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# The interaction of rat liver mitochondrial monoamine oxidase with clorgyline plus *d*-amphetamine

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Copeland *et al.* [1] have reported clorgyline to promote the binding of a spin-labelled amphetamine derivative to the soluble fraction of monoamine oxidase (MAO) activity from rat liver. This enhancing effect was found to occur rapidly in a time-scale corresponding to the initial non-covalent interaction between clorgyline and the enzyme. This initial interaction has been shown to be competitive with respect to the substrate and selective towards the A-form of MAO [2, 3]. *d*-Amphetamine has also been shown to be a competitive inhibitor with selectivity towards MAO-A [4], but the apparent synergism reported by Copeland *et al.* [1] would imply that both these compounds should be able to bind to the enzyme simultaneously. Since the ability of one inhibitor to enhance the binding of another could have important consequences for the therapeutic use of mixtures of inhibitors we have investigated the phenomenon using *d*-amphetamine and clorgyline as reversible inhibitors of rat liver mitochondrial MAO.

*Materials and methods*

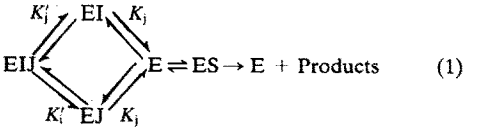
Rat liver mitochondria, prepared as previously described [5] and stored frozen, were used as the source of the enzyme. For studying the activity towards MAO-A the B-form was inhibited by preincubating samples of the mitochondria for 60 min at 37° with $3 \times 10^{-7}$ M *l*-deprenyl [5]. The mitochondria were then extensively washed by sedimentation and resuspension to remove any excess inhibitor. For studies on the activity towards MAO-B, the activity of the A-form was inhibited by treatment in a similar manner with $10^{-7}$ M clorgyline [5]. Assay of the preparations treated in these ways indicated that no detectable activity of the susceptible form remained whereas the activity of the remaining form had not been significantly affected.

Monoamine oxidase activity was determined at 37° by a modification [6] of the method of Otsuka and Kobayashi [7] using 100 $\mu$M 5-hydroxytryptamine-(side-chain-2-$^{14}$C) creatinine sulphate as a substrate for MAO-A and 10 $\mu$M 2-phenethylamine-[ethyl-1-$^{14}$C] hydrochloride as a substrate for MAO-B. In all cases the assay was initiated by adding the enzyme sample to the buffer, substrate, inhibitor mixture so that only the initial phase of inhibition was determined. Care was taken to restrict the time of the assay to the period where product formation was a linear function of time so that no significant time-dependent, irreversible, inhibition by cloryline was occurring.

The radioactive substrates were obtained from Amersham International p.l.c. Amersham, U.K. Clorgyline hydrochloride and *l*-deprenyl hydrochloride were kind gifts from May & Baker, Dagenham, Essex, U.K. and from Professor J. Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary, respectively. *d*-Amphetamine was a kind gift from Smith, Kline & French Ltd., Welwyn Garden City, Herts, U.K.

*Analysis*

Since both clorgyline and *d*-amphetamine can bind to the free enzyme as competitive inhibitors but the results suggesting synergism between their binding [1] indicate that they must both be able to bind to the enzyme at the same time, the simplest model that could account for this behaviour would be:



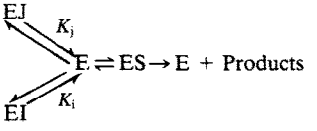

(1)

where I and J represent clorgyline and *d*-amphetamine respectively and $K_i$, $K_j$ etc. are dissociation constants. This mechanism gives rise to an initial rate equation of the form:

$$v = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i} + \frac{[J]}{K_j} + \frac{[I][J]}{K_iK_j'}\right)} \quad (2)$$

Where $v$ and $V$ are the initial and maximum velocities, respectively, and the square brackets denote concentrations.

Alternatively if the two inhibitors bound to the enzyme at the same site, a mechanism that would not allow for synergism between them, the system could be represented by:




(3)

the initial-rate equation would be

$$v = \frac{V}{1 + \frac{K_m}{[S]}\left(1 + \frac{[I]}{K_i} + \frac{[J]}{K_j}\right)} \quad (4)$$

If the inhibitors are mixed together in a fixed ratio, such that $x[I] = [J]$, equation (2) can be written in reciprocal form as:

$$\frac{1}{v} = \frac{1}{V} + \frac{K_m}{V[S]}\left(1 + \frac{[I]}{K_i} + \frac{x[I]}{K_j} + \frac{x[I]^2}{K_iK_j'}\right) \quad (5)$$

which would predict a non-linear dependence of the reciprocal of the initial velocity upon the concentration of the mixture (a Dixon [8] plot). In contrast equation (4) will not contain a squared term of concentration under these conditions and thus predicts a linear Dixon plot [see 9].

