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Changes in Dimethylaniline *N*-Oxidase Activity of Mouse Liver and Kidney Induced by Steroid Sex Hormones

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SUMMARY

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Activity of the flavin-containing monooxygenase (EC 1.14.13.8, *N,N*-dimethylaniline *N*-oxidizing) was measured in liver, kidney, and lung homogenates obtained from control; gonadectomized; and testosterone-, β-estradiol-, and progesterone-pretreated CF-1 mice. The sex-related differences in liver microsomal *N*-oxidase activity described by Wirth and Thorgeirsson [*Biochem. Pharmacol.* **27**:601–603 (1978)] appeared to be due primarily to testosterone repression of the liver enzyme. Activity, low in the adult male, rose significantly upon castration but returned to control levels upon testosterone replacement therapy. Activity of female liver, only marginally affected by ovariectomy, was markedly depressed by testosterone but rose slightly upon progesterone administration. In contrast to liver, testosterone appears to increase the amount of monooxygenase in kidney. Activity in this organ in sexually intact males was depressed by castration or by estradiol administration. The total amount of enzyme also increased following testosterone administration to gonadectomized or sexually intact females. Sex-related differences in lung *N*-oxidase activities could not be detected, and activity in this organ was not affected by gonadectomy or hormone pretreatment.

INTRODUCTION

The flavin-containing monooxygenase purified to homogeneity from hog liver (1) catalyzes oxygenation of diverse types of nucleophilic organic nitrogen and sulfur compounds (2). The monooxygenase preferentially catalyzes oxidative attacks on the heteroatom, yielding products readily distinguished from those produced by other microsomal monooxygenases. NADPH- and oxygen-dependent oxygenations catalyzed by the flavin-containing monooxygenase can be further differentiated from cytochrome P-450-dependent oxidations by differences in optimal pH, by use of selective inhibitors (3, 4), or by differential thermal inactivation of the flavin-containing enzyme (5). Although specificity of the latter monooxygenase is quite broad, its activity is usually determined by measuring the rate of *N,N*-dimethylaniline *N*-oxidation.

Dimethylaniline *N*-oxidase activity has been detected in a variety of mammalian tissues (6). In many species the enzyme is most concentrated in liver, although the studies of Devereux and Fouts (7) suggest that the activity of rabbit lung microsomes is equal to that of liver microsomes. While the *N*-oxygenation of dimethylaniline cannot be induced in hepatic tissue by pretreating animals with foreign compounds, age and sex differences have been described in rats (8, 9). Wirth and Thorgeirsson (10) have also shown that the activity is higher in liver from adult female mice than in liver from male mice.

This report describes studies on the effect of steroid sex hormones (17β-estradiol, testosterone, and progesterone) on the dimethylaniline *N*-oxidase activity of liver, kidney, and lung from CF-1 mice.

MATERIALS AND METHODS

White mice, ages 4–6 months, from the CF-1 strain were housed under controlled environmental conditions and allowed standard rations (Purina Lab chow) and water ad libitum. Male and female mice, separated at weaning, were housed in separate rooms with a daily 14-hr light/10-hr dark cycle. Female mice isolated from males remain largely in diestrus and exhibit low serum estradiol and progesterone concentrations (11).

N,N-dimethylaniline obtained from Eastman Chemi-

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cals Rochester, N. Y., was purified by gas chromatography prior to use. All other reagents used in the enzyme assays were of the highest purity available from commercial sources. 17β-Estradiol, progesterone, and testosterone were purchased from Sigma Chemical Company, St. Louis, Mo., and medical grade Silastic tubing used to construct subdermal hormone capsules was obtained from Dow Corning Corporation, Midland, Mich.

Hormone administration. Hormones were administered to both intact and gonadectomized mice from subdermally implanted, sustained-release capsules (12). To ensure consistent dose release, capsules were equilibrated in other mice for at least 2 weeks before use in experimental animals. The dimensions of the capsules were as follows, in millimeters outside diameter × inside diameter × length. The latter dimension refers to length of packed crystalline hormone. β-Estradiol, 1.96 × 1.47 × 25; testosterone, 3.18 × 1.98 × 25; and progesterone, 3.18 × 1.98 × 25. Two capsules implanted in gonadectomized mice released the hormones at the following approximate rates, expressed in micrograms per day per animal: 17β-estradiol, 42; testosterone, 230; and progesterone, 250. The capsules were implanted 2 weeks after gonadectomy and left in place for 14 days prior to killing the animals. Sexually intact males and females were also treated for 2 weeks with estradiol (42 μg/day) or with testosterone (690 μg/day) released from subdermally implanted capsules. Both control gonadectomized and intact animals received implants of empty capsules for the same period of time. The body weights of the animals used were: males, 35–40 g; females, 32–38 g.

