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SEX STEROIDS AND DRUG METABOLISM

A SEX-RELATED DIFFERENCE IN HEPATIC MICROSOMAL ETHOXYRESORUFIN-*O*-DEETHYLATION IN SPRAGUE–DAWLEY RATS

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Abstract—The effect of sex steroids and pregnenolone-16α-carbonitrile (PCN) on hepatic microsomal monooxygenase activity was examined in male and female Sprague–Dawley rats. Male rats had a greater cytochrome(s) P-450 content and ethoxycoumarin-*O*-deethylase (ECOD) and NADPH–cytochrome *c* reductase (CcR) activities than females. Ethoxyresorufin-*O*-deethylation (EROD) in hepatic microsomes from females, however, was greater than from males, suggesting that the female rats possessed inherently higher cytochrome P-448-mediated monooxygenase activity. Testosterone propionate and PCN administration resulted in stimulation of ECOD and CcR activities in female rats, and PCN also increased the cytochrome(s) P-450 content. Estradiol benzoate decreased ECOD and CcR in male rats but enhanced EROD in both sexes. Neither testosterone nor PCN had an effect on EROD activity. In castrated male rats, testosterone increased, and estradiol decreased, ECOD activity without affecting cytochrome(s) P-450 content. Estradiol pretreatment of castrated males resulted in an increase in EROD activity, with testosterone having no effect. Thus, testosterone and estradiol may be, in part, responsible for the inherently higher and lower cytochrome(s) P-450-related activities in male and female rats, respectively, and estradiol may be responsible for the elevated cytochrome P-448-type activity observed in the females.

In the rat, androgen-mediated sex differences exist in a variety of cytochrome P-450-dependent drug-metabolizing enzyme systems, with males possessing inherently higher enzymic activity than females (for review, see Refs. 1 and 2). Whether similar sex differences exist in the metabolism of cytochrome P-448-type‡ substrates has not been investigated as thoroughly.

A number of investigators have suggested that hepatic microsomal aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase (AHH) activity in male rats is greater than in female rats and that androgens stimulate, and estrogens inhibit, this activity [3, 4]. Wiebel and Gelboin [5], however, have demonstrated in rats two types of hepatic AHH activities that were distinguished by their sensitivities to 7,8-benzoflavone. The type that predominated in the livers of adult male rats was inducible by phenobarbital, while in the livers of adult females a second type of AHH predominated that was inducible by polycyclic aromatic hydrocarbons in mature animals of either sex. Furthermore, 3-methylcholanthrene (3-MC), a cytochrome P-448-type inducer, has been shown to decrease the androgen-dependent drug-metabolizing enzyme activities of rat liver microsomes but to increase markedly those which are androgen-independent [6].

By using purified preparations of cytochromes P-448 and P-450 from livers of rats pretreated with 3-MC and phenobarbital, respectively, it has been demonstrated that the *O*-deethylation of ethoxyresorufin is accomplished almost exclusively by cytochrome P-448, with negligible deethylation being catalyzed by cytochrome P-450 [7–9]. Since different types of AHH may exist, and since this activity is not a highly specific indicator of cytochrome P-448-mediated monooxygenation [5, 10, 11], we have further investigated whether a sex difference in cytochrome P-448-type activity exists by examining the *O*-deethylation of ethoxyresorufin in hepatic microsomes from male and female Sprague–Dawley rats.

For purposes of comparison, hepatic microsomal NADPH–cytochrome *c* reductase and ethoxycoumarin-*O*-deethylase activities, as well as the cytochrome(s) P-450 content, were also measured, since these variables have been demonstrated to be greater in male than in female rats [1, 2, 12]. The

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‡ The term "cytochrome P-448" is used in this text to refer to that constitutive hemoprotein(s) P-450 that is responsible for the microsomal *O*-deethylation of ethoxyresorufin and that is inducible by 3-MC [7–9]. In this series of experiments, however, it is not possible to distinguish between cytochromes P-448 and P_1-450 as no induction studies were carried out using polycyclic aromatic hydrocarbons.

effects of testosterone propionate, estradiol benzoate, and pregnenolone-16α-carbonitrile (PCN), the synthetic steroidal cytochrome P-450-type inducer were also examined, as well as the effect of sex steroid treatment on hepatic microsomal monooxygenase activity in castrated male rats.

