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# KINETICS OF ETHYLMORPHINE *N*-DEMETHYLASE AT LOW SUBSTRATE CONCENTRATIONS AND ITS IMPLICATIONS CONCERNING THE ROLE OF THE STIMULATION OF NADPH–CYTOCHROME P-450 REDUCTASE ACTIVITY IN MIXED-FUNCTION OXIDASE ACTIVITY*

JOHN A. THOMPSON† and JORDAN L. HOLTZMAN‡

Clinical Pharmacology Section of the Medical Service, Veterans Administration Hospital, Minneapolis, MN 55417; and the Departments of Pharmacology, Medicine, and Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55455, U.S.A.

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**Abstract**—The 1:1 stoichiometry observed in previous studies between the stimulation of NADPH–cytochrome P-450 reductase activity and ethylmorphine *N*-demethylase would imply that the stimulation of reduction is the rate-limiting process in the *N*-demethylation. In the present study, we have found that double reciprocal plots of the *N*-demethylase activity of hepatic microsomes from male and female rats is linear for ethylmorphine concentrations from 2.5 $\mu$M to 2 mM. Kinetic analysis suggests that these linear plots are not compatible with the stimulation of the reductase being the rate-limiting process.

Since our initial studies on the role of NADPH–cytochrome P-450 reductase in controlling the rate of the microsomal hydroxylases, it has been recognized that the reduction of cytochrome P-450 is the rate-limiting step in the hepatic, mixed-function oxidases [1, 2]. Although the exact chemical nature of the hydroxylating species is unknown, the stoichiometry of the overall reaction does indicate that peroxyl-like species must be formed [3, 4]. Hence, when NADPH is used as the sole source of reducing equivalents, two electrons must be transported through the cytochrome P-450 to provide the necessary reducing equivalents to form the "active oxygen" which is inserted into the substrate. At the present time it is unclear whether it is the insertion of the first or second electron which is rate limiting.

In spite of the controversy concerning whether the insertion of the first or second electron is rate limiting, there is general agreement on the broad outlines of the reaction sequence (Fig. 1). Concurrent studies by Gigon *et al.* [5, 6] and Schenkman [7] suggested that the initial step in hydroxylation was the activation of the NADPH–cytochrome P-450 reductase activity. It was felt that this activation was due to the conversion of the cytochrome to a high spin form which is more readily reduced. Further, it was only the activated cytochrome which was active in hydroxylation.

There are several lines of evidence suggesting that the reduction of the activated cytochrome is the rate-limiting process. The first is that Gigon *et al.* [5, 6] found that there is a 1:1 stoichiometry between the *N*-demethylation of ethylmorphine and the increase, Δ, in the NADPH–cyochrome P-450 reductase activity. This observation has been confirmed in our laboratory [8–12], as well as by Cohen and Mannering [13]. Further, in our studies, we found that the inhibition by $D_2O$ of the hydroxylation of ethylmorphine was equal to the inhibition of the activated reductase activity [8, 9], suggesting that the activated substrate cytochrome complex truly controlled the rate of the reaction.

At the present time there is much stronger evidence indicating that the activity of this activated reductase is not rate limiting. Studies from Estabrook's laboratory [14–16] and by Correia and Mannering [17, 18] have shown that NADH has a marked stimulatory effect on ethylmorphine *N*-demethylase without affecting the rate of cytochrome P-450 reduction. This suggested that the rate-limiting activity was the insertion of the second electron.

In a similar vein, concurrent studies by Schenkman [19] and ourselves [20] found that the heat of activation for the reductase was considerably less than that for the hydroxylase, suggesting that this reduction is not the sole rate-limiting process. We have come to a similar conclusion on the basis of the small isotope effect observed with the *N*-demethylation of ($^2H_3$-methyl)-ethylmorphine [21]. Finally, Peterson and Mock [22] have suggested, on the basis of stop flow kinetic data, that the reductase activity is actually greater than the hydroxylase activity and that the stoichiometry observed by ourselves and others [5, 6, 8–13, 20] is erroneous.

