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STUDIES ON THE SELECTIVE INHIBITION OF MEMBRANE-BOUND RAT LIVER MONOAMINE OXIDASE

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Abstract—Serotonin and benzylamine oxidising activities of membrane-bound rat liver monoamine oxidase have been distinguished according to their sensitivities towards 5-phenyl-3-(*N*-cyclopropyl)ethylamine-1,2,4-oxadiazole (PCO). Tyramine, tryptamine and dopamine deamination have been shown to exhibit dual sensitivities to PCO inhibition, corresponding to these monoamines undergoing oxidation at both the PCO sensitive and insensitive sites responsible for serotonin and benzylamine oxidation respectively. The biphasic inhibition of tyramine deamination by PCO is shown to result from 'fast' and 'slow' pseudo-first order reactions with the enzyme. Both 'fast' and 'slow' reactions are shown to have two components, of which the slower is quantitatively the most important. The corresponding 3-nitrophenyl compound (3-nitro-PCO) preferentially inhibits tyramine oxidation at low concentrations. This is shown to result from a reversal of the relative rates of attack, by this inhibitor, on the two centres of deamination. PCO has been shown to be a potent instantaneous competitive inhibitor of the enzyme. With serotonin as substrate a K_i of 10^{-7} M was obtained. An enzyme with serotonin oxidation completely blocked by PCO or 2-chloro-PCO, but retaining approximately half the tyramine deaminating activity has been prepared. The kinetic parameters for the oxidation of tyramine, tryptamine and dopamine by such partially inhibited preparations have been determined.

Several compounds are known that possess the ability to block preferentially the deamination of particular substrates by membrane-bound monoamine oxidase [1]. Johnston [2] originally described clorgyline, a propargylamine derivative, which is a highly potent inhibitor of serotonin oxidation but which shows a biphasic inhibition of tyramine oxidation. Low concentrations of clorgyline inhibit the oxidation of serotonin and a proportion of the activity towards tyramine whereas higher concentrations are necessary to inhibit the oxidation of benzylamine and the remainder of the activity towards tyramine [3]. Johnston proposed [2] that the two activities, clorgyline sensitive and insensitive, represent two distinct enzymes, which he designated A and B respectively.

Deprenil (phenylisopropylmethylpropynylamine) has been shown to behave in the reverse manner to clorgyline, by inhibiting benzylamine oxidation at lower concentrations than those required to block serotonin oxidation [4]. The inhibition of tyramine oxidation is again biphasic.

Johnston's original suggestion, that membrane-bound preparations of monoamine oxidase contain two distinct enzymes, has received little support from studies with solubilized preparations of the enzyme [5, 6]. While multiple forms of solubilized monoamine oxidase have been demonstrated by gel filtration [7, 8], gel electrophoresis [9, 10] and density gradient electrophoresis [11], none of these separable forms has been convincingly demonstrated to correspond to either of the hypothetical A or B enzymes. It has recently been suggested that the multiple forms obtained after polyacrylamide gel electrophoresis of the rat liver enzyme [9] result from the binding of different amounts of membrane material to a single enzyme species [5]. If such an explanation applies to other preparations of monoamine oxidase, which exhibit multiple forms, as seems the case for the human brain enzyme [12], it is hardly surprising that a satisfactory separation of serotonin and benzylamine oxidising activities has not been achieved [10, 13]. In order to examine the binary nature of the membrane-bound enzyme we have studied the interaction of four members of a novel series of substrate selective inhibitors [14] with a membrane-bound preparation of rat liver monoamine oxidase.

MATERIALS AND METHODS

Materials. [1-^{14}C]serotonin creatinine sulphate, [1-^{14}C]tyramine hydrochloride, [1-^{14}C]dopamine hydrochloride and [G-^{3}H]tryptamine hydrochloride were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. Serotonin creatinine sulphate, tyramine hydrochloride and benzylamine were obtained from B.D.H. Ltd, Poole, Dorset, U.K. Dopamine and tryptamine hydrochlorides were obtained from Sigma, London. Anisole was purchased from Koch Light Ltd, Colnbrook, Bucks, U.K. All other reagents were of the highest purity available. The inhibitors used were synthesized in the Chemistry Department, Roche Products Ltd [14]. The structure of the parent

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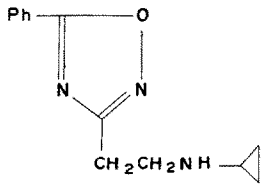
compound, 5-phenyl-3-(*N*-cyclopropyl)-ethylamine-1,2,4-oxadiazole (PCO), is shown below.



As the other three compounds of this series used in the present study were substituted in the phenyl ring these will be referred to as 2-chloro-PCO, 3-nitro-PCO and 3-chloro-PCO.

