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SEX DIFFERENCES IN THE KINETIC CONSTANTS FOR THE *N*-DEMETHYLATION OF ETHYLMORPHINE BY RAT LIVER MICROSOMES*

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Abstract—The sex difference in the metabolism of drugs by rat liver microsomes is not entirely explained by differences in the amount of enzyme. For the *N*-demethylation of ethylmorphine there is also a sex difference in the apparent affinity constant (K_m) as well as the maximal velocity (V_m). No sex difference in either K_m or V_m was found in immature rats. Maximal velocities are apparently increased by male sex hormones, but K_m values are controlled by other factors.

IT IS WELL known that various steriods and drugs are metabolized more rapidly by liver microsomal enzymes from male than by those from female rats. For example, microsomes from males are about three times as active as those from females in the metabolism of hexobarbital, pentobarbital, aminopyrine and morphine.[1]

It is generally thought that the production of androgens is the dominant factor in causing the sex variations in the metabolism of drugs because castration impairs the drug-metabolizing systems in liver microsomes of male rats,[2, 3] but does not significantly alter the systems in females.[3] In addition, the rate of metabolism can be increased by treating animals with testosterone and other anabolic steroids.[1–5] In contrast, the rate of metabolism of drugs is decreased by pretreatment of male rats with estradiol.[1]

Recently Schenkman *et al.*[6] found that the sex difference in the rate of metabolism of hexobarbital by liver microsomes may be due to differences in the apparent dissociation constant (K_m) as well as in the maximal velocity (V_m). The present study shows that there is a similar sex difference in the K_m for the *N*-demethylation of ethylmorphine by rat liver microsomes. Castration, however, did not alter the K_m value in either male or female rats.

MATERIALS AND METHODS

Animals. Rats of the Sprague–Dawley strain were used throughout these studies. The rats were about 60 days old (180 g, males; 160 g, females) unless otherwise stated. The immature rats were 21 days old and weighed about 50 g. Sham-castrated and castrated rats were obtained from Hormone Assay Laboratories, Inc., Chicago, Ill.

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and were usually kept for 21 days before use. The animals were maintained on Purina chow diet and had free access to water.

Protein estimations. Microsomal protein concentrations were determined by the method of Lowry *et al.*[7] Crystalline serum albumin was used as a standard.

Enzyme preparation. The rats were killed by cervical dislocation. The livers were removed and homogenized in 4 vol. of ice-cold 1·15% (w/v) KCl solution with a Teflon-glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a refrigerated Servall angle centrifuge. The supernatant fraction was then centrifuged at 78000 *g* for 1 hr in a Spinco model L preparative ultracentrifuge. All *g* values were calculated for the center of the centrifuge tube. The 78000 *g* supernatant was decanted and the microsomal pellet was suspended in 1·15% KCl containing 0·02 M Tris buffer (pH 7·4). The microsomal suspension, which contained the equivalent of 200 mg liver per ml of solution (about 4·5 mg microsomal protein per ml), was used immediately for the determination of kinetic constants.

Enzyme assays. The *N*-demethylation of ethylmorphine was carried out at 37° in a medium containing 50 mM Tris-HCl buffer (pH 7·4), 5 mM $MgCl_2$, 0·33 mM NADP, 8 mM isocitrate and 1 unit of isocitric dehydrogenase in a final volume of 3·0 ml. Nicotinamide was omitted because it inhibits the metabolism of at least some drugs by a competitive mechanism.[8] The microsomal protein content varied from 1·0 to 1·6 mg/ml and the substrate concentration ranged from 0·1 to 2·0 mM. The incubation period was 10 min. Under these conditions ethylmorphine demethylation was linear with respect to time and protein concentration.

The reaction was terminated by the addition of 1·0 ml of a 5% solution of anhydrous zinc sulfate, followed by the addition of 1·5 ml of saturated barium hydroxide solution and 0·5 ml of saturated sodium borate solution to give a final pH of 8·4. The mixture was centrifuged for 10 min at 4000 rpm, and 3·0-ml aliquots of the supernatant were assayed for formaldehyde according to the method of Nash,[9] except that the reagent consisted of 4 M ammonium acetate, 0·04 M acetylacetone and 0·1 M acetic acid.

