Sex Differences in Drug Metabolism by Rat Liver Microsomes

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SUMMARY

Sex differences in drug oxidase activity of liver microsomes from rats have been shown to be related to a difference in substrate affinity for the mixed function oxidase reaction, and not to a difference in the content of cytochrome P-450. These results, as well as spectral studies of substrate interaction with microsomal cytochrome, showed that microsomes isolated from livers of male rats had over twice the magnitude of substrate (hexobarbital and aminopyrine) binding than did microsomes isolated from the livers of female rats. Similar studies with aniline indicated only a small difference in V_{max} or substrate induced spectral change between microsomes isolated from livers of male or female rats.

INTRODUCTION

It has long been known that there is a sex difference in the response of rats to treatment of drugs, i.e., male rats are more resistant to the effects of several drugs than female rats (1, 2). This difference in response is due to a higher rate of drug metabolism in the livers of male rats (3) associated with an increased activity of the hydroxylating microsomal enzyme system (4–9). The higher drug-oxidizing activity seen in the liver microsomal fraction of male rats appears to be due to an androgenic effect, since Murphy and DuBois (10) have shown that castration of male rats decreases the rate of metabolism of Guthion, while administration of testosterone to female rats enhances the metabolism of Guthion (10) and hexobarbital (4). This type of sex specificity is unique for the rat and has not been observed in other animal species (2). Kato and Gillette (11), Schmidt-Volkmar (12), and Schmidt-Volkmar and Remmer (unpublished observations) have shown that the sex dependent difference of drug metabolism disappears during starvation of rats; drug-oxidizing activity is increased in liver microsomes prepared from starved female rats and decreased in the liver microsomes isolated from starved male rats. A similar effect (13) occurs upon treatment of rats with morphine. Morphine administration increases the *in vitro* drug oxidation rate by liver microsomes of female rats, but has the opposite effect on microsomes of male rats (13). In addition, treatment of rats with different hormones has been shown (14) to affect the enzyme activity of microsomes prepared from male rat liver, but not from female rat liver.

Not all drugs are oxidized at a faster rate by microsomes isolated from male rat liver. For example, Kato and Gillette (14) have found no sex difference in the metabolism of aniline and Zoxazolamine. In addition they found (14) that various treatment of rats, such as starvation or hormone administration, which affect the metabolism of drugs in the male animal, were

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without effect on the metabolism of aniline and Zoxazolamine.

Recently it has been shown that a microsomal cytochrome, P-450, is involved in steroid hydroxylation in adrenal cortex microsomes (15) and in liver microsomes (16–18). Remmer and Merker (19) and Orrenius and Ernster (20) found the activity of the liver microsomal drug oxidizing enzyme system to parallel the microsomal cytochrome P-450 both in content and in time course of induction after treatment of rats with phenobarbital, using female and male rats, respectively.

The addition of substrates of the liver microsomal mixed function oxidase to a microsomal suspension has recently been found to cause two types of spectral changes (21, 22) as well as a modification of the electron paramagnetic resonance (ESR) spectrum (23) associated with substrate interaction with some microsomal pigment. The magnitude of the spectral changes is dependent on the amount of substrates and of microsomal particles added, suggesting a substrate affinity to the enzyme. The pigment with which these substrates interact was shown to be cytochrome P-450 by relating the effect of substrates on the CO-complex of this terminal oxidase (24).

The present study was undertaken to determine whether the higher rate of drug oxidation observed with microsomes isolated from livers of male rats was due to a difference in the content of the microsomal mixed function oxidase or to differences in substrate affinity of this enzyme system.

METHODS

Microsomal fractions were prepared at 0° from 0.25 M sucrose homogenates of rat liver as described elsewhere (25). The microsomes were washed with 0.15 M KCl to remove adventitious hemoglobin, and resuspended in 0.15 M KCl containing 60 mM Tris-chloride buffer of pH 7.5, to a protein concentration of about 20 mg/ml. The assay medium contained 50 mM Tris-chloride buffer, pH 7.5, 0.33 mM TPN^+ (unless otherwise stated), 5 mM $MgCl_2$, 8 mM sodium isocitrate, and 15 μg/ml Sigma type IV isocitric dehydrogenase. Substrate concentrations were as indicated, as were microsome concentrations. The rate of aminopyrine oxidation was determined from the rate of formaldehyde production, while the rate of aniline oxidation was measured by determining the rate of *p*-aminophenol formed. Hexobarbital oxidation was determined by substrate disappearance as described previously (26). Other methods of assay, both enzymic and spectral, have been reported previously (24). Adult male rats and adult female rats of the Holtzman strain (Wisconsin) were used as indicated.