Enzyme preparation. Male Wistar rats weighing approx 180 g were stunned by a blow on the head and exsanguinated. The livers were removed and placed immediately into ice-cold 250 mM sucrose, blotted dry, weighed and homogenised using a glass hand homogenizer in 250 mM sucrose. After centrifuging the homogenate at 600 *g* for 10 min, the carefully decanted supernatant was centrifuged at 10,000 *g* for 10 min. The crude mitochondrial pellet so obtained was washed twice with 67 mM sodium potassium phosphate buffer, pH 7·4, and resuspended in the same medium to give a protein concentration of approx 20 mg/ml. This suspension was frozen in aliquots and thawed immediately before use.

Determination of enzyme activity. Monoamine oxidase activity towards tyramine, serotonin, dopamine and tryptamine was determined by the method described by Otsuka and Kobayashi for tyramine [15]. The protein concentration of the enzyme suspension was adjusted to approx 0·8 mg/ml to give a linear reaction rate during the period of incubation. Incubations were performed in a shaking water bath, with air as gas phase. The assay mixture consisted of 67 mM phosphate buffer, pH 7·4 (0·8 ml), 20 mM EDTA in buffer (0·2 ml), non-radioactive substrate (0·4 ml) in buffer and radioactive substrate (0·1 ml; 60,000 dis/min) in buffer. The reaction was started by the addition of 0·5 ml of enzyme and terminated after 30 min by adding 0·5 ml of 2M citric acid. For experiments in which the enzyme activity was determined after preincubation with inhibitor, the reaction was started by the addition of the non-radioactive substrate followed within 30 sec by the addition of radioactive substrate. The deaminated products were extracted into anisole (10 ml) containing 0·6% 2,5-diphenyl-oxazole (PPO) by vigorous shaking for 1 min. After centrifuging to separate the layers, the aqueous layer was frozen and the anisole decanted directly into a vial for liquid scintillation counting. Dis/min were calculated by the channels ratio method. No quenching was observed. Blanks, obtained by adding citric acid to the incubation mixture before the enzyme were negligible with tyramine, serotonin and dopamine as substrates. With tryptamine as substrate, however, the blank was significant and was subtracted from the observed dis/min.

The activity of monoamine oxidase towards benzylamine was determined by the spectrophotometric method of Tabor *et al.* [16].

Preparation of partially inhibited enzyme. The enzyme suspension (approx 20 mg protein/ml) was incubated for 20–30 min at 35° with a concentration of PCO or 2-chloro-PCO (approx 0·1–0·3 μM) sufficient to block the activity towards serotonin completely, but the activity towards tyramine by only about 50 per cent. At the end of the period of incubation the mixture was cooled in ice, immediately diluted with ice-cold 67 mM phosphate buffer (5 vol) and centrifuged at 10,000 *g* for 10 min. The pellet was resuspended in cold buffer with the aid of a loose-fitting glass homogeniser and centrifuged as before. This procedure was repeated three times and the final pellet made up to half the original volume in buffer and assayed for tyramine and serotonin deaminating activity. Such preparations retained 40–50% of their original activity towards tyramine, but were inactive towards serotonin. Washing the partially inhibited enzyme preparation did not reverse the inhibition.

Protein concentration. Protein concentration was determined by the method of Lowry *et al.* [17] with bovine serum albumin as standard.

Treatment of data. The relationship between the non-radioactive substrate S and product P concentrations, and the incubated (60,000) and extracted (N) dis/min may be written;

$$\frac{S}{60{,}000} = \frac{P}{N}$$

since the concentration of radioactive substrate was negligible compared to that of the non-radioactive substrate. A linear time-activity relationship was demonstrated to be operative under the assay conditions used, hence the initial rate was calculated as;

$$v_0 = \frac{dP}{dt} = \frac{S.N.}{60{,}000}$$

no correction being made for incomplete extraction of deaminated products. Statistically derived kinetic parameters were obtained by the method of Wilkinson [18]. These agreed well with values determined graphically.

RESULTS

Substrate dependence of the biphasic response. Figure 1 shows the effect on monoamine oxidase of preincubating increasing concentrations of PCO with the enzyme prior to assaying enzyme activity towards serotonin, tyramine, dopamine and tryptamine. It can be seen that inhibition of both tryptamine and dopamine deamination shows the biphasic response, previously demonstrated with tyramine as substrate [14].