Hence, we are faced with the question of whether the stimulation of the reductase activity may be only

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† Present address: School of Pharmacy, University of Colorado, Boulder, CO.

‡ Send reprint requests to: Dr. Jordan L. Holtzman, Chief, Clinical Pharmacology Section, Veterans Administration Hospital, 54th Street and 48th Avenue South, Minneapolis, MN 55417.

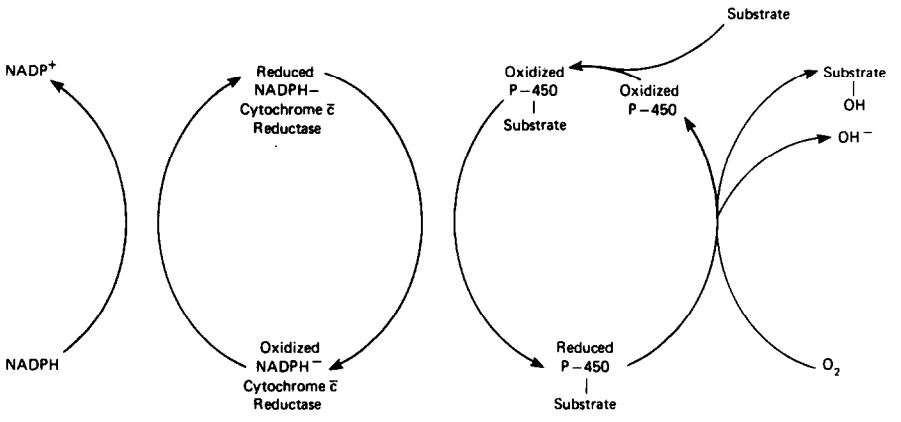


Fig. 1. General scheme for hepatic mixed-function oxidases.

an interesting property of the microsomal *N*-demethylases which may happen to have a 1:1 stoichiometry at 37° and saturating concentrations of substrate but which plays no real role in the *N*-demethylations, or whether the stimulation of the reductase activity may be important in controlling the rate of *N*-demethylation. In order to assess the role of the stimulation in controlling the *N*-demethylations, we have examined, by a new radiometric assay [23], the kinetics of this activity over a much wider range of substrate concentrations than has previously been possible (2.5 μM to 2 mM). Our studies indicate that the activation of the reductase activity is probably not rate limiting.

*Kinetic basis of the study.* Previous studies in our laboratory have demonstrated that the activation of the reductase activity is controlled by activation sites which are kinetically distinct from the catalytic site [10–12]. In the male rat, there are two sites with $K_s$ values equaling 29 μM and 1.0 mM, while in the female there is a single site with a $K_s = 67$ μM [12]. If this activated reductase activity is truly the rate-limiting component, then one would anticipate that, as the concentration of substrate is reduced below the $K_s$ for the stimulatory site, the activity of the hydroxylase should show a significant deviation from the standard Michaelis–Menten kinetics. And indeed, if we use the derivations given by Dixon and Webb [24], we find that such is the case. Taking the model which has been implied from our previous studies [10–12], we find:

$$E + S \rightleftarrows ES \qquad K_s' \qquad (1)$$

$$E + S \rightleftarrows ESa \qquad K_s \qquad (2)$$

$$ESa + S \rightleftarrows ESaS \qquad K_c \qquad (3)$$

$$ESaS \rightarrow E + S + P \qquad (4)$$

where $K_s'$ is the dissociation constant of the substrate for the catalytic site of the unstimulated enzyme, $E$; $K_s$, the dissociation constant for the stimulatory site of the unstimulated enzyme, $E$; and $K_c$, the dissociation constant for the catalytic site of the activated enzyme, $ESa$. If we assume that $K_c \simeq K_m$, then the rate equation for this process is:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m}(1 + K_s/K_s')\frac{1}{S} + \frac{K_s \cdot K_m}{V_m} \cdot \frac{1}{S^2} \qquad (5)$$

If we take $K_s' = \infty$, that is that the unstimulated enzyme does not bind the substrate at the catalytic site, then this reduces to:

$$\frac{1}{V} = \frac{1}{V_m} + \frac{K_m}{V_m} \cdot \frac{1}{S} + \frac{K_s \cdot K_m}{V_m} \cdot \frac{1}{S^2} \qquad (6)$$

This assumption is not necessary but does simplify the calculations and further gives the limiting case where only the stimulated enzyme is active, as is suggested by the studies cited above [5–13, 20]. As the substrate concentration goes below $K_s$, the quadratic component will predominate and there should be a marked upward curving of the double reciprocal plots (Fig. 2, panels a and b).

