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Neonatal Imprinting and the Turnover of Microsomal Cytochrome P-450 in Rat Liver

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SUMMARY

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Administration of [3,5-^{3}H]δ-aminolevulinic acid to immature and adult male and female rats led to the incorporation of radioactivity into liver microsomal cytochrome P-450. A biphasic decrease was observed in the radioactivity incorporated into the hemeprotein, and the half-lives were 7-8 and 42-46 hr in all cases, suggesting that at least two forms of cytochrome P-450 exist in rat liver microsomes. The ratio of the fast-phase component to the slow-phase component was 3.4-4.4:1 in the immature and adult female and the immature male rat. This ratio changed to 1.9:1 in the adult male, indicating an age and sex difference in the ratio of the two forms of the cytochrome. Castration of the male at 4 weeks of age did not prevent the change in ratio that occurred during the next 4 weeks. However, castration of male rats at birth prevented the age-dependent change in ratio that occurred after 4 weeks of age and resulted in a biphasic turnover of cytochrome P-450 that was indistinguishable from that of the adult female rat. These changes in cytochrome P-450 were correlated with changes in the activity of liver microsomal enzymes that hydroxylate testosterone at the 7α, 16α, and 6β positions. Different ratios of activities of the three hydroxylation reactions are characteristic of the immature and adult male and female rat. Castration of male rats at birth caused the development of a female pattern of testosterone hydroxylation, whereas castration at 4 weeks of age resulted in a pattern of hydroxylation that was intermediate between the adult male and female.

INTRODUCTION

Oxidative metabolism of drugs and steroids by rat liver microsomes is influenced by a variety of factors, including the age and sex of the animals (1, 2). Liver microsomes from male rats metabolize a wide variety of drugs and steroids more rapidly than do those from female rats. In addition, the magnitude of the sex difference in metabolism varies considerably, depending on the substrate metabolized.

Quinn *et al.* (3) showed that adult female rats, in response to an injection of hexobarbital, slept longer than adult males. This finding was coincident with a significantly higher plasma level of the drug in the female and a lesser ability of liver microsomes to oxidize the drug *in vitro* compared to males. This sex difference became apparent only after the animals reached 4-5 weeks of age, the age of puberty in male rats. Until recently, relatively little was known about factors regulating age and sex differences in drug and steroid metabolism. Several studies have

shown that castration of male rats reduces sex differences, but the magnitude of the changes in oxidative metabolism caused by castration varies with the substrate used (2, 4–9). Recently DeMoor and Denef (10) have shown that the development of the male cortisol metabolism pattern in liver homogenates is determined by a gonadal androgen in early postnatal life and that postpubertal castration of male rats has relatively little effect on this metabolite pattern. Gustafsson and co-workers (11, 12) confirmed this phenomenon of "neonatal imprinting" in a study on the metabolism of a variety of steroids by male rat liver microsomes. Similar results were obtained by Chung (13) for the oxidative metabolism of aniline and aminopyrine by the liver microsomal mixed-function oxidase system. Since cytochrome P-450, the terminal oxidase for the metabolism of drugs and steroids, has been shown to turnover biphasically *in vivo* with a turnover of 7–8 and 46–48 hr (14–16), it was of interest to determine whether age and sex differences in drug and steroid metabolism in the rat were related to differences in the ratios of the two forms of cytochrome P-450. The results presented in this paper indicate that differences in the ratios of the fast and slowly turning over forms of cytochrome P-450 present in liver microsomes are age- and sex-dependent and that these differences are neonatally imprinted.

METHODS

Long-Evans rats (Blue Spruce Farms, Altamont, N. Y.) were housed in stainless steel cages and fed a commercial diet (Purina laboratory chow) and water ad libitum. Newborn male rats (between 6 and 12 hr old) were castrated under light ether anesthesia. The animals were returned to their mothers until they reached 21 days of age. Sham-operated animals were handled in an identical manner. Other male rats were castrated at 4 weeks of age. Male and female rats 3½–4 weeks old were designated immature animals, and adult animals were 8 weeks of age.

[3,5-^{3}H]δ-Aminolevulinic acid (2.2 Ci/mmole) was diluted with unlabeled δ-aminolevulinic acid (final specific activity, 550 mCi/mmole) and injected into the tail vein in 0.9% NaCl. All animals received 0.25 mg of δ-aminolevulinic acid per kilogram of body weight. The animals were killed at various times (three rats per time period) after injection of the isotope. To rule out any variation that might have occurred in methodology from experiment to experiment, animals of different age and sex were cross-matched in each experiment. For example, in one experiment, immature male and adult male rats were used simultaneously. The next experiment was performed on adult males and adult females. Thus all six different animals sets (immature males and females, adult males and females, and rats castrated as neonates or castrated at 4 weeks of age) were cross-matched in different experiments. At least three complete experiments were performed on each set of animals with the exception of immature females, which were used once (matched against adult females).

