NON-MICHAELIAN MONOOXYGENASE KINETICS: STUDIES USING COMPETITIVE INHIBITORS

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1. Introduction

Microsomal monooxygenase activity has been found frequently to depart from Michaelis-Menten kinetics in that Lineweaver-Burk plots for the oxidation of a number of xenobiotics have exhibited downwards curvature at high substrate concentrations [1–4]. These results are consistent with catalysis being effected by more than one enzyme, each with a different K_m value, and this possibility has received some support in the finding that endoplasmic reticulum contains multiple monooxygenases [2,3,5]. Such kinetic behaviour could however be due to a single enzyme possessing multiple interacting sites [6,7] and it has been proposed that microsomal monooxygenase activity may be regulated by a ligand concentration-dependent mechanism exhibiting negative cooperativity [8].

Here, we have shown that Michaelis-Menten kinetics did not describe the kinetics of *O*-demethylation of *p*-nitroanisole by a rat liver microsomal preparation when enzyme activity was measured over a 1300-fold range of substrate concentration. In addition, results of studies with 3 competitive inhibitors precluded multiple independent catalytic sites as the source of the observed non-Michaelian kinetics of ether cleavage thereby lending support to the hypothesis that negative cooperativity may be a feature of the control of microsomal monooxygenase activity.

2. Method and results

The animals used in these studies were sexually mature, normal, male Wistar rats supplied by the University Central Animal Breeding Facility. They were fed a stock laboratory diet then starved for 1 day prior to sacrifice by decapitation. Microsomal suspensions were prepared from their livers by the method in [9] and the protein concentration of each preparation was determined by the Folin–phenol method [10]. Aliquots of each preparation were dispensed into polyethylene vials, frozen in liquid nitrogen and stored at −75°C until required for use in the studies of monooxygenase kinetics. Monooxygenase activity was determined spectrophotometrically at 430 nm by following the *O*-demethylation of *p*-nitroanisole using a method developed from that in [11]. Measurements were carried out at 37°C using a Varian Superscan 3 twin-beam recording spectrophotometer set at 1.0 nm spectral bandwidth and a full scale deflection of 0.1 absorbance units. This demethylation reaction is a useful method for monitoring monooxygenase activity as the product, *p*-nitrophenol, has a high molar absorptivity (10 277) which permits accurate determination of initial rates at low substrate concentrations. Moreover, *O*-demethylation of this substrate is the only reaction catalysed by monooxygenase which obviates the complexities of interpreting reaction kinetics where this is not the case [12]. The major modifications which were made to the original method were the omission of nicotinamide from the reaction mixture and the inclusion of NADP in both blank and sample cuvettes. The reasons for making these changes to such an assay system have been discussed [13] and the details of the method used are in the legend to fig.1. At a high [substrate] the reaction rate was proportional to [protein] over 0.25–2.0 mg/ml and all subsequent studies of monooxygenase kinetics were carried out at 1 mg protein/ml and with substrates 2.7 μM–3.6 mM; 2.7 μM being the minimum value at which an accurate measurement could be made of the reaction rate and 3.6 mM being con-

