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THE NATURE OF MICROSOMAL MONOOXYGENASE INHIBITION BY CIMETIDINE

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Abstract—A kinetic investigation of the inhibitory effect of cimetidine on the O-dealkylation of 7-ethoxyresorufin by rat liver microsomes has yielded linear Lineweaver–Burk plots which intersect in the second quadrant Though technically compatible with non-competitive inhibition, the results are shown to be readily explained by the more realistic molecular concept of competitive inhibition by invoking the involvement of cytochrome P-450 isoenzymes with widely different K_I values Microsomal monooxygenase heterogeneity is also shown to provide a plausible explanation of other published results signifying the departure of chloramphenicol and phenacetin from the concept of competitive inhibition despite competition with substrate for the active-site haem group of cytochrome P-450

Since spectral studies of the interaction between cimetidine and hepatic microsomes signify involvement of the haem prosthetic group of cytochrome P-450 [1], it seems reasonable to consider that inclusion of this drug in microsomal reaction mixtures should lead to competitive inhibition of monooxygenase activities towards substrates whose oxidative breakdown also involves their interaction with the haem group [2]. In this regard the microsomal *N*-demethylation of morphine [3] and α-hydroxylation of metoprolol [4] certainly seem to be competitively inhibited by cimetidine, but results obtained with benzo[*a*]pyrene [5], aminopyrine [6] and 7-ethoxycoumarin [7] as substrates have been of the general form ascribed to non-competitive inhibition [8]. The aim of the present communication is to report a similar inhibition pattern for the effect of cimetidine on the microsomal O-dealkylation of 7-ethoxyresorufin, and to reconcile this finding with a molecular interpretation in terms of competitive inhibition

MATERIALS AND METHODS

A stock microsomal suspension prepared by the Guengerich method [9] from the livers of adult male, random outbred Wistar rats was stored at −75° prior to use. The microsomal cytochrome P-450 content, determined by the Estabrook procedure [10] with an Aminco DW-2a spectrophotometer in the dual wavelength mode, was 0 82 nmoles/mg protein. In kinetic studies, the O-dealkylation of 7-ethoxyresorufin was followed spectrofluorimetrically [11] in mixtures containing microsomal pigment (0.26 μM), substrate (0 08–1 3 μM), cimetidine (0, 187 or 467 μM) and NADPH (250 μM) in 0.1 M Tris–chloride buffer, pH 7 6. These assays, conducted at 27° and initiated by addition of the NADPH, were linear for at least 4 min, an observation which implies that the cytochrome P-450 isoenzymes catalyzing the oxidation of 7-ethoxyresorufin must constitute a very minor proportion of the total microsomal pigment

Cimetidine was a gift from Smith Kline and French (Australia) Ltd., while 7-ethoxyresorufin was obtained from Pierce Chemical Co. (Rockford, IL) NADPH was a product of Sigma Chemical Co. (St. Louis, MO) The sample of resorufin used for calibrating the response of an Aminco SPF-500 spectrofluorimeter was supplied by Aldrich-Chemie (Steinheim, F R.G.)

RESULTS AND DISCUSSION

Results of the kinetic experiments are summarized in Fig. 1A, about which the following points are noted. First, O-dealkylation of 7-ethoxyresorufin is adequately described by Michaelis–Menten kinetics with values of 0.27 ± 0 05 μM and 35 ± 3 pmol/min for the Michaelis constant, K_m, and maximal velocity, V_m, respectively. These estimates (±2 S.E.M) were obtained with a modification of the method described by Duggleby [12] for non-linear regression analysis of the untransformed (v, [S]) data Secondly, the results obtained in the presence of cimetidine do not conform with the classical concept of competitive inhibition in that the essentially linear Lineweaver–Burk plots intersect in the second quadrant rather than at the ordinate axis. This behaviour, which has also been observed in other studies of monooxygenase inhibition by cimetidine [6, 7], nicotinamide [13], ranitidine [14, 15] and imidazole [16], is described diagnostically either as mixed inhibition [6, 7, 13, 14], a mechanistically meaningless term, or as non-competitive inhibition [8] In addition, classical non-competitive inhibition has been reported for the effects of cimetidine on microsomal benzo[*a*]pyrene oxidation [5], of nizatidine on aminopyrine oxidation [16], and of imidazole on dimethylaniline dealkylation [17] It should be realized, however, that any classification of the inhibition as non-competitive implies the formation of ternary enzyme–substrate–inhibitor complexes, and hence the existence of separate binding