Animals were killed by decapitation, and liver microsomes were prepared and washed as previously described (14). The final microsomal pellets were layered with 3 ml of buffer (0.1 M KH_2PO_4–K_2HPO_4, pH 7.4) and stored at −15° for 1–7 days before use. Previous studies from our laboratory have shown that storage of liver microsomes in this manner for as long as 14 days does not lead to any significant change in cytochrome P-450 content or drug or steroid metabolism (17). Immediately before use the microsomal pellets were thawed at room temperature, suspended in 0.1 M potassium phosphate buffer at pH 7.4 (microsomes equivalent to 200 mg of liver, wet weight, per milliliter), and incubated with 0.2% steapsin for 1 hr at 37° to solubilize cytochrome b_5 selectively as previously reported (14, 18). The pellet obtained after centrifugation for 60 min at 160,000 × *g* contained cytochrome P-450 (converted to the inactive form of the heme protein, cytochrome P-420) as its sole heme constituent (18). The pellet was resuspended in 0.1 M potassium phosphate buffer, and the radioactivity was quantified in a liquid scintillation spectrometer. Previous studies from several laboratories

showed that the radioactivity in the final pellet (CO-binding particles) after treatment with steapsin or subtilopeptidase (Nagarse) was present exclusively as radioactive heme whether measured by solvent extraction, solvent extraction followed by chromatography, or recrystallization of the heme (14–16, 19). In addition, several other studies, using a variety of techniques, demonstrated that the radioactive heme present in rat liver microsomes after an injection of radioactive δ-aminolevulinic acid *in vivo* is associated only with cytochrome P-450 and cytochrome b_5, and that this radioactive heme in microsomal cytochrome P-450 is not exchangeable (14, 16, 20–22).

The metabolism of [4-^{14}C]testosterone by rat liver microsomes was determined as previously described (17). Cytochrome P-450 was measured by the method of Omura and Sato (18), using an extinction coefficient of 91 mM^{-1} cm^{-1}, and protein was determined by the method of Lowry *et al.* (23).

RESULTS

Effect of age, sex, and castration on turnover of cytochrome P-450. Figure 1 shows the rate of disappearance of radioactive heme from the CO-binding particles (microsomes devoid of cytochrome b_5) of immature and adult male rats. The disappearance of cytochrome P-450 heme was

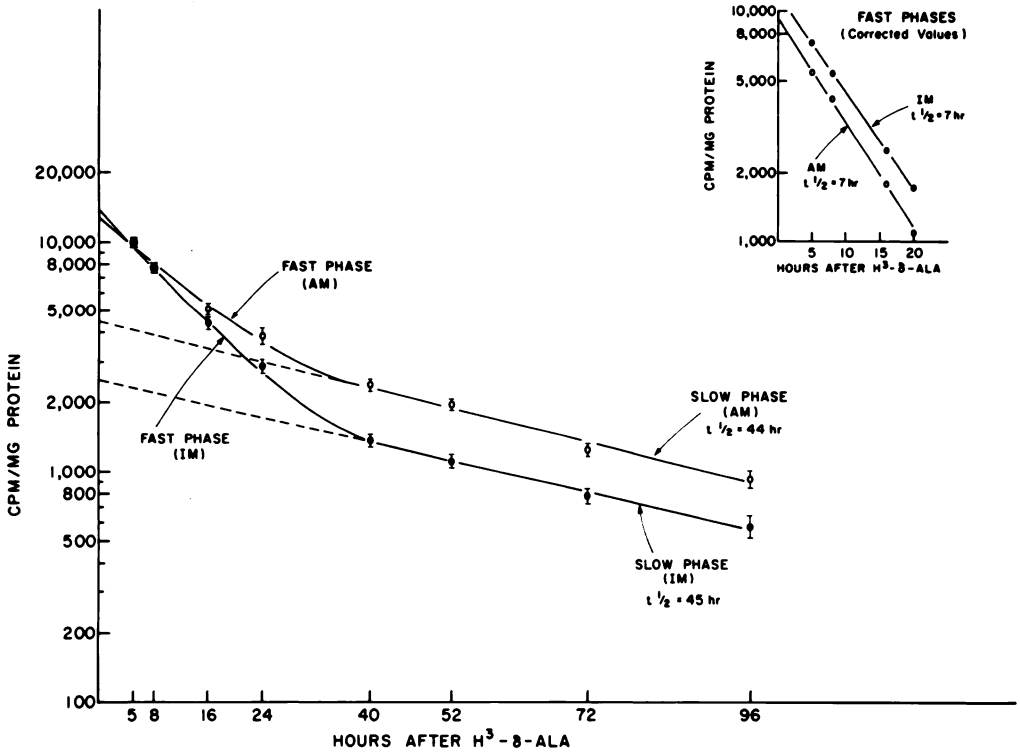

FIG. 1. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from immature (IM) and adult male (AM) rats*