## MATERIALS AND METHODS

All animals used in these studies were CD rats obtained from Charles River, Inc. (North Willmington, MA). Male rats weighed 180–200 g, while the females were 160–180 g. The animals were given water and a standard laboratory chow diet *ad lib.*

The [*N*-$^3$H-methyl]ethylmorphine was prepared by the method of Abdel-Monem and Portoghese [25]. In this method, the *N*-phenylcarbamate of norethylmorphine was reduced with LiAl $^3H_3$ and [$^3$H]ethylmorphine purified on Silica gel GF thin-layer plates using ethylacetate–methanol–ammonium hydroxide (17:2:1).

The animals were sacrificed by cervical fracture, and the livers excised, chilled on ice, and homogenized with a motor-driven Teflon–glass homogenizer in 3 vol. KCl–Tris (150 mM, 50 mM; pH 7.4) at 4°. The homogenate was centrifuged at 9000 *g* for 15 min in a Sorval RC2-B centrifuge with an SS-34 rotor. The supernatant was centrifuged at 165,000 *g* for 40 min in a Beckman L2-65 centrifuge with a 50 Ti rotor. The pellet was resuspended in KCl–Tris to give the equivalent of 2 g liver/ml. The protein was determined by the phenol reagent method of Sutherland *et al.* [26].

The activity of the microsomal ethylmorphine *N*-demethylase was determined as previously described [23]. In this method, varying amounts of [*N*-$^3$H-methyl]ethylmorphine were added to serum vials to give final concentrations of 2.5 μM to 2 mM

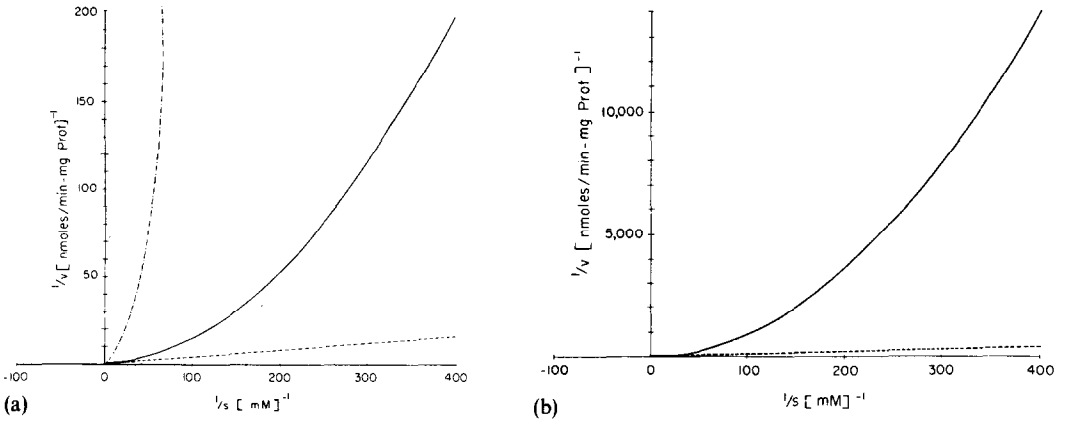


Fig. 2. Panel a: theoretical double reciprocal plots of ethylmorphine *N*-demethylase in hepatic microsomes from male rats where $K_m = 0.25$ mM and $V_m = 6.03$ nmoles formaldehyde/min·mg of protein for $K_s = 1.0$ (–·–), $K_s = 0.029$ mM (——) and $K_s = \infty$ (– – –). Panel b: Theoretical double reciprocal plots of ethylmorphine *N*-demethylase in hepatic microsomes from female rats where $K_m = 0.64$ mM, $V_m = 0.52$ nmole formaldehyde/min·mg of protein and $K_s = 0.067$ mM (——) and $K_s = \infty$ (– – –).