METHODS

Steroids. Testosterone propionate and estradiol benzoate were obtained from the Sigma Chemical Co. (St. Louis, MO). PCN was provided by the Upjohn Co. (Kalamazoo, MI).

Treatment. Intact male and female Sprague–Dawley (Madison, WI) rats weighing 150–180 g were acclimated for 1 week to a daily 6:00 a.m. to 6:00 p.m. photoperiod and were supplied with food and water *ad lib.* They were injected twice daily i.p. with 0.5 ml corn oil, or with testosterone propionate (25 mg/kg), estradiol benzoate (2.5 mg/kg), or PCN (12.5 mg/kg) in 0.5 ml corn oil, for 4 days. Pharmacological doses of testosterone propionate and estradiol benzoate were chosen to overcome the effects of endogenous sex steroids, if any, on the measured variables. In a second group of experiments, juvenile (4 weeks of age), male Sprague–Dawley rats were castrated and maintained in a 6:00 a.m. to 6:00 p.m. daily photoperiod for 3 weeks prior to steroid administration. They were then injected daily s.c. with 0.2 ml corn oil, or with testosterone propionate (1 mg/kg) or estradiol benzoate (5 μg/kg) in 0.2 ml corn oil, for 4 days. These doses of steroids are representative of standard physiological replacement therapy in castrated animals for the maintenance of secondary sexual characteristics [13]. All injection solutions were prepared on the day of administration.

Preparation of microsomes. Rats were killed by cervical dislocation, and the livers were excised, weighed, and rinsed in 0.15 M KCl. The livers were scissor-minced and washed three times with 0.15 M KCl. After washing, the tissues were homogenized in 3 vol. of 0.25 M sucrose. Homogenates were centrifuged at 8500 *g* for 20 min using a Sorvall RC-5 Superspeed centrifuge, the supernatant fraction was decanted and centrifuged at 165,000 *g* for 60 min in a Beckman L5-65 ultracentrifuge; the resultant microsomal pellet was washed in 20 ml of 0.15 M KCl and recentrifuged at 165,000 *g* for 60 min. The washed pellet was resuspended in SET buffer [20 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose and 5.4 mM EDTA] to a concentration equivalent to 1 g wet weight of liver/ml suspension. All operations were performed at 0–4° and, except for electrophoresis, the microsomes were utilized on the day of preparation.

Assays and electrophoresis. NADPH and 7-hydroxycoumarin (umbelliferone) were obtained from the Sigma Chemical Co. 7-Ethoxycoumarin was purchased from the Aldrich Chemical Co. (Milwaukee, WI). Ethoxyresorufin was synthesized by Dr. S. R. Challend, Wellcome Research Labs, Beckenham, Kent, England, from resorufin obtained from Eastman Kodak, Ltd. All other reagents were of the highest grade commercially available.

Ethoxycoumarin-*O*-deethylation (ECOD), ethoxyresorufin-*O*-deethylation (EROD), and NADPH–cytochrome *c* reductase (CcR) assays were performed by published procedures [7, 14, 15]. The cytochrome(s) P-450 content was determined by the procedure of Omura and Sato [16] using an Aminco DW-2a spectrophotometer, and microsomal protein content was estimated by a modification of the method of Lowry *et al.* [17].

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed essentially by the method of Laemmli and Favre [18] using 3% and 7.5% (w/v) total acrylamide concentrations in the stacking and resolving gels, respectively, with SDS at a concentration of 0.1% (w/v). Gels were stained with Coomassie Brilliant Blue R-250 and densitometric scans of the stained electrophoretograms were obtained at 550 nm using an ISCO gel scanner accessory in combination with an ISCO Type 6 optical unit and Type UA-5 absorbance monitor. Molecular weight standards electrophoresed simultaneously with microsomal samples included ovalbumin (mol. wt = 43,000), α-amylase (45,000), hexokinase (51,000), carnitine acetyltransferase (55,000), catalase (58,000), and bovine serum albumin (68,000).

Statistical comparisons between groups were carried out using Student's *t*-test.

RESULTS

Pretreatment of male rats with testosterone propionate or PCN resulted in a significant decrease in the yield of microsomal protein/g liver, whereas estradiol benzoate had no effect on this variable. Estradiol did, however, increase the liver weight/body weight ratio in male rats. In female rats, steroid pretreatment had no effect on the yield of microsomal protein per unit wet weight of liver. Estradiol benzoate and PCN, however, significantly increased liver weight/body weight ratios. In castrated male rats, estradiol decreased the yield of hepatic microsomal protein. There was no effect of either steroid on liver weight/body weight ratios.