Tissue preparation. Animals were routinely killed by decapitation between 8:00 and 8:30 a.m., but, because of the number of measurements per animal, no more than four animals were processed per day. Liver, lungs, and kidneys were rapidly excised and chilled in 0.25 M sucrose on ice. Rapid cooling was essential, since the flavin-containing monooxygenase is unusually sensitive to thermal inactivation (3, 13). All subsequent steps in tissue preparation were carried out at 0–4°. The organs from each animal were weighed, minced, and then homogenized in eight volumes of 0.25 M sucrose with a glass-Teflon tissue grinder. The volume of each homogenate was recorded and all homogenate assays were carried out within 4 hours after the animals were killed. The 9,000 × *g* pellet, microsomal, and cytosol fractions were also prepared from 10 ml of liver homogenate of selected animals. The 9,000 × *g* pellets, sedimented at 12,000 rpm for 10 min in a Spinco 40 rotor, were resuspended in 8 ml of 0.25 M sucrose and resedimented. The washed pellets were resuspended in 0.25 M sucrose to a final volume of 4.0 ml. The supernatant fraction, combined with the original 9,000 × *g* supernatant, was centrifuged at 40,000 rpm for 1 hr to sediment the microsomes. The microsomal pellets were washed once and resuspended in 2.0 ml of 0.25 M sucrose. To minimize mechanical loss the pellets were resuspended directly in the centrifuge tubes with a Teflon pestle attached to a 3-mm steel shaft. The volume of the final suspension was calculated from mass. The density of the resuspending medium at 4° was 1.033 and the density of the packed pellets was assumed to be no more than 1.10. The weight of the dry centrifuge tubes did not differ by more than ±2 mg before and after use.

Analytical methods. Protein concentration was determined by the biuret method (14). The *N*,*N*-dimethylaniline *N*-oxidase activity of homogenate and subfractions was routinely measured at pH 8.4 and 37° as described earlier (15). The rate of dimethylaniline *N*-oxide formation was linear for 10 min, and this product was quantitatively recovered after a 10-min incubation period with up to 5 mg/ml of homogenate or 3 mg/ml of microsomal protein. Steroid hormones, added either as concentrated suspensions in water or dissolved in ethanol, did not affect *N*-oxidation of dimethylaniline *in vitro*.

The effects of different pretreatments on *N*-oxidase activities were evaluated by one-way analysis of variance, and group means were compared by the Newman-Keuls procedure (16).

RESULTS

Plots of activity, as a function of pH, for mouse tissues were quite similar to those reported for hepatic tissue from other species (1, 3, 15). The optimal pH was between 8.4 and 8.6, and activity at the optimum was nearly twice that measured at 7.4. However, in contrast to hog and hamster, stimulation of mouse liver *N*-oxidase by *n*-octylamine was pH-dependent, as is shown in Fig. 1. The degree of stimulation by 3 mM octylamine, 10% or less at pH 8.5, increased with decreasing pH and was most pronounced below pH 7.4. In this respect, mouse liver dimethylaniline *N*-oxidase differs from the purified hog liver enzyme, which is stimulated approximately 2-fold by 3 mM *n*-octylamine at all hydrogen ion concentrations. Because stimulation by *n*-octylamine was marginal at the pH optimum, all *N*-oxidase activities of mouse tissues listed in subsequent sections were measured in the absence of this primary amine. However, in all tissues and at all pH values tested, *n*-octylamine never inhibited *N*-oxygenation of dimethylaniline, demonstrating that cytochrome P-450 does not contribute significantly to the

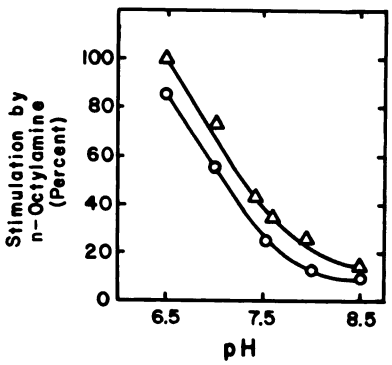

FIG. 1. *Stimulation of mouse liver dimethylaniline N-oxidase by n-octylamine as a function of pH*

