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SHORT COMMUNICATIONS

Dependence of K_m and V_{max} on substrate concentration for rat hepatic microsomal ethylmorphine *N*-demethylase*

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A COMMON method of studying alterations in the activity of the hepatic microsomal mixed-function oxidase system is a Michaelis–Menten analysis of the initial reaction rates.[1] The basic requirements of this method, reaction rates linear with time and protein concentration, can be met by most substrates of the microsomal enzyme system. Although the Michaelis–Menten method is generally adequate for isolated, purified enzymes, many restrictions are encountered in its application to the more complex microsomal mixed-function oxidase system.[2] Since the microsomal system has resisted attempts at purification, a relatively impure, poorly defined preparation, consisting of particulate membrane fragments, must be used in the kinetic studies. The microsomal enzymes are generally considered to be structurally and functionally integrated with the membrane of the endoplasmic reticulum. A Michaelis–Menten kinetic study of the microsomal enzyme system is further complicated by the fact that it is multi-substrate, requiring NADPH, O_2, and an oxidizable substrate. Moreover, there is little specificity for the oxidizable substrates, which may represent a vast array of exogenous and endogenous compounds. Still other substances such as ascorbic acid,[3] magnesium,[4] certain hormones[5] and vitamin K_3,[6] may exert an influence on the activity of the system. Thus, it can be assumed that suboptimal concentrations of any of the many compounds which influence the enzyme system could alter the values of the kinetic constants obtained. Of further importance in considering the usefulness of Michaelis–Menten kinetics is that the drug oxidase system is composed of several individual enzymes integrated into a complex system of electron transport and oxidation.

On the other hand, estimations of the kinetic parameters have been widely used and may be informative, provided their limitations are recognized. Estabrook[2] has considered the apparent K_m of the microsomal enzyme system to reflect simply some rate-limiting step. Thus a change in the K_m of the total enzyme system may reflect some type of qualitative change of an enzyme catalyzing a critical step.

In our previous studies on the effect of certain factors on the microsomal mixed-function oxidase system,[7] we used substrate concentrations for ethylmorphine (EM) *N*-demethylase as low as 10% that of other investigators[8] in an attempt to reflect more accurately the possible liver concentrations of foreign compounds *in vivo*. As an apparent consequence, the K_m-values obtained were approximately one order of magnitude lower than those reported by others. We then initiated further experiments to examine the relationship of substrate concentration with K_m determinations.

Sprague–Dawley derived male rats (150–200 g) were used. On procurement, they were housed individually in stainless steel wire-bottom cages and were provided tap water and Wayne Lab-Blox (Allied Mills, Inc.) *ad lib*. The animals were sacrificed between 7:00 a.m. and 8:00 a.m. about 1 week after procurement and microsomes were prepared as previously described.[7]

The kinetic parameters for the *N*-demethylation of EM were determined as previously described,[7] except that different substrate concentration ranges were employed. In a typical experiment, 20 substrate concentrations were used which spanned a range from 0·001 to 10·0 mM. Initial reaction rates were independent of incubation times (up to 10 min) and protein concentration (0·4–1·6 mg/ml) for the selected substrate concentrations of 0·004, 0·006, 0·008, 0·010, 0·020, 0·100, 1·00 and 4·00 mM. Intermediate concentrations were assumed to be independent of both protein concentration and time. The small amounts of product produced over short time periods at the two concentrations below 0·004 mM, however, precluded similar verification of the independence. Microsomal protein was estimated by the method of Lowry *et al.*[9] The K_m and V_{max} parameters applicable to each concentration range were calculated using the computer program of Cleland.[10]

Presented in Table 1 are the apparent kinetic parameters for arbitrarily selected narrow concentration ranges. Some of these ranges, by choice, overlap in order to obtain more closely spaced points.

