Biochem. J. (1973) **135**, 735–750
Printed in Great Britain

The Reaction Pathway of Membrane-Bound Rat Liver Mitochondrial Monoamine Oxidase

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(*Received* 18 *June* 1973)

1. A preparation of a partly purified mitochondrial outer-membrane fraction suitable for kinetic investigations of monoamine oxidase is described. 2. An apparatus suitable for varying the O_2 concentration in a spectrophotometer cuvette is described. 3. The reaction catalysed by the membrane-bound enzyme is shown to proceed by a double-displacement (Ping Pong) mechanism, and a formal mechanism is proposed. 4. KCN, NaN_3, benzyl cyanide and 4-cyanophenol are shown to be reversible inhibitors of the enzyme. 5. The non-linear reciprocal plot obtained with impure preparations of benzylamine, which is typical of high substrate inhibition, is shown to be due to aldehyde contamination of the substrate.

Monoamine oxidase (EC 1.4.3.4) catalyses the reaction

$$RCH_2NH_2 + O_2 + H_2O = RCHO + NH_3 + H_2O_2$$

Bernheim (1931) proposed that this reaction proceeded by way of an imine intermediate and indirect evidence has been provided to support this view (Tipton & Spires, 1972). The enzyme has been shown to be a flavoprotein and it is believed that the flavin is reduced or partly reduced during the reaction (see, e.g., Gomes *et al.*, 1969; Tipton, 1968*c*).

Monoamine oxidase preparations from ox thyroid (Fischer *et al.*, 1968), pig brain (Tipton, 1968*b*) and ox liver (Oi *et al.*, 1970) have been shown to obey a double-displacement kinetic mechanism when acting on amine substrates, although the oxidation of hydrazones by pig brain monoamine oxidase has been shown to obey a kinetic mechanism that does not involve the formation of a free modified form of the enzyme (Tipton & Spires, 1971).

All these enzyme preparations had been subjected to vigorous procedures to bring the enzyme into solution (Fischer *et al.*, 1968; Tipton, 1968*a*; Gomes *et al.*, 1969). Monoamine oxidase has been shown to be firmly bound to the outer membrane of the mitochondrion (Schnaitman *et al.*, 1967; Tipton, 1967). This membrane environment might be expected to convey certain allotopic properties on the bound enzyme (see, e.g., Greenawalt, 1972; Coleman, 1973) and the attachment of lipid fragments to partly purified preparations of the enzyme has been shown to affect its temperature stability and inhibitor sensitivity (Houslay & Tipton, 1973). Interaction with membrane material has also been shown to be capable of modifying the kinetic properties of some enzymes (see, e.g., Gawron *et al.*, 1966). The present paper reports an attempt to investigate the kinetics of membrane-bound monoamine oxidase from rat liver.

Methods

Preparation of an outer-membrane fraction

Mitochondria were prepared from 48g of freshly excised livers from female rats by the method of Chappell & Hansford (1969), except that no EGTA [ethanedioxybis(ethylamine)tetra-acetate] was included in the homogenization medium.

Outer membranes were then prepared by the shrink–swell method of Sottocasa *et al.* (1967), all operations being carried out at +4°C. The 'light' and 'soluble' supernatants (terminology of Sottocasa *et al.*, 1967) were combined and dialysed overnight (approx. 16h) against 10litres of 0.01M-Tris–HCl buffer, pH7.2. Then $(NH_4)_2SO_4$ was added to 40% saturation and the mixture was stirred for 20min before being centrifuged at 23000*g* for 30min. The precipitate was washed carefully with 0.075M-potassium phosphate buffer, pH7.2, and resuspended in the same buffer to give a protein concentration of 1–3mg/ml. This preparation was then dialysed against two changes of 2 litres of 0.075M-potassium phosphate buffer, pH7.2. The results of this procedure are summarized in Table 1.

Enzyme assays

All determinations of enzyme activity were carried out at 30°C and specific activities are expressed as μmol of product formed/mg of protein. Spectrophotometric assays were carried out with a Beckman DU monochromator with a Gilford 240 power source and photomultiplier which was connected to either a

Table 1. *Purification of the outer-membrane fraction*

Fraction	Volume (ml)	Total protein (mg)	Monoamine oxidase: Total (units)	Monoamine oxidase: Specific activity (munits/mg)	Yield (%)	Purification
Mitochondria	50	550	4.2	7.6	(100)	–
Discontinuous-gradient-centrifugation supernatants	105	136.4	3.8	27.5	90	3.6
0–40% $(NH_4)_2SO_4$, dialysed precipitate	40	48.0	2.1	43.3	50	5.7

Vitatron or a Rikadenki variable-input recorder. With this apparatus it was possible to obtain a scale expansion of 0–0.05 extinction unit on a 25.4cm (10in) recorder chart. Under operating conditions the instrumental drift was less than 5×10^{-5} extinction units/min. In no experiments was the extinction of the assay mixture greater than 1.8, and Beer's Law was shown to be obeyed with NADH at 340nm and benzaldehyde at 250nm up to extinction values of 2.0.

Monoamine oxidase activity was measured by following the change in extinction at 250nm when benzylamine was converted into benzaldehyde (Tabor *et al.*, 1954). The assays were carried out in silica cuvettes with a 1cm light-path and a total volume of 1.4ml. The assay mixture contained, in a total volume of 1.0ml, enzyme, benzylamine and 0.12M (final concn.)-potassium phosphate buffer, pH7.2. The extinction coefficient E_{250}^{1cm} for benzaldehyde in the assay buffer was $13.8\times10^3 M^{-1}\cdot cm^{-1}$. The coupled assay of monoamine oxidase with aldehyde dehydrogenase was used as previously reported (Houslay & Tipton, 1973).

Unless otherwise stated O_2 concentrations were varied by passing O_2–N_2 gas mixtures through the reaction vessel as shown in Fig. 1. Micromite valves were of the all-brass type obtainable from Hoke International Ltd., New Barnet, Herts., U.K., and low-pressure gas-flow meters measuring from zero to 6 litres/h for N_2 and O_2 were from G.A.P. Meters Ltd., Basingstoke, Hants., U.K. The 1.4ml internal volume, 1cm light-path silica cell (type 6140) was obtained from Hellma Ltd., Westcliff-on-Sea, Essex, U.K.

Gas mixtures were bubbled through the cuvette for 5min while it was in a water bath at 30°C. Additions could be made by injecting through the stainless-steel cannula 'gas-escape' tube in the rubber bung. The Portex tube supplying the gas mixture to the cuvette could be raised through the cannula tube, so as to eject the gas mixture directly on to the surface of the reaction mixture while the cuvette was placed in the spectrophotometer-cell holder. The thin Portex tubing was routed between the top of the cell housing and housing cover; this effected a 'light-tight' seal while keeping the reaction mixture in equilibrium with the gas mixture. This apparatus was capable of delivering a preset mixture at a steady rate for at least 45min, with less than 4% change in the absolute concentration of O_2.

The absolute value of O_2 concentration was determined by using a Clark Electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.), set up as described by Tipton & Spires (1971), coupled to a variable-input Rikadenki recorder.

Aldehyde dehydrogenase (EC 1.2.1.3) was assayed by following the increase in extinction at 340nm in a reaction mixture containing 0.12M-potassium phosphate buffer, pH7.2, 0.5mM-NAD^+, 100μM-benzaldehyde and enzyme in a total volume of 1.0ml. Glucose 6-phosphatase (EC 3.1.3.9) was assayed by a coupled-assay system in which the glucose produced was converted back into glucose 6-phosphate and ADP in the presence of hexokinase (EC 2.7.1.1) and ATP. The ADP produced in this reaction reacted with phosphoenolpyruvate in the presence of pyruvate kinase (EC 2.7.1.40) to regenerate ATP and pyruvate, and this pyruvate was used to oxidize NADH in the presence of lactate dehydrogenase (EC 1.1.1.27) (Warren, 1973). The reaction mixture contained enzyme, 1.0mM-ATP, 6.0mM-$MgSO_4$, 100mM-KCl, 5.0M-K_2HPO_4, 1.0mM-phosphoenolpyruvate, 0.1mM-NADH, 0.05M-triethanolamine hydrochloride and 3.0 EC units each of pyruvate kinase, hexokinase and lactate dehydrogenase. The total volume of the assay mixture was 1.0ml and the final pH was 7.2. The reaction was monitored by following the decrease in extinction at 340nm.

Citrate synthase (EC 4.1.3.7) activity was assayed at 412nm in a mixture containing 0.2mM-acetyl-CoA, 0.25mM-5,5′-dithiobis-(2-nitrobenzoic acid) and 0.20mM-oxaloacetic acid in 1ml of 10mM-Tris–HCl buffer, pH8.0.