Rats were injected with [3,5-^{3}H]δ-aminolevulinic acid (ALA) (0.25 mg/kg) and killed at various times thereafter. Since essentially the same amount of [^{3}H]δ-aminolevulinic acid was incorporated into cytochrome P-450 in all animal groups 5 hr after administration of the isotope, the 5-hr time period was set equal to 10,000 cpm/mg of protein so that results from different experiments could be easily compared. Values used for the determination of the corrected half-life of the fast phase (inset) were obtained by first extrapolating the slow phase to zero time and then subtracting the values of the extrapolated slow phase from the uncorrected fast phase portion of the curve. Values represent the means ± standard errors of four complete half-life curves, using three animals per time period in each experiment (n = 12).

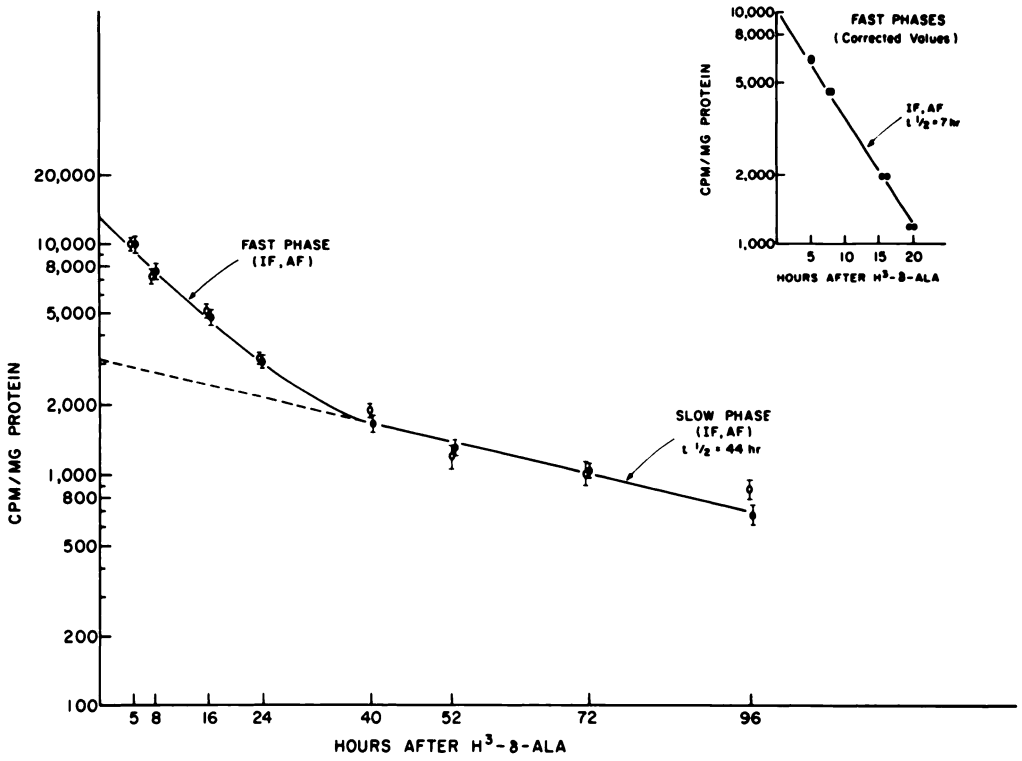

FIG. 2. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from immature (IF) and adult female (AF) rats*

Injection of isotope and calculations were performed as stated in Fig. 1. Values represent the means ± standard errors of three complete half-life curves for adult females and one curve for immature females, using three animals per time period in each experiment.

biphasic, indicating the existence of at least two forms of the hemeprotein, as previously reported (14). The half-lives of the fast- and slow-phase hemeproteins were 7 and 44–45 hr, respectively, in both the immature and adult male rats. However, there was a significant difference in the ratio of the two forms in immature and adult male Long-Evans rats. The ratio of the fast-phase to the slow-phase hemeprotein in the immature male rats was 4.4, which is similar to the value of 3.8 previously reported for the same animals (14). Between 4 and 8 weeks of age the ratio of the fast-phase to slow-phase hemeprotein decreased to 1.9. Thus, in the male rat, the proportion of fast and slowing turning over cytochrome P-450 changes with age.