Table 1 compares the hepatic microsomal drug-metabolizing enzyme activity and the cytochrome(s) P-450 content of corn oil-pretreated male and female rats. ECOD and CcR activities were significantly higher in males than in females. EROD activity, expressed both as a function of protein and cytochrome(s) P-450 content, was significantly higher in microsomes from female rats.

Hepatic microsomal cytochrome(s) P-450 content from steroid-pretreated male, female and castrated male rats is shown in Table 2. PCN significantly increased the cytochrome(s) P-450 content in female rats, whereas testosterone and estradiol had no effect on this variable. In males, estradiol treatment decreased microsomal cytochrome(s) P-450 levels. Treatment of male rats with testosterone or PCN did not alter the cytochrome(s) P-450 content when compared to controls, and no differences were noted in the cytochrome(s) P-450 levels with either sex steroid pretreatment in the castrated males. No shift of the Soret maximum of the carboxyferrohemoprotein complex was observed with steroid pretreatment in

Table 1. Comparison of hepatic microsomal monooxygenase activity and cytochrome(s) P-450 content in male and female Sprague–Dawley rats

Assay	Males*	Females*
Ethoxycoumarin-*O*-deethylation		
nmoles product formed·min^{-1}·(mg protein)$^{-1}$	1.67 ± 0.13†	0.54 ± 0.03
nmoles product formed·min^{-1}·(nmole P-450)$^{-1}$	2.07 ± 0.14†	0.87 ± 0.08
Ethoxyresorufin-*O*-deethylation		
nmoles product formed·min^{-1}·(mg protein)$^{-1}$	0.14 ± 0.01	0.20 ± 0.02†
nmoles product formed·min^{-1}·(nmole P-450)$^{-1}$	0.15 ± 0.01	0.32 ± 0.02†
NADPH–cytochrome *c* reductase		
nmoles reduced·min^{-1}·(mg protein)$^{-1}$	79.5 ± 3.6†	59.5 ± 1.4
Cytochrome(s) P-450 content		
nmoles/mg protein	0.91 ± 0.05†	0.63 ± 0.04

* Values are means ± S.E.M.; N = 4.
† Significantly different from opposite sex ($P < 0.05$).

Table 2. Cytochrome(s) P-450 content in hepatic microsomes from male, female and castrated male rats pretreated with selected steroids

	Cytochrome(s) P-450 content† (nmoles/mg protein)		
Treatment*	Males	Females	Castrated males
Corn oil	0.91 ± 0.05	0.63 ± 0.04	0.46 ± 0.03
Testosterone propionate	0.86 ± 0.05	0.63 ± 0.02	0.52 ± 0.01
Estradiol benzoate	0.58 ± 0.05‡	0.51 ± 0.02	0.46 ± 0.02
PCN	1.20 ± 0.10	1.29 ± 0.07‡	

* Refer to text for treatment regimens for intact male and female and castrated male animals.
† Values are means ± S.E.M.; N = 4 for each of the three groups.
‡ Significantly different from corn oil-pretreated controls ($P < 0.05$).

Table 3. NADPH–cytochrome *c* reductase activity in hepatic microsomes from male, female, and castrated male rats pretreated with selected steroids

	NADPH–cytochrome *c* reductase activity† [nmoles·min^{-1}·(mg protein)$^{-1}$]		
Treatment*	Males	Females	Castrated males
Corn oil	79.5 ± 3.6	59.9 ± 1.4	44.9 ± 3.5
Testosterone propionate	82.2 ± 4.6	98.3 ± 4.5‡	51.1 ± 4.8
Estradiol benzoate	57.6 ± 2.4‡	47.5 ± 2.2‡	61.3 ± 2.8‡
PCN	70.1 ± 5.9	123.0 ± 6.9‡	

* See text for treatment regimens for intact male and female and castrated male animals.
† Values are means ± S.E.M.; N = 4 for each of the three groups.
‡ Significantly different from corn oil-pretreated controls ($P < 0.05$).