RESULTS

A comparison of the content of cytochrome P-450 in microsomes isolated from the livers of adult male and immature or adult female rats, surprisingly, did not reveal differences in magnitude similar to those expected from the observed differences in the drug oxidase activities. For

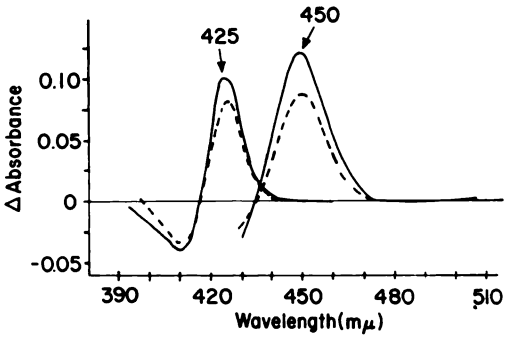

FIG. 1. *Comparison of cytochrome b_5 and cytochrome P-450 content in liver microsomes from male and female rats*

Liver microsomes of male (——) or female (- - - - -) rats were suspended to 2 mg/ml in 50 mM Tris buffer, pH 7.5, and added to two cuvettes. A 0.01-ml aliquot of a 2% DPNH solution was added to one cuvette to obtain the difference spectrum of cytochrome b_5 (425 mμ peak). In a separate experiment cytochrome P-450 was determined by bubbling CO for 1 min into one of a similar pair of cuvettes, and $Na_2S_2O_4$ was then added to both cuvettes. This action eliminates the contribution of cytochrome b_5 to the spectrum. Difference spectra were obtained with a split-beam recording spectrophotometer.

TABLE 1

Rats	Number of rats, n	Body weight (g)	Liver weight (% of body weight)	Microsomal protein (mg/g liver)	Formaldehyde formed from aminopyrine (mμmoles/mg protein/min)	$\Delta E \times 10^{-3}$/mg protein[a]			Ratio of the rate formaldehyde formed per Cyt P-450[c]
						Cyt b_5	Cyt P-450	Cyt P-450 hexobarbital complex	
Male	16	231 ± 37[d]	4.4 ± 0.8	18.5 ± 1.9	8.5 ± 0.8	50 ± 4.5	59 ± 7	7.5 ± 2.0	0.14
Male (castrated)[b]	10	200 ± 37	4.5 ± 0.4	18.6 ± 1.9	5.7 ± 0.5	46 ± 7.5	46 ± 5	4.8 ± 0.9	0.12
Female	9	189 ± 10	3.8 ± 0.5	15.7 ± 2.2	4.1 ± 0.8	54 ± 8	51 ± 6	2.8 ± 0.9	0.08
Female (ovariectomized)[b]	8	193 ± 12	4.1 ± 0.4	13.7 ± 2.1	3.5 ± 1.0	48 ± 12	45 ± 8	2.6 ± 0.6	0.08

[a] The change in absorbance observed on addition of DPNH (cytochrome b_5: 425 mμ–490 mμ), sodium dithionite plus CO (cytochrome P-450: 450 mμ–490 mμ) or 2 mM hexobarbital (hexobarbital complex: 420 mμ–445 mμ) to microsomal suspension as described in Methods.

[b] Results obtained 1 week after castration or ovariectomy.

[c] Expressed as millimicromoles of HCHO/min/$\Delta E \times 10^{-3}$.

[d] Standard deviations are indicated by ±.

