albumin (68,000 mol wt), glutamate dehydrogenase (53,000 mol wt), ovalbumin (45,000 mol wt), aldolase (40,000 mol wt), and chemotrypsinogen A (25,000 mol wt). The gels were stained overnight in 0.1% (w/v) Coomassie Brilliant Blue which was dissolved in 10% (v/v) acetic acid and 25% (v/v) 2-propanol and destained in 10% (v/v) acetic acid and 25% (v/v) 2-propanol for 30 min followed by 10% (v/v) acetic acid and 10% (v/v) 2-propanol for 30 min.

Preparation of radioactively labeled cytochrome P-450 from hepatic microsomes. Hepatic cytochromes P-450 were labeled with ^{14}C-δ-ALA, 55 mCi/mmole (New England Nuclear Corporation, Boston, Mass.), *in vivo* according to the procedures described (30). ^{14}C-δ-ALA was dissolved in 0.9% NaCl and administered to rats through the jugular vein under ether anesthesia at a dose of 1.87 μmoles/kg of body weight. The animals were killed 2 or 48 hr after ^{14}C-δ-ALA treatments. Hepatic microsomes were isolated, solubilized, and fractionated through a DEAE-cellulose column according to procedures described (18). The major cytochrome P-450 fractions (Peak II), which contained a sex-dependent and neonatally imprinted form of cytochrome P-450, were pooled, dialyzed against Buffer W, and concentrated by membrane ultrafiltration to 16–28 nmoles of cytochrome P-450 per milliliter.

Immunization of rabbits with partially purified cytochrome P-450. Adult New Zealand White female rabbits were immunized with the partially purified cytochrome P-450 from adult male rats according to procedures described by Vaitukaitis *et al.* (31). The antigen consisted of cytochrome P-450 (7.2–9.4 nmoles/mg), diluted with 0.9% NaCl, and emulsified thoroughly with complete Freund's adjuvant at a ratio of 1:1 (v/v). Rabbits were immunized with this partially purified cytochrome P-450 via intradermal and subcutaneous routes at multiple sites along both sides of the spinal cord. About 0.1–0.2 ml was injected per site for a total of 2.5–3.0 ml. Two to four subsequent injections of antigen emulsified with incomplete Freund's adjuvant were given at 2-week intervals. Each rabbit received a total of 280–360 μg of cytochrome P-450 during the entire course of immunization (200 μg for the initial immunization and 40 μg for each subsequent booster injection). Seven to nine days after the last immunization, blood samples for antisera were obtained from the ear veins of the animals. The blood was allowed to clot (30 min at 37° and overnight at 2°), and antisera were collected after centrifugation at 10,000 × *g* for 5 min.

IgG was subsequently purified from total rabbit antiserum by ammonium sulfate precipitation followed with DEAE-Affi-Gel Blue column chromatography (Bio-Rad). Briefly stated, crude IgG was first precipitated in 1.75 M ammonium sulfate and dialyzed overnight against 50–100 volumes of 0.02 M Tris-HCl and 0.028 M NaCl (pH 8.0). The dialyzed sample was subsequently eluted by the same buffer through a DEAE-Affi-Gel Blue column (7.5 ml of gel per milliliter of rabbit serum). Purified IgG was collected from the unbound fraction and concentrated by membrane ultrafiltration (Amicon, Lexington, Mass.) to a protein concentration of 7.8 mg/ml.

The presence of cytochrome P-450-specific antibody in the purified IgG fraction was analyzed by three methods. First, Ouchterlony double-diffusion plates were used to determine the specificity of purified IgG by placing IgG in the center wells and the partially purified cytochrome P-450 from neonatally imprinted (adult males and adult males castrated at 28 days) and non-imprinted (adult females and adult males castrated at birth) rats in the surrounding wells as described (28). Second, the purified IgG and the appropriately immunoadsorbed IgG were used to study the testosterone hydroxylation or benzphetamine *N*-demethylation reaction (or both) in both intact microsomes and reconstituted systems as described (18, 28). Finally, IgG was used to quantify the amount of immunoprecipitable cytochrome P-450 previously labeled with ^{14}C-δ-ALA *in vivo*. In these experiments, a constant amount of ^{14}C-labeled cytochrome P-450 (7.5 nmoles/ml), eluted as Peak II from the DEAE-cellulose column, was incubated with varying concentrations of IgG (0–3.9 mg/ml) in a final volume of 0.6 ml. After overnight incubation at 2°, 0.5 ml of the goat antirabbit IgG (14.8 mg of antibody per milliliter as

determined by precipitin analysis using rabbit serum in the test; Miles-Yeda Ltd., Jerusalem, Israel) was added to the mixture. After a 2-hr incubation at 2°, the mixture was centrifuged at 10,000 × *g* for 10 min. Total cytochrome P-450 and radioactivity in the supernatant fractions were determined.