*Results and discussion*

A mixture of clorgyline and *d*-amphetamine each present at 25 times its $K_i$ value for MAO-A was prepared and the initial velocity of 5-hydroxytryptamine oxidation was determined in the presence of varying quantities of this mixture. Figure 1(a) shows that the Dixon plot of these data shows no significant deviation from a straight line. Similarly when a mixture of the two inhibitors was used, each at its $K_i$ concentration for MAO-B, and the activity towards 2-phenethylamine was determined there was no evidence of curvature (Fig. 1b).

Since both these inhibitors were present at the same fixed proportion of their $K_i$ values the intercept on the horizontal axis in Figs. 1(a) and (b) should correspond to a value of $(1 + [S]/K_m)/2$. The published $K_m$ values for rat liver mitochondrial monoamine oxidase A and B respectively oxidising 5-hydroxytryptamine and 2-phenethylamine are $161 \pm 34$ and $20 \pm 6\ \mu M$ respectively under the conditions used here [10]. Thus at the assay concentration of 100 $\mu$M 5-hydroxytryptamine the theoretical value of the intersection point in Fig. 1(a) should be $0.81 \pm 0.08$ whereas that in Fig. 1(b), determined at 10 $\mu$M 2-phenethylamine should be $0.75 \pm 0.11$. These theoretically-predicted values are in good agreement with the experimentally observed values of $0.78 \pm 0.11$ and $0.69 \pm 0.1$ determined by linear regression analysis of the data in Figs. 1(a) and (b) respectively, providing further evidence that the binding of either one of these inhibitors has no significant effect on the $K_i$ value of the other.

The data presented above show that the binding synergism reported by Copeland *et al* [13] is not reflected in the reversible inhibitory potencies of *d*-amphetamine and clorgyline. Although the mechanism presented in equation (1) represents the simplest that could account for binding synergism of the type reported by Copeland *et al.* [1] more complex mechanisms would also give rise to non-linear behaviour in the kinetic plots used in the present experiments. The reason for this discrepancy is unclear although the possibilities that the spin-labelled amphetamine derivative used in the binding studies [1] interacted with an additional site as well as, or instead of, the active site occupied by *d*-amphetamine itself or that the minor soluble MAO component used in that study differs from the mitochondrial activity in its interactions with inhibitors cannot be excluded.

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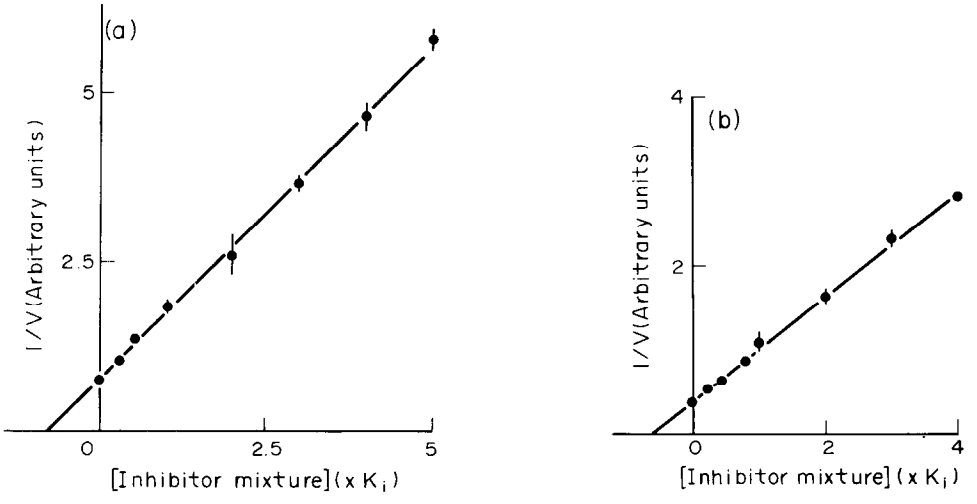


Fig. 1. The inhibition of monoamine oxidase activity by a mixture of clorgyline plus *d*-amphetamine. The reversible inhibition of the activity towards MAO-A and MAO-B was determined using a mixture of the two inhibitors each at an equivalent concentration relative to its $K_i$ value as a reversible inhibitor. The $K_i$ values for inhibition of the two forms by *d*-amphetamine and clorgyline respectively were taken from Mantle *et al.* [4] and Fowler *et al.* [3]. The concentration of the mixture is expressed relative to the $K_i$ value of one component. (a) Activity of MAO-A assayed with 100 $\mu$M 5-hydroxytryptamine; (b) activity of MAO-B assayed with 10 $\mu$M 2-phenethylamine. All values are mean ± S.E.M. for at least three separate determinations.