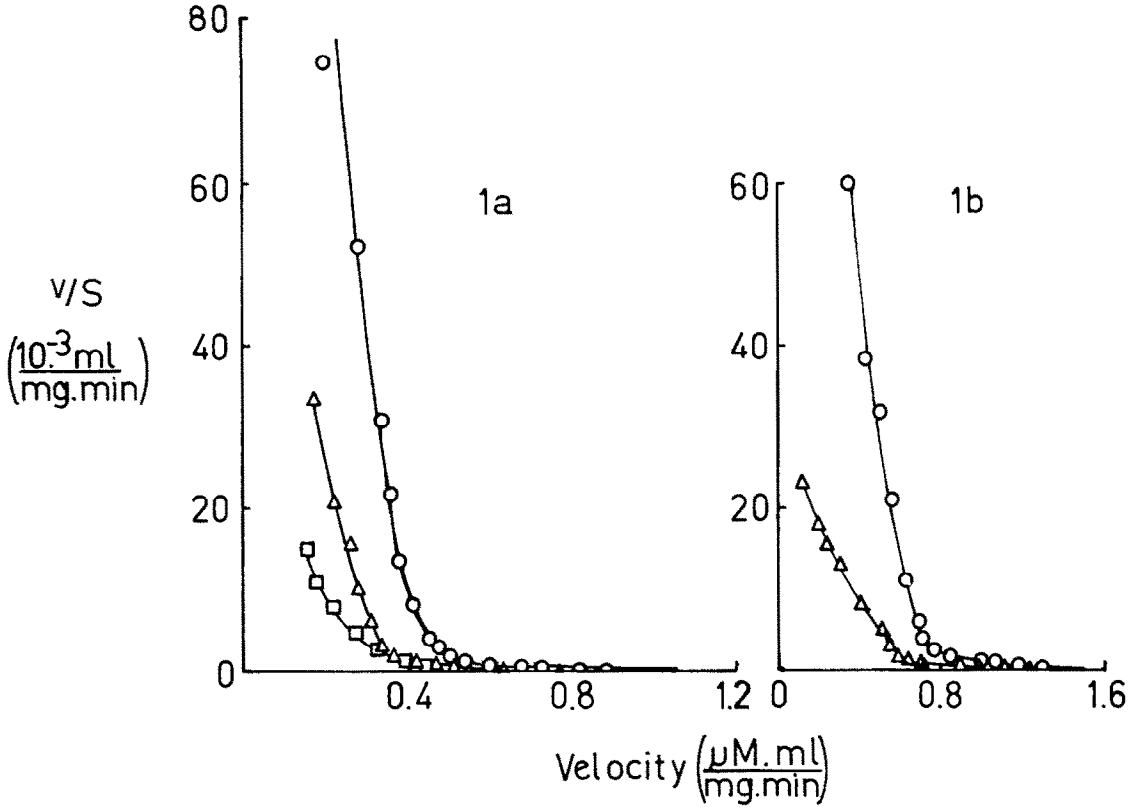

Fig.1. Scatchard analysis of rat-liver monooxygenase catalysed *O*-demethylation of *p*-nitroanisole. All monooxygenase assays were done at 37°C in a 3.7 ml total vol. in 1 cm pathlength stoppered matched quartz cuvettes. Both blank and sample reaction mixtures contained M/15 phosphate buffer (pH 7.9), 2.5 mM glucose 6-phosphate, 100 μM NADP, *p*-nitroanisole at 2.7 μM–3.6 mM and 1 μl (0.14 U) glucose 6-phosphate:NADP oxidoreductase (EC 1.1.1.49), this last component being added to the sample cuvette only. In the assays where the effect of chloramphenicol on monooxygenase activity was determined aliquots of a 4.5 mM stock solution of the antibiotic, prepared in the M/15 phosphate buffer, were dispensed into both blank and sample cuvettes together with these other reaction components. Similar stock solutions of phenacetin and *p*-methoxybenzaldehyde were used in the same way when their inhibitory effects were being determined. After equilibration at 37°C to allow for generation of reduced NADP in the sample cuvette the *O*-demethylation reaction was started by the simultaneous addition, and thorough mixing, of equal aliquots of stock microsomal suspension to both blank and sample cuvettes to give a final conc. 1.0 mg protein/ml. All the solutions of reagents were prepared fresh for use each day and were maintained in equilibrium with room air, and held at room temperature except those of NADP and glucose 6-phosphate which were stored on ice, and that of *p*-nitroanisole which was maintained at 37°C in a water bath. The linear increases in absorbance were recorded over 3–5 min periods, and these rates were converted to rates of change of molarity of *p*-nitrophenol using the value of 10 277 obtained for its molar absorptivity under these assay conditions. Each point is the mean of at least 3 assays, the standard error of each point being contained within its dimensions. (a) shows results obtained using monooxygenase prep. 1 with no additions (○) and in the present of 0.1 mM chloramphenicol (△) or 0.25 mM phenacetin (□). (b) shows results obtained using monooxygenase prep. 2 with no additions (○) and in the presence of 50 μM *p*-methoxybenzaldehyde (△). Best-fit lines using the appropriate parameter values in tables 1 and 2 are shown.

strained by the solubility of the substrate.

The non-Michaelian kinetic behaviour of the monooxygenase-catalysed *O*-demethylation of *p*-nitroanisole is clearly evident from the data depicted by ○ in fig.1a,b. which present the results in the form of a Scatchard [14] plot for 2 separate rat liver microsomal preparations. The 2 most plausible explanations of these results were that catalysis was being effected either by a minimum of 2 non-interacting catalytic sites with widely differing K_m values, or by at least 2 interacting sites exhibiting negative cooperativity. Inhibition studies were undertaken to determine which of these possibilities was responsible for the observed kinetic properties.