Other analytical methods. Protein was determined by the method of Lowry *et al.* (32), using bovine serum albumin as the reference standard. Cytochrome P-450 was determined by its characteristic of reduced CO-difference spectrum according to the procedures of Omura and Sato (33). NADPH-cytochrome *c* reductase was determined by the method described by Phillips and Langdon (34).

RESULTS

Previous studies from our laboratory indicated the presence of a sex-dependent and neonatally imprinted form of cytochrome P-450 in hepatic microsomes of imprinted animals (18). This form of cytochrome P-450 can

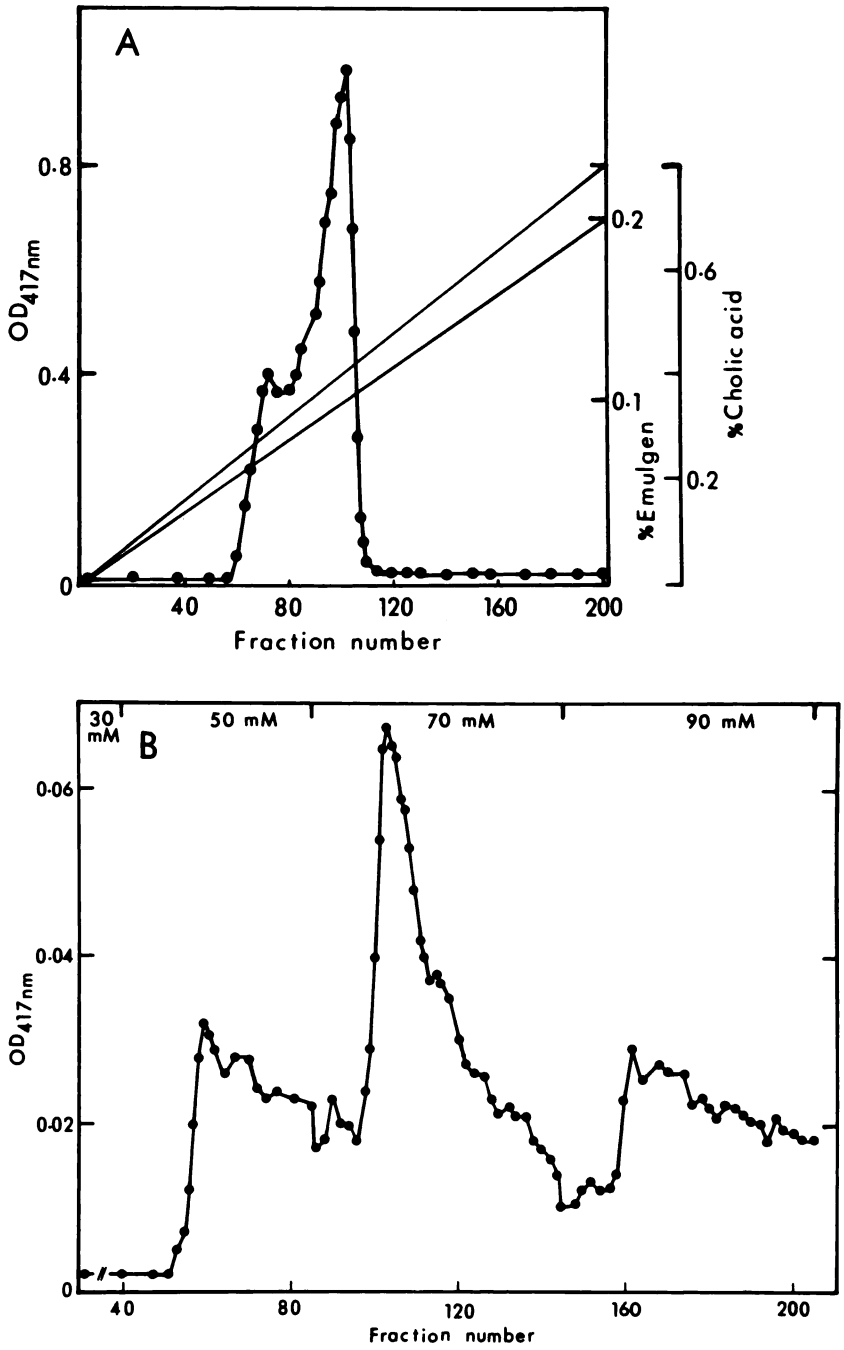


FIG. 1. *Elution profiles of hepatic cytochrome P-450*

A. Octylaminoagarose elution pattern of cytochrome P-450 isolated from hepatic microsomes of untreated adult male rats.

B. Elution profiles of hepatic cytochrome P-450 from a CM-Sephadex column by stepwise gradients consisting of 30, 50, 70, and 90 mM potassium phosphate-GED buffer (pH 7.25).

be eluted from a DEAE-cellulose column with an NaCl concentration of approximately 80 mM. By utilizing Scheme 1, we have partially purified this form of hepatic cytochrome P-450 from adult male rats. Cytochrome P-450 was eluted as one major peak with a shoulder from an octylaminoagarose column (Fig. 1A). The cytochrome P-450 subsequently was eluted by stepwise gradients of potassium phosphate-GED buffer. The bulk of cytochrome P-450 with the highest specific activity of testosterone 16α-hydroxylase appeared in the 70 mM potassium phosphate-GED buffer eluate (Fig. 1B). Table 1 summarizes the specific contents and recoveries of cytochrome P-450 during various steps of purification. The specific content and recovery of the final partially purified cytochrome P-450 in this experiment were 9.4 nmoles/mg and 8%, respectively. This partially purified cytochrome P-450 exhibited one major band on SDS-polyacrylamide gel electrophoresis (Fig. 2). Based on the specific content of cytochrome P-450, this represents a 6-fold purification from Peak II of the DEAE-cellulose column eluate (Table 1). The estimated molecular weight of this protein is 50,000.