The rate of *N*-oxide formation by liver homogenates from male (△——△) and female (○——○) mice was measured in the presence and absence of 3 mM octylamine. Percentage stimulation was calculated from the difference ± octylamine divided by rate in absence of octylamine × 100. The pH listed at each point was measured at 37° in the reaction vessel with the complete reaction mixture.

formation of this product. Octylamine is known to inhibit reactions catalyzed by cytochrome P-450 (17).

Effects of gonadectomy and steroid hormones on N-oxidase activity. In agreement with the earlier report by Wirth and Thorgeirsson (10), dimethylaniline *N*-oxidase activity was higher in liver from female mice than in liver from male mice. This was reflected in both a higher specific activity (Table 1) and total activity in this organ (Fig. 2). After gonadectomy, activity increased significantly in males and decreased somewhat in females. However, after gonadectomy, hepatic activity of testosterone-treated males and progestone-treated females was restored to values observed with intact animals. Estradiol administered to gonadectomized females further depressed hepatic activity, but the change over gonadectomy alone was not significant. However, in the castrate male, estradiol decreased hepatic activity to levels not significantly different from those of sexually intact animals.

Changes in total hepatic activity did not exactly parallel changes in specific activity, since the organ size was also altered by gonadectomy and hormone treatment (Table 2). For example, in castrate males, estradiol decreased specific activity by 45% but it also increased liver weight by 32%. On the other hand, testosterone depressed specific activity 52%, but liver size increased only 20%. In both sexes the testosterone-dependent decrease in specific activity was considerably greater than the increase in liver weight, resulting in significant enzyme repression.

The *N*-oxidase activity of kidney was also affected by steroid sex hormones, although not as dramatically as activity of liver. Hormone-dependent changes in total amount of enzyme (Fig. 2) were due primarily to changes in organ size (Table 2) rather than specific activity (Table 1). In the female, neither ovariectomy nor hormone treatment significantly changed dimethylaniline *N*-oxidase activity per milligram of tissue homogenate protein. However, testosterone increased organ size by more than 70%. This latter general anabolic effect appeared respon-

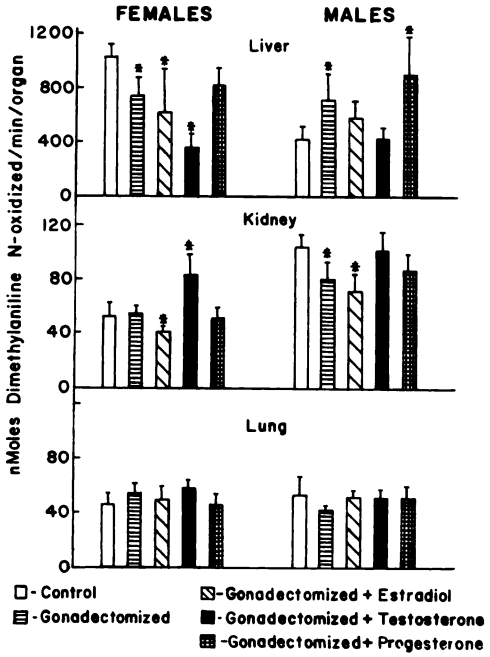

FIG. 2. *Changes in total organ dimethylaniline N-oxidase activities of mice following gonadectomy and hormone pretreatment*

The experimental conditions were as described in Table 1. Enzymatic activity was measured on an aliquot of the tissue homogenate as described under Materials and Methods, and total organ activity was calculated from original volume of homogenate. Values represent mean values ± standard error for at least four mice. Asterisks indicate averages significantly different from those of sexually intact (control) mice ($p < 0.05$).

TABLE 1

Changes in dimethylaniline N-oxidase activity of mouse tissues after gonadectomy and steroid hormone treatment

Hormones were administered for 2 weeks from subdermally implanted, sustained-release capsules at the following approximate dose per day per animal: 17β-estradiol, 42 μg; testosterone, 230 μg; and progesterone, 250 μg. Animals were gonadectomized 2 weeks before beginning hormone treatment. Control (sexually intact) and untreated gonadectomized mice received implants of empty capsules 2 weeks prior to killing. All values are expressed as means ± standard error for tissues from at least four animals. *N*-Oxidase activities were measured at pH 8.4 and 37°.