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TABLE 1. ESTIMATIONS OF APPARENT KINETIC CONSTANTS FOR ETHYLMORPHINE *N*-DEMETHYLASE AT VARIOUS SUBSTRATE CONCENTRATION RANGES*

Substrate conc. range (mM)	K_m (mM × 10^3)	V_{max}†
0·001–0·010	0·379 ± 0·065	33·1 ± 5·9
0·006–0·040	12·5 ± 3·2	84·0 ± 22·7
0·010–0·100	29·0 ± 6·4	133 ± 39
0·020–0·200	64·8 ± 10·5	150 ± 27
0·060–0·400	89·0 ± 7·0	192 ± 44
0·200–2·000	120 ± 15·5	172 ± 18
0·600–4·000	162 ± 48·0	212 ± 46
0·800–10·000	212 ± 29·0	186 ± 18

* Twenty different substrate concentrations were used and then arbitrarily divided into concentration ranges. Data in body of table represent means ± S.E. of at least three experiments.

† Nanomoles formaldehyde per milligram of microsomal protein per 10 min.

With one exception, the kinetic parameters vary with substrate concentration, as can be shown either with the conventional Lineweaver–Burk plot of $1/V$ vs $1/S$ (curvilinear downward at higher substrate concentration) or as plots of K_m vs S and V_{max} vs S (Fig. 1). That one exception is exhibited by the estimation of V_{max} above 0·23 mM substrate concentration, where the estimation for V_{max} remains independent of the concentration.

Shown in Table 2 are the estimates of the minimum and maximum values for the apparent kinetic parameters. The y intercepts were used to estimate the minima. The estimate for the maximum V_{max} was experimentally found to be approximately 190 nmoles formaldehyde × mg protein^{-1} × 10 min^{-1}. If at least two distinct systems are assumed (discussed below), the maximum for the V_{max} (190 nmoles formaldehyde × mg protein^{-1} × 10 min^{-1}) must be reduced by the minimum V_{max} (71 nmoles formaldehyde × mg protein^{-1} × 10 min^{-1}) in order to obtain the estimate (119 nmoles formaldehyde × mg protein^{-1} × 10 min^{-1}) for the system operative in the presence of high substrate concentration.

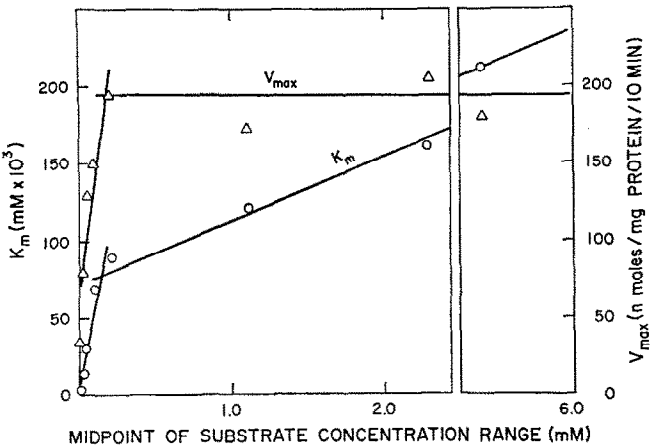

FIG. 1. Kinetic parameters as functions of substrate concentrations; V_{max} = Δ, K_m = 0. Estimation of maxima and minima shown in Table 2.

TABLE 2. ESTIMATED THEORETICAL MAXIMUM AND MINIMUM VALUES FOR APPARENT V_{max} AND APPARENT K_m FOR EACH SYSTEM*

	Minimum	Maximum
Apparent K_m ($M \times 10^3$)	0·0082 ± 0·0113†	0·169 ± 0·027‡
Apparent V_{max} (nmoles formaldehyde × mg protein^{-1} × 10 min^{-1})	70·9 ± 26·6†	190 ± 8§

* All data (except maximum for V_{max}) represent term a of the general equation $y = a + bx$ with standard error, $S_{y.x}$; substrate concentration (or its reciprocal) substituted for x and appropriate kinetic parameter substituted for y.

† Obtained with data in Table 1 from regression of S vs K_m (or V_{max}); values for S represent midpoints of the first five ranges.

‡ Obtained with data in Table 1 from regression of $1/S$ vs $1/K_m$; values for S represent midpoints of the last five ranges.