Adenylate kinase (EC 2.7.4.3) was assayed by a system similar to that used for the detection of the ADP formed in the assay of glucose 6-phosphatase. The reaction mixture contained, in a final volume of

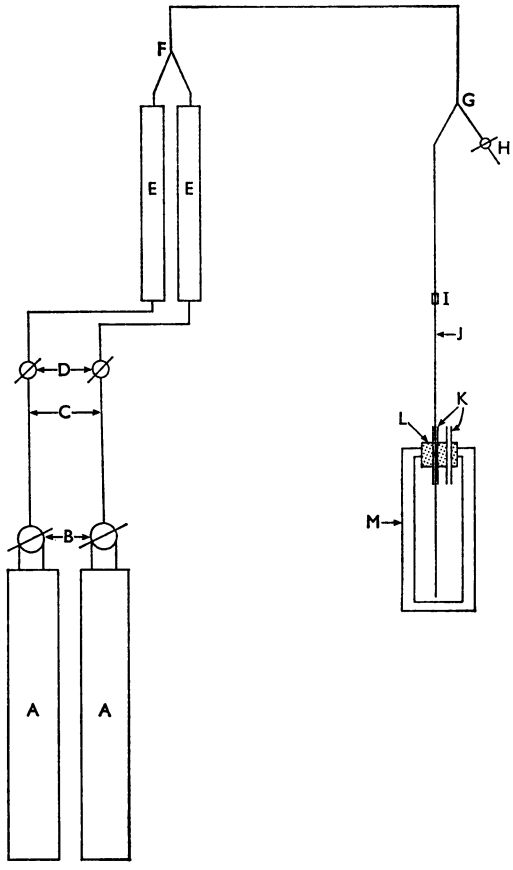


Fig. 1. *Apparatus for O_2 kinetics*

The basic units for assembling such a system are given below; connexions and fittings can easily be modified to suit availability and an individual's requirement. A, O_2 and N_2 compressed-gas cylinders. B, Reduction valves. C, 6.35mm (¼in) rubber or brass tubing. D, Hoke Micromite valves, allowing flow rates from zero to 7 litres/h. E, G.A.P. low-pressure gas-flow meters. F, Y-piece, mixing junction. G, Y-piece, stream-splitting junction. H, Valve, e.g. Hoke Micromite gas tap; for altering gas flow through cuvette. I, Junction of Portex type 7E tube (J), to rubber–brass tube or Y-piece (G); can be made by rubber stopper or Araldite adhesive connexion, to suit. K, Stainless-steel cannula needle tubing. L, Rubber stopper. M, Cuvette.

1.0ml: enzyme, 50mM-glycylglycine–NaOH buffer, pH7.2, 1mM-phosphoenolpyruvate, 0.2mM-NADH, 5.0mM-ATP, 10mM-$MgSO_4$, 2.0mM-AMP, 3.0 EC units of pyruvate kinase and 5.0 EC units of lactate dehydrogenase. ATPase* activity was assayed in the same system as that used for adenylate kinase except that AMP was omitted.

Glutamate dehydrogenase (EC 1.4.1.2) activity was assayed in a mixture containing 1.0mM-EDTA–NaOH, pH7.2, 13.7mM-α-oxoglutarate, 0.35mM-NADH, 0.2mM-NH_4Cl and enzyme in 0.12M-potassium phosphate buffer, pH7.2. The final volume of the mixture was 1.0ml and the reaction was followed at 340nm. NH_3 concentrations were determined by using a similar assay mixture in which the added NH_4Cl was omitted and 2.25 EC units of glutamate dehydrogenase were included.

Succinate dehydrogenase (EC 1.3.99.1) was assayed by following the reduction of 2,6-dichlorophenol-indophenol at 600nm in a reaction mixture containing, in a total volume of 1.0ml: 0.5mg of bovine serum albumin, 0.9mM-KCN, 8.0μM-dichlorophenol-indophenol, 10mM-sodium succinate and enzyme in 0.01M-potassium phosphate buffer, pH7.4.

Catalase activity (EC 1.11.1.6) was assayed polarographically by using a Clark O_2 electrode. The reaction mixture contained enzyme, 0.021mM-H_2O_2 and 0.15M-potassium phosphate buffer, pH7.2, in a total volume of 2.5ml.

Other methods

Protein concentrations were determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard. The concentration of the mitochondrial outer-membrane preparation is expressed in mg of protein/ml.

Sonication was carried out with a Dawe Soniprobe (Dawe Instruments Ltd., London W.3, U.K.) and pH measurements were made with a Radiometer PHM 22r pH meter (Radiometer A/S, Copenhagen, Denmark). Glass-distilled water was used in all experiments.

Materials

Benzylamine hydrochloride and *N*-methylbenzylamine hydrochloride were prepared by treating their respective bases, suspended in ether, with conc. HCl. The filtered precipitate was twice recrystallized from acetone–water (10:1, v/v). The hydrochloride solutions were made up freshly before use.

Benzaldehyde was redistilled at 1.3×10^4Pa (100 mmHg) under N_2 and the fraction distilling at 112–115°C was collected. This was kept in a tightly sealed darkened bottle under N_2 at +4°C.

Benzaldehyde, benzyl cyanide and 4-cyanophenol solutions of 1mM concentration were made by stirring the appropriate quantities in distilled water which had been degassed for 10min at the filter pump. They were then kept only over the period of assays (<5h)

* Abbreviation: ATPase, adenosine triphosphatase.

at +4°C in darkened glass vessels. The absolute concentration of benzaldehyde was determined by using ox liver aldehyde dehydrogenase as described by Houslay & Tipton (1973).

Benzyl cyanide, 4-cyanophenol, benzylamine and *N*-methylbenzylamine were obtained from Ralph Emanuel Ltd., Wembley, Middx., U.K.

ATP, phosphoenolpyruvate (Na^+ salt), oxaloacetate (Na^+ salt), NAD^+, NADH, hexokinase, glutamate dehydrogenase, catalase, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Corp. (London) Ltd., London W.5, U.K. O_2 and O_2-free N_2 (white spot) were obtained from the British Oxygen Co., Wembley, Middx., U.K. Sepharose and Sephadex gels were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K.

All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., and were of the highest quality available.

Results and discussion

Properties of the outer-membrane preparation

The partial purification of a typical mitochondrial outer-membrane preparation is summarized in Table 1. Specific activity is expressed in munits/mg of protein.

Monoamine oxidase activity was directly proportional to added protein between zero and 100μg/ml final concentration. As a routine, concentrations of 70–85μg/ml were used in these kinetic studies.

Linear increases in absorption on addition of samples of the preparation to 0.12M-potassium phosphate buffer, pH7.2, from zero to 100μg/ml (final concns.), were obtained at 250nm and 520nm, indicating that no gross aggregation–disaggregation phenomenon was occurring. Additions of benzylamine, to give final concentrations in the range 2–10mM, to a suspension of 80μg of membrane protein/ml in 0.12M-potassium phosphate buffer, pH7.4, caused no perturbation in the absorbance at 520nm (<0.0005 absorbance unit/min) (see Chappell & Crofts, 1966). Neither sonication at 5A output for 5min at +4°C, nor preincubation at 30°C with any of the detergents 0.002% sodium deoxycholate, 0.001% sodium deoxycholate or 0.1% Triton X-100, altered the activity of the enzyme towards benzylamine (1mM final concn.). These results suggest that the enzyme is freely accessible to substrate, i.e. no kinetically significant permeability barriers exist.

Spectra of 1.5mg/ml solutions of the outer membrane preparation suspended in 0.2M-mannitol, 5mM-Tricine[*N*-tris(hydroxymethyl)methylglycine]–NaOH buffer, pH7.2, were determined between 400 and 600nm at a full scale of 0.2 extinction unit by using a Johnson Foundation split-beam spectrophotometer. Spectra of oxidized, dithionite-reduced or dithionite-reduced and CO-treated samples indicated the presence of a b_5-type cytochrome with absorption maxima at 552, 558 and 420nm (see, e.g., Lindenmeyer & Weiner, 1958; Tipton, 1967).

The absence of any gross contamination by cytochromes *a* and *c*, and the enzyme systems characteristic of the inner mitochondrial membrane, soluble matrix and microsomal fraction (Table 2), indicate that the majority of the preparation consists of outer mitochondrial membranes (cf. Schnaitman *et al.*, 1967). After centrifugation at 100000*g* for 1h all monoamine oxidase activity was found in the pellet. Gel filtration on Sepharose 4B showed that particulate matter containing some 90% of the monoamine oxidase activity did not enter the gel column but remained on the top surface; that entering was eluted in a fraction corresponding to the void volume of the column (determined by Dextran Blue), no activity being found in the fractions characteristic of the molecular weight of 'solubilized' monoamine oxidase (Houslay & Tipton, 1973). These results indicate that the monoamine oxidase in this preparation of outer membranes is indeed firmly bound to those membranes (see, e.g., Greenawalt, 1972; Schnaitman *et al.*, 1967).

Kinetic investigation

Reciprocal plots (Lineweaver & Burk, 1934) obtained when the initial rates of benzylamine oxidation were determined at different benzylamine concentrations and a series of fixed O_2 concentrations exhibited a series of parallel lines (Fig. 2*a*). Similar parallel reciprocal plots were obtained when the O_2 concentration was varied at a series of fixed benzylamine concentrations (Fig. 2*b*). Plots of apparent K_m values against apparent maximum velocities for both varied substrates (Slater, 1955) passed through the origin. Secondary intercept plots were

Table 2. *Contaminating enzymes*

For details see the Methods section.