Table 1
The effects of chloramphenicol and phenacetin on the kinetic parameters of microsomal *O*-demethylase activity

Inhibitor (mM)	V_{max_1} ($\mu M \cdot min^{-1} \cdot ml^{-1} \cdot mg\ protein^{-1}$) ± SEM	V_{max_2}	V_{total}	app. K_{m_1} (μM) ± SEM	app. K_{m_2} (mM) ± SEM
0	0.387 ± 0.011	0.668 ± 0.019	1.053 ± 0.022	2.35 ± 0.341	0.896 ± 0.094
Chloramphenicol					
0.10	0.264 ± 0.016	0.883 ± 0.029	1.14 ± 0.031	2.14 ± 0.915	0.969 ± 0.126
0.30	0.195 ± 0.013	1.214 ± 0.079	1.40 ± 0.084	1.36 ± 0.921	1.69 ± 0.252
0.50	0.160 ± 0.014	1.250 ± 0.106	1.41 ± 0.110	1.62 ± 1 22	1.84 ± 0.332
Phenacetin					
0.25	0.218 ± 0.020	0.832 ± 0.022	1.05 ± 0.024	4.79 ± 2.03	0.669 ± 0.084
0.50	0.125 ± 0.022	0.885 ± 0 032	1.01 ± 0.035	1.90 ± 3.15	0.732 ± 0.109

Equation (1), which represents the sum of two hyperbolic relationships, was fitted to each substrate saturation curve to yield the values and standard errors shown in the table. Fitting was performed using a non-linear regression program based on the Gauss-Newton method. The program was written in FORTRAN and was run on a PDP-11/03-L computer. The values for the parameters represent the slopes and intercepts of the asymptotes to the curves shown in fig.1a, as well as other curves which, for clarity, are not shown in the figure

Table 2
The effect of *p*-methoxybenzaldehyde on the kinetic parameters of microsomal *O*-demethylase activity

Inhibitor (μM)	V_{max_1} ($\mu M \cdot min^{-1} \cdot ml^{-1} \cdot mg\ protein^{-1}$) ± SEM	V_{max_2}	V_{total}	app. K_{m_1} (μM) ± SEM	app. K_{m_2} (mM) ± SEM
0	0.667 ± 0.025	0.869 ± 0.058	1.54 ± 0.062	5.88 ± 0.799	1.33 ± 0.310
50	0.502 ± 0.037	1.107 ± 0.010	1.60 ± 0.039	15.14 ± 3.068	1.72 ± 0.527

Equation (1), which represents the sum of two hyperbolic relationships, was fitted to each substrate saturation curve to yield the values and standard errors shown in the table. Fitting was performed using a non-linear regression program based on the Gauss-Newton method. The program was written in FORTRAN and was run on a PDP-11/03-L computer. The values for the parameters represent the slopes and intercepts of the asymptotes to the curves shown in fig.1b

Substrate saturation curves were determined in the presence of 3 inhibitors of monooxygenase activity; chloramphenicol (△, fig.1a), phenacetin (□, fig.1a) and *p*-methoxybenzaldehyde (△, fig.1b). Each saturation curve, as well as others not depicted in fig.1, were analysed by fitting eq. (1) to the data:

$$v = \frac{V_{max_1} S}{K_{m_1} + S} + \frac{V_{max_2} S}{K_{m_2} + S} \qquad (1)$$

using the Gauss-Newton method of non-linear regression. The resulting values and standard errors for V_{max_1}, V_{max_2}, K_{m_1} and K_{m_2} for all the kinetic studies undertaken are given in tables 1 and 2. The lines in fig.1 are the best fit lines based on these parameter values. In all cases a good fit was obtained which indicated that eq. (1) represents an accurate description of the data.