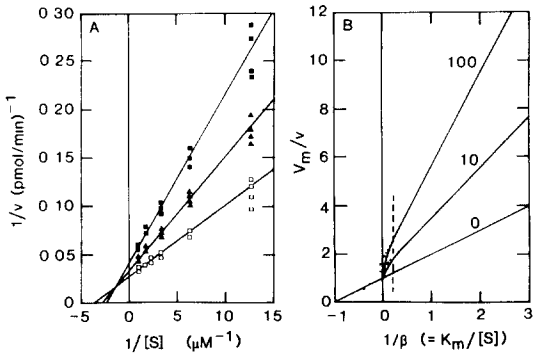

Fig 1 Inhibitory effect of cimetidine on the O-dealkylase activity of rat liver microsomes towards 7-ethoxyresorufin (A) Lineweaver–Burk plots of results obtained in the presence of zero (□), 187 μM (▲) and 467 μM (■) cimetidine (B) Theoretical plots for competitive systems described by equation (2) with $f = 0\,5$ and the indicated values of $[I]/K_I$ the vertical broken line corresponds to $[S] = 5\,K_m$, the likely limit of experimental studies

sites for inhibitor and monooxygenase substrate on the cytochrome P-450 haem group. This situation is considered to be rendered most unlikely by the fact that cimetidine has been shown to compete for the active site in inhibition studies with morphine [3] and metoprolol [4] as monooxygenase substrates, and also by the fact that cimetidine, an imidazole derivative, exhibits a "type II" spectral interaction with this pigment, a phenomenon universally ascribed to the direct binding of ligand by the haem moiety of cytochrome P-450 [18]. Moreover, direct competition between substrate and inhibitor for the sixth coordination position of the haem iron has been demonstrated by N M.R spectroscopy [17]. We therefore seek a molecular explanation whereby competitive inhibition of microsomal monooxygenase catalysis by cimetidine may lead to its diagnostic classification [8] as non-competitive (Fig. 1A)

The question at issue is not the correctness of Cleland's rules for the classification of inhibitors according to the location of intersection points for Lineweaver–Burk plots [8]; but rather the validity of their application to the microsomal monooxygenase system, which comprises an array of cytochrome P-450 isoenzymes with widely different catalytic characteristics towards any given substrate Furthermore, fractionation studies [19, 20] signify that monooxygenase activity towards a substrate such as 7-ethoxyresorufin is not necessarily confined to a single cytochrome P-450 isoenzymic species. Thus, instead of the K_m and V_m values obtained from Fig. 1A being regarded as the kinetic parameters for the catalytic O-dealkylation of 7-ethoxyresorufin by a single monooxygenase species, they should be considered to reflect weighted means for a number of cytochrome P-450 isoenzymes with fairly similar catalytic characteristics towards this substrate in the concentration range examined As demonstrated previously [21], resort to a sufficiently wide range of substrate concentration may well detect a second population of monooxygenase species with markedly different affinity for substrate. Even though several microsomal cytochrome P-450 isoenzymes may exhibit sufficiently similar K_m values for their catalytic effects to be describable by a single Michaelis constant, that does not mean that a single dissociation constant, K_I, necessarily describes their interactions with a competitive inhibitor, I In this regard it is noted that rat liver microsomal cytochrome P-450 exhibits a wide range of affinities for cimetidine [1, 22]; and that although K_I values in the micromolar range are now being detected [3, 4], most inhibition constants for cimetidine are in the millimolar range [1, 5–7, 14, 23, 24] We therefore explore the possibility that inhibition patterns of the type shown in Fig. 1A may reflect competitive inhibition of two classes of cytochrome P-450 isoenzymes with the same (or similar) Michaelis constant for ethoxyresorufin oxidation but with different K_I values for their interactions with cimetidine