	Dimethylaniline *N*-oxidized/min/mg homogenate protein		
	Liver	Kidney	Lung
	nmoles		
Females			
Control	2.38 ± 0.076	0.77 ± 0.015	0.90 ± 0.032
Gonadectomized	1.77 ± 0.072	0.78 ± 0.016	0.68 ± 0.022
+ Estradiol	1.30 ± 0.120[a]	0.67 ± 0.021	1.03 ± 0.043
+ Testosterone	0.69 ± 0.045[a, b]	0.66 ± 0.039	0.82 ± 0.043
+ Progesterone	1.92 ± 0.095	0.71 ± 0.015	0.89 ± 0.023
Males			
Control	0.90 ± 0.060	1.04 ± 0.020	1.01 ± 0.054
Gonadectomized	1.83 ± 0.085[a]	1.20 ± 0.060	0.72 ± 0.070
+ Estradiol	1.00 ± 0.073[b]	0.99 ± 0.018	0.99 ± 0.023
+ Testosterone	0.88 ± 0.063[b]	0.88 ± 0.025	0.77 ± 0.055
+ Progesterone	2.02 ± 0.138[a]	1.13 ± 0.020	0.75 ± 0.030

[a] Significantly different from control of same sex ($p < 0.05$).
[b] Change induced by hormone treatment significantly different from gonadectomized animals of same sex ($p < 0.05$).

TABLE 2

Changes in organ weights of mice induced by gonadectomy and steroid hormone treatment

After chilling in 0.25 M sucrose, the organs were blotted dry and weighed. The values listed are mean wet weights ± standard error for tissues from at least four animals. Left and right kidneys and both lungs were combined before weighing. Hormones were administered as described in Table 1.

	Organ weight		
	Liver	Kidney	Lung
	g		
Females			
Control	1.79 ± 0.022	0.41 ± 0.011	0.26 ± 0.005
Gonadectomized	1.80 ± 0.030	0.42 ± 0.008	0.37 ± 0.016
+ Estradiol	2.41 ± 0.11[a, b]	0.43 ± 0.010	0.25 ± 0.013
+ Testosterone	2.29 ± 0.018[a, b]	0.73 ± 0.008[a, b]	0.36 ± 0.021
+ Progesterone	1.66 ± 0.055	0.40 ± 0.008	0.22 ± 0.005
Males			
Control	2.14 ± 0.028	0.61 ± 0.016	0.29 ± 0.020
Gonadectomized	1.86 ± 0.070	0.44 ± 0.010[a]	0.32 ± 0.018
+ Estradiol	2.45 ± 0.010[a, b]	0.47 ± 0.010[a]	0.25 ± 0.008
+ Testosterone	2.26 ± 0.11	0.69 ± 0.023[b]	0.32 ± 0.015
+ Progesterone	2.25 ± 0.045[b]	0.48 ± 0.005[a]	0.33 ± 0.010

[a] Significantly different from controls of same sex ($p < 0.05$).
[b] Change induced by hormone treatment significantly different from gonadectomized animals of same sex ($p < 0.05$).

sible for the increased total amount of kidney N-oxidase induced by testosterone. Hormone-dependent changes in the total kidney activity in males followed the same pattern observed with females and were due primarily to changes in organ size (Table 2).

The total amount of dimethylaniline N-oxidase activity of lung tissue was not significantly affected by gonadectomy or hormone treatment (Fig. 2). The small changes in specific activity (Table 1) were not statistically significant. The large individual variations within each group may have been due to differences in the amount of blood remaining in the lungs after decapitation. Variable amounts of blood protein would affect specific activity, but it should not markedly change estimations of the total amount of enzyme present in the whole organ. As is indicated in Fig. 2, there was no significant sex-dependent difference in total amount of N-oxidase present in lung tissue.

Effects of hormones on liver and kidney N-oxidase in sexually intact mice. As is shown in Fig. 3, testosterone reduced the total dimethylaniline N-oxidase in liver from intact females almost 70%, and activity per milligram of homogenate protein decreased from 2.3 ± 0.08 to 0.92 ± 0.04 nmoles of N-oxide formed per minute. Although testosterone did not significantly change specific activity in female kidney tissue, there was a significant increase in total enzyme activity owing to increased organ size.