Determination of kinetic constants. As used in this paper, K_m is defined as that concentration of ethylmorphine which results in half the maximal activity (V_m); it thus should be regarded as an apparent K_m and does not necessarily represent the affinity of the substrate for the enzyme.

Kinetic constants (K, V) were obtained by a least squares method in which the data were fitted to the Michaelis–Menton model:

$$v = \frac{s}{s + K} V.$$

The expected velocity $E(v_i)$ at any given concentration of substrate (s_i) is given by

$$E(v_i) = \left(\frac{s_i}{s_i + K_m} \right) V_m$$

Least squares estimates of K_m and V_m were obtained by minimizing

$$\Phi = \sum_i [v_i - E(v_i)]^2.$$

Such a minimum is obtained by solving the equations $\delta\Phi/\delta V_i = 0$, $\delta\Phi/\delta V = 0$ for K, V. Since these equations are not linear in K_m and V_m, a Taylor series expansion

about 0 was used, iterating until the change in V and K was less than a predetermined amount (0·001). Initial values of K and V (K_0, V_0) were found by fitting a straight line to the reciprocal plot of the data,

$$\frac{1}{V} = a + b\left(\frac{1}{S}\right) \text{ where } a = \frac{1}{V_0} \text{ and } b = \frac{K_0}{V_0}.$$

We also calculated the standard errors of the K_m and V_m values, which are the square roots of the maximum likelihood estimates of the variances of K_m and V_m, assuming that K_m and V_m are distributed approximately bivariate normal. Experiments giving standard errors greater than 10 per cent were discarded.

Statistical variations among the experiments were evaluated by the Student t-test or by an analysis of variance.

RESULTS

Sex difference in K_m *and* V_m. As shown in Table 1, there is a sex difference in both the K_m and the V_m for the N-demethylation of ethylmorphine by liver microsomes from mature rats. The K_m obtained with microsomes from male rats was about one-third of the K_m obtained with those of females, whereas the V_m value for the enzyme in males was about 3·5 times as large as that in females.

TABLE 1. SEX DIFFERENCES IN THE N-DEMETHYLATION OF ETHYLMORPHINE BY RAT* LIVER MICROSOMES

	$K_m \pm$ S.E. (mM)	$V_m +$ S.E. (mμmoles HCHO formed/mg protein/10 min)
Male (10)†	0·26 ± 0·02	116 ± 7
Female (10)†	0·66 ± 0·06	32 ± 3
P value	< 0·001	< 0·001

* Mature rats, 60 days old.
† Number of animals.

Lack of evidence for the presence of endogenous competitive inhibitors or activators. A variety of studies have failed to reveal the presence of endogenous activators or inhibitors in liver microsomes from either female or male rats. The K_m values were not altered either by treatment of microsomes from males with the soluble fraction from female rats or by treatment of microsomes from females with the soluble fraction from males (Table 2). These findings thus suggest that the soluble fractions do not contain large amounts of inhibitors or activators. It seemed possible that activators or inhibitors might be loosely bound to liver microsomes, but washing the microsomes with Tris-KCl buffer (0·02 M, pH 7·4) did not alter the K_m values obtained with liver microsomes from either male or female rats (Table 3). Moreover, the data reported in Table 2 suggest that proteins in the soluble fraction from male rats cannot bind appreciable amounts of possible inhibitors reversibly bound to liver microsomes from females.

It seemed possible that female microsomes might contain an inhibitor that is specifically and reversibly bound to the active site of the enzyme which catalyzes ethylmorphine demethylation, but treatment of 9000 *g* supernatants with 3 mM ethylmorphine, which is 6–12 times the K_m concentration, failed to alter the K_m

TABLE 2. LACK OF EVIDENCE FOR ENDOGENOUS COMPETITIVE ACTIVATOR OR INHIBITOR IN SOLUBLE FRACTION*

Microsomes	Soluble fraction used to wash microsomes	K_m† + S.E. (mM)
Male	None	0·25 ± 0·01
Male	Male	0·27 ± 0·02
Male	Female	0·31 ± 0·02
Female	None	0·44 ± 0·02
Female	Female	0·45 ± 0·03
Female	Male	0·45 ± 0·03

* Microsomes were resuspended in buffer, male soluble fraction or female soluble fraction as indicated in the table. The microsomes were then centrifuged down and suspended in Tris-KCl buffer as outlined in Materials and Methods. In the enzyme assay system, isocitrate dehydrogenase and not the soluble fraction was used for the NADPH-generating system.