In contrast to the age difference in the turnover of cytochrome P-450 in the male rat, no significant change occurred with age in the female rat between 4 and 8 weeks of age (Fig. 2). Again, half-lives of 7 and 44 hr were obtained for the two forms of the hemeprotein. In contrast to the male rat, a ratio of 3.4 was observed for the proportion of fast to slowly turning over cytochrome P-450 in both the immature and adult female rat. This value is similar to the ratio obtained for immature male rats. As can be seen in Fig. 3, however, adult male and adult female rats have significantly different ratios of the two forms of the hemeprotein. These age and sex differences in the ratios of the two forms of cytochrome P-450 are in good agreement with results on the metabolism of many drugs and steroids by liver microsomes, which have shown that no significant sex difference in metabolism occurs until after 4 weeks of age (1, 3, 4, 24).

Castration of male rats at 4 weeks of age

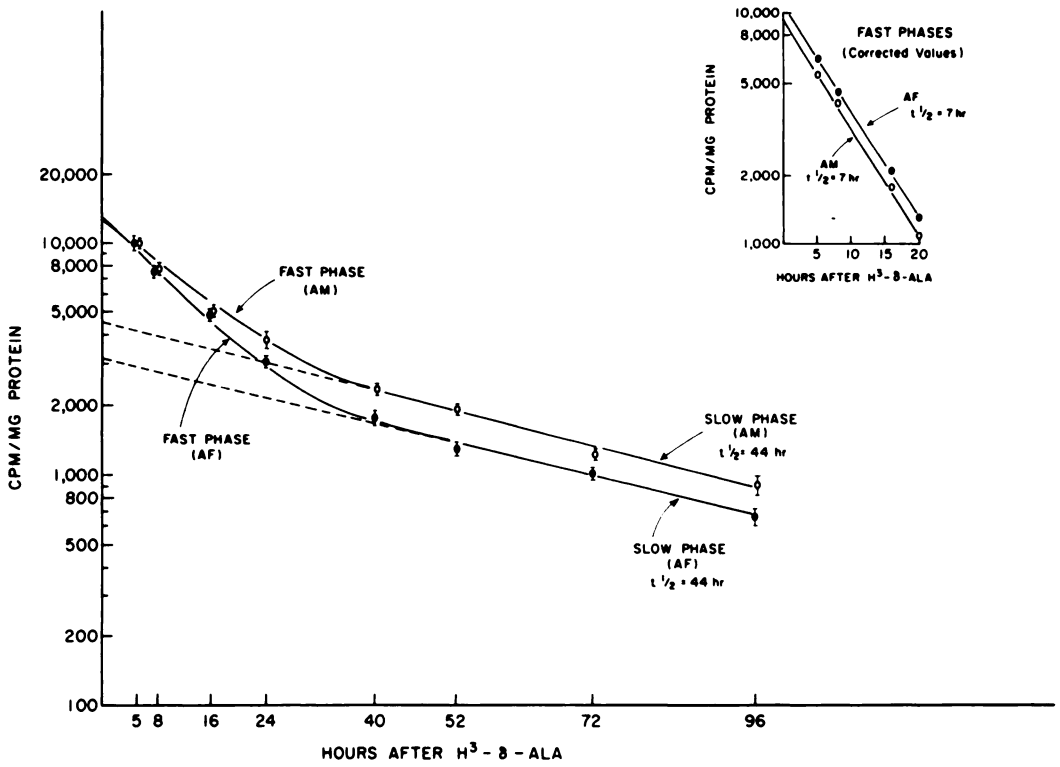

FIG. 3. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from adult male (AM) and female (AF) rats*

These curves were replotted from the data in Figs. 1 and 2.

did not prevent the change in ratio that occurred between the two forms of cytochrome P-450 during the next 4 weeks (Fig. 4). These results indicate that the change in the proportions of cytochrome P-450 with age is independent of the circulating levels of androgens in the male rat. Both adrenalectomy and castration of rats at 4 weeks of age also had no influence on the age-dependent change in cytochrome P-450 that occurred over the next 4 weeks (data not shown), further demonstrating the lack of effect of circulating androgens on the ratio of the two forms of cytochrome P-450. These results were surprising, since castration of male rats at 4 weeks of age results in a change in the liver microsomal oxidative metabolism of many drugs and steroids toward the female pattern of metabolism (1, 2, 4–9). However, the recent studies of DeMoor and Denef (10), Einarsson *et al.* (11), and Chung (13) indicated that other metabolic pathways which exhibit a sex difference are only slightly changed by postpubertal castration of the male rat; only castration at birth resulted in a complete change to the female pattern of metabolism for these pathways of metabolism (10, 11, 13).

The effect of castration of the male rat at birth on the turnover of cytochrome P-450 at 8 weeks of age is shown in Fig. 5. As can be seen, castration of rats 6–12 hr after birth prevented the change in ratio of the two forms of cytochrome P-450 that occurred between 4 and 8 weeks of age. There was a significant difference in the ratio of the fast- and slow-phase hemeprotein fractions in adult male rats castrated at birth compared to the control adult male rats (3.4 vs. 1.9, respectively). While no significant differences in turnover rates were observed, the ratios of the two forms of the hemeprotein observed after castra-

tion at birth were identical with those seen in the female rat and similar to those seen for the immature male rat (Fig. 6).