Figure 2 illustrates the results of a similar experiment with an enzyme suspension that had been sonicated in 250 mM-sucrose at 6 A for 80 min at 5°. Prolonged sonication, necessary for assaying activity with benzylamine as substrate, did not effect the activity of the enzyme or the complex inhibition curves obtained with tyramine and tryptamine as substrates. These results indicate that there are no rate limiting permeability barriers in the non-sonicated enzyme preparation. Serotonin remained sensitive to PCO inhibition and followed a classical sigmoidal dose–response curve (pI_{50} = 7·2). The inhibition of benzylamine oxidation also followed a sigmoidal dose–response curve, however this activity was much less susceptible to the inhibitor (pI_{50} = 5·2) and corresponds

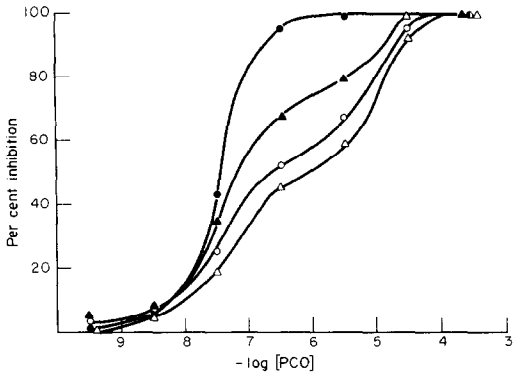


Fig. 1. Effect of PCO on the activity of membrane bound monoamine oxidase. Enzyme was pre-incubated for 30 min at 35° with the concentrations of PCO indicated prior to the addition of non-radioactive substrate (final concn 100 μM), followed by the appropriate radioactive substrate (60,000 dis/min). After 30 min the reaction was terminated by the addition of 2 M-citric acid and the deaminated products extracted as described in the text. The substrates used were tyramine (○), tryptamine (△), serotonin (●) and dopamine (▲). Control experiments indicated that no loss of activity occurred under the pre-incubation conditions in the absence of PCO.

to the second phase of the inhibition of tyramine and tryptamine deamination (pI_{75} = 5·2). Johnston has discussed [2] the use of pI_{25} and pI_{75} in such situations.

Kinetics of inactivation by PCO and derivatives. Varying the period of preincubation of the enzyme with PCO and its derivatives showed that, in all cases, the inhibition was time dependent. Figure 3 shows the time course of inactivation by 0·15 μM PCO. It can be seen that the serotonin oxidising activity was completely inactivated after 45 min preincubation. The same period of preincubation abolished approx half the tyramine oxidizing activity, there being no

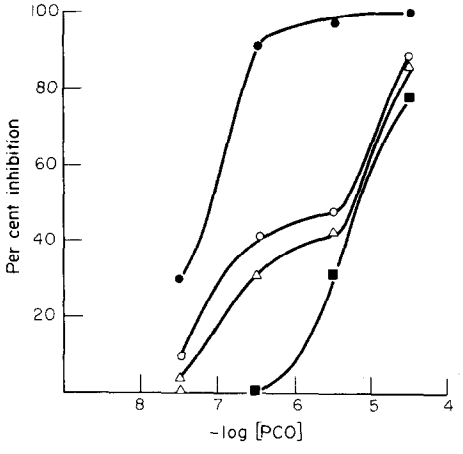


Fig. 2. Effect of PCO on the monoamine oxidase activity of a sonicated enzyme preparation. The enzyme preparation (sonicated as described in the text) was pre-incubated for 30 min at 35° with the concentrations of PCO indicated prior to the addition of substrate (final concn 100 μM). The deamination of serotonin (●), tyramine (○) and tryptamine (△) was assayed radioisotopically as described in the text. Benzylamine (■) deamination was measured by following the increase in extinction at 250 nm.

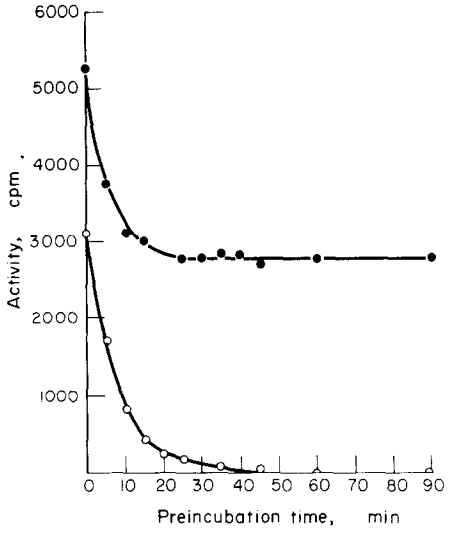


Fig. 3. Time dependence of inhibition of monoamine oxidase by PCO. Samples of the enzyme were incubated at 35° with 0·15 μM PCO. After the times indicated serotonin (○) or tyramine (●) was added (final concn 100 μM), followed by the addition of the corresponding radioactive substrate. Enzyme activity was determined radioisotopically as described in the text.