Table 4. Ethoxycoumarin-*O*-deethylase activity in hepatic microsomes from male, female, and castrated male rats pretreated with selected steroids

	Ethoxycoumarin-*O*-deethylation†					
	Males		Females		Castrated males	
Treatment*	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmole P-450)$^{-1}$]	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmole P-450)$^{-1}$]	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmole P-450)$^{-1}$]
Corn oil	1.67 ± 0.13	2.07 ± 0.14	0.54 ± 0.03	0.87 ± 0.08	0.82 ± 0.09	1.79 ± 0.20
Testosterone propionate	1.59 ± 0.12	1.83 ± 0.06	0.96 ± 0.11‡	1.66 ± 0.18‡	1.05 ± 0.08§	2.01 ± 0.15
Estradiol benzoate	1.15 ± 0.13‡	1.96 ± 0.13	0.54 ± 0.01	1.05 ± 0.07	0.61 ± 0.02‡	1.33 ± 0.06‡
PCN	1.85 ± 0.10	1.80 ± 0.11	0.83 ± 0.09‡	0.92 ± 0.03		

* Refer to text for treatment regimens for intact male and female and castrated male animals.
† Values are means ± S.E.M.; N = 4 for each of the three groups.
‡ Significantly different from corn oil-pretreated controls ($P < 0.05$).
§ Significantly different from corn oil-pretreated controls ($P < 0.07$).

Table 5. Ethoxyresorufin-*O*-deethylase activity in hepatic microsomes from male, female, and castrated male rats pretreated with selected steroids

	Ethoxyresorufin-*O*-deethylation†					
	Males		Females		Castrated males	
Treatment*	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmole P-450)$^{-1}$]	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmole P-450)$^{-1}$]	[nmoles·min^{-1}·(mg protein)$^{-1}$]	[nmoles·min^{-1}·(nmoles P-450)$^{-1}$]
Corn oil	0.14 ± 0.01	0.15 ± 0.01	0.20 ± 0.02	0.32 ± 0.02	0.05 ± 0.01	0.12 ± 0.02
Testosterone propionate	0.17 ± 0.01	0.20 ± 0.02	0.22 ± 0.03	0.34 ± 0.04	0.07 ± 0.01	0.13 ± 0.01
Estradiol benzoate	0.29 ± 0.01‡	0.50 ± 0.05‡	0.24 ± 0.01	0.47 ± 0.04‡	0.08 ± 0.01‡	0.17 ± 0.01‡
PCN	0.15 ± 0.01	0.14 ± 0.01	0.23 ± 0.03	0.26 ± 0.02		

* Refer to text for treatment regimens for intact male and female and castrated male animals.
† Values are mean ± S.E.M.; N = 4 for each of the three groups.
‡ Significantly different from corn oil-pretreated controls ($P < 0.05$).

any group, nor was a difference noted in the λ_{max} between male and female animals.

Pretreatment of female rats with testosterone or PCN resulted in an increase in hepatic microsomal CcR activity when compared to corn oil-injected controls. These steroids had no effect on reductase activity in male animals. Estradiol significantly decreased CcR activity in both male and female rats when compared to their respective controls (Table 3). In castrated males, estradiol stimulated reductase activity, whereas testosterone had no effect.

Table 4 shows ECOD activity in microsomes from steroid-pretreated male, female, and castrated male rats. Testosterone pretreatment had no effect on male rats, but significantly increased ECOD activity in female rats when compared to controls. Treatment of male rats with estradiol resulted in a decrease in ECOD activity when expressed as a function of microsomal protein, but no difference was noted when the data were expressed as a function of the cytochrome(s) P-450 content, probably due to the effect of estradiol on the cytochrome(s) P-450 content in male rats (Table 2). Estradiol treatment of female rats did not change ECOD activity from control levels. Male rats pretreated with PCN exhibited no change in ECOD activity, whereas females showed elevated ECOD levels compared to controls when expressed as a function of microsomal protein. This difference was not observed when the data were expressed per nmole cytochrome P-450, most likely due to the stimulatory effect of PCN on cytochrome(s) P-450 content in female rats (Table 2). Testosterone pretreatment of the castrated male animal resulted in a slight but not very significant ($P < 0.07$) increase in ECOD activity when expressed per mg protein, and estradiol caused a decrease in the deethylation of ethoxycoumarin in castrated males regardless of how the data are expressed.