3. Discussion and conclusions

Rat liver monooxygenase exhibits non-Michaelian kinetic behaviour when catalyzing the oxidative *O*-demethylation of *p*-nitroanisole (○, fig.1a,b). The kinetic behaviour which is seen is qualitatively consistent with catalysis by a minimum of 2 independent

enzymes with widely different K_m values or by a single enzyme with >1 site which interact in a negatively cooperative fashion. In the absence of added inhibitors the data are described well as the sum of 2 hyperbolic relationships (eq. (1)) but this fact does not necessarily imply that there are 2 independent enzymes; negative cooperativity between interacting sites on a single enzyme will yield a saturation curve with the same general form as eq. (1). We have sought to use inhibitors of monooxygenase to distinguish between these possibilities.

Since chloramphenicol and phenacetin are substrates for monooxygenase [5,15], and *p*-methoxybenzaldehyde has a close structural similarity to *p*-nitroanisole, these 4 compounds would be expected to be mutual competitive inhibitors of monooxygenase activity. Thus, if 2 independent enzymes were responsible for catalysis, it would be expected that the presence of a competitive inhibitor would increase the app. K_m of one or both enzymes without affecting the V_{max} values. In fact, the consistent observation is a decrease in V_{max_1} and an increase in V_{max_2}. The K_m values are also affected but no regular pattern can be discerned. For example, chloramphenicol had no significant effect on K_{m_1} but caused an increase in K_{m_2} while *p*-methoxybenzaldehyde caused K_{m_1} to increase without affecting K_{m_2}. The decrease in V_{m_1} might be explained by a non-competitive component in the inhibition but it is more difficult to interpret the apparent activation of the high-K_m form of monooxygenase. The most reasonable comprehensive explanation of these observations would be that the enzyme possesses several active sites which exhibit negatively cooperative interactions and that the peculiar effect which these inhibitors appear to have on V_{max_2} is symptomatic of the fact that the data have been fitted to the wrong model.

Data similar to those shown in fig.1 which were obtained in the absence of inhibitor, have previously been reported for the oxidation of a number of xenobiotics [1–4] and in some cases [2,3] the results have been used in support of the argument that several different enzymes may have been responsible for catalysing the metabolism of those substrates. From these results with *p*-nitroanisole it would appear that this should be regarded as only one possible explanation for the observed kinetics of monooxygenase activity, and that corroborative evidence would be needed to establish its validity in each case. Apparently different forms of cytochrome P450 have been isolated from rat liver microsomes [16,17], which indicates that endoplasmic reticulum probably does contain multiple monooxygenases. These findings do not however rule out the possibility that cooperativity may be a feature of monooxygenase activity with respect to some particular substrates.

The suggestion that monooxygenase activity may show cooperative interactions came first as an explanation for the results of studies on the competitive inhibition of the metabolism of a number of xenobiotics [8]. In [18], for example, it was shown that catalysis of *N*-demethylation of ethylmorphine was competitively inhibited by phenobarbital, chlorpromazine, zoxazolamine, phenylbutazone and acetanilide; in [19], a number of steroids were shown to competitively inhibit the oxidation of phenobarbital and ethylmorphine and in [8], octane and aniline were both shown to competitively inhibit benzphetamine metabolism. Since these interactions were demonstrated with such markedly different chemical types of compound it was suggested that a common enzyme showing poor substrate specificity was involved and it was proposed [8] that monooxygenase activity may be due to multiple catalytic sites which also act as effector sites conferring allosteric control of the negatively cooperative type over substrate oxidation. While we do not fully accept this interpretation, these results support the basic proposal that rat liver microsomal monooxygenase may contain multiple sites which interact to yield negatively cooperative kinetics. Clearly more work is required to clarify the number, nature and interactions of the ligand binding sites of monooxygenase and continuing studies in this laboratory are directed to this end.

An early report [20] that chloramphenicol is a non-competitive inhibitor of rat liver microsomal monooxygenase activity can be rationalized in the light of information given in table 1 and fig.1a. In that study acetanilide was used as the model xenobiotic substrate for measuring monooxygenase activity over the narrow and low substrate range of 4–16 μM. From table 1 it is apparent that when inhibition by chloramphenicol of monooxygenase activity is examined under analogous conditions with *p*-nitroanisole as substrate the antibiotic does appear to produce non-competitive inhibition kinetics as app. K_{m_1} is largely unaffected while V_{max_1} is significantly decreased. It is clear from this that the absolute substrate concentration can have a marked effect on the

apparent kinetics of inhibition of monooxygenase activity and a wide substrate concentration range should be employed to yield the most meaningful and informative results.

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