further decrease in activity with up to 90 min preincubation. The results of a more detailed examination of the time course of inactivation by PCO are shown in Fig. 4. Without preincubation there was no inhibition by 0·01 μM or 0·033 μM PCO. However in the presence of 0·1 μM PCO serotonin oxidation was inhibited 20 per cent without preincubation. Similar effects were observed at higher concentrations of PCO with tyramine as substrate. The time course of inactivation of tyramine deamination by 0·33 μM PCO showed a rapid inactivation to 50 per cent inhibition as in the previous experiment (Fig. 3). However in the presence of 10 μM and 20 μM PCO the tyramine deaminating activity was reduced to half the original value without preincubation. The further time dependent inactivation observed on preincubating at these concentrations of PCO was therefore presumably due to inactivation of the 'inhibitor resistant' tyramine deaminating activity, the inhibitor sensitive activity being lost instantaneously under these conditions. These kinetically distinguishable inactivations will be referred to as occurring at 'fast' and 'slow' reaction centres on the enzyme. Semi-log plots of the data shown in Fig. 4 showed that the inactivation by PCO was complex with respect to both substrates, but in all cases could be interpreted as being composed of two first order curves. The pseudo-first order and bimolecular rate constants for the major and minor (uncorrected) reactions are given in Table 1. The equivalence of the bimolecular rate constants, derived at different concentrations of PCO, for the major and minor components of the inactivation of serotonin deamination, and for the major and minor components of both the 'fast' and 'slow' inactivations of tyramine deamination are in agreement with simple second order kinetics. Semi-log plots for the inactivation of the enzyme by 3-nitro-PCO could also be interpreted as being composed of two first order curves.

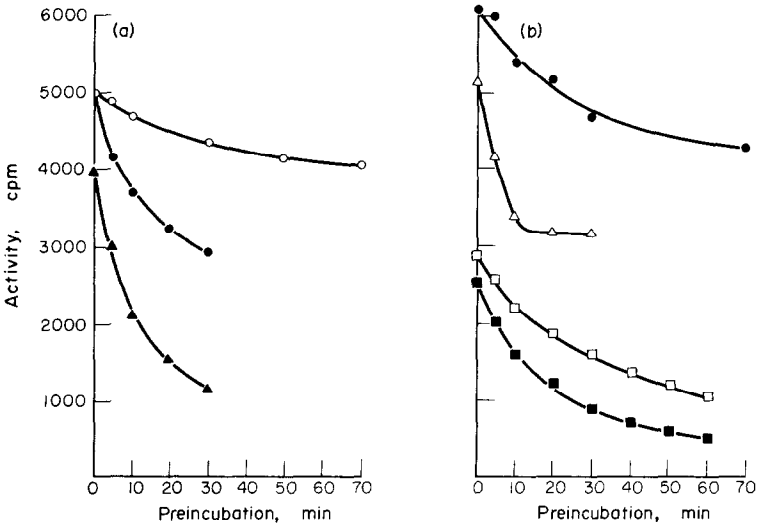


Fig. 4. Effect of PCO concentration on the rate of inhibition of monoamine oxidase. Samples of the enzyme were incubated at 35° with 0·01 μM (○), 0·033 μM (●), 0·1 μM (▲), 0·33 μM (△), 10 μM (□) and 20 μM (■) -PCO. After the time intervals indicated non-radioactive substrate (final concn 100 μM) was added, followed by the corresponding radioactive substrate (60,000 dis/min). Enzyme activity was determined radioisotopically as described in the text. Fig. 4(a) shows results obtained with serotonin, and Fig. 4(b) with tyramine.

In agreement with the observation that this compound, at low concentrations, preferentially inhibits tyramine deamination [14], using low inhibitor concentrations the bimolecular rate constant against tyramine deamination is twice that calculated with serotonin as substrate (Table 2). With the two higher concentrations of 3-nitro-PCO used considerable inhibition occurred during the period of assay, so that although the pseudo-first order rate constants are not directly proportional to the inhibitor concentration, this apparent deviation from simple second order kinetics is probably an artefact. As with PCO inactivation the faster reaction (minor component) was responsible for only a small percentage of the total inactivation. The inactivation of serotonin deamination by 3-chloro-PCO followed straightforward pseudo-first order kinetics. The rate constants are shown in Table 3.