Estradiol at the dose administered did not change hepatic N-oxidase activity of sexually intact males. However, this hormone did decrease kidney size, resulting in a 50% decrease in the total amount of enzyme present (Fig. 3). As observed with gonadectomized animals, the

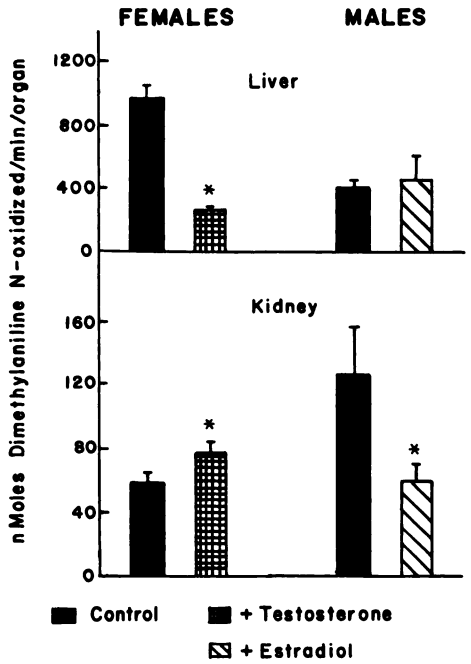

FIG. 3. *Effect of testosterone and estradiol on total organ N,N-dimethylaniline N-oxidase activity of sexually intact mice*

Animals were pretreated with hormones as described under Materials and Methods, and total organ activities were calculated as described in legend to Fig. 2. Asterisks indicate values significantly different from those of control mice ($p < 0.05$).

N-oxidase activity of lung tissue was not changed in estradiol- or testosterone-treated, sexually intact animals (data not shown).

Subcellular distribution of hepatic activity. Although most of the dimethylaniline N-oxidase activity of hepatic tissue is present in the microsomal fraction, some is also present in other organelles (18). To determine whether hormones affected subcellular distribution, the dimethylaniline N-oxidase activity was determined with hepatic 9000 × g and microsomal pellets separated as described under Materials and Methods. In sexually intact animals, specific activity in both particulate fractions from males was consistently lower than that in fractions from females. However, the sex-related difference in the microsomal fraction was greater than that in the 9000 × g pellet. This difference results in a significantly higher recovery of tissue activity in the 9000 × g fraction from males than in that from females. The higher activity in liver homogenates from females appears to be due primarily, but not entirely, to the increased activity of the microsomal fraction. This also appears to be the case following gonadectomy, although sex-dependent differences were much less pronounced.

Testosterone treatment further depressed activity of both particulate fractions in gonadectomized females. The 4-fold decrease in the microsomal fraction was much greater than in the 9000 × g pellet (2-fold). Testosterone administration to castrate males reversed the effects of gonadectomy; all values listed in Table 3 for this group were not significantly different from those for control mice.

Neither gonadectomy nor testosterone treatment affected the distribution of protein between the two particulate fractions (Table 3). Somewhat more than half of the homogenate protein was recovered in these fractions, and 37%–40% was recovered in the cytosol fractions. Some mechanical loss of protein undoubtedly occurred, since recovery in the three fractions was consistently less than 100%. However, the loss was never more than 8%, which should not substantially affect interpretation of treatment-related differences observed.

DISCUSSION

Sex difference in mouse liver dimethylaniline N-oxidase activity is primarily mediated by testosterone. Both specific activity (Table 1) and total organ activity (Fig. 2) increase in castrate males, and the changes are reversed by testosterone therapy. Testosterone pretreatment also depresses hepatic activity in both ovariectomized (Fig. 2) and sexually intact females (Fig. 3). Although testosterone repression is primarily responsible for the lower hepatic N-oxidase in males, the ovaries also have a minor effect. The N-oxidase activity, consistently lowered by ovariectomy, is partially restored by progesterone treatment (Fig. 2). However, it is unlikely that this hormone alone accounts for the higher activity of intact females. Serum progesterone concentrations probably do not exceed 0.3 μg/ml in female mice kept separate from males (11). Other possible ovarian factors involved were not identified.

In contrast to liver, total kidney N-oxidase activity increases in the kidneys of gonadectomized animals (Fig.