TABLE 2

Rats	Number of rats, n	Body weight (g)	Liver weight (% of body weight)	Microsomal protein (mg/g liver)	Aniline-hydroxylation (mμmoles/mg protein/min)	$\Delta E \times 10^{-3}$/mg protein[a]			Ratio of rate aniline hydroxylation per Cyt P-450 content[c]
						Cyt P-450	Cyt b_5	Cyt P-450 aniline-complex	
Male	4	248 ± 36[d]	3.1 ± 0.01	17.8 ± 2	0.72 ± 0.06	54 ± 2.6	56 ± 2.4	16.7 ± 3.1	0.0133
Male (castrated)[b]	5	271 ± 50	3.1 ± 0.02	17.8 ± 2.5	0.58 ± 0.07	43 ± 4.4	49 ± 1.7	13.4 ± 1.7	0.0135
Female	5	237 ± 12.9	3.4 ± 0.32	18.5 ± 1.7	0.62 ± 0.06	44 ± 4.5	46 ± 6.2	14.4 ± 1.3	0.014
Female (ovariectomized)[b]	5	281 ± 34	3.3 ± 0.36	17.7 ± 0.98	0.56 ± 0.08	44 ± 2.9	39 ± 3.7	12.2 ± 0.9	0.013

[a] The change in absorbance observed on addition of DPNH (cytochrome b_5: 425 mμ–490 mμ), sodium dithionite plus CO (cytochrome P-450: 450 mμ–490 mμ) or 15 mM aniline (aniline complex: 430 mμ–450 mμ) to microsomal suspensions as described in Methods.

[b] Results obtained 1 week after castration or ovariectomy.

[c] Expressed as millimicromoles of *p*-aminophenol formed/min/$\Delta E \times 10^{-3}$.

[d] Standard deviations are indicated by ±.

example, while the rates of oxidation of aminopyrine, codeine, and many other compounds are 2–3 times faster with microsomes isolated from livers of male rats compared to microsomes isolated from livers of female rats, the content of cytochrome P-450 is only about 20% higher. Figure 1 shows a comparison of the spectral contributions of cytochrome b_5 and cytochrome P-450 in microsomes isolated from male and female rat livers. The cytochrome b_5 content ranges from equal to about 20% higher in microsomes from male rat liver, while the cytochrome P-450 content is significantly 15–20% higher (Tables 1 and 2). This latter small difference is insufficient to explain the differences observed in mixed function oxidase activity.

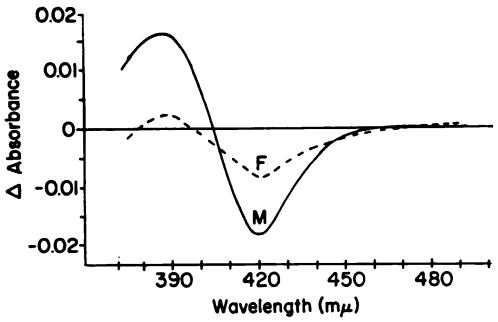

FIG. 2. *Comparison of the magnitude of the type I spectral change obtained with microsomes from livers of male and female rats*

Sodium hexobarbital (2 mM) was added to one of a pair of cuvettes containing 2 mg of protein per milliliter of liver microsomes from male (———) or female (-----) rats; the difference spectra were recorded.

An examination of the magnitude of spectral change caused by different substrates (Fig. 2) indicated that the magnitude of type I spectral change (trough 420 mμ, peak 385 mμ) (21, 24), caused by substrates like aminopyrine and hexobarbital, is much greater with microsomes isolated from male than from female rat liver. When expressed on a per milligram protein basis, the magnitude of interaction between hexobarbital and the pigment of microsomes from male rat liver was about 2.7 times greater than that seen with microsomes from female rat liver (Table 1).