Contaminant	Specific activity (munits/mg of protein)
Glucose 6-phosphatase	28 ± 1
Catalase	3000
Succinate dehydrogenase	Zero (<0.05)
Citrate synthase	Zero (<0.1)
Alcohol dehydrogenase	Zero (<0.1)
Aldehyde dehydrogenase	Zero (<0.1)
Adenylate kinase	Zero (<0.1)
ATPase	Zero (<0.1)
Glutamate dehydrogenase	Zero (<0.1)

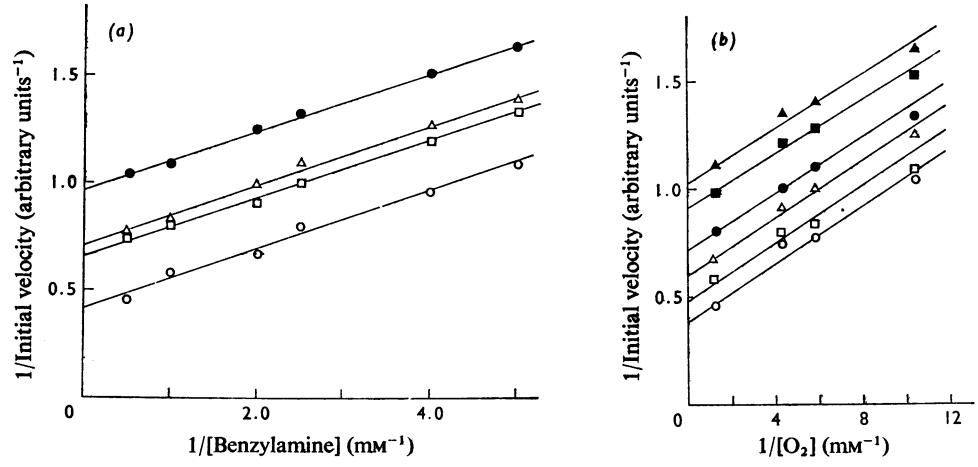


Fig. 2. *Kinetics of oxidation of benzylamine by rat liver monoamine oxidase*

(*a*) Reciprocal plots of initial velocities against benzylamine concentrations at a series of fixed O_2 concentrations. O_2 concentrations were 0.095mM (○), 0.17mM (□), 0.23mM (△) and 0.74mM (●). (*b*) Reciprocal plots of initial velocities against O_2 concentrations at a series of fixed benzylamine concentrations. Benzylamine concentrations were 0.2mM (○), 0.25mM (□), 0.4mM (△), 0.5mM (●), 1mM (■) and 2mM (▲). Throughout initial velocities are expressed in arbitrary units. One arbitrary unit is defined as a change in extinction of 0.001 extinction unit/min at 250nm (i.e. production of 724 pmol of benzaldehyde/min).

linear and yielded values of 475μM for $K_m^{\text{benzylamine}}$ and 156μM for $K_m^{O_2}$.

Such reciprocal plots, exhibiting lines of equal slope which is independent of the concentration of the second substrate, are indicative of a mechanism proceeding through a free modified form of the enzyme in which a series of binary complexes are formed, without the occurrence of any kinetically significant amounts of a ternary complex. This type of mechanism is known as a double-displacement or Ping Pong mechanism. However, the occurrence of these parallel reciprocal plots, although indicative of such a mechanism, is not conclusive, as any irreversible step between the binding of the two substrates will lead to such results and such a step need not necessarily be the release of a product. In addition it is very difficult to tell whether the lines in such reciprocal plots are truly parallel or weakly intersecting, that is, whether in the general kinetic equation for two substrate reactions:

$$v = \frac{V_{max.}}{1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]} + \frac{K_s^A K_m^B}{[A][B]}}$$

(in which K_s^A is the apparent dissociation constant of the EA complex and K_m^A and K_m^B are Michaelis constants) the term $K_s^A K_m^B/[A][B]$ is equal to zero or is simply very small.

Evidence in support of the absence of the $K_s^A K_m^B/[A][B]$ term can be obtained if the two substrates A (benzylamine) and B (O_2) are varied in a constant ratio (see, e.g., Cleland, 1963). In such a case if $[A] = y[B]$ the following relationship holds:

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{1}{[B]}\left(\frac{K_m^A}{y} + K_m^B\right)\frac{1}{V_{max.}}$$

Hence a reciprocal plot of initial velocities (v) against the concentration of B will yield a linear result. By a similar process it can be shown that if $K_s^A K_m^B/[A][B]$ is finite:

$$\frac{1}{v} = \frac{1}{V_{max.}} + \frac{1}{[B]}\left(\frac{K_m^A}{y} + K_m^B + \frac{K_s^A K_m^B}{y[B]}\right)\frac{1}{V_{max.}}$$

This will give rise to a non-linear reciprocal plot of initial velocities against the concentration of B.

Fig. 3 shows such a mixed-substrate experiment where benzylamine and O_2 concentrations are varied in a constant ratio of 2:1. The reciprocal plot exhibits a linear relationship between initial velocities and O_2 concentration, as would be expected of a double-displacement mechanism.

It would thus seem likely that rat liver mitochondrial monoamine oxidase, being a flavoprotein (Sourkes, 1968), proceeds in a similar fashion to the mitochondrial monoamine oxidases of pig brain (Tipton, 1968*b*), ox liver (Oi *et al.*, 1970) and bovine

thyroid (Fischer *et al.*, 1968) where the free modified form of the enzyme is that with its flavin reduced.

Product inhibition

The inhibition by the products benzaldehyde and NH_3 was studied at a concentration of one substrate which was of the same order as its K_m value (approx. $2 \times K_m$) and at a series of concentrations of the other substrate. Reciprocal plots indicate benzaldehyde to be a mixed inhibitor with respect to both benzylamine and O_2 (Fig. 4). Such a pattern could be accommodated in terms of a double-displacement mechanism if it were assumed that benzaldehyde could bind both to the oxidized and to the reduced forms of the enzyme (see Tipton, 1968*b*). Dixon (1953) plots of these data were non-linear, as shown in Fig. 5. This result would be expected if benzaldehyde were capable of reversing the reaction:

$$E + \text{amine} \rightleftharpoons E + \text{benzaldehyde} + NH_3$$

Such a reversal would not be expected under initial rate conditions unless there was either sufficient NH_3 in the assay medium to allow the reaction to proceed as indicated above or the NH_3 remained bound to the reduced enzyme after the release of the aldehyde.

Although the assay mixtures used were found to be normally contaminated with about 0.2mM-NH_3 the high K_i value calculated from Dixon plots ($K_i = 140$mM; Fig. 6) and the observation that the addition of NH_4Cl to give concentrations of 100–400μM had no detectable effect on the curvature of Dixon plots of aldehyde inhibition would argue against NH_3 being released before aldehyde.

Reciprocal plots showed NH_3 to be a mixed inhibitor with respect to both substrates (Fig. 7). Such an inhibition pattern could be rationalized by

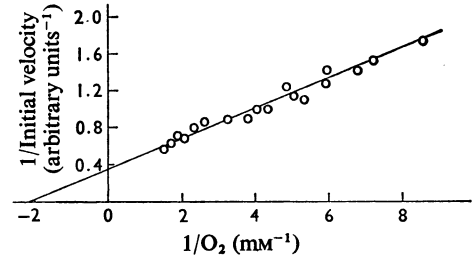


Fig. 3. *Mixed-substrate experiment*

Benzylamine and O_2 concentrations were varied in a constant ratio of 2:1; the reciprocal of initial velocities of benzylamine oxidation are plotted against the reciprocal of the O_2 concentration. O_2 concentration was varied as described by Tipton & Spires (1971), by using dithionite or H_2O_2, both in the presence of 520 units (final concn.) of catalase/ml.

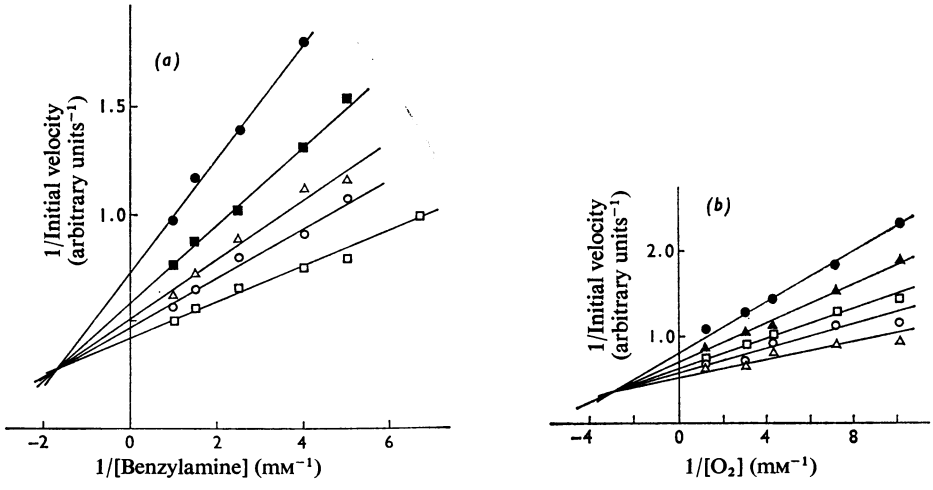


Fig. 4. *Reciprocal plots of the inhibition of monoamine oxidase by benzaldehyde*

(*a*) Reciprocal plots of initial velocities against benzylamine concentration at different inhibitor concentrations. The O_2 concentration was 0.23mM and the inhibitor concentrations were 0.004mM (○), 0.006mM (△), 0.01mM (■), 0.014mM (●) and zero (□). (*b*) Reciprocal plots of initial velocities against O_2 concentration at different inhibitor concentrations. The benzylamine concentration was 1mM throughout and the inhibitor concentrations were 0.003mM (○), 0.005mM (□), 0.007mM (▲), 0.009mM (●) and zero (△).