In separate experiments using this protocol, we also have partially purified cytochrome P-450 from the livers of adult male rats, adult female rats, adult male rats castrated at birth, and adult male rats castrated at 28 days. The specific contents of these partially purified cytochromes P-450 were 5.0, 5.5, 6.7, and 4.6 nmoles/mg, respectively. Figure 3 shows gel electrophoretic patterns of these partially purified cytochromes P-450 from neonatally imprinted and non-imprinted rats. Comparison of these electrophoretic patterns indicates that the 50,000 mol wt protein (*arrows*) is present only in the neonatally imprinted rats and virtually absent from the neonatally non-imprinted rats. Catalytic activities of these partially purified cytochromes P-450 were reconstituted using testosterone and benzphetamine as substrates. The results (Fig. 4) indicate that the neonatally imprinted testosterone 16α-hydroxylase activity is associated with the reconstituted activity of cytochrome P-450 isolated from neonatally imprinted animals; comparatively low reconstituted testosterone 16α-hydroxylase activity was detected in the cytochrome P-450 isolated from non-imprinted animals. In contrast, measurement of non-imprintable enzyme activities such as testosterone 7α- and 6β-hydroxylases and benzphetamine *N*-demethylase, in the same reconstituted system, failed to a correlation with the imprinting states of the animals (Fig. 4).

We have used partially purified cytochrome P-450 from untreated adult male rats as antigen to produce antisera in rabbits. IgG isolated from these antisera reacted with partially purified cytochrome P-450 from both neonatally imprinted and non-imprinted rats (Fig. 5a). Visible single and double precipitin lines were detected in wells containing cytochrome P-450 partially purified from neonatally non-imprinted and imprinted animals, respectively. When IgG was immunoadsorbed against the partially purified cytochrome P-450 from neonatally non-imprinted rats, a single immunoprecipitin line was observed between the residual IgG and cytochrome P-450 isolated from neonatally imprinted animals (Fig. 5b). An