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# Different effects of carbon tetrachloride toxicity and cirrhosis on substrate binding to rat hepatic microsomal cytochrome P-450

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In humans, impaired drug metabolism is often associated with hepatic disease, especially cirrhosis, and this may lead to toxic complications of drug therapy [1]. Levels of hepatic cytochrome P-450, the principal component of the mixed function oxidase system which is active in drug metabolism, are lowered in experimental animals rendered cirrhotic by chronic exposure to carbon tetrachloride ($CCl_4$) [2]. However, attempts to use this model to study altered drug metabolism in cirrhosis may be complicated by the acute destructive effects of $CCl_4$ on cytochrome P-450 [3]. In the present study, the binding of representative Type I and Type II substrates [4] to cytochrome P-450 has been examined in cirrhotic and control rat liver microsomes. In addition, the binding changes observed in cirrhotic liver have been compared with those found in microsomes from rats acutely exposed to $CCl_4$.

## *Methods*

*Animals.* Hepatic cirrhosis was produced in male Wistar rats (from the Institute of Clinical Pathology and Medical Research, Westmead, N.S.W.) receiving drinking water containing 0.1% sodium phenobarbitone (PB) by exposure to $CCl_4$. This was administered by inhalation twice weekly for ten weeks, essentially as described elsewhere [5]. Animals were sacrificed ten days after the final exposure to $CCl_4$ and PB. Control animals received PB-water for the same period of time as cirrhotic rats.

Acute $CCl_4$ intoxication was produced in rats receiving PB-water by three exposures, for a period of 10 min each, in six days. Unlike the procedure used to generate cirrhosis, acutely poisoned rats were not permitted to recover after the third exposure to $CCl_4$. Instead, PB was stopped, access to normal drinking water was restored, and forty-eight hours later rats were killed and microsomal fractions were prepared. Controls received PB for the same length of time.

*Preparation of microsomal fraction.* Washed hepatic microsomal fractions were prepared by differential centrifugation as described previously [6]. Microsomes were stored as pellets at −20° and were used within three weeks of preparation.

*Assays.* Cytochrome P-450 was determined by the method of Omura and Sato [7] employing an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the cytochrome P-450 ferrous carbonyl spectral complex.

*Difference spectra.* Optical difference spectra were determined in an Aminco Chance DW-2a spectrophotometer operating in the split beam mode. Microsomal pellets were resuspended in 0.1 M potassium phosphate buffer (pH 7.4) to a final protein concentration of 1 mg/ml, divided equally between two cuvettes, and a baseline of equal light absorbance established. Ethylmorphine HCl (McFarlan Smith Ltd., Edinburgh, Scotland) or metyrapone (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were selected as examples of Type I and Type II substrates, respectively, and were introduced to the sample cuvette in microlitre quantities from stock solutions in 0.1 M phosphate buffer. The difference spectrum was recorded between 380 and 500 nm. Spectral dissociation constants ($K_s$; μM) and maximal absorbance changes ($\Delta A_{max}$; absorbance units/nmoles cytochrome P-450) were determined from the ordinate and abscissa intercepts, respectively, of double reciprocal plots of ligand concentration vs $\Delta A$ (peak to trough; 386–417 nm for ethylmorphine and 426–392 nm for metyrapone; 4–6 ligand concentrations).

As changes in affinity ($K_s$) and capacity ($\Delta A_{max}$) may occur independently, the ratio of $\Delta A_{max}/K_s$ was calculated to assess the net effect of substrate binding alterations. The significance of this ratio, the efficiency of binding, is analogous to that which the ratio of $V_{max}/K_m$ has in catalytic studies [9].

*Statistics.* Differences between mean values ($K_s$, $\Delta A_{max}$ and cytochrome P-450 content) were assessed using the unpaired Student's *t*-test (two-tailed).

## *Results and discussion*

*Changes in substrate binding to cirrhotic microsomes.* Changes in the binding of Type I (EM) and Type II (MET) substrates to cytochrome P-450 varied between microsomes from cirrhotic rat liver and those from rat liver acutely poisoned with $CCl_4$ (Table 1). In cirrhotic liver microsomes, the $K_s$ values for both EM and MET were identical to those in control liver microsomes. In contrast, there was a significant decrease in the extent of the interaction ($\Delta A_{max}$) of both compounds in cirrhotic microsomes compared with controls. Thus, it is apparent that the affinity of substrate binding to oxidised cytochrome P-450 is unaltered in hepatic cirrhosis but that the proportion of cytochrome P-450 undergoing the spectral interaction is reduced.

The extent of the decrease in substrate binding capacity ($\Delta A_{max}$) was 35% for the Type I substrate (EM) and 20% for the Type II compound (MET) (Table 1). The percentage decrease in binding capacity for the Type I interaction is almost twice that of the decrease in the Type II interaction, a finding that supports the assertion that the microsomal Type I and Type II sites are distinct. It seems apparent that the two binding sites (apoprotein and haem sites) are affected to a different extent by the cirrhotic process.

The values of the ratio $\Delta A_{max}/K_s$ for both Type I and Type II interactions with cytochrome P-450 in cirrhotic rat liver microsomes were lower than those in control microsomes. This reflects the reduced capacity of the cirrhotic