and warmed to 37°. To each vial was added 3 ml of an incubation mixture containing glucose 6-phosphate (5.5 mM), NADP (0.3 mM), glucose 6-phosphate dehydrogenase (Sigma Type XI, Sigma Chemical Co., St. Louis, MO) (0.67 units/ml), and 1 mg/ml of microsomal protein in a KCl–Tris–$MgCl_2$ buffer (150 mM, 50 mM, 5 mM, pH 7.4) warmed to 37°. The samples were incubated for 10 min and the reaction was terminated by the addition of 1 ml of 5% $ZnSO_4$. One ml of the incubation mixture was placed on a 1 × 8 cm column XAD-2 resin to remove the labelled substrate and allowed to flow onto the column. The column was washed with 6 ml of water. The first 2 ml was discarded and 5 ml of the effluent was added to 15 ml of a scintillation mixture containing dioxane (417 ml), 2-methoxyethanol (83 ml), naphthalene (25 g), 2,5-diphenyloxazole (5 g) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.25 g) [27]. The samples were counted in a Beckman LS-100C liquid scintillation counter, and the absolute activity was determined by the addition of [$^3$H]toluene as an internal standard.

All incubations were run in duplicate. The data were fit to a double reciprocal plot by eye and the kinetic parameters confirmed by a least squares calculation.

## RESULTS

When we incubated the microsomes from both male and female rats with concentrations of ethylmorphine ranging from 2.5 μM to 2 mM, we observed excellent linearity over three orders of magnitude range of substrate concentrations (Fig. 3, panels a and b). The $K_m$ in the male rat was 0.25 mM, while in the female it was 0.64 mM. Utilizing the kinetic parameters ($K_m$ and $V_m$) obtained in this study and the $K_s$ values obtained in previous studies [12], we can calculate from Equation 6 theoretical values for the ethylmorphine *N*-demethylase activities for microsomes from male and female rats (Fig. 2, panels a and b). It would seem reasonable to use the $K_s$ values from the previous studies, since in those studies we used animals of the same strain, provided by the same

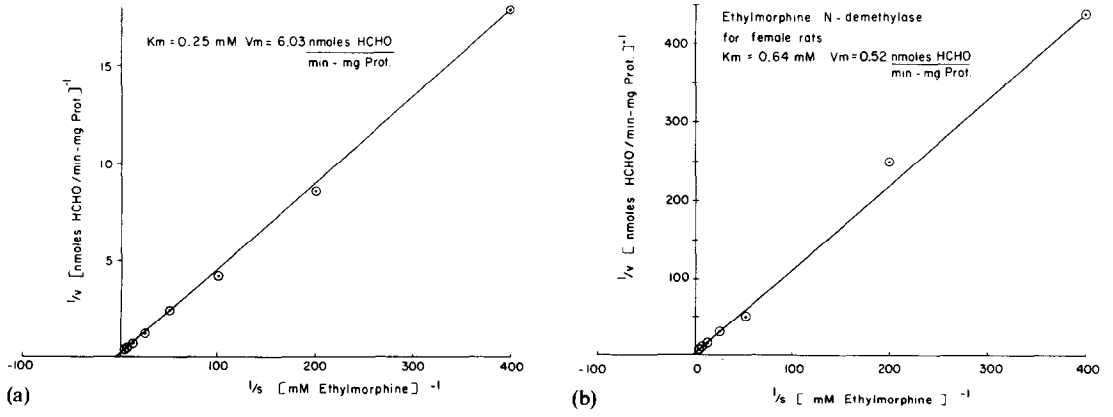


Fig. 3. Double reciprocal plot of ethylmorphine *N*-demethylase in hepatic microsomes from (a) male and (b) female rats using substrate concentrations of 2.5 μM to 2.0 mM. Preparations and assays are given in the text.

supplier, and of the same weight as in the present investigations. Further, the $K_s$ values were highly reproducible over a period of several months.

