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A Steady-State Kinetic Study of the Reaction Catalysed by the Secondary-Amine Mono-oxygenase of *Pseudomonas aminovorans*

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1. Secondary-amine mono-oxygenase (proposed EC group 1.14.99.–) was partially purified from trimethylamine-grown *Pseudomonas aminovorans* by $(NH_4)_2SO_4$ fractionation, gel filtration, hydrophobic chromatography on 5-aminopentylamino-Sepharose, and affinity chromatography on Sepharose-bound NADH. 2. Some problems in the affinity-chromatography step are discussed. 3. A steady-state kinetic analysis varying substrate, oxygen and electron-donor concentrations was performed, which, over the concentration range studied, gave a series of families of approximately parallel double-reciprocal plots. From secondary and tertiary plots, Michaelis constants of 0.160mM, 0.086mM and 0.121 mM were obtained for dimethylamine, NADPH and oxygen respectively. 4. Product-inhibition studies supported the postulated Hexa Uni Ping Pong (triple-transfer) reaction mechanism.

The catabolism of methylamines by the facultative methylotroph *Pseudomonas aminovorans* is thought to involve a series of enzymes catalysing the successive oxidative removal of methyl groups (Large, 1971; Jarman & Large, 1972*a*). One of these enzymes catalyses the oxidation of secondary alkylamines with the stoicheiometry of a mono-oxygenase (Eady *et al.*, 1971) and we have proposed for it the name secondary-amine, NAD(P)H–oxygen oxidoreductase (*N*-dealkylating) (proposed EC group 1.14.99.–) (Brook & Large, 1975). Partially purified preparations of this enzyme contained haem, flavin, non-haem iron and acid-labile sulphur (Eady *et al.*, 1971). The enzyme catalyses the oxidation of short-chain secondary alkylamines to the corresponding primary amine and aldehyde, and is usually studied with dimethylamine as substrate (eqn. 1):

$$(CH_3)_2NH_2^+ + NAD(P)H + H^+ + O_2 \rightarrow CH_3NH_3^+ + HCHO + NAD(P)^+ + H_2O \quad (1)$$

NADH and NADPH are both effective electron donors, although NADH has the lower apparent K_m. The enzyme's most noteworthy property is its extreme sensitivity to CO (Brook & Large, 1975).

The present paper describes an improved purification procedure and reports a steady-state kinetic analysis of the reaction, which aims at elucidating the mechanism of action of the enzyme. In addition, product-inhibition patterns are examined which suggest a sequence of substrate binding compatible with the conclusion drawn from the kinetics in the absence of inhibitors.

Materials and Methods

Materials

Oxygen and nitrogen (oxygen-free) were obtained from BOC Ltd., Gases Division, Brentford, Middx., U.K. Sepharose 4B and Sephadex G-75 were obtained from Pharmacia (G.B.) Ltd., London W.5, U.K., and CNBr was from Aldrich Chemical Co., Gillingham, Dorset, U.K. NAD^+, NADH, $NADP^+$, NADPH, 1,5-diaminopentane, 6-aminohexanoic acid, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulphonate, bovine serum albumin (fraction V) and streptomycin sulphate were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, U.K. Other chemicals were from Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K., and were of analytical grade where available.

Synthesis of Sepharose derivatives

Sepharose 4B was activated with CNBr as described by Cuatrecasas (1970). It was then treated with either 1,5-diaminopentane (for the hydrophobic chromatography step; Shaltiel & Er-el, 1973) or with 6-aminohexanoic acid for the affinity-chromatography medium (Barry & O'Carra, 1973). The 5-carboxypentylamino-Sepharose formed by the latter reaction was then coupled with NAD^+ by the procedure of Barry & O'Carra (1973), except that the coupling agent used was 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulphonate. The NAD^+-Sepharose was then reduced to NADH-Sepharose as described by Larsson & Mosbach (1971).

Table 1. *Partial purification of the secondary-amine mono-oxygenase from trimethylamine-grown Pseudomonas aminovorans*

In this preparation 40g wet wt. of cells was used as starting material.