Differences were also observed in the substrate dependence of both spectral change and enzyme activity (Fig. 3). The Lineweaver-Burk plots in Fig. 3A show differences in the substrate dependence of hexobarbital oxidase, as reflected by the different K_m values obtained with microsomes isolated from livers of male or female rats. The V_{max} value is about three times higher with microsomes from livers of male rats (6.0 mμmoles/min/mg protein) than microsomes from livers of female rats (1.8 mμmoles/min/mg protein). Figure 3B shows reciprocal plots relating the hexobarbital dependence of type I spectral change, as determined at 420 mμ relative to 445 mμ, with suspensions of microsomes isolated from male and female rat livers. The maximal optical density value for this type of spectral change is 2.5 times higher in the microsomes from male liver, in agreement with the V_{max} value obtained for enzyme activity (see also Table 1). The intercept on the abscissa, termed "spectral dissociation constant" (21, 24), was higher with microsomes isolated from livers of female than male rats. Although there is not a direct correlation between the absolute values of K_m as determined by enzymatic assay and K_s as determined spectrophotometrically, there is a similar trend in the differences between the two types of microsomes. The differences in the values of the constants obtained here presumably reflect the differences in the concentration of hexobarbital used for the two types of experiments, i.e. it was not possible to measure hexobarbital concentrations in such a low range in the enzymic assay for the K_m determination. Thus the greater tolerance or resistance to drugs by male rats may be due to a greater affinity of the drug to the enzyme (lower K_m and K_s), coupled together with the increased ability of the mixed function oxidase system to metabolize the drug.

A similar series of comparisons of substrate dependence for aminopyrine demethylase activity and aminopyrine induced spectral change revealed similar differences between microsomes isolated from livers of male and female rats. The magnitude of

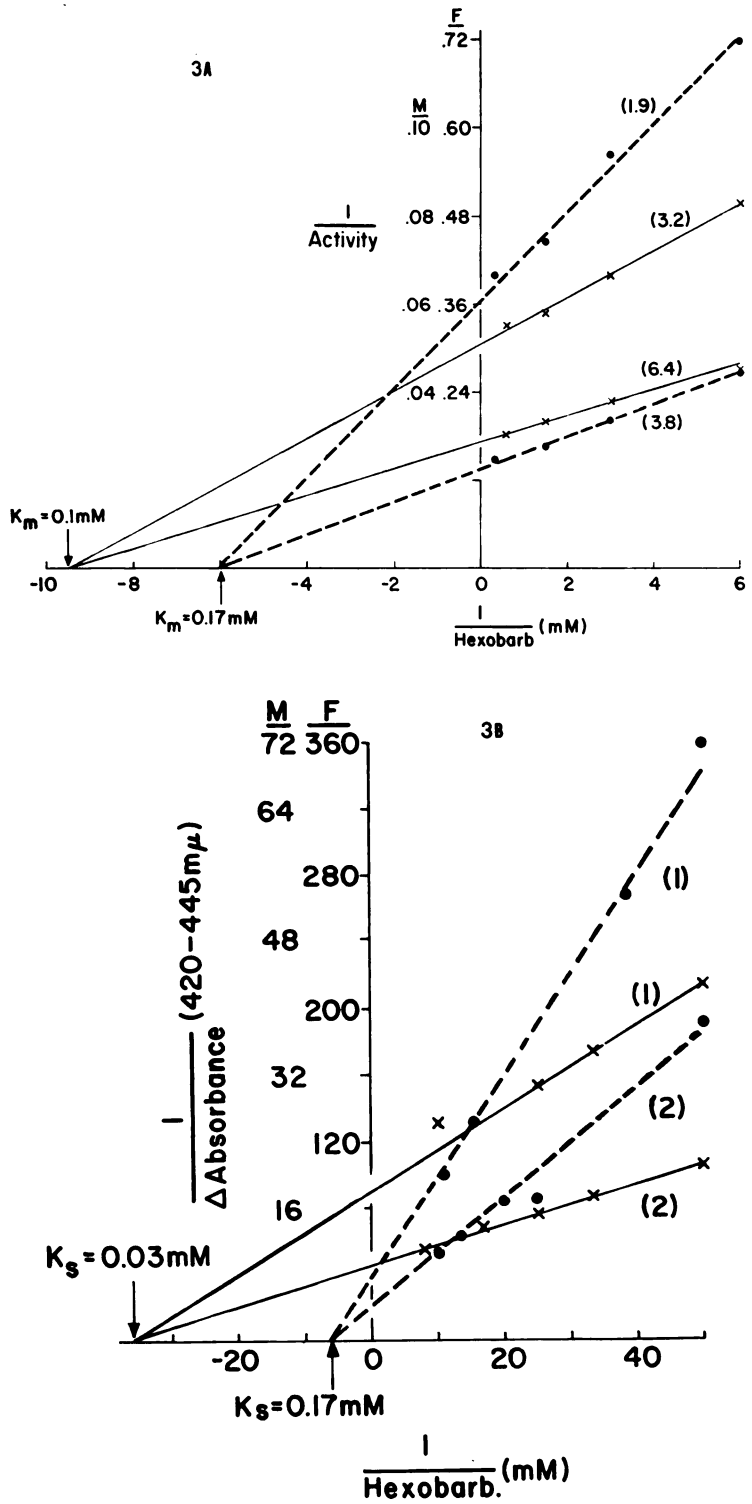

FIG. 3. *Comparison of the hexobarbital dependence of hexobarbital metabolism (A) and type I*