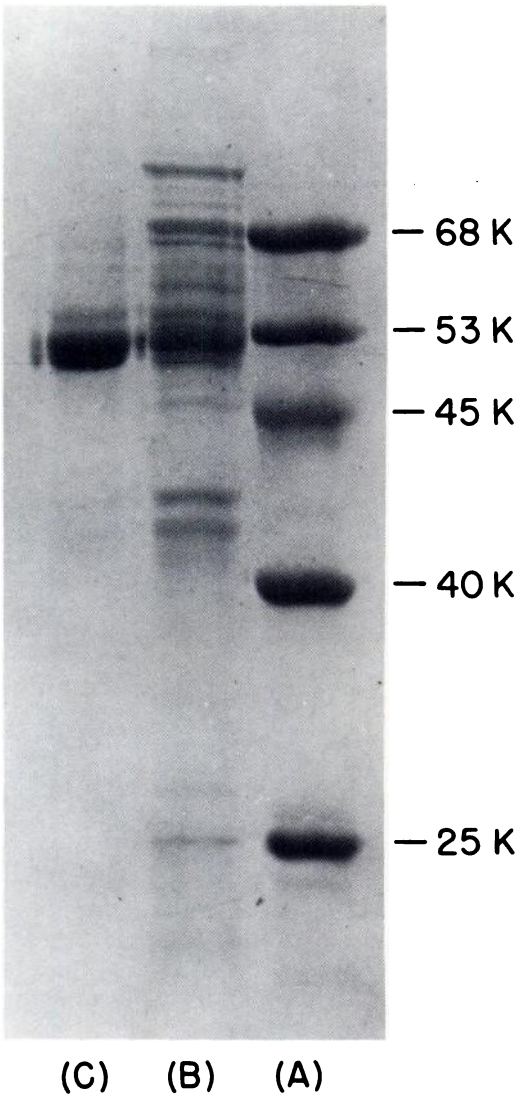


FIG. 2. *Polyacrylamide gel electrophoretic pattern of partially purified cytochrome P-450 from untreated adult male rats*

Track *A* represents protein standards. Track *B* represents 6 μg of protein of cytochrome P-450 from the DEAE-cellulose fraction. Track *C* represents 4 μg of protein of cytochrome P-450 from the CM-Sephadex fraction (eluted with 70 mM potassium phosphate-GED buffer).

TABLE 1

Partial purification of hepatic cytochrome P-450 from untreated adult male rats

Partial purification steps are described in Scheme 1. The starting material for this purification was derived from Peak II of the DEAE-cellulose eluate which contained a sex-dependent and neonatally imprinted form of hepatic cytochrome P-450 (18).

Purification step	Total cytochrome P-450 content	Total protein content	Specific content	Fold of purification	Recovery of cytochrome P-450
	nmoles	*mg*	*nmoles/mg*		%
DEAE-cellulose	446	279	1.6	1.0	100
Hydroxylapatite	320	160	2.0	1.3	72
Octylaminoagarose	179	75	2.4	1.5	40
CM-Sephadex	34	3.6	9.4	5.9	8

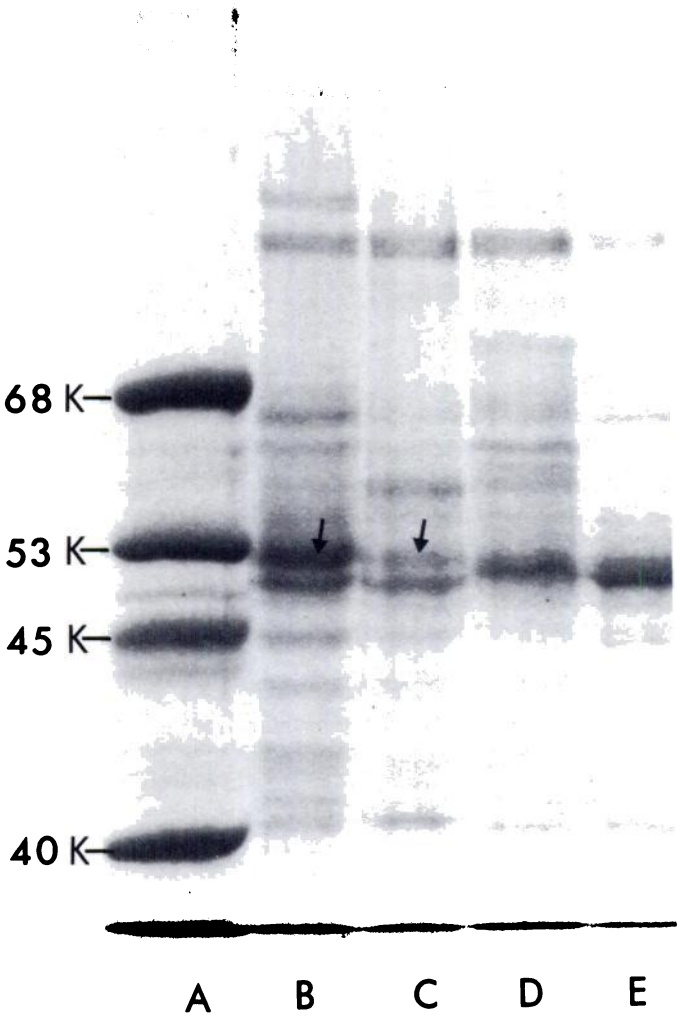


FIG. 3. *Polyacrylamide gel electrophoretic pattern of partially purified cytochrome P-450 from neonatally imprinted and non-imprinted rats*