Step no. and fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
1. Crude extract	60.0	144.0	3900	0.036	1	100
2. Supernatant after streptomycin sulphate treatment	60.0	174.0	2496	0.070	1.9	121
3. Redissolved ppt. after 40–52%-satd.-$(NH_4)_2SO_4$ fractionation	6.2	172.4	614	0.270	7.5	119
4. Combined Sephadex G-75 eluate	20.0	185.0	480	0.385	10.7	128
5. Combined hydrophobic column fractions	29.0	169.0	178	0.95	26.4	117
6. Combined NADH-Sepharose fractions	20.0	45.4*	10	4.54	126.0	31

* Only 63 units (of the 169 applied) bound to the column, and of these 72% were recovered as above.

After use, the NAD^+-carboxypentylamino-Sepharose columns were washed with 3M-KCl, reduced and re-equilibrated with starting buffer ready for re-use. After being used two or three times the material showed a marked decrease in binding capacity, and was discarded.

Maintenance and growth of the organism

These have been described previously (Eady *et al.*, 1971; Boulton *et al.*, 1974). Large quantities of bacteria were grown on trimethylamine hydrochloride (2g/litre) by the Microbial Products Section, M.R.E., Porton Down, Salisbury, Wilts., U.K.

Partial purification of the secondary-amine mono-oxygenase

All steps were performed at 4°C. A typical purification is shown in Table 1.

Steps 1 *to* 3: *Preparation of extract and* $(NH_4)_2SO_4$ *treatment.* Trimethylamine-grown *Pseudomonas aminovorans* was suspended in 2 vol. of 0.05M-sodium/potassium phosphate buffer, pH7.5, and disrupted by using a 100W Dawe Soniprobe ultrasonic disintegrator (Dawe Instruments Ltd., London W.3, U.K.) with cooling in ice, for a total time of 2min at full power. The suspension was centrifuged at 4°C, 40000*g* for 20min, to remove cell debris. To the supernatant was added, with stirring, ethanol at 0°C to a final concentration of 5% (v/v). Then 20% (w/v) streptomycin sulphate solution in 50mM-sodium/potassium phosphate, pH6.8, was added to a final concentration of 2% (w/v). The precipitated nucleic acid was removed by centrifuging as above and the supernatant was fractionated with solid $(NH_4)_2SO_4$. The precipitate obtained between 40% and 52% saturation was removed by centrifuging (27000*g*, 15min) and redissolved in a small volume of 0.05M-sodium/potassium phosphate buffer, pH6.8, containing 5% (v/v) ethanol. Ethanol was included at this concentration in all subsequent buffers to stabilize the enzyme (Eady *et al.*, 1971).

Step 4: *Gel filtration.* The redissolved sample, usually 5–7ml, was then applied to a column (2.5cm×30cm) of Sephadex G-75 equilibrated with the same buffer. The enzymically active fractions, only slightly retarded behind the position of the void volume, were collected and combined.

Step 5: *Hydrophobic chromatography.* The combined fractions were then applied to a column (1.0cm×10.0cm) of 5-aminopentylamino-Sepharose. The column was washed with several column-volumes of 0.05M-phosphate buffer, pH6.8, containing 5% (v/v) ethanol. When all the unbound protein had been washed off (i.e. when the E_{254} returned to less than 0.05) the column was eluted stepwise with phosphate buffer containing, successively, 50mM-, 150mM- and 300mM-KCl. The latter concentration eluted the mono-oxygenase, and active fractions were collected and combined.

Step 6: *Affinity chromatography.* The above fractions were then applied to a freshly synthesized NADH-Sepharose 4B column. Any enzyme activity that failed to bind to the column was retained and used again. After application of the sample, the column was washed with several volumes of 0.05M-phosphate buffer, pH6.8, containing 5% (v/v) ethanol. The enzyme activity was then eluted with buffer containing either NADH (10mg/ml) or

further KCl (1M), both proving suitable eluting agents giving approximately equal resolution. The enzyme preparation was divided into small batches and stored in liquid N_2.

The present work was carried out with a single batch of purified enzyme by using KCl in the affinity-chromatography step. Each sample was thawed and used within 1.5–2h so as to minimize loss of activity during the course of the experiment. Control assays were performed on each sample at the beginning and end of every experiment and no significant loss of activity was observed. The specific activity of the sample used throughout the work described below was 48mkat/kg of protein (Commission on Biochemical Nomenclature, 1973).