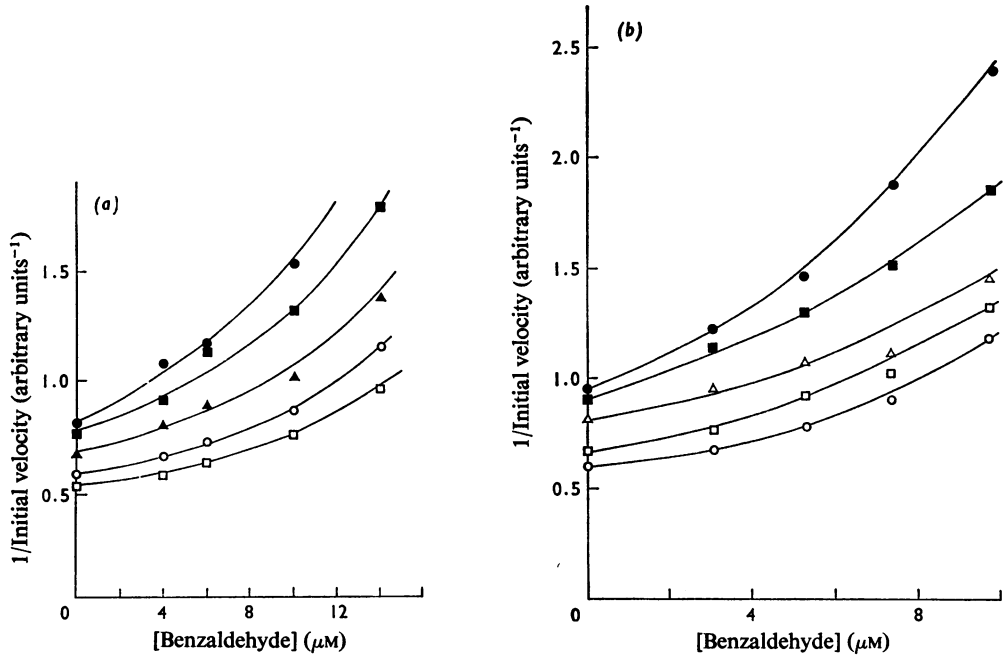


Fig. 5. *Inhibition of monoamine oxidase by benzaldehyde*

Dixon (1953) replots of the data presented in Fig. 4. (*a*) Reciprocal of the initial velocities against inhibitor concentration at a series of benzylamine concentrations. The O_2 concentration was 0.23 mM throughout. Benzylamine concentrations were 0.2 mM (●), 0.25 mM (■), 0.4 mM (▲), 0.67 mM (○) and 1.0 mM (□). (*b*) Reciprocal of the initial velocities against inhibitor concentration at a series of O_2 concentrations. The benzylamine concentration was 1.0 mM throughout. O_2 concentrations were 0.098 mM (●), 0.137 mM (■), 0.23 mM (△), 0.32 mM (□) and 0.77 mM (○).

Schemes 1(*a*) and 1(*b*) where NH_3 is liberated before O_2 binding, or by Scheme 2 where liberation is effected after O_2 binds. However, as Dixon plots for inhibition by NH_3 were linear (Fig. 6) and very high ('saturating') concentrations of the amine cause the inhibition to be uncompetitive with respect to O_2 (Fig. 8), only Scheme 2 becomes acceptable. In addition, if Scheme 1(*b*) were obeyed then Dixon (1953) plots of NH_3 inhibition, in the presence of aldehyde, would yield a non-linear result; however, linear plots are obtained.

The mechanism shown in Scheme 2 would, in the absence of products, give rise to the steady-state kinetic equation:

$$v = \frac{V_{max.}}{\frac{K_m^{amine}}{[amine]} + \frac{K_m^{O_2}}{[O_2]} + 1}$$

where K_m^{amine} is the Michaelis constant for amine at infinite concentration of O_2 and $K_m^{O_2}$ is the Michaelis constant for O_2 at infinite concentration of amine.

During aldehyde inhibition the occurrence of a dead-end complex is postulated (Scheme 2):

$$E_{ox.} \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} E_{Ox.}^{aldehyde}$$
$$\text{aldehyde}$$

Steady-state treatment yields:

$$v = V_{max.} \Bigg/ \left\{ 1 + \frac{K_m^{amine}}{[amine]} \left(1 + \frac{[aldehyde]}{K_{si}^{aldehyde}} \right) + \frac{K_m^{O_2}}{[O_2]} \times \left(1 + \frac{[aldehyde]}{K_{mi}^{aldehyde}} \right) + \frac{[aldehyde] K_s^{amine} K_m^{O_2} K_{si}'^{aldehyde}}{[O_2]\,[amine]} \times \left(1 + \frac{[aldehyde]}{K_{si}^{aldehyde}} \right) \right\} \quad (1)$$

And for NH_3 inhibition:

$$E_{ox.} \underset{k_{-9}}{\overset{k_{+9}}{\rightleftharpoons}} E_{Ox.}^{NH_3}$$
$$NH_3$$

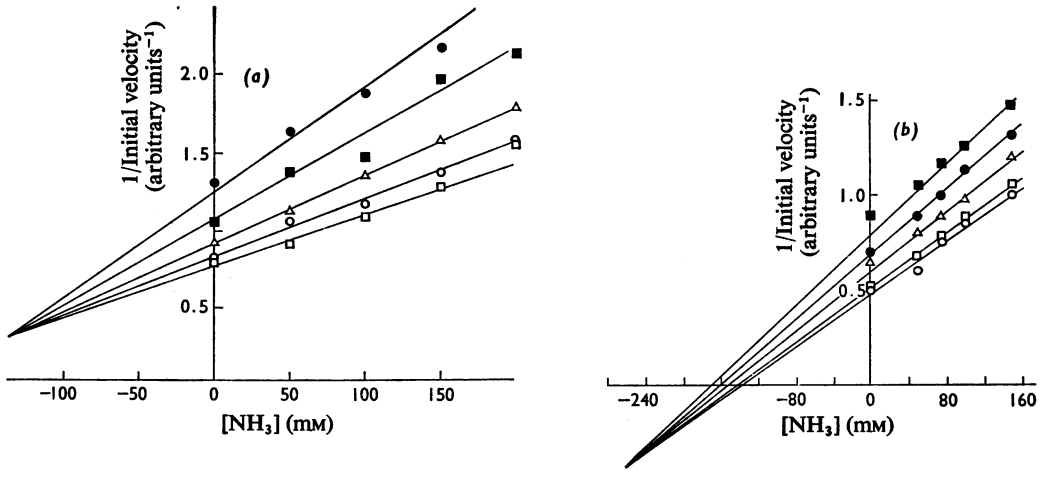


Fig. 6. *Inhibition of monoamine oxidase by NH_3*

(*a*) Dixon (1953) plots of the reciprocal of the initial velocities of benzylamine oxidation against NH_3 concentration at a series of fixed benzylamine concentrations. The O_2 concentration was 0.23 mM throughout and benzylamine concentrations were 0.2 mM (●), 0.3 mM (■), 0.5 mM (△), 1.0 mM (○) and 2.0 mM (□). (*b*) The reciprocal of the initial velocities of benzylamine oxidation against NH_3 concentration at a series of fixed O_2 concentrations. The benzylamine concentration was 1.0 mM throughout and O_2 concentrations were 0.094 mM (■), 0.132 mM (●), 0.175 mM (△), 0.23 mM (□) and 0.294 mM (○). During the course of all experiments where the NH_3 concentration was varied, both pH and ionic strength were kept constant by using solutions of KOH and KCl. NH_4Cl solution was used as the NH_4^+ source for all inhibition studies.