Track *A* represents protein standards. Tracks *B*, *C*, *D*, and *E* represent 10.8, 6.8, 9.1, and 6.1 μg of protein of the partially purified cytochrome P-450 (70 mM potassium phosphate-GED buffer eluate from the CM-Sephadex column) derived from adult males, adult males castrated at 28 days, adult males castrated at birth, and adult female rats, respectively.

optimal concentration of antigen/antibody (2.5 nmoles of cytochrome P-450 per milligram of IgG before immunoadsorption) is required for formation of this immunoprecipitin line; this line was not observed when the ratio of antigen to antibody (nanomoles per milligram) was decreased to less than 0.25 (28). The apparent homology of these immunoprecipitin materials between adult males and adult males castrated at 28 days is indicated by the fusion of these lines (Fig. 5c). These immunoprecipitin lines disappeared when IgG previously adsorbed by partially purified cytochrome P-450 from the neonatally imprinted adult rats was used (data not shown).

The specific immunoprecipitation of the above residual IgG to the neonatally imprinted form of hepatic cytochrome P-450 was further substantiated by experiments in which residual IgG inhibited a certain testosterone hydroxylase activity, including the neonatally imprintable testosterone 16α-hydroxylase activity, in both intact microsomes and the reconstituted system. Similar residual IgG obtained after immunoadsorption against the partially purified cytochrome P-450 from neonatally imprinted animals showed no such inhibition. Figure 6b indicates that the unadsorbed IgG inhibited hepatic microsomal testosterone 16α- and 7α-hydroxylase activities but not 6β-hydroxylase activities in adult male rats in a concentration-dependent manner. IgG that previously had been immunoadsorbed against the partially purified cytochrome P-450 from neonatally imprinted rats failed to inhibit significantly all testosterone hydroxylase activities tested (Fig. 6c). However, IgG that had been immunoadsorbed against the cytochrome P-450 isolated from neonatally non-imprinted animals retained inhibitory activity against both testosterone 16α- and 7α-hydroxylase without significantly impairing 6β-hydroxylase activities (Fig. 6d). It should be noted that preimmunized IgG also inhibited the testosterone hydroxylation reaction (6a). We have observed a similar degree of inhibition of preimmunized IgG by utilizing IgG prepared from rabbits which were immunized with rat prostatic α-protein.[5] However, IgG inhibited all testosterone hydroxylase activities (16α, 7α, and 6β) in a reconstituted system using partially purified material from hepatic microsomes of adult males as a source of cytochrome P-450 (Table 2). The inhibitory effects of IgG appear to be heat-sensitive (28).

To characterize further the turnover rates of specific forms of cytochrome P-450 which can be immunoprecipitated by IgG, cytochrome P-450 previously labeled *in vivo* with ^{14}C-δ-ALA for either 2 or 48 hr were isolated by DEAE-cellulose column chromatography. The isolated cytochromes P-450 were then mixed with increasing concentrations of IgG. Figure 7 shows that IgG effectively precipitated the cytochrome P-450 from Peak II of the DEAE-cellulose eluate of adult male rats. However, under these experimental conditions IgG failed to remove any radioactivity that is associated with either the fast- or slow-turnover species of hepatic cytochrome P-450 (7, 30).

DISCUSSION

The purpose of the present investigation was to provide immunochemical evidence for the presence of a sex-dependent and neonatally imprinted form of hepatic cytochrome P-450. Antisera prepared from rabbits immunized with partially purified cytochrome P-450 from adult male rats inhibited the imprintable testosterone 16α-hydroxylase activity in both intact microsomes and reconstituted systems. The inhibition by IgG of other testosterone hydroxylase activities, i.e., testosterone 7α-hydroxylase activity in intact microsomes and testosterone 7α- and 6β-hydroxylase activities in the reconstituted system, may be due to the overlapping substrate specificities of the isolated neonatally imprintable form of cytochrome P-450. Alternatively, it is possible that there are different forms of hepatic cytochrome P-450 present

[5] H. Chao and L. W. K. Chung, unpublished results.