Effect of age, sex, and castration on metabolism of testosterone by rat liver microsomes. The hydroxylations of testosterone in the 7α, 16α, and 6β positions have been shown to be cytochrome P-450-mediated reactions (25). The effects of microsomal enzyme inducers and inhibitors and of age and sex on these three hydroxylations of testosterone indicated that more than one cytochrome P-450 in liver microsomes is responsible for testosterone hydroxylation (26). Table 1 shows the effect of age, sex, and castration on the metabolism of testosterone. As previously reported, a significant decrease in the 7α-hydroxylation of testosterone occurs with age in the male rat (7). Similar results were obtained with the female rat, and no significant sex differences were observed in this pathway in rats of the same age. Castration of the male rat at birth or at 4 weeks of age did not prevent this decrease in 7α-hydroxylation of testosterone with age.

In contrast to the above results with 7α-hydroxylase, an age-dependent increase in the hydroxylation of testosterone in the 6β position was observed in male rats, as previously reported (7). However, the 6β-hydroxylation pathway decreased in female rats with increasing age. Castration of male rats at 4 weeks of age inhibited the age-dependent increase in this pathway, and the 6β-hydroxylase activity in these animals at 8 weeks of age was intermediate between those of the adult male and the adult female rat. Castration at birth resulted in 6β-hydroxylase activity identical with that seen in the adult female rat.

The 16α-hydroxylation of testosterone in

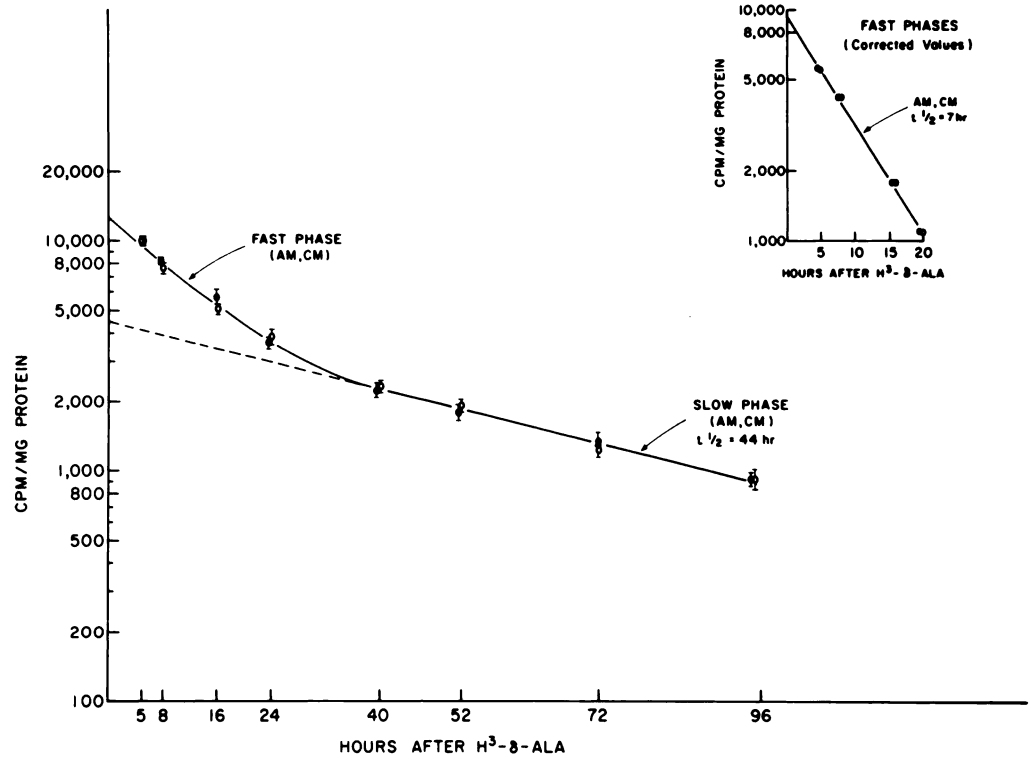

FIG. 4. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from adult male rats castrated at 4 weeks of age (CM) and adult male rats (AM)*

Injections of isotope and calculations were carried out as stated in Fig. 1. Values represent the means ± standard errors of four complete half-life curves for each animal group, using three animals per time period in each experiment.