Enzyme assays

Assay method. Enzyme activity was measured at 25°C by following, on a Unicam SP.1800 recording spectrophotometer with Unicam A25 linear recorder, the dimethylamine-dependent oxidation of NADPH at 340nm. The reaction mixtures contained: 67mM-sodium/potassium phosphate buffer, pH6.8, enzyme preparation (12μg of protein), NADPH (17–128μM), dimethylamine hydrochloride (20–100μM) and water, in a total volume of 3ml. It was not possible to use higher concentrations of NADPH or dimethylamine, because of substrate inhibition. The reaction was started by addition of dimethylamine. The dissolved oxygen concentration was varied as described below. The reaction rate was linear for 3–4min, except at very low substrate concentrations. A unit of enzyme activity is defined as the amount required to catalyse the dimethylamine-dependent oxidation of 1μmol of NADPH/min at 25°C.

Variation of oxygen concentration in assay mixtures. The kinetic studies used a modified form of the gas-mixing apparatus of Houslay & Tipton (1973). The gas-flow meters used were of the Rotameter series 1100 type (flow rate 600–7200ml/h) (G.E.C.–Elliott Process Instruments Ltd., Croydon CR9 4PG, U.K.) and the control valves were supplied by G. A. Platon Ltd. (Basingstoke, Hants., U.K.). O_2 concentration was varied by altering the respective flow rates of air and N_2 through the sample cuvettes to give mixtures of the appropriate percentage (v/v) composition. The assays were carried out in 3ml disposable polystyrene cuvettes (Hughes and Hughes Ltd., Romford, Essex, U.K.), light-path 1cm, fitted with Suba-seal caps through which passed two cannula tubes. The gas mixtures were bubbled through the incubation mixtures for 5min in the spectrophotometer cell holder at 25°C. Additions were then made to the cuvette by injection with microlitre syringes through the gas-escape tube in the rubber seal. The Portex tube supplying the gas mixture to the cuvette was then raised through the cannula tube so as to eject the gas mixture directly on to the surface of the reaction mixture during the time of the assay. The dissolved oxygen concentration at each gas-flow rate was measured by using a Beckman model 777 oxygen analyser calibrated by the method of Robinson & Cooper (1970).

Data processing. For experiments in the absence of product (Table 2 and Figs. 1, 2, 3 and 4), determinations were performed in duplicate, and agreement between duplicates was within 5%. The slope of the spectrophotometer trace was measured and converted into μmol of NADPH oxidized/min per ml, i.e. mM/min. Reciprocal velocities and reciprocal substrate concentrations were then calculated, and the best fit of the data to a straight line was determined by linear-regression analysis on a Hewlett–Packard model 9810 calculator by using a program which gave the correlation coefficient and the values of slope and intercept for each line, with their standard errors. In the product-inhibition experiments (Fig. 5), the lines were fitted to the data by eye, and confidence limits are thus lower.

Protein concentration

This was measured by the method of Lowry *et al.* (1951) using bovine serum albumin dried *in vacuo* at 20°C as standard.

Results

Affinity chromatography

Various possible ligands were tested as media for affinity chromatography. Dimethylamine and *NN*-dimethylhydrazine were coupled to Sepharose 4B directly, or via a six-carbon spacer arm. None of these derivatives bound the enzyme. Inclusion of either NADH or NAD^+ in the column buffers failed to assist in the binding process. Attempts were made to carry out the affinity-chromatography step under anaerobic conditions by using buffers equilibrated with O_2-free N_2 and treated with low concentrations of sodium dithionite, but no binding of the enzyme to amine-coupled media was observed. NAD^+ coupled directly to Sepharose or via a 5-carboxypentylamino spacer arm failed to adsorb the enzyme, but when the NAD^+-carboxypentylamino-Sepharose was reduced with dithionite (Larsson & Mosbach, 1971), it bound the enzyme. Directly coupled NAD^+-Sepharose, when reduced with dithionite, however, was ineffective.

The procedure for coupling NAD^+ to the carboxypentylamino-Sepharose described in the Materials and Methods section was more reproducible than the pyridine method using dicyclohexylcarbodi-imide described by Larsson & Mosbach (1971). Certain batches of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodi-imide metho-*p*-toluenesulphonate from Sigma were ineffective.

Once optimal-binding conditions had been established for affinity chromatography, the conditions for elution were examined. Inclusion of dimethylamine, monomethylamine, formaldehyde or NAD^+ at concentrations up to 100mM failed to elute the activity. If, however, NADH was added to the elution buffer, the enzyme was eluted. The most effective concentration of NADH was 10mg/ml, which eluted 70–80% of the bound activity with an increase in specific activity up to tenfold. Below this concentration, less than 50% of the activity was eluted: above 10mg/ml, other proteins were eluted, decreasing the specific activity. Non-substrate-specific elution could be obtained by using high concentrations of KCl in the elution buffer. Subsequent preparations on NADH-carboxypentylamino-Sepharose were eluted with either NADH or 1M-KCl, depending on whether or not the enzyme was required in the absence of NADH (which is a stabilizing agent). In the present study KCl was used to elute the enzyme so that the NADPH concentration in the kinetic work could be accurately controlled.