† K_m for *N*-demethylation of ethylmorphine by liver microsomes isolated from mature rats (60 days old). The data are expressed as the average value of 3 experiments ± S.E.

TABLE 3. LACK OF EVIDENCE FOR ENDOGENOUS COMPETITIVE INHIBITOR IN LIVER MICROSOMES OF RATS*

	K_m† ± S.E. (mM)	
	Male	Female
Microsomes	0·28 ± 0·03	0·52 ± 0·03
Microsomes washed with buffer‡	0·26 ± 0·03	0·51 ± 0·05
Microsomes washed with a solution of ethylmorphine§	0·26 ± 0·04	0·60 ± 0·05

* Mature rats, 60 days old.

† K_m for *N*-demethylation of ethylmorphine. The data are expressed as the average of 3 experiments ± S.E.

‡ The microsomes were isolated by centrifugation for 60 min at 78000 *g*, then washed with a 1·15% KCl solution containing Tris buffer (0·02 M, pH 7·4), reisolated by centrifugation and suspended in the Tris-KCl buffer as described in Methods.

§ Twenty-five ml of the 9,000 *g* supernatant fraction was shaken for 15 min with 0·5 ml of a 150 mM ethylmorphine HCl solution (final concentration 3 mM). The microsomes were isolated by centrifugation for 60 min at 78000 *g*, then washed with a 1·15% KCl solution containing Tris buffer (0·02 M, pH 7·4), reisolated by centrifugation and suspended in the Tris-KCl buffer as described in Methods.

value for ethylmorphine demethylation by microsomes from either male or female rats (Table 3). Thus there was no evidence that ethylmorphine displaces an inhibitor from the active site into the medium.

It also seemed possible, however, that an inhibitor or activator might be bound not only to the active site but also to phospholipids, which constitute a major part of the liver microsomes. According to this view, ethylmorphine could have displaced an inhibitor from the active site but the inhibitor in turn became bound to nonspecific sites in the phospholipid matrix of microsomes rather than displaced into buffer. However, the K_m values were not significantly changed by treatment of the 9000 *g* supernatant fraction from liver of female rats with boiled liver microsomes from male rats or by treatment of the 9000 *g* supernatant fraction from liver of male rats with boiled liver microsomes from female rats (Table 4). Thus any activator or inhibitor

TABLE 4. LACK OF EVIDENCE FOR NONSPECIFICALLY BOUND ACTIVATORS OR INHIBITORS IN MICROSOMES OF MALE AND FEMALE RATS

Microsomes	Treatment*	$K_m \pm$ S.E. (mM)
Male	None	0·28 ± 0·03
Male	Boiled microsomes	0·26 ± 0·04
Female	None	0·52 ± 0·12
Female	Boiled microsomes	0·54 ± 0·13

* The 9000 *g* supernatant fraction (24 ml) was extracted with washed, boiled microsomes obtained from 6 g liver; the boiled microsomes were removed by centrifugation at 1000 *g* for 10 min and the unboiled microsomes were isolated as described in Methods. The data are expressed as the average of 3 experiments ± S.E.

present in unboiled microsomes did not diffuse into the boiled microsomes, nor did any activator or inhibitor present in boiled microsomes diffuse into the unboiled microsomes. Although these findings do not preclude the possibility that liver microsomes contain very highly bound substances, it is noteworthy that treatment of 9000 *g* supernatant fractions of liver with boiled microsomes is known to remove such highly bound substances as SKF 525 A.[10]

Metabolism by immature rats. Quinn *et al.*[1] found no sex difference in the metabolism of hexobarbital by liver microsomes of immature rats. Accordingly, the K_m values for both sexes are about 0·51 mM for the metabolism of ethylmorphine by liver microsomes from 21-day-old rats (see Table 5). Thus the K_m value apparently decreases with age in males, but increases in females.

TABLE 5. *N*-DEMETHYLATION OF ETHYLMORPHINE BY LIVER MICROSOMES FROM MATURE AND IMMATURE RATS

	Apparent Michaelis constant ± S.E. (mM)	
	Male	Female
Immature (6)*	0·51 ± 0·02	0·52 ± 0·02
Mature (10)	0·26 ± 0·02	0·66 ± 0·06†

* Number of animals used is given in parentheses.
† $P < 0·001$ as compared with males.