In a mixture with substrate concentration $[S]$ and inhibitor concentration $[I]$ the initial velocity v, under such circumstances is given by

$$v = \frac{fV_m[S]}{[S] + K_m\{1 + ([I]/K_1)\}} + \frac{(1-f)V_m[S]}{[S] + K_m\{1 + ([I]/K_2)\}} \quad (1)$$

where f denotes the fractional maximum velocity contributed by enzymic species with inhibition constant K_1 for inhibitor, and the second term on the right-hand side refers to the contribution by the remaining enzyme, for which the interaction with inhibitor is characterized by dissociation constant K_2. By expressing $[S]$ in terms of K_m, i e $[S] = \beta K_m$, equation (1) may be rewritten as

$$(v/V_m) = \frac{f\beta}{\beta + \{1 + ([I]/K_1)\}} + \frac{(1-f)\beta}{\beta + \{1 + ([I]/K_2)\}} \quad (2)$$

which allows examination of the effects of varying f, K_1 and K_2 without the need to assign specific values to $[S]$ and K_m

Figure 1B presents normalized Lineweaver–Burk plots (V_m/v versus $1/\beta$) calculated on the basis of equation (2) with $f = 0\,5$ and the indicated values of $[I]/K_1$ for a system with $K_2 = 100\,K_1$ The first point to note is that apart from the situation where $[I]/K_1 = 0$, which refers to the system in the absence of inhibitor, the Lineweaver–Burk plot is curvilinear and intersects the ordinate axis at $(V_m/v) = 1$, as required for competitive inhibition Secondly, it should be noted that this curvature becomes evident at values of β ($=[S]/K_m$) that are higher than those commonly employed in experimental enzyme kinetic studies, for which a realistic upper limit of 5 (the vertical broken line in Fig 1B) is likely to apply Disregard of the experimentally inaccessible regions of the theoretical plots leads to the extrapolations shown as dotted lines in Fig 1B There is a striking similarity between the general form of these Lineweaver–Burk plots and that of their experimental counterparts in Fig 1A It is therefore con-

cluded that the action of cimetidine on the O-dealkylation of 7-ethoxyresorufin by rat liver microsomes, though technically compatible with non-competitive inhibition [8], finds ready explanation in terms of the more realistic molecular concept of competitive inhibition by invoking the involvement of isoenzymic species with widely different K_I values. Although the existence of three parameters of unknown magnitude in equation (1) precludes its use to obtain unique identification of f, K_1 and K_2, numerical simulations signify the experimental results to be described reasonably well by systems with K_1 = 20–30 μM, f = 0 45–0.55 and K_2 some 50- to 150-fold greater than K_1

Although Fig 1B points to the feasibility of a competitively inhibited system giving rise to Lineweaver–Burk plots that intersect in the second quadrant, it does only refer to one combination of the parameters f, K_1 and K_2. Other numerical examples have shown that the position of the intersection is displaced to the left by, for example, increasing the ratio of the inhibition constants (K_2/K_1). By this means it is possible to generate Lineweaver–Burk plots which intersect in the vicinity of the abscissa, and which thus approach the form ascribed to classical non-competitive inhibition. As noted above, such a phenomenon has been reported for the action of cimetidine on several microsomal monooxygenase activities [5, 6, 17].