Instantaneous inhibition of monoamine oxidase by PCO. Figure 5 and 6 show double reciprocal plots for serotonin and tyramine deamination respectively, in the presence and absence of PCO. The inhibition was competitive, the slope replot (inset Fig. 5) showing that PCO was a potent linear competitive inhibitor (after the Cleland monenclature [19]) of serotonin deamination with a K_i of 0·1 μM. However with tyramine as substrate PCO appeared to be a hyperbolic competitive inhibitor [19]. Unfortunately data at higher concentrations of PCO could not be obtained as time dependent inactivations would be expected to accompany the instantaneous inhibition. Hyperbolic competitive kinetics can arise from competitive

Table 1. Pseudo-first order and bimolecular rate constants for the inactivation of monoamine oxidase by PCO

[PCO]μM	Minor component $10^3k_i'$ (min^{-1})	Minor component $10^{-5}k_i$ ($M^{-1}min^{-1}$)	Major component $10^3k_i'$ (min^{-1})	Major component $10^{-5}k_i$ ($M^{-1}min^{-1}$)
(a) Serotonin as substrate				
0·01	9·2	9·2	1·1	1·1
0·033	27·6	8·3	11·5	3·5
0·1	62·1	6·2	29·9	3·0
(b) Tyramine as substrate				
Fast				
0·033	9·2	3·0	2·3	0·7
0·33	43·7	1·3	3·4	0·1
Slow				
10·00	27·6	0·027	16·1	0·016
20·00	50·6	0·025	30·0	0·015

Pseudo-first order rate constants (k_i') were calculated for the minor (uncorrected) and major components from the semi-log plots, where the slope is: $-k_i'/2{\cdot}303$. The bimolecular rate constant (k_i) for the reaction $E + I \xrightarrow{k_i} EI$ was calculated from the relationship: $k_i' = k_i[I]$.

Table 2. Pseudo-first order and bimolecular rate constants for the inactivation of monoamine oxidase by 3-nitro-PCO

[PCO]μM	(a) $10^3k_i'$ (min^{-1})	(a) $10^{-4}k_i$ (M^{-1}min^{-1})	(b) $10^3k_i'$ (min^{-1})	(b) $10^{-4}k_i$ (M^{-1}min^{-1})
0·5	5·7	1·1	11·5	2·3
2·0	13·8	0·69	16·1	0·8
6·6	18·4	0·26	20·7	0·29

Pseudo-first order (k_i') and bimolecular (k_i) rate constants were calculated as described in Table 1, for the major component. Results are shown for serotonin (a) and tyramine (b) as substrates.

Table 3. Pseudo-first order and bimolecular rate constants for the inactivation of monoamine oxidase by 3-chloro-PCO

[PCO]μM	$10^3k_i'$ min^{-1})	$10^{-5}k_i$ (M^{-1}min^{-1})
0·17	17·2	1·0
0·33	34·5	1·0
3·3	225·0	0·7

Pseudo-first order (k_i') and bimolecular (k_i) rate constants were calculated for the single component observed with serotonin as substrate, as described in Table 1.

inhibition of two sites by an inhibitor with different affinities for the two sites [20], and extrapolation of hyperbolic slope replots have been shown, under certain conditions, to give reasonable first approximations to the real K_i values [20]. Too few concentrations of PCO were used to obtain an estimate of the K_i for the low affinity site, however an apparent K_i (K_i app) of 0·3 μM was calculated for the high affinity site.

Effect of enzyme concentration of inhibition by PCO. A plot of velocity against enzyme concentration in the presence and absence of inhibitor was suggested by Ackermann and Potter [21] as a test for irreversibility. Plots in the absence of inhibitor, or in the presence of a reversible inhibitor pass through the origin, while an irreversible inhibitor (see Discussion for a necessary qualification) ideally produces plots parallel to the control, the intersection on the enzyme concentration axis being proportional to the amount of 'irreversibly' bound enzyme. Fig. 7 shows Ackermann–Potter plots of PCO inhibition of tyramine and serotonin deamination. By the criteria of Ackermann and Potter PCO is an irreversible inhibitor of serotonin deamination but a reversible inhibitor of tyramine deamination.

Kinetics of PCO-partially inhibited enzyme. A double reciprocal plot of the tyramine deaminating activity of the partially inhibited enzyme gave a K_m of 240 μM. This was similar to that obtained with untreated preparations of the enzyme (130 μM). Tryptamine, benzylamine and serotonin showed competitive kinetics against tyramine deamination catalysed