spectral change caused by aminopyrine as determined by enzymic assay, as well as the V_{max} value, were higher with the microsomes isolated from male rats. The spectral dissociation constant K_s, as well as the K_m for aminopyrine as determined by enzymic assay, were higher with the microsomes isolated from female rats. Thus the pattern of differences described above with hexobarbital is likewise observed with the substrate aminopyrine.

In contrast to the results described above, there was only a small difference in the rate of *p*-hydroxylation of aniline when microsomes from male or female rats were used (Table 2). This is in agreement with the reported lack of sex difference in aniline oxidation (11). The present studies show that microsomes from male rats hydroxylated aniline only 18% faster than microsomes from livers of female rats. It is noteworthy that nearly the same difference was observed in the magnitude of type II spectral change (21) caused by aniline addition to microsomal suspensions (Fig. 4) and in the amount of cytochrome P-450 present (Table 2).

Figure 5 shows reciprocal plots of the aniline dependence of the type II spectral change for microsomes from male and female rat livers. No significant difference

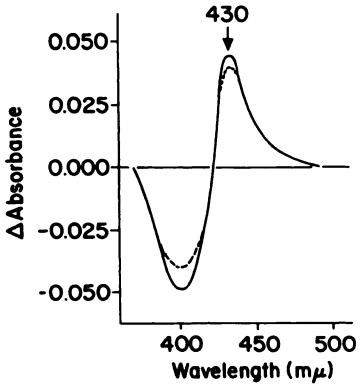

FIG. 4. *Comparison of type II spectral change obtained with microsomes from livers of male and female rats*

Aniline (final concentration about 15 mM) was added to one of a pair of cuvettes containing 2.8 mg protein per milliliter of liver microsomes from male (———) or female (- - - - -) rats; the difference spectra were recorded.

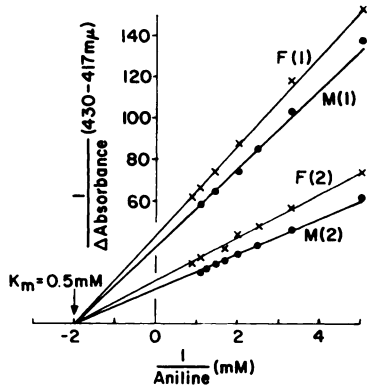

FIG. 5. *Comparison by reciprocal plots of aniline dependence of the type II spectral change, using microsomes isolated from livers of male and female rats*

The change in absorbance was measured at 430 mμ minus 417 mμ with an Aminco-Chance dual wavelength spectrophotometer. Microsome concentrations employed are indicated in parentheses for males (●) and females (×).

spectral change (B) between liver microsomes of male and female rats as expressed by reciprocal plots

(A) The microsome concentrations employed are indicated in parentheses for female (●) and male (×) rats. Since the rate of enzyme activities differed, two ordinates were used: M = scale for males; F = scale for females. Activities are expressed as millimicromoles of hexobarbital oxidized per milliliter of reaction medium per minute. The rate of hexobarbital oxidation was determined by the removal of 0.5–1.5 ml aliquots from the incubation medium at 0, 10, and 20 min. The aliquots were added to 30 ml of petrol ether, and the extent of substrate disappearance was determined spectrophotometrically as described previously (24).