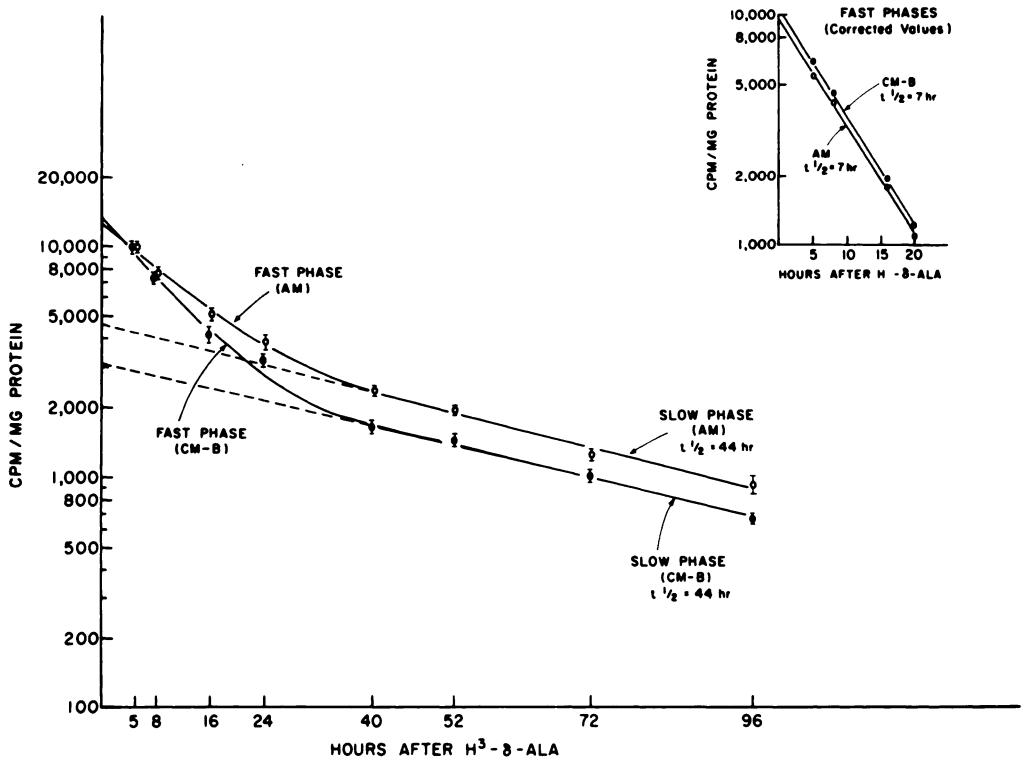

FIG. 5. *Disappearnace of labeled cytochrome P-450 heme from CO-binding particles obtained from adult male rats castrated at birth (CM-B) and adult male rats (AM)*

Injections of isotope and calculations were carried out as stated in Fig. 1. Values represent the means ± standard errors of four complete half-life curves for each animal group, using three animals per time period in each experiment.

male rats did not appear until after 4 weeks of age. This pathway was undetectable in both the immature and adult female rat. Castration of the male rat at 4 weeks of age only partially prevented the development of the 16α-hydroxylation of testosterone. However, castration of male rats at birth completely prevented the development of this pathway of testosterone metabolism in the adult male rat. Thus a comparison of the adult female rat and the adult male rat castrated at birth or at 4 weeks of age revealed that only males castrated at birth gave a pattern of testosterone hydroxylation in the 6β, 7α, and 16α positions identical with that of the adult female. Adult male rats castrated at 4 weeks of age metabolized testosterone at the 7α, 16α, and 6β positions at rates somewhat intermediate between those seen in the adult male and adult female rat. Similar results were observed when over-all hydroxylation and reduction of testosterone by liver microsomes were evaluated (Table 2); that is, the formation of the total hydroxylated metabolites of testosterone showed age and sex differences which were reversed only upon neonatal castration of the male rat. Interestingly, the reductive metabolism of testosterone by non-cytochrome P-450 enzymes increased dramatically in the neonatally castrated male rat, to levels very similar to that seen in the adult female rat, while the male rat castrated at 4 weeks of age showed only a small increase in reductive pathways of metabolism of testosterone. These results are in good agreement with the changes in ratios of the two forms of cytochrome P-450 observed in liver microsomes.