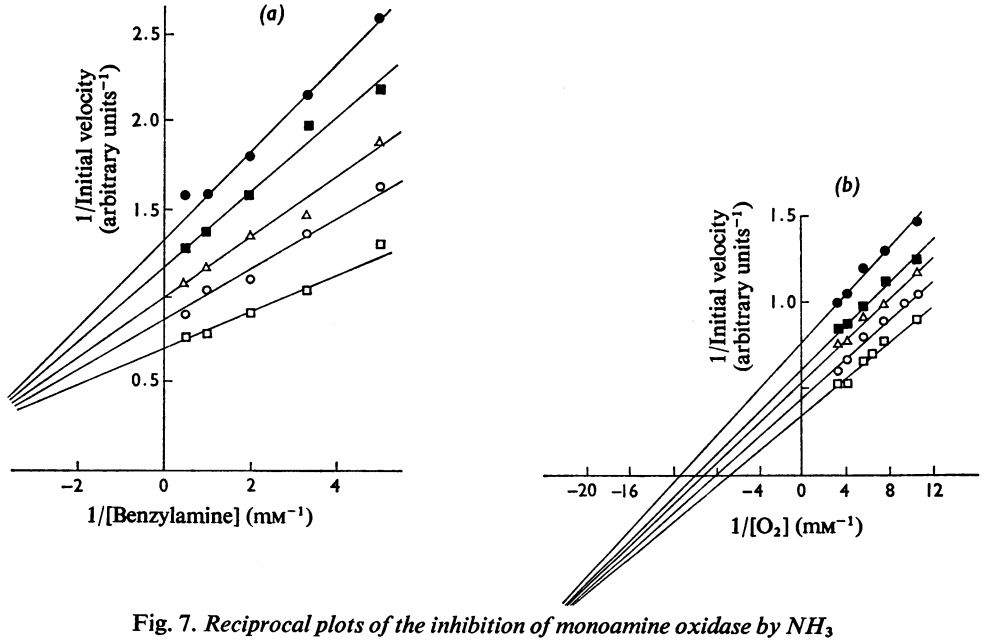
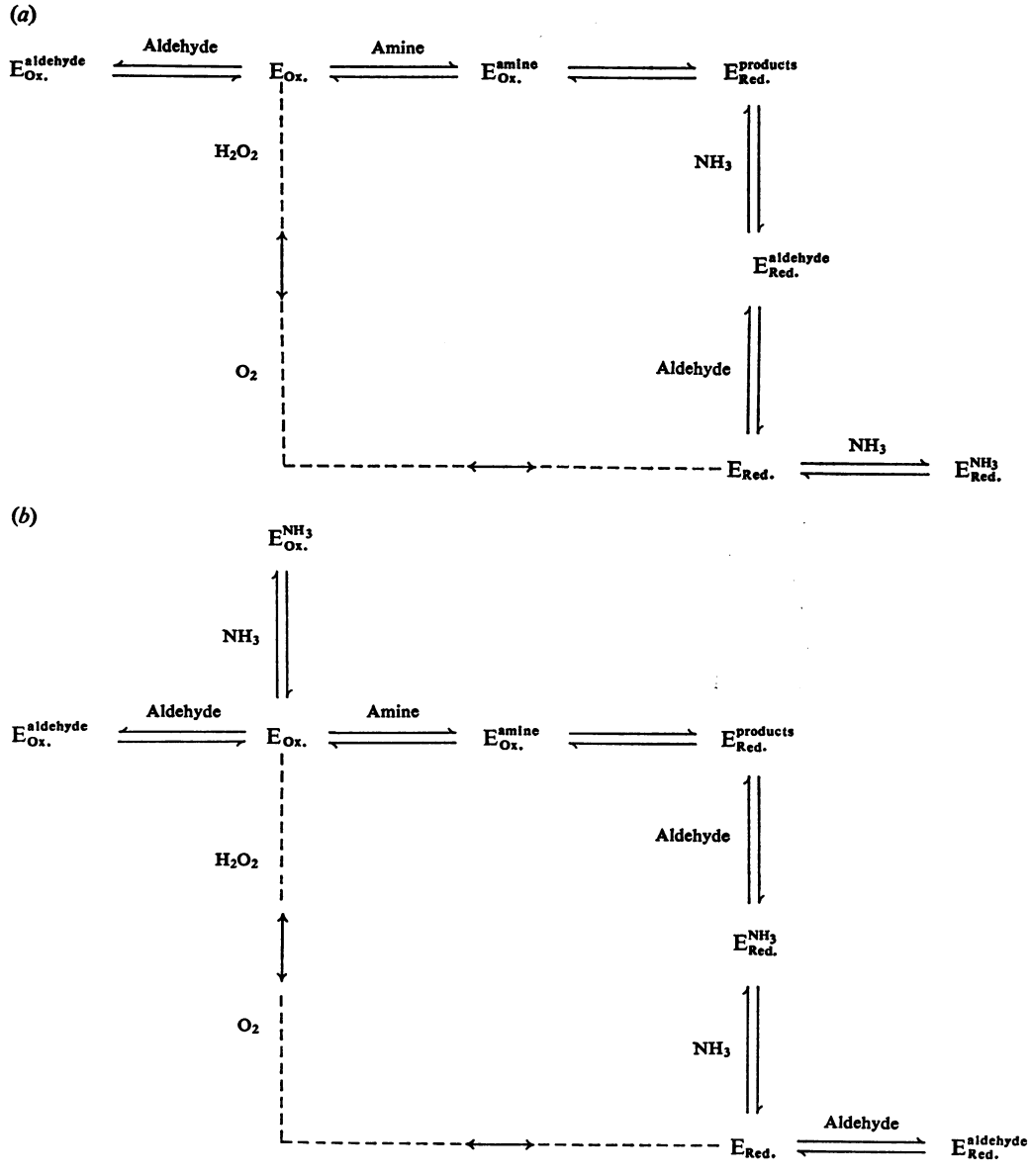


Fig. 7. *Reciprocal plots of the inhibition of monoamine oxidase by NH_3*

(*a*) Reciprocal plots of initial velocities against benzylamine concentration at a series of fixed inhibitor concentrations. The O_2 concentration was 0.23 mM throughout and the inhibitor concentrations were 50 mM (○), 100 mM (△), 150 mM (■), 200 mM (●) and zero (□). (*b*) Reciprocal plots of initial velocities against O_2 concentration at a series of fixed inhibitor concentrations. The benzylamine concentration was 1.0 mM throughout and the inhibitor concentrations were 50 mM (○), 75 mM (△), 100 mM (■), 150 mM (●) and zero (□).

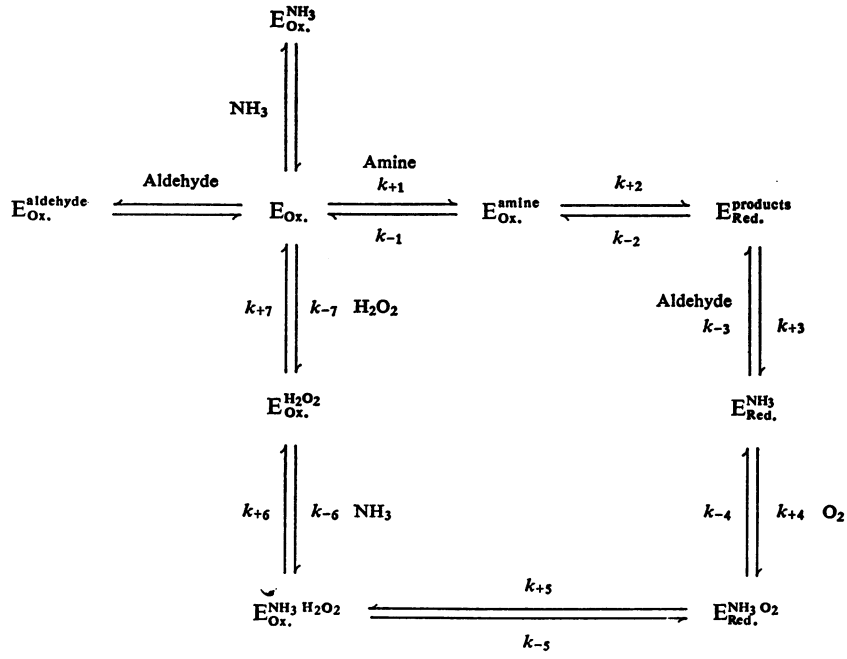


Scheme 1. *Possible reaction pathways for monoamine oxidase in which NH_3 is liberated before O_2 binding*

Steady-state treatment yields:

$$v = V_{max.} \Big/ \left\{ 1 + \frac{K_m^{amine}}{[amine]} \left(1 + \frac{[NH_3]}{K_{si}^{NH_3}} \right) + \frac{1}{[O_2]} (K_m^{O_2} + K_s^{O_2} K_{si}'^{NH_3} [NH_3]) + [NH_3] (K_{si}'^{NH_3} + K_{si}''^{NH_3}) \right\}$$

Further information on the proposed kinetic mechanism (Scheme 2) could have been obtained if it were possible to study the product inhibition of the reaction by H_2O_2. Unfortunately a large amount of catalase activity was found associated with the membrane fraction (Table 2). This activity was inhibited by KCN (1mM), Na_2S (1mM), hydroxylamine (1mM), CO (at water-saturating concentration, at 22°C), but not by the catalase inhibitors 2,4-dichlorophenol (1mM) or 3-amino-1,2,4-triazole (10mM) in the presence of H_2O_2 (approx. 10mM).



Scheme 2. *Reaction pathway of monoamine oxidase in which NH_3 release follows O_2 binding*

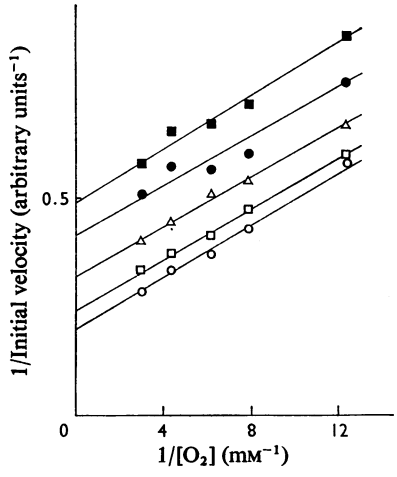


Fig. 8. *Inhibition by NH_3 at saturating benzylamine concentrations*

A reciprocal plot of initial velocities of benzylamine oxidation against O_2 concentration at several fixed NH_3 final concentrations. The benzylamine concentration was $10\times[K_m^{amine}]-5$ mM throughout and the NH_3 concentrations were 50 mM (□), 100 mM (△), 150 mM (●), 200 mM (■) and zero (○).