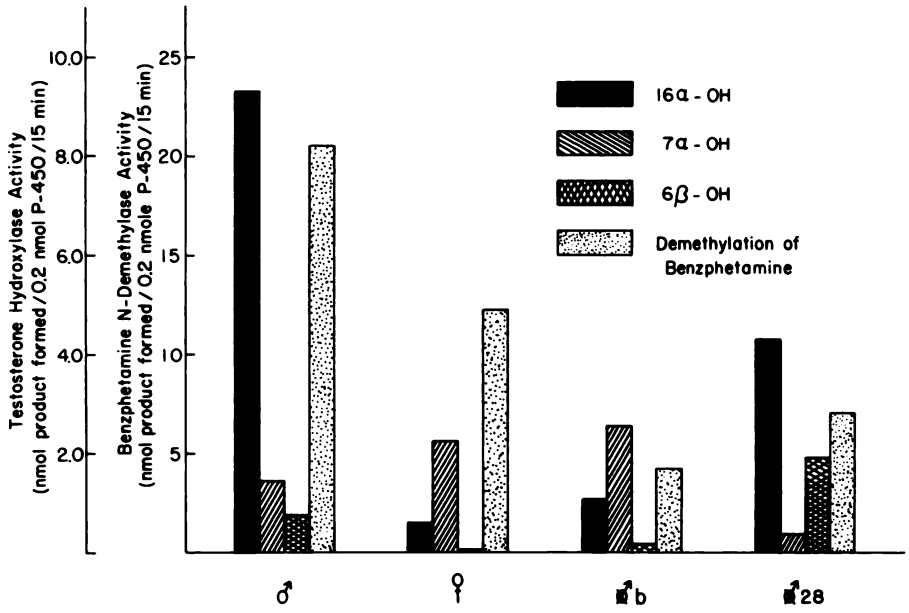


FIG. 4. *Reconstituted testosterone hydroxylase and benzphetamine N-demethylase activities from hepatic cytochrome P-450 partially purified from adult males (♂), adult females (♀), adult males castrated at birth (♂b), and adult males castrated at 28 days (♂28)*

Note that 16α-hydroxylase activity is associated with ♂ and ♂28 samples.

in the partially purified preparation which resulted in the production of multiple species of IgG molecules with their specific inhibitory action against different testosterone hydroxylase activities. In the present study, we cannot differentiate between these two possibilities. The fact that antisera inhibited only testosterone 16α- and 7α-hydroxylase activities in the intact microsomes, whereas all three testosterone hydroxylase activities (16α, 7α, and 6β) were inhibited in the reconstituted system, suggests that testosterone 6β-hydroxylase may be more deeply imbedded in the membrane and thus less accessible to antibody inhibition.

Reconstitution of the partially purified cytochrome P-450 from neonatally imprinted and non-imprinted animals resulted in a correlation between the reconstituted testosterone 16α-hydroxylase activity and the imprinting state of the animal. For example, the imprintable testosterone 16α-hydroxylase activity is present in abundance only with cytochrome P-450 isolated from neonatally imprinted animals; no correlation was found between the imprinting state of the animal and the non-imprintable enzyme activities such as testosterone 7α- and 6β-hydroxylase activities and benzphetamine *N*-demethylase activity. It has been established that neonatal imprinting affects cytochrome P-450 rather than NADPH-cytochrome *c* reductase (18, 28).

The characteristic immunoprecipitin line associated with cytochrome P-450 isolated from neonatally imprinted animals was detectable in an Ouchterlony double-diffusion chamber. The specificity of this immunoreaction is confirmed further by the effectiveness with which this immunoadsorbed IgG (immunoadsorbed against ♀ and ♂b P-450) inhibits the neonatally imprinted testosterone 16α-hydroxylase activity.