In addition to studies of the effect of cimetidine on microsomal monooxygenase activity in which non-competitive [8] inhibition patterns have been reported [5–7, 16, 17], there have been others [1, 25] in which the inhibition is considered to be competitive but with K_I apparently a function of cimetidine concentration. This situation, which is clearly incompatible with competitive inhibition of a single enzyme, can readily be generated by the present concept that widely different K_I values apply to different cytochrome P-450 isoenzymes responsible

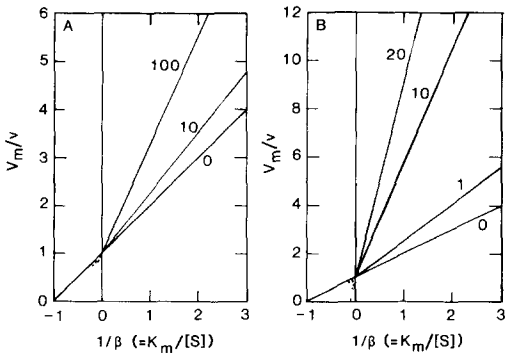

Fig 2 Theoretical Lineweaver–Burk relationships illustrating the ability of the present competitive model to accommodate other reported inhibitory effects of cimetidine on microsomal monooxygenase kinetics (A) Plots, calculated from equation (2) with $K_2 = 100\,K_1$, f = 0 1 and the indicated values of $[I]/K_1$, that seemingly signify competitive inhibition except that K_I would be a function of inhibitor concentration [1, 25] (B) Classical competitive inhibition plots [3], generated by equation (2) with $K_2 = 5\,K_1$, f = 0 5 and the indicated values of $[I]/K_1$

for the substrate oxidation under examination. Figure 2A presents theoretical Lineweaver–Burk plots for the same system considered in Fig. 1B ($K_2 = 100\,K_1$) except that the enzymic species with higher affinity for I contributes a smaller proportion of the maximal velocity (f = 0 1, cf. 0.5). These theoretical Lineweaver–Burk plots seemingly comply with the requirements of classical competitive inhibition, but their interpretation in such terms would lead to a value from the relationship for $[I]/K_1 = 100$ some twofold greater than that pertaining to the lower inhibitor concentration ($[I]/K_1 = 10$) Such behaviour is qualitatively reminiscent of the report [1] that competitive inhibition of microsomal ethoxycoumarin O-dealkylase activity is governed by K_I values of 0.22 mM and 0.43 mM in the presence of 0.25 mM and 0 75 mM cimetidine respectively In similar vein, results reported for the effect of cimetidine on pentobarbital hydroxylation by rat liver microsomes [25] seemingly signify respective K_I values of 38 μM and 270 μM from experiments with 59 μM and 590 μM inhibitor present.

Since atypical behaviour of inhibitors with microsomal monooxygenase systems seems to be the norm rather than the exception, it seems logical to question whether the fact that the inhibition by cimetidine of rat liver microsomal morphine demethylase does comply with classical competitive inhibition [3] may be taken to indicate the involvement of a single cytochrome P-450 isoenzyme Such interpretation is, however, dispelled by Fig 2B, which presents theoretical plots that also seemingly conform with classical competitive inhibition despite the fact that they have been generated, via equation (2), for a system with maximal velocity divided equally between enzymic species with a fivefold difference in inhibition constant ($K_2 = 5K_1$). Thus, although microsomal morphine demethylase in rat liver could be a single isoenzymic species, it could equally well comprise a series of cytochrome P-450 isoenzymes with sufficiently similar affinities towards substrate and cimetidine for their combined effects to be describable by a single K_m and a single K_I respectively, the net result being that cimetidine's action is manifested experimentally as classical competitive inhibition.