§ Since V_{max} estimations for last five ranges shown in Table 1 were not a function of S concentration ($P < 0.05$), the value presented is the mean ± S.E. for the five ranges.

Several approaches were used to obtain the best estimates of these parameters, such as using different points, different estimates of the most appropriate finite substrate concentration within each substrate range, and various procedures of reciprocal relationships. The estimates presented in Table 2 are the best fits as shown by generation of a hypothetical curve of

$$V_{total} = \frac{(V_{max_a})S}{K_{m_a} + S} + \frac{(V_{max_b})S}{K_{m_b} + S}$$

where substitution of the minimum and maximum values is represented by the subscripts a and b respectively.

Several interpretations may account for this phenomenon. Two or more enzyme systems, or rate-limiting components of the same system, could be involved and would be consistent with the findings of Pederson and Aust,[11] who reported kinetic evidence for multiple enzyme systems for the metabolism of aminopyrine. Observations which support the existence of at least two systems for the data presented in this report are that: (1) the differences in the regression slopes between the low and high substrate concentration ranges are highly significant ($P < 0.01$) for both the V_{max} and K_m parameters; and (2) generation of the theoretical curve (V_{total} vs S) for the composited systems essentially agrees with the velocities experimentally determined for the various substrate concentrations.

One apparent explanation for two systems is that ethylmorphine may be metabolized not only by the cytochrome P-450 mediated mixed-function oxidase system but also by a mixed-function amine oxidase described by Zeigler *et al.*[12] not requiring cytochrome P-450. However, Zeigler *et al.*[12] showed that aminopyrine was not metabolized by the mixed-function amine oxidase system; yet this substrate still produces a nonlinear function similar to ethylmorphine in our system.* The fact that these two mixed-function oxidases cannot explain nonlinearity for aminopyrine would also suggest that they would not account for the ethylmorphine data shown here.

An alternative explanation is that some component of the mixed-function oxidase system may, by association with an accessory constituent, exist either with or without that constituent and consequently manifest two separate kinetic properties. One possibility is that cytochrome P-450, which has been shown to require phosphatidylcholine (PC) for binding and/or metabolism of type I substrates,[13–15] could exist either with or without that constituent. Previous data from this laboratory[7,16,17] have also implicated cytochrome P-450 with and without an association with PC as an explanation for the kinetics of binding and metabolism observed after phenobarbital induction and/or dietary protein deprivation. The generation of a $1/V$ vs $1/S$ function would curve concave downward, since this situation would be analagous to two or more enzymes catalyzing the same reaction, as discussed by Plowman.[18] Explanations other than phosphatidylcholine effects could, of course, be suggested, such as the involvement of more than one ionization state of the enzyme and substrate-induced alteration in the activity of an endogenous modifier other than phosphatidylcholine. The possibility that the two suggested forms of cytochrome P-450 (denoted as P-450 and P_1-450 by some authors[19] or as P-450 and P-448 by others[20,21]) may account for the proposed systems is not considered likely in that:

(1) we are unable to detect P-448 in the control microsomes; (2) 3-methylcholanthrene-induced animals, which show elevated levels of P-448, do not exhibit significant alterations in the apparent ratios of the systems reported here*; and (3) P-448 is not known to catalyze the metabolism of ethylmorphine.

Recently, other laboratories have observed relationships similar to that reported here. For example, Wada *et al.*[22] and Hansen and Fouts[23] have both published Lineweaver–Burk plots for the *p*-hydroxylation of aniline which are not linear. Also, Hansen and Fouts,[23] who used aniline and benzphetamine, and Schenkman,[24] who used aniline and several type I substrates, have reported Lineweaver–Burk plots of substrate-induced microsomal difference spectra which curve downward at the higher substrate concentrations. We have also seen similar phenomena for substrate-induced difference spectra using ethylmorphine as the ligand.* Although the relationship between difference spectra and enzymatic metabolism is admittedly not completely understood, such a similarity is of considerable interest to the data reported here. Clearly, employment of kinetic parameters as a means of determining the effects of exogenous treatments on microsomal enzyme activities is more complex than originally envisioned and must be interpreted with care.

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