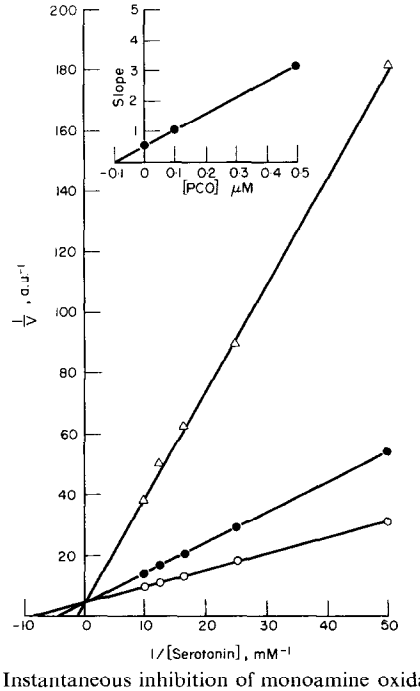


Fig. 5. Instantaneous inhibition of monoamine oxidase by PCO using serotonin as substrate. A double reciprocal plot of the initial rate of serotonin deamination against substrate concn in the presence of 0·1 μM (●) and 0·5 μM (△)-PCO, and control (○). Enzyme activity was determined radioisotopically as described in the text. The inset is a plot of the slope (arbitrary units) against inhibitor concn (Cleland, [19]).

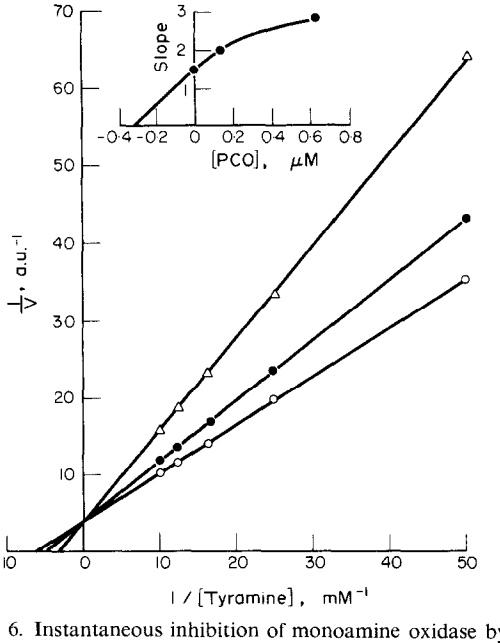


Fig. 6. Instantaneous inhibition of monoamine oxidase by PCO using tyramine as substrate. A double reciprocal plot of the initial rate of tyramine deamination against substrate concentration in presence of 0·125 μM (●) and 0·625 μM (△)-PCO, and control (○). Enzyme activity was determined radioisotopically as described in the text. Inset is a plot of the slope against inhibitor concentration (Cleland [19]).

Table 4. Kinetic parameters for untreated and partially inhibited preparations of monoamine oxidase

Substrate	Untreated enzyme K_m (μM)	Untreated enzyme V_{max} (arb. units)	Partially inhibited enzyme K_m (μM)	Partially inhibited enzyme V_{max} (arb. units)
Dopamine	210 + 13	11·3 ± 0·34	270 ± 13	4·9 ± 0·12
Tryptamine	8·6 ± 0·4	6·2 ± 0·12	25·9 ± 2·4	3·5 ± 0·2
Tyramine	114 ± 9	18·8 ± 0·9	276 ± 24	16·7 ± 1·2

Enzyme, partially inhibited by 2-chloro-PCO, was prepared as described in the text. The untreated enzyme was taken through the same procedure except that inhibitor was omitted from the incubation. Kinetic parameters were calculated by the method of Wilkinson [18]. Assays were conducted at 35° in 67 mM phosphate (pH 7·4).

by the PCO-partially inhibited enzyme. The K_i values obtained were 20 μM for tryptamine, 100 μM for benzylamine and 2·4 mM for serotonin.

Kinetics of 2-chloro-PCO-partially inhibited enzyme. Two aliquots of an enzyme suspension were taken through the procedure for preparation of partially inhibited enzyme, except that the control was incubated with phosphate buffer rather than 3·3 μM 2-chloro-PCO. Both the control and 2-chloro-PCO resistant activity were then assayed with varying concentrations of tyramine, tryptamine and dopamine. The kinetic parameters derived from the linear double reciprocal plots obtained for the control and partially inhibited preparations are shown in Table 4. Serotonin competitively inhibited tyramine deamination (K_i 3 mM) catalysed by the partially inhibited preparation.

DISCUSSION

PCO inhibition of membrane bound rat liver monoamine oxidase distinguishes a PCO sensitive activity that catalyses the deamination of serotonin and an activity relatively insensitive to PCO that catalyses the deamination of benzylamine. Both activities deaminate tyramine, tryptamine and dopamine, as shown by the biphasic dose response curves obtained with these substrates. These results are in agreement with the substrate specificities of the clorgyline sensitive and insensitive activities [3].