Effects of castration on K_m and V_m in mature rats. As shown in Table 6, castration had no effect on the K_m value of the liver microsomal enzyme in male rats but diminished the V_m value to about 50 per cent of control. In contrast, castration of female rats did not alter either the K_m or the V_m values. Thus it appears that the androgenic sex steroids regulate the amount of enzyme but appear to have no effect on the apparent affinity constant (K_m).

TABLE 6. EFFECT OF CASTRATION ON THE *N*-DEMETHYLATION OF ETHYLMORPHINE BY RAT* LIVER MICROSOMES

Sex	Treatment	$K_m \pm$ S.E. (mM)	$V_m \pm$ S.E. (mμmoles HCHO formed/mg protein/10 min)
Male	Control (10)†	0·26 ± 0·02	116 ± 7
	Sham-castrated (6)	0·28 ± 0·01	112 ± 8
	Castrated (6)	0·28 ± 0·01	59 ± 7‡
Female	Control (10)	0·66 ± 0·06	32 ± 3
	Sham-castrated (6)	0·59 ± 0·02	37 ± 2
	Castrated (6)	0·66 ± 0·05	31 ± 3

* Animals were sacrificed 21 days after surgery.
† Number of animals used is given in parentheses.
‡ Significantly different from control and sham-castrated animals $P < 0·001$.

TABLE. 7. *N*-DEMETHYLATION OF ETHYLMORPHINE BY LIVER MICROSOMES FROM CASTRATED MALE RATS AT VARIOUS TIME INTERVALS AFTER SURGERY*

		Sham-castrated		Castrated	
		K_m (mM)	V_m† (mμmoles)	K_m (mM)	V_m† (mμmoles)
Experiment 1					
0 (control)	(10)	0·26 ± 0·02	116 ± 7	0·26 ± 0·02	116 ± 7
9	(2)	0·94 – 0·50‡	95 – 103	0·42 – 0·56‡	49 – 53‡
21	(6)	0·29 ± 0·01	102 ± 3	0·28 ± 0·01	54 ± 6‡
Experiment 2					
0 (control	(3)	0·31 ± 0·02	127 ± 5	0·31 ± 0·02	127 ± 5
3	(2)	0·26 – 0·28	132 – 148	0·28 – 0·29	103 – 117
8	(2)	0·29 – 0·30	114 – 182	0·26 – 0·27	103 – 107
10	(2)	0·26 – 0·30	135 – 185	0·27 – 0·34	84 – 92‡

* The figures in parentheses represent the number of animals used. When 2 animals were used, the values are given; when more than 2 animals were used, the results are expressed as the mean ± S.E.
† Formaldehyde formed per mg microsomal protein in 10 min.
‡ $P < 0·01$ (K_m compared with controls; V_m compared with sham-castrated).

In some studies, rats were sacrificed at various times after castration (Table 7). At 9 days after surgery, the K_m value of the demethylase from male rats occasionally was increased in both castrated rats and the sham-castrated control animals (Table 7, Experiment 1). At 21 days, however, the K_m value for both groups had decreased to that obtained with unoperated controls. The elevation of the K_m was not consistent; in most experiments there was no change in the K_m at any time (Table 7, Experiment 2). The reason for the inconsistency is not known, but it may be important that starvation of male rats causes an increase in the K_m value (unpublished results).

DISCUSSION

Studies described in the present paper show that the sex difference in the *N*-demethylation of ethylmorphine by rat liver microsomes is due not only to differences in the amount of enzyme (V_m) but also to differences in the apparent affinity of the enzyme for ethylmorphine as measured by the K_m. At first, it seemed possible that variations in K_m could be caused by the presence of an endogenous activator or inhibitor of the *N*-demethylase; for example, pretreatment of animals with SKF 525 A (β-diethylaminoethyl diphenylpropylacetate) competitively inhibits the demethylation of ethylmorphine and thus causes an apparent increase in the K_m value (H. A. Sasame, unpublished results). Our studies, however, have failed to reveal the presence of reversibly bound endogenous activators or inhibitors in liver preparations. The finding that treatment of liver microsomes from male or female rats with the soluble fraction from either sex does not alter the K_m value suggests not only that the soluble fraction does not contain appreciable amounts of reversible activators or inhibitors but also that proteins in the soluble fractions do not displace inhibitors or activators from liver microsomes. Moreover, the finding that the K_m value was not altered by washing the microsomes with KCl or high concentrations of ethylmorphine suggests that no activator or inhibitor was bound specifically to the active sites of the microsomal enzyme. In addition, treatment of the 9000 *g* supernatant fraction with boiled microsomes before the isolation of liver microsomes did not alter the K_m value, even though this treatment is known to remove a number of highly bound substances from liver microsomes.