In our previous study we found that in the male rat the stimulation of the NADPH–cytochrome P-450 reductase is controlled by two kinetically distinct sites with $K_s$ values of 29 $\mu$M and 1.0 mM for ethylmorphine. When each of these values are substituted into Equation 6, double reciprocal plots of the calculated *N*-demethylase activities show marked deviations from linearity over the substrate concentration range used in this study (Fig. 2a). The $K_s = \infty$ plot in Fig. 2a (dashed line) has the same numerical values as the plot of the incubation in Fig. 3a, only the scale of the ordinate of the latter figure has been expanded 10-fold to better illustrate the data.

Similarly for microsomes from female rats, we can calculate values for the rate of ethylmorphine *N*-demethylase from Equation 6, using the $K_m$ and $V_m$ from this study and the value of 67 $\mu$M from our previous study. Again the line calculated from Equation 6 shows a marked upward curve over the ethylmorphine concentration range used in the incubations in this study (Figs. 2b and 3b). As in the study with microsomes from male rats, the $K_s = \infty$ (dashed line, Fig. 2b) has the same slope and intercepts as the line in Fig. 3b.

## DISCUSSION

The kinetic scheme of hepatic mixed-function oxidases represented in Equations 1–6 has been one of the major, albeit controversial, working models for the study of this system. The primary assumption of this model is that the rate-limiting process of this enzymatic activity is the stimulation of the NADPH–cytochrome P-450 reductase activity. This assumption is quite attractive, since it suggests that there is a close parallel between the control of the microsomal mixed-function oxidases by substrates like ethylmorphine and the well-defined tight coupling to the presence of ADP of mitochondrial respiration. Yet as discussed above, the results of studies bearing on this question, even from our own laboratory, have been highly contradictory. The current study appears to argue against this model.

The data from this study clearly indicate that the *N*-demethylation is quite linear over three orders of magnitude of substrate concentrations. Yet, our kinetic analysis of Equations 1–6 suggests that there should be a marked nonlinearity in the double reciprocal plots over this range of concentrations. This contradiction between our observed results and our prediction strongly indicates that the stimulation of the reductase activity does not control any process before the first irreversible step of the *N*-demethylase reaction, since in a sequential reaction no process after an irreversible step will have a major effect on the kinetic parameters. Clearly this is a cyclic rather than a sequential reaction, but some of these same considerations will enter into the development of a model.

These considerations viewed in the light of the 1:1 stoichiometry between the stimulation of the reductase and the *N*-demethylation might suggest an alternative model for the mixed-function oxidases. It may be that the stimulation of the reductase activity may have nothing to do with the insertion of the first electron, but rather provides the second. In such a case, the first would be provided by a very rapidly turning over cytochrome P-450 which is unaffected by the presence of substrate. This is in line with the concept that NADH stimulates the reaction without affecting the rate of the reduction by providing an alternative source of second electrons [14–18]. Similarly, the discrepancy between the temperature dependence of the reductase and the *N*-demethylase may indicate that the activation energy is the sum of the activation energies of two steps, the reduction and then the transfer from the reduced cytochrome of the second electron to the partially activated oxygen. Similarly, if the stimulated cytochrome reductase provides the second electron, the $K_s$ for stimulation would not have to affect the $K_m$ for the *N*-demethylase, since the transfer of the second electron would only limit $V_m$. In such a case, the binding to the catalytic site would be determined by Equation 1 rather than Equation 3. Verification of this hypothesis will have to await further studies.

Finally, it should be noted that our observation of linearity is in contrast to the results of Pederson and Aust [28] and Hayes *et al.*[29] who reported nonlinear kinetics for aminopyrine and ethylmorphine *N*-demethylase respectively. The former workers used a different substrate which could account for the discrepancy. But, the latter workers used the same substrate and a similar strain of rats. Yet, these workers utilized a colorimetric method to determine activities at very low substrate concentrations. In our hands, the colorimetric assay has not proven sufficiently sensitive to go below 100 $\mu$M. Even at 100 $\mu$M of substrate and up to 10 mM, we have never observed any nonlinearity in the curves in the innumerable studies in which we have examined the substrate-dependent kinetics.

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