(B) The changes in absorbance (420–445 mμ) were measured with an Aminco-Chance dual wavelength spectrophotometer. Two ordinate scales are used, since spectral changes are much greater with microsomes from male rats. M = scale for males; F = scale for females. Microsomal protein concentrations for males (×) and females (●) are indicated in parentheses in the figure.

was observed in the spectral dissociation constant between the two types of microsomes. In a similar manner the dependency of the rate of aniline hydroxylation on the concentration of aniline indicated no significant difference ($K_m = 0.04$ mM) between microsomes from livers of male and female rats. As noted previously (24), aniline is one of the few substrates so far examined where a direct correlation of the enzymically determined K_m and the spectrally determined K_s do not correspond.

One week after castration, the rate of oxidation of aminopyrine and the change of absorbance obtained with hexobarbital (420–445 mμ) did not yet reach the respective values obtained with microsomes isolated from livers of female rats. However the cytochrome P-450 content (Table 1) decreased a little and corresponded to the amount of cytochrome P-450 found in microsomes from female rats. By 2 weeks after castration of male rats the K_m value obtained for aminopyrine demethylation increased to a value intermediate between that found with liver microsomes isolated from normal male and female rats. Four weeks after castration, the change of absorbance after hexobarbital was added to microsomes did not differ significantly from the value determined with microsomes from livers of female rats (Table 3).

TABLE 3
Magnitude of hexobarbital-induced spectral change

Results are expressed as the change in absorbance observed at 420 mμ relative to 445 mμ ($\Delta E \times 10^{-3}$ per milligram of protein) observed on addition of 2 mM hexobarbital. Standard deviations are indicated by ±.

Conditions	*n*	Male	*n*	Female
Before castration	21	7.9 ± 1.5	16	3.0 ± 0.91
After castration				
1 week	10	4.7 ± 1.75	6	2.5 ± 0.59
4 weeks	10	2.9 ± 0.95	9	2.8 ± 0.67

Ovariectomy, however, did not change the rate of aminopyrine demethylation, the cytochrome P-450 content, or the magnitude of the type I spectral change of liver microsomes (Table 1). The aniline hydroxylation rate and the change of the spectral absorbancy, after aniline was added to male microsomes, decreased a little 3 weeks after castration and approached the values found with microsomes from livers of female rats. This indicates that the small sex difference in the rate of aniline hydroxylation, the cytochrome P-450 content, and in the aniline-induced spectral changes are significant.

DISCUSSION

The often observed greater tolerance of male rats to different drugs, and the greater rate of drug oxidation by male rats, can now be explained as due to a greater affinity of drugs for the terminal oxidase, cytochrome P-450, present in liver microsomes, and to a greater maximal extent of binding of substrate by these microsomes.

Microsomes isolated from livers of male rats were found to oxidize aminopyrine and hexobarbital at more than twice the rate of microsomes isolated from livers of female rats. This increase in activity correlated with the increase in the maximal spectral change occurring upon drug interaction with the microsomal hemoprotein, cytochrome P-450. The striking difference in activity can not be due to an increased content of cytochrome P-450, since microsomes from livers of male rats contain no more than a 20% higher content of cytochrome P-450 per milligram of microsomal protein compared to microsomes isolated from livers of female rats. In addition, a greater affinity of the terminal oxidase of microsomes from livers of male rats for these drugs was seen from the relative magnitudes of the spectral dissociation constants (K_s) and the K_m obtained when substrate dependence on both spectral change and enzyme activity were determined. The physiological significance of this latter point is at once apparent when one considers the concentration of drug which may actually reach the enzyme system in the liver of the intact rat. Since the enzyme system may not be functioning at saturation levels of substrate, the lower K_m and K_s values for the system in the

male rat increase the effective differences in the rate of metabolism between the sexes, i.e., at saturation level the male rat would only metabolize a given drug twice as fast as the female rat, but with any given substrate level below saturation, the percentage of enzyme interacting with a substrate will be even greater for the male.

In contrast to studies with hexobarbital or aminopyrine, only a small difference in activity could be demonstrated between microsomes from livers of male and female rats when aniline was used as substrate. The 20% higher rate of hydroxylation of aniline observed with microsomes from livers of male rats (Table 2), as well as the slightly greater magnitude of the maximal spectral change seen after aniline was added to microsomes from male rats (Table 2 and Figs. 4 and 5) is presumably due to the 20% higher content of cytochrome P-450 associated with these microsomes. No difference in the affinity of aniline for the enzyme system of microsomes from livers of male and female rats has been observed when the affinity was determined either by measuring the dependence of the rate of hydroxylation or the magnitude of the spectral change on the aniline concentration, i.e., the K_m and K_s values, respectively.