Repeated centrifugation and suspension in fresh buffer or re-fractionation on discontinuous sucrose density gradients (Sottocasa *et al.*, 1967) did not remove any significant amounts of the activity. Thus it was found impossible to inhibit or remove this activity to an extent sufficient to make it kinetically insignificant.

Inhibition by NaN_3

NaN_3 was an uncompetitive inhibitor with respect to both substrates (Fig. 9). Since it seems reasonable to assume that this compound might act as an analogue of NH_3, the most plausible way of interpreting its action is to assume that like NH_3 it binds to the $E_{Ox.}^{H_2O_2}$ form of the enzyme, but that it is unable to bind the $E_{Ox.}$ enzyme alone. When initial rates are measured NH_3 release can be considered as an irreversible step and thus uncompetitive inhibition would be expected with respect to both substrates. The steady-state rate equation accounting for this would be of the form:

$$v=\frac{V_{max.}}{\frac{K_m^{amine}}{[amine]}+\frac{K_m^{O_2}}{[O_2]}+\frac{[NaN_3]}{K_i^{NaN_3}}+1}$$

where $E_{Ox.}^{H_2O_2}+NaN_3 \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} E_{Ox.}^{H_2O_2NaN_3}$

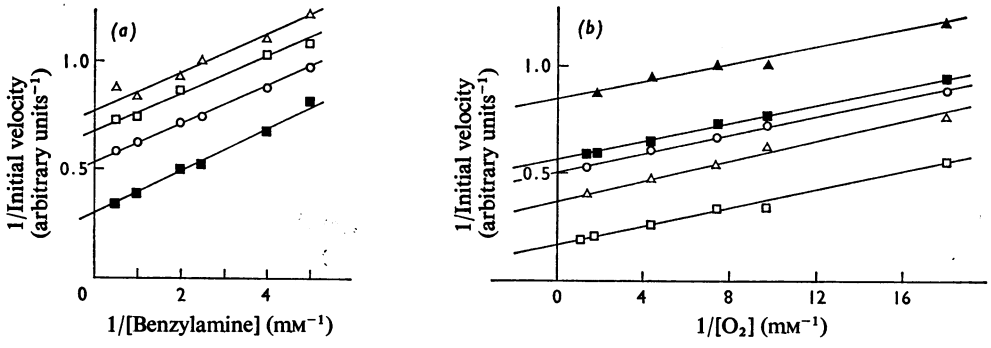


Fig. 9. *Inhibition of monoamine oxidase by NaN_3*

(*a*) Reciprocal plots of initial velocities against benzylamine at a series of fixed inhibitor concentrations. The O_2 concentration was 0.23mM throughout and the inhibitor concentrations were 1.0mM (○), 1.5mM (□), 2.0mM (△) and zero (■). (*b*) Reciprocal plots of initial velocities against O_2 concentration at a series of fixed inhibitor concentrations. The benzylamine concentration was 1.0mM throughout and the inhibitor concentrations were 1.0mM (△), 1.5mM (○), 2.0mM (■), 3.0mM (▲) and zero (□).

If, like NH_3, NaN_3 had bound to the $E_{ox.}$ form of the enzyme as well, then reciprocal plots with benzylamine concentration varied would have shown mixed inhibition, whereas if O_2 were the varied substrate an uncompetitive type of inhibition would have resulted.

Inhibition by KCN

Reciprocal plots at sub-saturating concentrations of second substrate yielded a competitive pattern when O_2 was the varied substrate (Fig. 10*b*) and an uncompetitive pattern when benzylamine concentration was varied (Fig. 10*a*). It would seem probable that KCN is acting as an O_2 analogue by combining with the free modified form of the enzyme:

$$E^{NH_3}_{Red.} \underset{k_{-8}}{\overset{k^{KCN}_{+8}}{\rightleftharpoons}} E^{NH_3KCN}_{Red.}$$

The steady-state rate equation for this inhibition would take the form:

$$v = \frac{V_{max.}}{\frac{K_m^{amine}}{[amine]} + \frac{K_m^{O_2}}{[O_2]}\left(1 + \frac{[KCN]}{K_i^{KCN}}\right) + 1}$$

Before this report KCN has not been considered an inhibitor of monoamine oxidase, although it is known that it irreversibly inhibits diamine oxidase by reaction with the pyridoxal phosphate cofactor [see, e.g., Zeller, 1963 (review); Zeller, 1940].

The KCN inhibition exhibited by this outer-membrane fraction, Triton X-100-solubilized enzyme and perchlorate-treated enzyme prepared as described by Houslay & Tipton (1973), is freely reversible by gel filtration or dilution, although the type of inhibition is pH-dependent, being mixed at pH8.2, assayed in 0.05M-glycine–NaOH buffer. This may be due to a pH-dependent change in mechanism, such as observed with the ox liver enzyme (Oi *et al.*, 1971).

As monoamine oxidase is able to deaminate *N*-substituted amines, whereas diamine oxidase is not (see, e.g., Blaschko, 1963; Tipton, 1973, for reviews), we investigated the action of our preparation on *N*-methylbenzylamine under standard assay conditions. The observed K_m was 714μM, and $V_{max.}$ relative to benzylamine oxidation (100%) was 91%. A mixed-substrate experiment (Dixon & Webb, 1964), where both benzylamine and *N*-methylbenzylamine were assayed together and separately at K_m concentrations, gave a value of 67% for the expression:

$$\frac{\text{Observed mixed rate of oxidation}}{\text{sum of individual rates of oxidation}} \times 100\%$$

This value is very close to the theoretical result of 66.7%, which would be expected for one enzyme acting on both substrates. It thus seems probable that it is indeed 'classical' monoamine oxidase that is degrading benzylamine in this preparation.

Experimentalists who carried out the initial studies on monoamine oxidase usually used minced liver extracts (e.g. Hare, 1928), and followed the stoicheiometry of the reaction, by allowing it to proceed to completion over a period of several hours (e.g. Hare, 1928; Kohn, 1937; Philpott, 1937). As KCN is a reversible inhibitor it will obviously not alter this but merely slow it down; because of this and the pH-dependence of the type of inhibition displayed it is perhaps easy to understand the

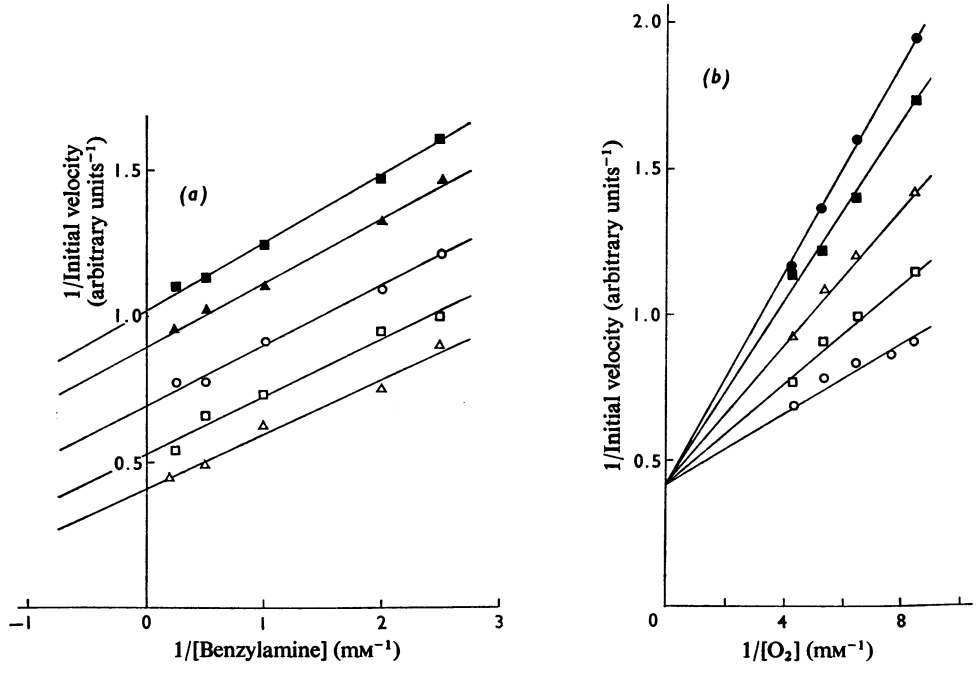


Fig. 10. *Inhibition of monoamine oxidase by KCN*

(*a*) Reciprocal plots of initial velocities against benzylamine concentration at a series of fixed inhibitor concentrations. The O_2 concentration was 0.23mM throughout and inhibitor concentrations were 0.5mM (□), 1.0mM (○), 1.5mM (▲), 2.0mM (■) and zero (△). (*b*) Reciprocal plots of initial velocities against O_2 concentration at a series of fixed inhibitor concentrations. The benzylamine concentration was 1.0mM throughout and inhibitor concentrations were 0.5mM (□), 1.0mM (△), 1.5mM (■), 2.0mM (●) and zero (○).

confusion in interpreting some observations at that period (Bernheim, 1931).