To this stage theoretical consideration has been restricted to systems in which enzymic catalysis is governed by a single K_m, and in view of the present supposition that monooxygenase isoenzymes exhibit markedly different affinities towards an inhibitor such as cimetidine, there is no reason *a priori* why such differences should not also extend to substrate affinities (K_m). In order to illustrate extension of the present model to this more general situation, a system governed by two Michaelis constants as well as two inhibition constants is considered In this regard it is noted that the simplest such situation involves identification of one Michaelis constant and one inhibition constant with each enzyme class, but that analysis of such a system by either the Scatchard or Eadie–Hofstee method signifies classical competitive inhibition of each enzyme class Accordingly, a system is envisaged wherein equation (2) with f = 0 5 and $K_2 = 100\,K_1$ applies to each of two enzyme populations (A and B) for which

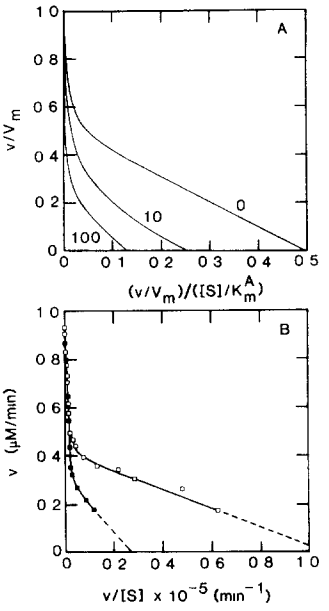

Fig 3 Extension of the present competitive model to include enzyme systems characterized by two Michaelis constants as well as two inhibition constants (A) Theoretical Eadie–Hofstee relationships for a system comprising enzymes A and B with Michaelis constants (K_m^A, K_m^B) and maximal velocities (V_m^A, V_m^B) such that $K_m^B = 100\ K_m^A$ and $V_m^A = V_m^B$, and for each of which the inhibition is described by equation (2) with $K_2 = 100\ K_1$ and $f = 0.5$. numbers adjacent to the curves denote the values of $[I]/K_1$ used for their computation (B) Experimental Eadie–Hofstee plots for the inhibitory effect of phenacetin on the O-demethylation of *p*-nitroanisole by rat liver microsomes, the results being reformulated from Fig 1a of a previous publication [21] □, results in the absence of inhibitor; ■, results in the presence of 0 25 mM phenacetin

$K_m^B = 100\ K_m^A$ and $V_m^B = V_m^A$. Figure 3A presents, in Eadie–Hofstee format, the theoretical dependence of velocity upon normalized substrate concentration for such a system The first point to note is the curvilinear nature of the plot in the absence of inhibitor ($[I]/K_1 = 0$), a feature which signifies non-conformity with Michaelis–Menten kinetics and thus distinguishes this model from the experimental and theoretical systems considered above. Secondly, consideration of these curvilinear plots as the sums of two linear contributions would lead to the conclusion that the stronger interaction with substrate is non-competitively inhibited, but that the weaker interaction undergoes compensatory activation such that the inhibition is competitive overall (V_m unchanged). From this viewpoint it is of interest that Fig 3A duplicates the findings of an experimental study on the inhibition of microsomal dealkylation of *p*-nitroanisole by chloramphenicol, *p*-anisaldehyde and phenacetin [21], the results for the last-named inhibitor being presented in Fig 3B. Although the non-competitive element of the inhibition was recognized to be an artifact [21], no molecular explanation of its origin was proposed The similarity between the general forms of Figs. 3A and 3B strongly implicates monooxygenase heterogeneity as the likely cause of the phenomenon.

In summary, this investigation serves to illustrate that, due to the heterogeneous nature of the hepatic microsomal monooxygenase system, kinetic investigations conducted over a relatively narrow substrate concentration range may often yield results that are seemingly symptomatic of non-competitive inhibition despite the fact that the inhibitor is competing with substrate for the active-site haem group of cytochrome P-450 It also serves to re-emphasize [21] the desirability of examining monooxygenase catalysis over a very wide range of substrate concentration in order that the competitive nature of the inhibition may be established unequivocally by failure to change V_m Finally, we conclude that evidence purporting to indicate microsomal inhibition by cimetidine as non-competitive is more realistically considered to be the consequence of microsomal cytochrome P-450 heterogeneity, and that cimetidine's inhibitory effects on monooxygenase catalyzed drug oxidation should therefore be viewed in terms of competitive inhibition

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