Ethoxyresorufin-*O*-deethylase activity of microsomes from steroid-pretreated male, female, and castrated male rats is illustrated in Table 5. Neither testosterone nor PCN had an effect on EROD activity in male, female, or castrated male rats when compared to their respective corn oil-pretreated controls. Treatment with estradiol, however, resulted in an increase in EROD in the male and castrated male. Estradiol also increased EROD activity in female rats when expressed as a function of cytochrome(s) P-450 content.

There were no significant differences noted in the SDS–PAGE protein profiles between male and female rats (Fig. 1), nor did the sex steroids have an appreciable effect on the banding pattern in male or female animals. Electrophoretic profiles of hepatic microsomes from PCN-pretreated rats of both sexes demonstrated the accentuation of a band with an apparent molecular weight of 44,000 in our system. This protein differs from that induced by phenobarbital (mol. wt = 48,000) or 3-MC (mol. wt = 52,000).

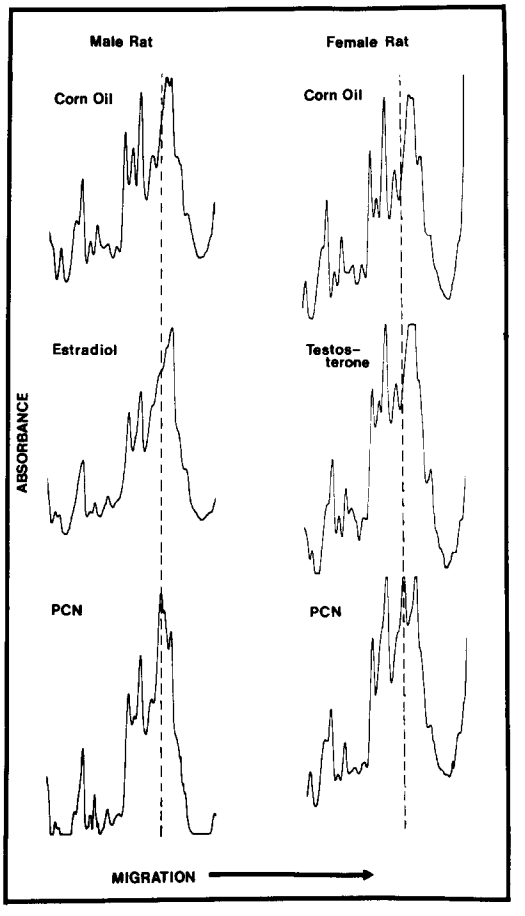

Fig. 1. Densitometric scans of electrophoretograms of hepatic microsomes from male and female Sprague–Dawley rats pretreated with selected steroids. All sample tracts contained 75 μg protein. The vertical broken line is 44,000 daltons.

DISCUSSION

The results of these experiments support those of previous investigators with respect to the effects of testosterone, estradiol, and PCN on hepatic cytochrome(s) P-450-type monooxygenase activity and on at least one component of the NADPH-dependent electron transport system in male and female rats. Specifically, testosterone stimulated ECOD and CcR activities without an effect on cytochrome(s) P-450 content. Estradiol administration to male rats decreased ECOD, CcR, and the hepatic cytochrome(s) P-450 content.

It has been suggested that the higher hepatic microsomal cytochrome(s) P-450 content in male rats, when compared to females, could not be solely responsible for the observed differences in monooxygenase activity, as this variable alone could not explain the magnitude of the sex difference seen in the metabolism of some substrates. Schenkman *et al.* [19] and Kato and Onoda [20] have demonstrated that the sex differences in monooxygenase activity in rats may be related to a difference in substrate affinity for cytochrome(s) P-450. Castration of male rats increased the apparent K_m values for aminopyrine-*N*-demethylation and hexobarbital hydroxylation, whereas the administration of testosterone to these animals decreased the K_m. Castration of males also increased the spectral dissociation constant (K_s) values for aminopyrine and hexobarbital,

with testosterone treatment reversing this effect. Kato and Onoda [20] have also shown that the administration of estradiol or diethylstilbestrol counteracted these androgen effects. Apparently then, androgens may regulate the capacity of the cytochrome(s) P-450 to interact with certain substrates, whereas estrogens may interfere with these binding capacities, as well as decrease the cytochrome(s) P-450 content.