Polyacrylamide gel electrophoresis indicated that a protein band migrating with an apparent molecular

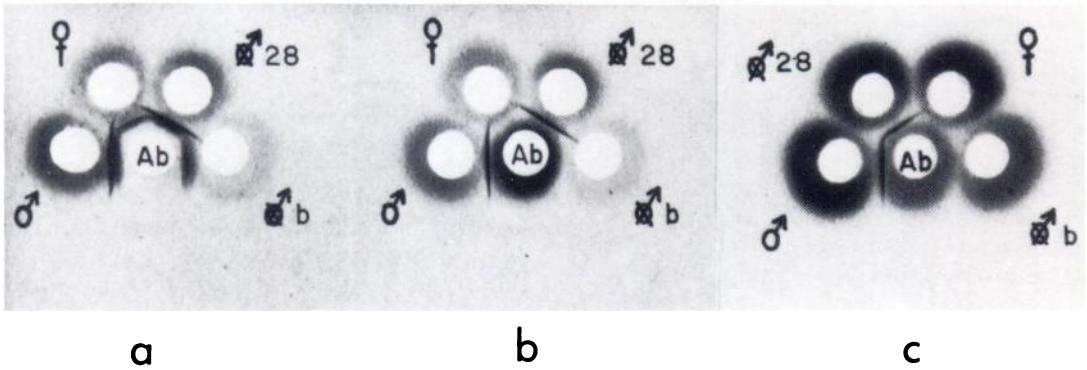


FIG. 5. *Ouchterlony double-diffusion plates*

a. Immunoprecipitation between partially purified cytochrome P-450 (surrounding wells) from adult males (♂), adult females (♀), adult males castrated at 28 days (♂28), and adult males castrated at birth (♂b), and the IgG (center well) prepared from immunized rabbit serum. Rabbits were immunized with partially purified cytochrome P-450 isolated from untreated adult male rats.

b. Same as a except that IgG was previously immunoadsorbed against partially purified cytochrome P-450 from neonatally non-imprinted animals.

c. Same as b except that the positions of two surrounding wells were switched.

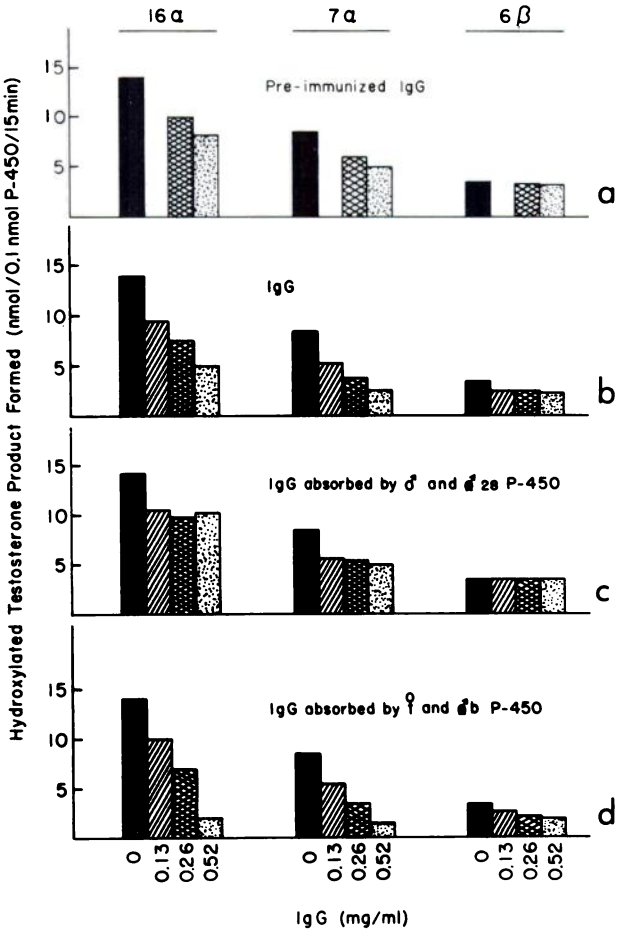


FIG. 6. *Inhibition of testosterone hydroxylase activities by preimmunized IgG and IgG prepared from rabbits previously immunized with partially purified hepatic cytochrome P-450 of untreated adult male rats*

Note that the inhibition of testosterone hydroxylase activities (16α and 7α) by IgG was abolished only by previously adsorbing IgG with cytochrome P-450 isolated from neonatally imprinted rats. Adsorption of IgG by cytochrome P-450 of the neonatally non-imprinted rats retained inhibitory effects against these testosterone hydroxylase activities. ♂28, Adult males castrated at 28 days; ♂b, adults males castrated at birth.