DISCUSSION

Liver microsomal cytochrome P-450 has been shown to decay biphasically *in vivo*

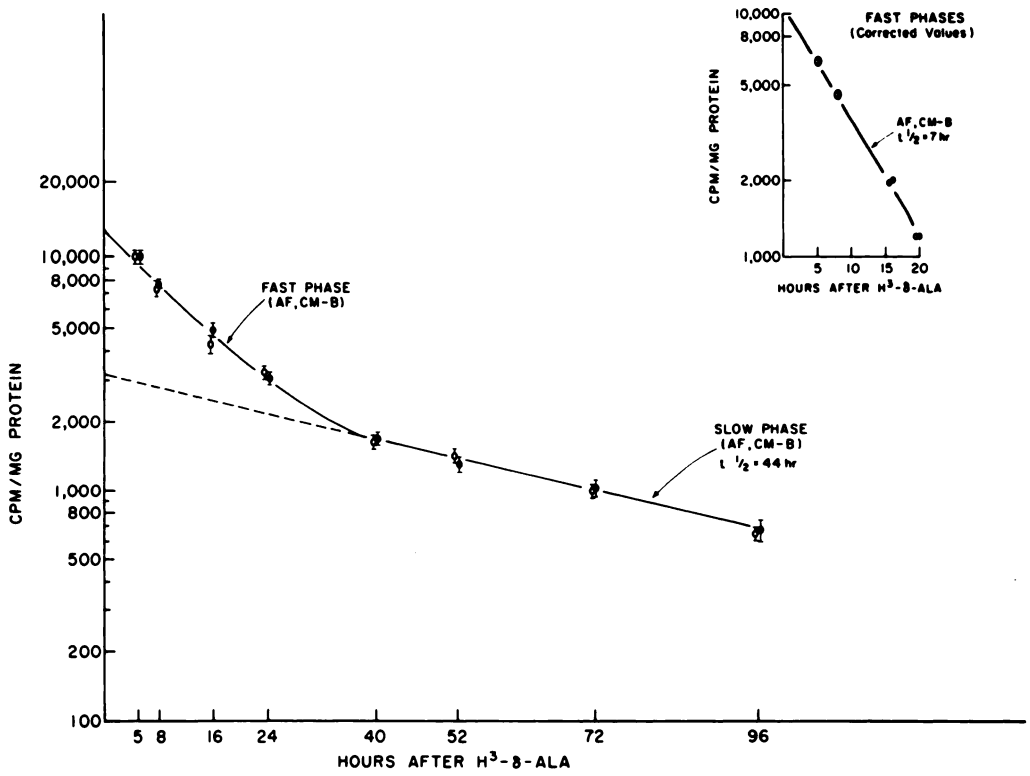

FIG. 6. *Disappearance of labeled cytochrome P-450 heme from CO-binding particles obtained from adult male rats castrated at birth (CM-B) and adult female rats (AF)*

These curves were replotted from the data in Figs. 2 and 5.

TABLE 1

Effect of age, sex, and castration on liver microsomal cytochrome P-450 and testosterone hydroxylation

Rat liver microsomes were prepared and analyzed for cytochrome P-450 and testosterone metabolism as described in METHODS. The ratio of the fast- and slow-phase heme proteins was obtained by first extrapolating the lines for the two phases to zero time (see figures) and then subtracting the zero-time intercept for the slow phase from the zero-time intercept of the uncorrected fast phase and dividing the result by the zero-time intercept for the slow phase [(fast phase intercept-slow phase intercept)/slow phase intercept]. The results are expressed as means ± standard errors of three to five determinations.

Treatment	Cytochrome P-450	Ratio of fast to slow component	Testosterone hydroxylation		
			7α	16α	6β
	nmoles/mg protein		*nmoles/mg protein/15 min*		
Immature male	0.79 ± 0.07	4.4 ± 0.5	2.18 ± 0.09	0	9.57 ± 0.80
Adult male	0.88 ± 0.03	1.9 ± 0.1	1.61 ± 0.04	2.77 ± 0.36	21.92 ± 0.72
Adult male, castrated at 4 weeks	0.93 ± 0.08	1.9 ± 0.3	1.50 ± 0.06	1.65 ± 0.23	13.35 ± 1.60
Adult male, castrated at birth	0.83 ± 0.06	3.4 ± 0.4	1.66 ± 0.10	0	1.65 ± 0.25
Immature female	0.73 ± 0.03	3.4	2.41 ± 0.09	0	8.06 ± 0.63
Adult female	0.76 ± 0.04	3.4 ± 0.3	1.71 ± 0.09	0	1.12 ± 0.05

with half-lives of 7–8 and 44–46 hr (14, 15). The ratio of the rapidly turning over cytochrome P-450 to the slowly turning over hemeprotein changed in the male rat from 4.4 in 4-week-old animals to 1.9 in 8-week-old animals. In contrast, no age-dependent change in the ratio of the two forms of cytochrome P-450 was observed in the

TABLE 2

Effect of age, sex, and castration on metabolism of testosterone

Rat liver microsomes were prepared and incubated with [4-^{14}C]testosterone as previously described (17). Polar and nonpolar metabolite formation from testosterone was quantified after separation of metabolites on Gelman thin-layer silica gel chromatography strips, using isooctane-chloroform (1:1) as the developing solvent system. Values represent the means ± standard errors of four determinations. The formation of polar metabolites is an indication of over-all hydroxylation of testosterone, whereas formation of nonpolar metabolites indicates reductive pathways of testosterone metabolism.