Although the lack of a sex difference in the K_m value in immature rats suggested that this kinetic parameter is controlled by sex hormones, castration did not alter the K_m value for the *N*-demethylase in either male or female rats even though it decreased the V_m value in males to about 50 per cent of the control. In addition, pretreatment of male rats with estradiol caused a 50 per cent decrease in the V_m value but no significant effect on the K_m value (unpublished results). Thus the sex steroids apparently control the maximal velocities of the sex-dependent enzyme in rat liver microsomes but not the K_m values.

It seemed possible that the K_m value reflects mainly alterations in the rate of diffusion of the substrate to the enzyme site rather than the dissociation constant of the enzyme–substrate complex. According to this possible mechanism, however, the apparent K_m value would increase with increasing V_m values, as shown by the following expression (see Addendum):

$$V_m/2k \text{ (diffusion)} = K_m \text{ (apparent)} - K_m \text{ (true)}.$$

If there were no sex difference in the true K_m, it would be necessary to postulate a marked sex difference in the diffusivity of the substrate into liver microsomes to account for our finding that the apparent K_m value with liver microsomes from males was lower than the K_m value from females, whereas the V_m of *N*-demethylation was greater in the microsomes from male rats. Even if there were a sex difference in diffusivity, however, it would still be difficult to account for the finding that castration of male rats decreases V_m without altering the K_m value. The rate-limiting step of the reaction, therefore, is probably not the rate of diffusion of the substrate into liver microsomes.

Our findings do not preclude the possibility that the sex differences are due to activators or inhibitors that are irreversibly bound to liver microsomes of either male or female rats. However, it is clear that the presence of an inhibitor that is irreversibly bound to the enzymically active site occupied by ethylmorphine cannot account for the sex difference in K_m values, for the presence of this kind of inhibitor would be manifested by a decrease in V_m but no change in the K_m value. Alternatively, it is possible that substances might be irreversibly bound to sites other than the active site and that these substances might alter the properties of the enzyme. Thus the sex difference might be due to allosteric differences in the structure of the drug-metabolizing enzyme system, but the validity of this view remains debatable.

ADDENDUM

In a diffusion-limited system at steady state, the concentration of a substrate in the immediate vicinity of an enzyme is given by the following equation:

$$\frac{dS}{dt} = S'k - Sk - v = 0 \tag{1}$$

where S' is the concentration of the substrate outside of the diffusion barrier, S is the concentration of the substrate in the immediate vicinity of the enzyme, k is the rate constant of diffusion and v is the rate of metabolism of the substrate. On rearrangement, equation 1 becomes:

$$k(S' - S) = v \tag{2}$$

But according to the Michaelis-Menton equation

$$v = \frac{V_m S}{S + K_m} \tag{3}$$

On substitution of equation 3 into equation 2

$$k(S' - S) = \frac{V_m S}{S + K_m} \tag{4}$$

On rearrangement, equation 4 becomes

$$V_m/k = S'[S + K_m)/S] - S[S + K_m)/S] \tag{5}$$

$$V_m/k = (S' + S'K_m/S) - (S + K_m) \tag{6}$$

At half maximal velocity

$$S' = S'K_m/S = K_m \text{ (apparent)} \tag{7}$$

and

$$S = K_m = K_m \text{ (true)} \tag{8}$$

On substitution of equations 7 and 8 into equation 6

$$V_m/k = 2\,K_m \text{ (apparent} - 2\,K_m \text{ (true)} \tag{9}$$

On rearrangement, equation 9 becomes

$$V_m/2k = K_m \text{ (apparent)} - K_m \text{ (true)}. \tag{10}$$

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