An examination of the kinetics of inactivation suggests that the differential sensitivities arise from different rates of attack by PCO on two distinct centres of amine oxidation. The major component of the 'fast' inactivation observed with tyramine as substrate, bimolecular rate constant $7 \times 10^4 M^{-1}$ min^{-1}, is underestimated because the insensitive centre contributes to the enzyme activity.* A more realistic estimate is obtained from the kinetics of inactivation of serotonin deamination for which a bimolecular rate constant of 3×10^5 M^{-1} min^{-1} is obtained. When compared with the bimolecular rate constant for the 'slow' inactivation of the PCO insensitive tyramine deaminating activity of $1{\cdot}5 \times 10^3$ M^{-1} min^{-1} it can be seen that the rate of attack against the sensitive activity (A-site) is 200 times faster than the rate of attack against the insensitive activity (the B-site). The nature of the minor kinetic components observed with PCO and 3-nitro-PCO inhibition of serotonin and tyramine deaminating activity is unclear, but may be related to the binary nature of the A and B activities of monoamine oxidase as previously noted from thermal stability [22] and inhibitor [23] studies. 3-nitro-PCO attacks the B-site more rapidly than the A-site, as indicated by the relative magnitudes of the bimolecular rate constants for the inactivation of tyramine and serotonin deaminating activity respectively. Such a result is in agreement with the observation that at low concentrations this inhibitor preferentially inhibits tyramine deamination [14].

The difference in the rates of attack against the two sites is probably reflected in the hyperbolic competitive kinetics shown with PCO inhibition of tyramine deamination. Unfortunately, higher concentrations of PCO could not be used to determine the apparent K_i (K_i app) for the postulated low affinity site [20] as the instantaneous competitive inhibition would be accompanied by time dependent inactivation at high PCO concentrations. However, the value of 0·3 μM calculated from the hyperbolic slope replot for the K_i app of the high affinity site corresponds well with the K_i of 0·1 μM obtained with serotonin as substrate. Instantaneous competitive kinetics and time dependent inactivation have been reported for pargyline inhibition of human liver

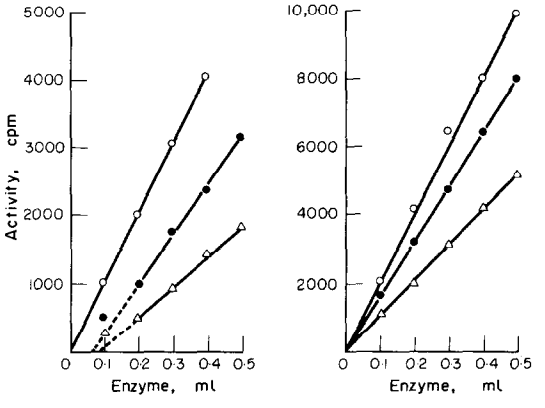


Fig. 7. The effect of enzyme concentration on inhibition by PCO. (a) The indicated vol of an enzyme suspension (1 mg protein/ml) made up to 0·5 ml with buffer were pre-incubated for 30 min at 35° with 0·1 μM (●) and 0·2 μM (△)-PCO, prior to adding substrate and assaying serotonin deaminating activity as described in the text. (b) The indicated volumes of the enzyme suspension made up to 0·5 ml with buffer were pre-incubated with 0·1 μM (●) and 1 μM (△)-PCO prior to adding substrate and assaying tyramine deaminating activity as described in the text. Control (○) was pre-incubated with buffer for 30 min.

* This can be corrected for to give a result in agreement with the value of 3×10^5 M^{-1} min^{-1} obtained from the kinetics of inactivation of serotonin deamination.

monoamine oxidase [24]. Pargyline showed a similar K_i (0·1 μM) and bimolecular rate constant ($2{\cdot}2 \times 10^5$ M^{-1} min^{-1}) with benzylamine as substrate as reported here with serotonin as substrate. Pargyline has been suggested to act like deprenil, in selectively blocking the oxidation of benzylamine [22].

The irreversible nature of the inhibition of MAO by PCO and derivatives was concluded from the fact that the inhibition was time dependent and not reversed by washing the inhibited enzyme preparation with buffer. This conclusion was supported by the Ackerman–Potter plots obtained with serotonin as substrate. However, with tyramine as substrate the Ackermann–Potter plots indicate a 'reversible mechanism'.