Although a number of hypotheses could be proposed for the observed differences in substrate binding properties and activities of the hepatic microsomal oxidase of male and female rats, for example, the presence of an endogenous inhibitor as suggested by Davies *et al.* (27) or the presence of two different forms or types of cytochrome P-450 as suggested by El Defrawy (28), a further understanding of the enzyme system and its components will be required before the question can be resolved regarding the existence of molecular differences between this enzyme system in male and female rats, and why such differences are absent or difficult to detect in similar systems from other species.

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REFERENCES

1. J. S. Nicholas and D. H. Barron, *J. Pharmacol. Exptl. Therap.* **46**, 125 (1932).
2. G. O. Holck, M. A. Kanan, L. M. Mills and E. L. Smith, *J. Pharmacol. Exptl. Therap.* **60**, 323 (1937).
3. E. Robillard, A. d'Orio and J. Pellerin, *Union Med. Can.* **83**, 853 (1954).
4. G. P. Quinn, J. Axelrod and B. B. Brodie, *Federation Proc.* **13**, 395 (1954).
5. A. N. Davison, *Biochem. J.* **61**, 203 (1955).
6. J. Axelrod, *J. Pharmacol. Exptl. Therap.* **117**, 322 (1956).
7. G. P. Quinn, J. Axelrod and B. B. Brodie, *Biochem. Pharmacol.* **1**, 152 (1958).
8. H. Remmer, *Arch. Exptl. Pathol. Pharmakol.* **233**, 173 (1958).
9. R. Kato, E. Chiesa and G. Frontino, *Biochem. Pharmacol.* **11**, 221 (1962).
10. S. D. Murphy and K. P. DuBois, *J. Pharmacol. Exptl. Therap.* **124**, 194 (1958).
11. R. Kato and J. R. Gillette, *J. Pharmacol. Exptl. Therap.* **150**, 279 (1965).
12. W. Schmidt-Volkmar, Inaugural Dissertation, Univ. of Tübingen, 1966.
13. H. Remmer and B. Alsleben, *Klin. Wochschr.* **36**, 322 (1958).
14. R. Kato and J. R. Gillette, *J. Pharmacol. Exptl. Therap.* **150**, 285 (1965).
15. R. W. Estabrook, D. Y. Cooper and O. Rosenthal, *Biochem. Z.* **338**, 741 (1963).
16. H. Remmer, "Proceedings of the European Society for the Study of Drug Toxicity," Vol. IV, Excerpta Medica International Congress Series No. 81 (1964).
17. D. Y. Cooper, S. Levin, S. Narasimhulu, O. Rosenthal and R. W. Estabrook, *Science* **147**, 400 (1966).
18. A. H. Conney, M. Ikeda, W. Levin, D. Y. Cooper, O. Rosenthal and R. W. Estabrook, *Federation Proc.* **26**, 462 (1967).
19. H. Remmer and H. J. Merker, *Ann. N.Y. Acad. Sci.* **123**, 79 (1965).
20. S. Orrenius and L. Ernster, *Biochem. Biophys. Res. Commun.* **16**, 60 (1964).
21. H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame, J. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Mol. Pharmacol.* **2**, 187 (1966).
22. Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.* **22**, 620 (1966).
23. W. Cammer, J. B. Schenkman and R. W.

Estabrook, *Biochem. Biophys. Res. Commun.* **23**, 264 (1966).
24. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113 (1967).
25. H. Remmer, H. Greim, J. B. Schenkman and R. W. Estabrook, *in* "Oxidation and Phosphorylation" (R. W. Estabrook and M. Pullman, eds.), Methods in Enzymology, Vol. 10, p. 703. Academic Press, New York, 1967.
26. H. Remmer, *Arch. Exptl. Pathol. Pharmakol.* **233**, 173 (1958).
27. D. S. Davies, P. L. Gigon and J. R. Gillette, *Federation Proc.* **26**, 461 (1967).
28. S. El Defrawy, *Federation Proc.* **26**, 462 (1967).