TABLE 3

Effect of gonadectomy and testosterone on distribution of N-oxidase activity in subfractions of mouse liver

The fractions were separated from homogenates by the procedure described under Materials and Methods. Pretreatment of animals was as described in Table 1. All values are listed as means ± standard error for no less than three animals; specific activity is expressed as nanomoles of dimethylaniline *N*-oxide formed per minute per milligram of protein at pH 8.4 and 37°.

	Homogenate SA	Specific activity (SA) and % homogenate activity recovered				% Homogenate protein recovered in	
		9000 × *g* pellet		Microsomes		9000 × *g* pellet	Microsomes
		SA	%	SA	%		
Females							
Control	3.10 ± 0.16	1.32 ± 0.050	16 ± 1.2	15.8 ± 0.60	71 ± 1.5	38 ± 1.5	14 ± 1.0
Gonadectomized	2.32 ± 0.09[a]	1.26 ± 0.045	22 ± 1.2	11.0 ± 0.38[a]	67 ± 7.2	40 ± 0.67	14 ± 1.1
+ Testosterone	0.84 ± 0.01[a]	0.61 ± 0.015[a]	28 ± 1.7[a]	3.7 ± 0.37[a]	60 ± 5.5	38 ± 3.0	14 ± 0.3
Males							
Control	1.13 ± 0.03	0.87 ± 0.037	31 ± 0.3	4.50 ± 0.40	53 ± 2.8	41 ± 2.3	14 ± 0.33
Gonadectomized	1.88 ± 0.02[a]	1.23 ± 0.068[a]	27 ± 1.2	7.09 ± 0.42[a]	60 ± 4.0	41 ± 0.66	14 ± 0.33
+ Testosterone	0.99 ± 0.09	0.69 ± 0.060	28 ± 0.88	4.22 ± 0.50	66 ± 2.3	40 ± 0.88	14 ± 0.57

[a] Significantly different from controls of same sex ($p < 0.05$).

2) and of sexually intact females (Fig. 3) treated with testosterone. However, this increase is due almost entirely to the androgen-induced increase in organ size (Table 2). Sex- or treatment-related changes in homogenate *N*-oxidase specific activity are not observed (Table 1).

Sex-dependent differences in hepatic *N*-oxidase are more pronounced in microsomal fractions than in either the whole homogenate or 9000 × *g* fraction. Activity of the latter fraction is only 2.5 times higher in the female than in the male, whereas the difference in the microsomal fraction is closer to 4 times (Table 3). A larger fraction of homogenate activity is also recovered in microsomes from females than males (71% versus 53%). The specific activity of the microsomal *N*-oxidase also appears to be more sensitive to gonadectomy and testosterone therapy than that of the 9000 × *g* fraction. For example, activity of the microsomes from females decreased more than 4-fold following ovariectomy and testosterone treatment, but the change in the 9000 × *g* fraction is only slightly more than 2-fold. The observed differences in specific activity or enzyme recovery in the particulate fractions are not due to marked changes in distribution of homogenate protein. The fraction of tissue protein recovered in the 9000 × *g* and microsomal fractions is relatively constant (Table 3). Whether sex-related differences in *N*-oxidase activity of the isolated subcellular fractions reflect real differences in subcellular localization or only differences in cell fragmentation or sedimentation properties is not known. Nevertheless, the data in Table 3 show that the microsomal fraction is not an accurate indicator of hepatic *N*-oxidase activity, and conclusions based only on activity measurements with isolated microsomes can be misleading.

Since mice are frequently used for studies on metabolism and toxicity of xenobiotics, sex differences in specific activity and organ distribution could be a factor in the metabolism of amine drugs (e.g., phenothiazines, guanethidine; cf. refs. 19 and 20) and organic sulfur compounds (5) largely or exclusively oxygenated by the flavin-containing monooxygenase.

The sex difference in hepatic dimethylaniline *N*-oxidase of mice is opposite that reported for rats. In rats this activity, highest in the male, is apparently induced by androgens (9). The *N*-demethylation of ethylmorphine, catalyzed exclusively by the cytochrome P-450 system, is also induced in rats but repressed in some strains of mice by androgens (21–23). It is surprising that the *N*-oxidation of dimethylaniline, catalyzed within detectable limits only by the flavin-containing monooxygenase, also appears to follow a similar pattern. Whether the responses of these two distinct monooxygenases to steroid sex hormones is also similar in other strains of mice or other species remains to be determined.

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