The confusion arises as a result of a misconception in the formulation of the test derived by Ackermann and Potter [21] to distinguish reversible and irreversible inhibitors. These workers based their approach on 'pseudoirreversible' inhibitors, i.e. inhibitors binding so tightly with the enzyme (E) that for most practical purposes the equilibrium;

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \qquad K_i = \frac{k_{-1}}{k_1}$$

approximates to;

$$E + I \xrightarrow{k_1} EI$$

as $k_1 \gg k_{-1}$. They then proceeded to derive the rate equation for the reversibly inhibited enzyme reaction, including a conservation of inhibitor equation as recommended by Goldstein [25] where enzyme binding significantly reduces the concentration of free inhibitor. From the resultant rate equation they demonstrated that as K_i tends to zero the enzyme activity is reduced by an amount (E_t) equivalent to the amount of inhibitor added (I_t), and that under this condition the v_0 vs enzyme concentration plot with inhibitor present is parallel to the control (inhibitor absent), intercepting the enzyme concentration axis at a point where $E_i = I_t$. Ackermann and Potter also pointed out that setting K_i to zero is an extreme case, and that as K_i becomes large the situation behaves like, in their nomenclature 'a reversible system', i.e. v_0 vs enzyme concentration plots pass through the origin with a reduced slope. However they failed to point out that genuine irreversible inactivations will also result in 'reversible' plots unless (a) the inhibitor has a high affinity (i.e. the pseudoirreversible case they discuss), or (b) in the case of a relatively slow inactivation the reaction is taken to completion—a task which in many cases would involve impractical periods of preincubation!

An intermediate case has been reported by Oreland and Ekstedt [26] for pargyline inhibition of purified pig liver MAO. Incubation for 40 min produced 'reversible' plots, but when preincubation with pargyline was allowed to go to completion (3–4 hr) 'irreversible' plots were obtained.

The misconception arises in the extension of inhibition resulting from very tight binding (K_i 10^{-10} – 10^{-9} M), which by definition is virtually instantaneous, to irreversible reactions of the type;

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E'I$$

where formation of the irreversibly inactivated species ($E'I$) will occur slowly if K_i is large and/or k_2 is small. In such cases, where the relatively slow inactivation of the enzyme is occurring under approximately first order conditions, at time t the total enzyme concentration (E_t) will be reduced by an amount $k^{**}E_t$, where $k^{**} = 1 - e^{-k'_i}$. As at constant substrate concentration the initial rate equation reduces to;

$$v_0 = k^*E_t \qquad \text{where } k^* = \frac{k_{cat}}{K_m + S}$$

after preincubation with inhibitor for time t the rate equation becomes;

$$v_0 = k^*E_t(1 - k^{**})$$

which is the condition (line passes through the origin with a decreased slope) for 'reversible' inhibition. It is only when the inactivation is very fast, such that at time t and at all concentrations of E_t a fraction EI_t is 'instantaneously' removed, that the 'irreversible' plot results. Therefore Ackerman–Potter plots do not discriminate, as originally suggested, between reversible and irreversible inhibitions "regardless of the exact nature of the irreversibility".

The intermediate case determined experimentally with serotonin as substrate, a line not parallel to the control but not passing through the origin either, is probably due to the high affinity of PCO for the A-site (K_i 0·1 μM) and represents 'pseudoirreversible' inhibition of an intermediate type. With tyramine as substrate the relatively weak binding of PCO at the B-site probably results in 'reversible' plots which mask the intermediate case observed when only the activity of the A-site is measured with serotonin.

A comparison of the kinetic parameters for tyramine, tyramine, tryptamine and dopamine deamination catalysed by the enzyme partially inhibited by 2-chloro-PCO and the untreated enzyme demonstrates the kinetic equivalence of the two sites for these substrates. Serotonin competitively inhibited the tyramine deaminating activity of the enzyme partially inhibited by PCO or 2-chloro-PCO, and gave K_i values of 2·4 and 3 mM respectively. These results show that serotonin can at least bind, albeit relatively weakly, at the B-site. Whether the B-site can oxidise serotonin is not known. However the hyperbolic double reciprocal plot for serotonin oxidation obtained by Sierens and D'Iorio [10] with a membrane bound preparation of the enzyme from rat liver might be interpreted as indicating that serotonin oxidation does occur at the B-site.

The present results are in agreement with Johnston's earlier suggestion [2] that membrane bound monoamine oxidase is a binary system. The origin of the two activities is, however, still far from clear. In the present study we have referred to the PCO sensitive and insensitive activities as being due to the action of A and B-sites respectively, rather than A and B enzymes as originally suggested by Johnston. The origin of the two activities observed with membrane bound preparations of the enzyme could arise from at least three situations; (1) one enzyme in different membrane environments, (2) two distinct enzymes (3) a two site enzyme. As the heterogenous solubilised

preparations of monoamine oxidase may well represent artefactual species [5, 27, 28], and as the physical separation of serotonin and benzylamine oxidising activities without a thorough characterisation of these fractions [10, 13] does not necessarily discriminate between situations (1), (2) and (3), it would seem that further studies with membrane bound monoamine oxidase are required to help characterise the enzyme in this state. Such studies would provide a realistic basis for comparisons with solubilised preparations of monoamine oxidase in order to assess the relevance of studies with such preparations for the physiological function of the enzyme.

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