A number of workers add fixed quantities of KCN to their assays (e.g. Creasy, 1956; Tipton, 1968*b*; Johnston, 1968). Thus the fraction $\frac{[KCN]}{K_i^{KCN}}$ will be a constant, and for most workers using 1mM-cyanide for the rat liver enzyme at pH7.2, it will be approx. 1. Thus the steady-state rate equation will simplify to:

$$v = \frac{V_{max.}}{\frac{K_m^{amine}}{[amine]} + \frac{2K_m^{O_2}}{[O_2]} + 1}$$

and reciprocal plots of initial velocities of amine oxidation against varied amine concentration at a fixed, non-saturating O_2 concentration will yield a value of apparent K_m^{amine}, which is less than the true value.

Benzyl cyanide (2-phenylacetonitrile) was found to reversibly inhibit benzylamine oxidative deamination by the outer-membrane fraction. A competitive-inhibition pattern resulted when benzylamine was the varied substrate (Fig. 11), and a K_i value of 10μM was obtained from Dixon (1953) replots which were linear. Benzyl cyanide was also found to be a competitive inhibitor of partly purified and perchlorate-treated monoamine oxidase (prepared as described by Houslay & Tipton, 1973) when benzylamine was the varied substrate. The K_i value obtained in 0.05M-Tris–HCl buffer, pH8.2, was 28μM in both cases. With the outer-membrane preparation benzyl cyanide was found to be an uncompetitive inhibitor with respect to O_2 at non-saturating concentrations of benzylamine (Fig. 11*b*). Benzyl cyanide would thus appear to be acting as an analogue of the amine substrate and steady-state treatment of Scheme 2, in which the equilibrium:

$$E_{Ox.} \underset{k_{-8}}{\overset{k_{+8}^{benzyl\ cyanide}}{\rightleftharpoons}} E_{Ox.}^{benzyl\ cyanide}$$

is inserted yields:

$$v = \frac{V_{max.}}{1 + \frac{K_m^{amine}}{[amine]}\left(1 + \frac{[benzyl\ cyanide]}{K_i^{benzyl\ cyanide}}\right) + \frac{K_m^{O_2}}{[O_2]}}$$

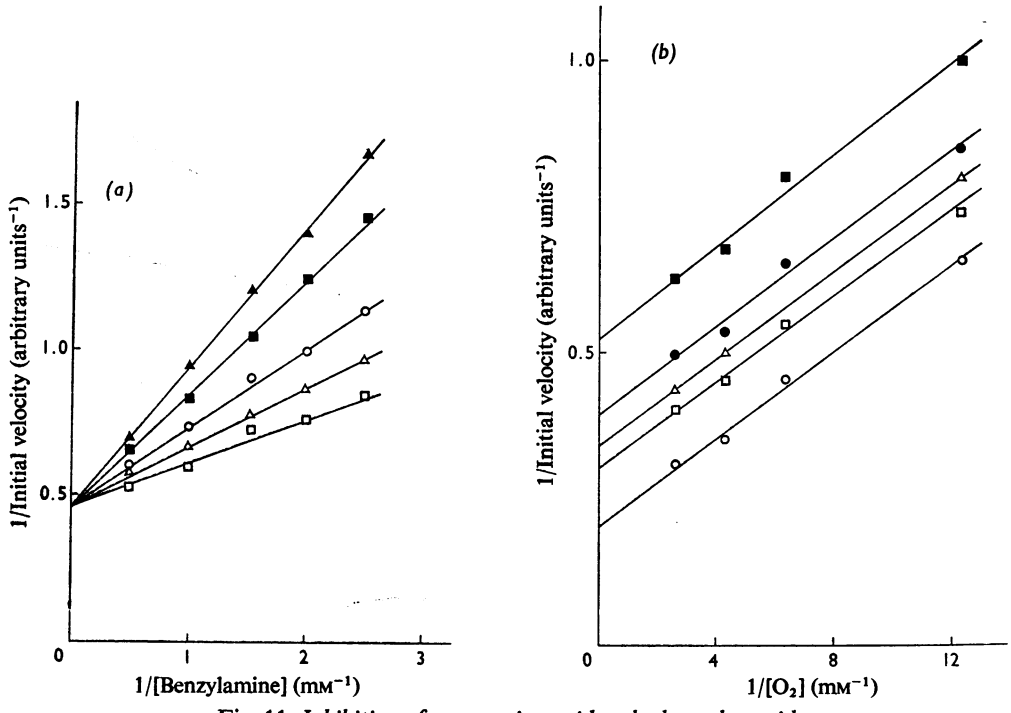


Fig. 11. *Inhibition of monoamine oxidase by benzyl cyanide*

(*a*) Reciprocal plot of initial velocities against benzylamine at a series of fixed inhibitor concentrations. The O_2 concentration was 0.23 mM throughout and inhibitor concentrations were 0.005 mM (△), 0.01 mM (○), 0.02 mM (■), 0.03 mM (▲) and zero (□). (*b*) Reciprocal plot of initial velocities against O_2 at a series of fixed inhibitor concentrations. The benzylamine concentration was 1.0 mM throughout and inhibitor concentrations were 0.01 mM (□), 0.02 mM (△), 0.03 mM (●), 0.05 mM (■) and zero (○).

4-Cyanophenol was found to inhibit the preparation in a similar fashion to benzyl cyanide, yielding a K_i of 30 μM.

Non-linearity in reciprocal plots

High substrate inhibition appears to be a common phenomenon among double-displacement-type enzyme systems (see, e.g., Cleland, 1963; Middleton, 1972), and is often observed with monoamine oxidase preparations [e.g. Tipton (1968*a*); Van Woert & Cotzias (1966); Huszti & Borsy (1966); Harada *et al.* (1971); Gabay & Valcourt (1968)].

When preparations of benzylamine (free base) in aqueous solution was used for assay systems, apparent high substrate inhibition resulted (Fig. 12*a*) when benzaldehyde production was followed by direct spectrophotometric assay (Tabor *et al.*, 1954). However, if the coupled assay with NAD^+-linked aldehyde dehydrogenase (Houslay & Tipton, 1973) was used no apparent substrate inhibition could be detected when the reaction was initiated by the addition of monoamine oxidase (Fig. 12*a*). With benzylamine hydrochloride as substrate, no apparent high substrate inhibition resulted whether the direct or coupled-assay systems were used, and reciprocal plots were linear from 10 μM to 10 mM final concentration of benzylamine. The cause of this apparent high substrate inhibition was found to be aldehyde contamination of the substrate, and this effect could be demonstrated by making the benzylamine hydrochloride solution 1% (mol/mol) with benzaldehyde, when apparent high substrate inhibition resulted (Fig. 12*b*) when the direct assay was used. The contamination of amine substrates by aldehydes can be checked as a routine by using aldehyde dehydrogenase as reported by Houslay & Tipton (1973). Commercial preparations of benzylamine free base (BDH, Ralph Emanuel) were found to contain 0.7–1.2% (mol/mol) of benzaldehyde, which is sufficient to affect reciprocal plots in the way shown in Fig. 12(*b*), giving apparent high substrate inhibition, a lower maximum velocity and a higher value for the apparent K_m^{amine}.

Contamination of substrates by products or inhibitors which are competitive or mixed inhibitors

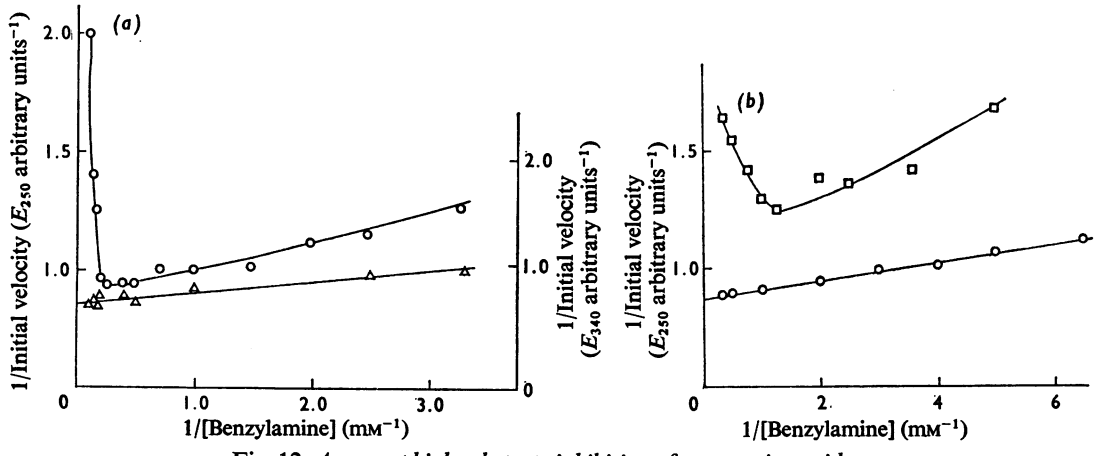


Fig. 12. *Apparent high substrate inhibition of monoamine oxidase*

(*a*) Reciprocal plot of initial velocities of benzylamine oxidation against benzylamine concentration. The O_2 concentration was 0.23 mM throughout and benzylamine free base was the source of the amine substrate. o shows the reaction followed by direct spectrophotometric assay (Tabor *et al.*, 1954). △ shows the reaction followed by the increase in extinction of NADH, by using the NAD^+-linked aldehyde dehydrogenase assay (Houslay & Tipton, 1973); all reactants except for monoamine oxidase were preincubated for 1 min (after which time there was no change in extinction at 340 nm) before starting the reaction with monoamine oxidase. The amine free base source was contaminated with 1.2% (mol/mol) benzaldehyde. (*b*) Reciprocal plot of initial velocities of benzylamine oxidation against benzylamine concentration. The O_2 concentration was 0.23 mM throughout and purified benzylamine hydrochloride was the source of the amine substrate. The reaction is followed by the direct spectrophotometric assay of Tabor *et al.* (1954), without (o) and with 1% (mol/mol) benzaldehyde added to the substrate (□). Where absorbances were greater than 2.0 units (e.g. 10 mM-benzylamine plus 10 mM-benzaldehyde), then corrections were made to the rates observed, as Beer's law is not strictly obeyed at absorbances greater than this.

with respect to one substrate of the reaction has been discussed in relation to other enzyme systems (see, e.g., Tubbs, 1962; Dalziel, 1962*a*,*b*).