Treatment	Testosterone metabolism	
	Total polar metabolites	Total nonpolar metabolites
	nmoles/mg protein/15 min	
Immature male	24.9 ± 1.8	20.0 ± 1.4
Adult male	49.8 ± 2.2	17.3 ± 3.5
Adult male, castrated at 4 weeks	31.3 ± 2.4	42.6 ± 4.8
Adult male, castrated at birth	7.5 ± 0.6	80.0 ± 6.1
Immature female	21.4 ± 1.6	34.6 ± 1.7
Adult female	7.4 ± 0.5	111.2 ± 7.0

female rat, which had a ratio of 3.4. Although castration of the male rat at 4 weeks of age did not prevent the change in ratio that occurs with age, castration at birth resulted in a ratio of the two forms of cytochrome P-450 identical with that seen in the female rat. These results were correlated with age and sex differences in the hydroxylation of testosterone. The 6β- and 16α-hydroxylation of testosterone increased with age in the male rat, and this development was only partially prevented by castration at 4 weeks of age. Only neonatal castration resulted in a completely female pattern of hydroxylation of testosterone at 8 weeks of age.

Although some changes in testosterone metabolism occurred at 8 weeks of age in male rats castrated when 4 weeks old, there was no detectable change in the ratio of the two forms of cytochrome P-450. This may be a result of the assay method used in the cytochrome P-450 turnover studies, which is less sensitive than the assay for the hydroxylation of testosterone. In addition, there may exist many forms of cytochrome P-450, several of which have similar turnover rates *in vivo*, and thus changes in a small number of hemeproteins could markedly alter catalytic activity without affecting the over-all ratio of the rapidly turning over cytochrome P-450 to the slowly turning over hemeprotein. However, castration at birth not only would result in the usual changes in the heme proteins associated with castration at any age, but would also cause changes in the forms of cytochrome P-450 that are neonatally imprinted, and thus a significant change in the ratio of different forms could be expected. Whatever the reasons for the lack of effect of castration at 4 weeks of age on the ratio of the fast and slowly turning over forms of the hemeprotein, it is apparent that changes do occur if the animals are castrated at birth, which display a female pattern of development, at least with respect to the different forms of the hemeprotein and for testosterone metabolism.

Recently Einarrson *et al.* (27) showed that pseudohermaphroditic rats, which are phenotypic females with an XY karotype and bilateral inguinal testes, metabolized androstene-3,7-dione and pregnene-3,20-dione to a pattern identical with that of female rats. Those authors suggested that this female pattern of hepatic metabolism in the male pseudohermaphroditic rat might be due to the lack of a neonatal testosterone "imprinting" on the liver, possibly because of end-organ insensitivity to testosterone or a deficient testicular biosynthesis of androgens.

Einarsson *et al.* (11) recently classified the hydroxylation of steroids into three classes. In class 1, hydroxylation reactions occur 2-3 times faster in adult male than in adult female rats; these reactions are under the control of continuous androgen production in the male. Castration of the adult male rat decreases these pathways, but treatment of the castrated rats with testosterone restores them to normal levels. In class 2, hydroxylations that occur in adult male rats are virtually absent in adult female rats. Postpubertal castration de-

creases these pathways, but only neonatal castration results in their complete loss. In class 3 are hydroxylations that show no sex difference. These pathways are probably regulated by nongonadal mechanisms. Based on these three classifications, it appears from the data presented in this paper that the 16α-hydroxylation of testosterone falls within the second category, and the 7α-hydroxylation of testosterone fits in the third classification. The 6β-hydroxylation of testosterone, however, appears to be under a different control from any described in the above categories. Only castration of the male rat at birth results in a completely feminine pattern of metabolism of testosterone at these three positions. This feminine pattern of metabolism in the neonatally castrated male rat parallels changes in the tunover of cytochrome P-450 in this animal.

Conney *et al.* (26), who studied factors regulating the hydroxylation of testosterone by rat liver microsomes, suggested that the term cytochrome P-450 may actually embrace a group of cytochromes with identical heme moieties bound to different proteins (26). Recently Lu and co-workers (28–30) resolved and partially purified the liver microsomal hydroxylation system into its component parts (cytochrome P-450, NADPH-cytochrome *c* reductase, and lipid) and showed that the substrate specificity for hydroxylation resides primarily in the cytochrome fraction rather than in the lipid or reductase fractions. There is an increasing body of evidence to suggest that multiple forms of cytochrome P-450 are present in liver microsomes obtained from the same animal (26, 31–34). The data presented in this paper provide further evidence for this concept and also show that there are sex differences in the content of at least two forms of cytochrome P-450 in rat liver microsomes.

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