TABLE 2

Inhibition of testosterone hydroxylase activities by rabbit preimmunized IgG and nonabsorbed IgG in a reconstituted system

The final assay mixture in the control sample contained partially purified cytochrome P-450 (0.05 nmole) from untreated adult male rats, NADPH-cytochrome *c* reductase (25 units), dilauroylphosphatidylcholine (3.75 μg), and $MgCl_2$ (2.5 μgmoles) in 0.25 ml. Preimmunized and immunized rabbit IgG were preincubated with cytochrome P-450 at 2° for 2 hr prior to assay for testosterone hydroxylase activities. Concentrations of immunoglobulins were expressed as micrograms per milliliter of the final assay volume.

Condition	Testosterone hydroxylase activities		
	16α	7α	6β
	% control activity		
Control	100	100	100
+Pre-IgG			
17 μg/ml	70	37	59
34 μg/ml	59	37	55
+IgG			
22.8 μg/ml	46	20	39
45.6 μg/ml	36	19	36

weight of 50,000 existed only in the cytochrome P-450 preparations from the neonatally imprinted animals and was absent from the neonatally non-imprinted animals. However, additional biochemical characterization is required before firm conclusions can be made on the exact molecular weight of this neonatally imprinted form of cytochrome P-450.

Studies of the turnover rates of this neonatally imprinted form of cytochrome P-450 by an immunoprecipitation method revealed that this form of cytochrome P-450 has a very slow turnover rate. No significant incorporation of ^{14}C-δ-ALA into this hemoprotein was found during the entire course of the pulse-labeling experiment for up to 48 hr. This observation is consistent with the early result of Levin *et al.* (7), who reported that both adult males and adult males castrated at 4 weeks have a higher proportion of slow to fast turnover forms of hepatic cytochrome P-450 than of the neonatally non-imprinted rats such as adult females and adult males castrated at birth. These early results can be explained by the preferential enrichment of a very slow turnover form(s) of cytochrome P-450 in the hepatic microsomes of neonatally imprinted rats.

In summary, the present investigation provides immunochemical evidence to document further the existence of a sex-dependent and neonatally imprinted form of hepatic cytochrome P-450 in rats. This form of cyto-

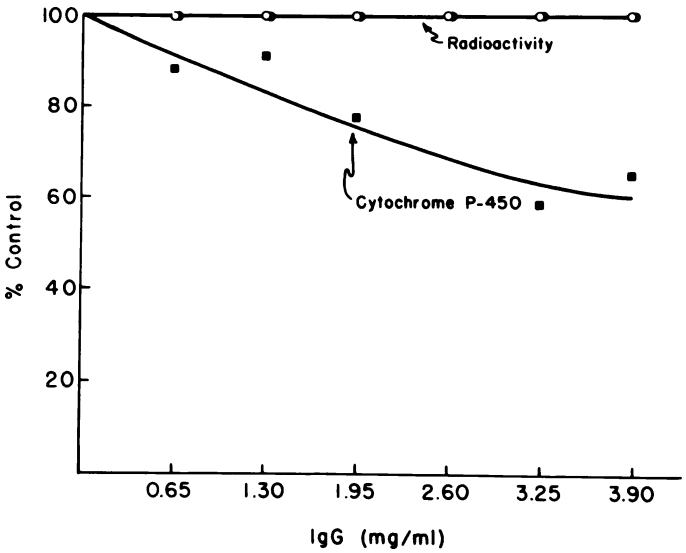


FIG. 7. *Immunoprecipitation of cytochrome P-450 by purified IgG*

Cytochrome P-450 (7.5 nmoles/ml) of untreated adult male rats was labeled *in vivo* with ^{14}C-δ-ALA for either 2 hr (○——○) or 48 hr (●——●). Purified IgG (against cytochrome P-450 partially purified from untreated adult male rats) in concentrations ranging from 0 to 3.9 mg/ml was added to the incubation mixture. The residual concentrations (nanomoles per milliliter) of cytochrome P-450 in the supernatant fractions were determined. Each point represents the average of two determinations with variation between determinations less than 10% (■——■). Control samples contained equivalent amounts of preimmunized IgG. Data were expressed as percentage of control.

chrome P-450 apparently is present in a small amount in hepatic microsomes and has a very slow turnover rate. The existence of this form of cytochrome P-450 in hepatic microsomes accounts for certain major sex-dependent and neonatally imprinted differences of drug and steroid metabolism in adult life.

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Send reprint requests to: Dr. Leland W. K. Chung, Pharmacology/ School of Pharmacy, University of Colorado, Boulder, Colo. 80309.