If we consider eqn. 1, then if the aldehyde concentration is a certain fixed fraction (x) of the amine concentration, we can make the substitution:

$$[\text{Aldehyde}] = x[\text{amine}]$$

which will yield an equation of the form:

$$v = V_{max.}\Bigg/\left\{1+\frac{K_m^{amine}}{[amine]}+\frac{K_m^{O_2}}{[O_2]}+x\frac{K_m^{amine}}{K_{si}^{aldehyde}}+x\frac{K_s^{amine}K_m^{O_2}K_{si}'^{aldehyde}}{[O_2]}+\frac{K_m^{O_2}[amine]}{[O_2]}\times\left(\frac{x}{K_{mi}^{aldehyde}}+\frac{x^2K_{si}'^{aldehyde}K_s^{amine}}{K_{si}^{aldehyde}}\right)\right\}$$

which is of the form for an enzyme exhibiting high substrate inhibition (Haldane, 1930). The concentration of amine, for which a maximum value of v ($V_{app.}$) will be observed will be given by:

$$[S] = \sqrt{\left(\frac{K_m^{amine}}{\frac{xK_m^{O_2}}{[O_2]K_{mi}^{aldehyde}}+\frac{x^2K_s^{amine}K_m^{O_2}K_{si}'^{aldehyde}}{K_{si}^{aldehyde}}}\right)}$$

and that maximal observed velocity:

$$V_{app.} = V_{max.}\Bigg/\left\{1+\frac{K_m^{O_2}}{[O_2]}+\frac{xK_m^{amine}}{K_{si}^{aldehyde}}+\frac{xK_{si}'^{aldehyde}K_s^{amine}K_m^{O_2}}{[O_2]}+\left(1+\frac{1}{[O_2]}\right)\sqrt{\left[K_m^{amine}\left(\frac{xK_m^{O_2}}{[O_2]K_{mi}^{aldehyde}}+\frac{x^2K_s^{amine}K_m^{O_2}K_{si}'^{aldehyde}}{K_{si}^{aldehyde}}\right)\right]}\right\}$$

For the pig brain enzyme (Tipton, 1968*a*), aldehyde inhibition produces an uncompetitive pattern when amine is the varied substrate and competitive when O_2 is varied (Tipton, 1968*b*). Thus, by using a similar substitution we derive:

$$v = \frac{V_{max.}}{\frac{K_m^{amine}}{[amine]}+\frac{K_m^{O_2}}{[O_2]}+\frac{x[amine]K_m^{O_2}}{[O_2]K_i^{aldehyde}}+1}$$

Reciprocal plots of initial velocities of amine oxidation against amine concentration will yield classical high substrate-inhibition patterns (Haldane, 1930).

The maximum value of v observed will be given by:

$$V_{app.} = \frac{V_{max.}}{\left(1+\frac{1}{[O_2]}\right)\sqrt{\left(\frac{xK_m^{amine}K_m^{O_2}}{K_i^{aldehyde}}\right)}+\frac{K_m^{O_2}}{[O_2]}+1}$$

at a concentration of amine equal to:

$$[S] = \sqrt{\left(\frac{K_m^{amine}K_i^{aldehyde}}{xK_m^{O_2}}\right)}$$

When aldehyde product inhibition is mixed with respect to both varied substrates, and Dixon plots are linear, as shown by the ox thyroid enzyme (Fischer *et al.*, 1968) and the ox liver enzyme when assayed at pH values greater than 10.3 (Oi *et al.*, 1971), aldehyde contamination of the amine substrate will yield apparent high substrate inhibition.

Substituting as before:

$$[\text{Aldehyde}] = x[\text{amine}]$$

then:

$$v = \frac{V_{max.}}{\frac{K_m^{amine}}{[amine]}+\frac{K_m^{O_2}}{[O_2]}+\frac{xK_m^{amine}}{K_i^{aldehyde}}+\frac{x[amine]K_m^{O_2}}{[O_2]K_i'^{aldehyde}}+1}$$

The concentration of amine yielding a maximal observed velocity will be given by:

$$[S] = \sqrt{\left(\frac{K_i'^{aldehyde}K_m^{amine}}{xK_m^{O_2}}\right)}$$

and the maximal value of v ($V_{app.}$) will be given by:

$$V_{app.} = \frac{V_{max.}}{\left(1+\frac{1}{[O_2]}\right)\sqrt{\left(\frac{xK_m^{O_2}K_m^{amine}}{K_i'^{aldehyde}}\right)}+\frac{xK_m^{amine}}{K_i^{aldehyde}}+\frac{K_m^{O_2}}{[O_2]}}$$

At pH values from 7.3–10.3, then, for the ox liver enzyme aldehyde inhibition is competitive with respect to amine as varied substrate; contamination of the substrate with an inhibitor in this situation has been discussed by Tubbs (1962).

It seems unlikely, however, that the marked sensitivity of this enzyme to aldehyde product inhibition will have any significant effect *in vivo*, as the aldehyde would no doubt be rapidly oxidized to the corresponding acid by NAD^+-linked aldehyde dehydrogenase (EC 1.2.1.3), which has very low K_m values for aromatic aldehydes, e.g. benzaldehyde [$<0.03\,\mu$M, horse liver (Feldman & Weiner, 1972); $<1\,\mu$M, ox liver (Houslay & Tipton, 1973); $<0.2\,\mu$M, pig brain (Duncan & Tipton, 1971)]. However, in many assay systems this will not be the case. Often when radioactively labelled substrates are used for study, they are diluted out with unlabelled benzylamine free base (see, e.g., Robinson *et al.*, 1968); the distinct possibility that such chemicals are contaminated with free aldehyde should be considered seriously. The accumulation of aldehyde during assay procedures which involve long incubation of the enzyme with substrate (see, e.g., Veryovkina *et al.*, 1972; Squires & Lansen, 1968; Robinson *et al.*, 1968; Jarrott, 1971; Yang *et al.*, 1972) could also result in a decrease from the initial rate from this cause. The addition of aldehyde dehydrogenase and NAD^+ to prevent such an accumulation (Yang *et al.*, 1972) may not ameliorate the situation in all cases because of the possible formation of complexes between amines and NAD^+ as shown for 5-hydroxytryptamine and tryptamine by Alivisatos *et al.* (1960).

As Oi *et al.* (1970) concluded, it would appear that although monoamine oxidases from various sources proceed by way of a double-displacement mechanism while oxidatively deaminating amines, their formal reaction mechanisms differ (Tipton, 1968*b*; Fischer *et al.*, 1968; Oi *et al.*, 1970). The change in the pattern of inhibition by KCN with pH may indicate that the mechanism for the membrane-bound rat liver enzyme described here may change with pH as does that of the solubilized ox liver heart enzyme (Oi *et al.*, 1971).

The catalase activity which was associated with the membrane may be an activity of the b_5-type cytochrome component. The inability to carry out H_2O_2-inhibition studies, to saturate with O_2 (water-saturating O_2 concentrations reach only $7–8 \times K_m^{O_2}$), or to investigate benzaldehyde inhibition at saturating benzylamine concentrations (extinctions of these solutions were too great for spectrophotometric analysis), leaves us to postulate a mechanism which seems the simplest one accounting for the observable experimental results. However, it would be of interest to complete a full kinetic analysis of highly purified solubilized mitochondrial monoamine oxidase from rat liver to observe if the kinetic relationships are altered on extraction of this protein from its membrane matrix.

None of the kinetic results indicated more than one enzyme acting on the benzylamine substrate; this would be in accord with the experiments performed on crude rat liver mitochondria by Oswald & Strittmatter (1963), who concluded that most if not all the amine oxidase activity in rat liver mitochondria was due to one enzyme. Similarly McEwen *et al.* (1969), in their studies on Triton extracts of human liver mitochondrial monoamine oxidase, could find no evidence of multiplicity.

We thank Mr. P. R. Rich for carrying out the spectral studies. M. D. H. was supported by an M.R.C. studentship. This work was partly supported by a grant from the Smith, Kline and French Foundation to K. F. T.

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