Other investigators have suggested that the observed sex differences may be due to differences in the activity of NADPH–cytochrome P-450 reductase [21, 22]. Specifically, these studies demonstrated that the substrate-enhanced rate of reduction of cytochrome(s) P-450 in male rats was greater than in female rats and could account for the sex differences observed in the *N*-demethylation of ethylmorphine.

Finally, in view of the fact that multiple forms of hemoproteins P-450 have been demonstrated in mammals, it is not unlikely that discrete specie(s) of cytochrome(s) may be responsible for the differences between males and females in the rate of metabolism of specific substrates. Recent data demonstrate that some species of cytochrome P-450 appear to be present in microsomes from male Wistar rats in quantities greater than those from females [12].

Our data on CcR activity support those of Kato *et al.* [23] who showed higher reductase activity in male rats, compared to females. Moreover, Hamrick *et al.* [24] have demonstrated a stimulatory effect of methyltestosterone on CcR activity in female rats.

The studies with castrated male rats indicate that ECOD activity was slightly enhanced by testosterone administration and depressed by estradiol treatment, although neither steroid had an effect on cytochrome(s) P-450 content. These data further support the observation that estradiol acts on the hepatic monooxygenase system independently of its proposed antagonism of androgen effects or inhibition of androgen biosynthesis in intact animals. We are unable at present to explain the enhancement of CcR activity by estradiol and the lack of effect of testosterone in castrated male animals. The castrated males received physiological doses of the steroids for a short period of time; it is possible that more prolonged replacement therapy would have produced more conclusive results. Indeed, several investigators have demonstrated increased cytochrome P-450-related enzyme activities in castrated male animals after testosterone administration [3, 20, 25]. Pharmacological doses of steroids, however, were used in these studies, and the duration of treatment was extended. Whether adrenal-derived sex steroids exerted an effect on the variables measured or interfered with the action of the administered steroids in this and previous investigations with castrated male animals must also be considered.

Pregnenolone-16α-carbonitrile increased cytochrome(s) P-450 content and stimulated ECOD and CcR activities in female but not in male rats. This synthetic steroid has been shown to have a substrate specificity different from that of either phenobarbital or 3-MC [26], and the species of cytochrome P-450 induced appear to differ immunochemically as well [27]. Furthermore, several investigators have demonstrated that PCN induces a unique cytochrome(s) P-450 protein profile upon electrophoresis [28–30]. Our data support these results in that PCN caused the intensification of a protein band different from those induced by either phenobarbital or 3-MC. PCN has been shown previously to increase cytochrome(s) P-450 content and to stimulate CcR, aminopyrine-*N*-demethylase, and ethylmorphine-*N*-demethylase activities in female rats [31–33]. Lake *et al.* [34] have demonstrated an increase in cytochrome(s) P-450 content and ethylmorphine-*N*-demethylase activity in male rats pretreated with PCN at 40 mg/kg for 7 days. This difference in treatment regimen may explain our lack of effect of PCN in male animals, although an increase in cytochrome(s) P-450 content and an effect on microsomal electrophoretic protein profiles in males are suggested by our data.

The data with respect to elevated EROD activity in female rats, compared to that in males, have not, to our knowledge, been reported previously. Estradiol administration increased EROD activity in male and castrated male rats when expressed per milligram of microsomal protein and in all groups when expressed as a function of cytochrome(s) P-450 content. Whether the effect of estradiol on EROD activity was due to the direct action of the hormone on the liver, or whether this effect was dependent on the presence of an intact pituitary gland, as has been demonstrated with the estrogen-mediated hepatic microsomal metabolism of a number of other drugs and steroids [2], remains to be determined.

Considering the fact that estradiol decreased cytochrome(s) P-450 content in male rats, it is plausible to think that hormone treatment enhanced substrate binding to cytochrome P-448, similar to the proposed effect of testosterone on substrate affinity for cytochrome(s) P-450. On the other hand, estradiol may have been stimulating the production of a particular hemoprotein that may account for only a small proportion of the total hemoproteins P-450 and that is highly specific for cytochrome P-448-type substrates. This hypothesis is supported by the data of Wiebel and Gelboin [5] who demonstrated that female rat AHH was primarily cytochrome P-448-type. Whether this estrogen-stimulated basal cytochrome P-448-type activity is related to enhanced rates of biotransformation/excretion of endogenous or exogenous substrates in female rats remains to be elucidated.

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