Steady-state kinetics

NADPH was chosen as electron donor for the kinetic experiments rather than NADH (which was used to assay the enzyme during purification) because NADPH has a higher apparent K_m (Eady *et al.*, 1971) and consequently a greater variation in rate could be observed over the concentration range of NADPH which was practicable in the spectrophotometric measurements (17–128μM).

The general rate equation for a three-substrate enzyme-catalysed reaction is:

$$\frac{e}{v} = \phi_0 + \frac{\phi_A}{[A]} + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{AB}}{[A][B]} + \frac{\phi_{AC}}{[A][C]} + \frac{\phi_{BC}}{[B][C]} + \frac{\phi_{ABC}}{[A][B][C]} \quad (2)$$

using the symbols of Dalziel (1969), where A, B and C are the three reactants involved and the square brackets denote their concentrations. The 'ϕ' terms are constants, and evaluation of several of them is possible by observation of the variation in initial reaction rate when one substrate is varied at several fixed concentrations of the other two substrates. From such primary data, if e/v is plotted against 1/[A], where A is the variable substrate, then the slope of the resulting line is given by eqn. (3):

$$S = \phi_A + \frac{\phi_{AB}}{[B]} + \frac{\phi_{AC}}{[C]} + \frac{\phi_{ABC}}{[B][C]} \quad (3)$$

and the intercept (I) of such a primary plot is given by eqn. (4):

$$I = \phi_0 + \frac{\phi_B}{[B]} + \frac{\phi_C}{[C]} + \frac{\phi_{BC}}{[B][C]} \quad (4)$$

For the remainder of this section, the symbol S will be used for the slope, and the symbol I for the intercept, with the numerical suffixes being the same as those used by Dalziel (1969).

Table 2 summarizes the primary data obtained when plots of $1/v$ against 1/[dimethylamine] are made at various concentrations of O_2 and different concentrations of NADPH. Dimethylamine (DM), NADPH and O_2 replace A, B and C respectively in the above equations. The primary plots are families of approximately parallel straight lines.

Secondary-plot data. Analysis of the slope and intercept data of Table 2 can be made by secondary plots of these parameters against the reciprocal of the concentration of one of the varying fixed substrates while the concentrations of the remaining substrates

Table 2. *Summary of primary double-reciprocal plot data*

All enzyme assays were performed in duplicate. Six different concentrations of the variable substrate (dimethylamine) were used, covering a fivefold range, with the varying fixed substrates at the concentrations given. Initial-velocity data (μmol of NADPH oxidized/min per ml by 4μg of enzyme/ml) were analysed by regression analysis as described in the Materials and Methods section, on a calculator which computed the slope and intercept for each double-reciprocal plot and the error in each.

[Oxygen] (mM)	[NADPH] (mM)	Slope (min)	Intercept (min·mM^{-1})
0.126	0.128	30±3	366±51
0.078	0.128	27±3	507±54
0.054	0.128	27±3	543±66
0.042	0.128	21±3	774±63
0.126	0.082	27±3	555±72
0.078	0.082	27±3	549±75
0.054	0.082	27±3	618±48
0.042	0.082	36±6	645±168
0.030	0.082	39±6	993±150
0.126	0.052	30±3	468±99
0.078	0.052	33±3	627±66
0.054	0.052	27±3	798±66
0.042	0.052	30±3	864±66
0.030	0.052	39±3	1176±57
0.126	0.037	24±3	690±48
0.078	0.037	21±3	861±33
0.054	0.037	33±3	654±63
0.042	0.037	30±3	939±63
0.030	0.037	24±3	1221±57
0.126	0.026	15±3	945±48
0.078	0.026	24±3	816±54
0.054	0.026	24±3	846±39
0.042	0.026	15±3	1281±66
0.030	0.026	15±3	1323±63
0.126	0.017	24±3	870±105
0.078	0.017	27±6	945±204
0.054	0.017	21±3	1278±105
0.030	0.017	27±6	1254±129

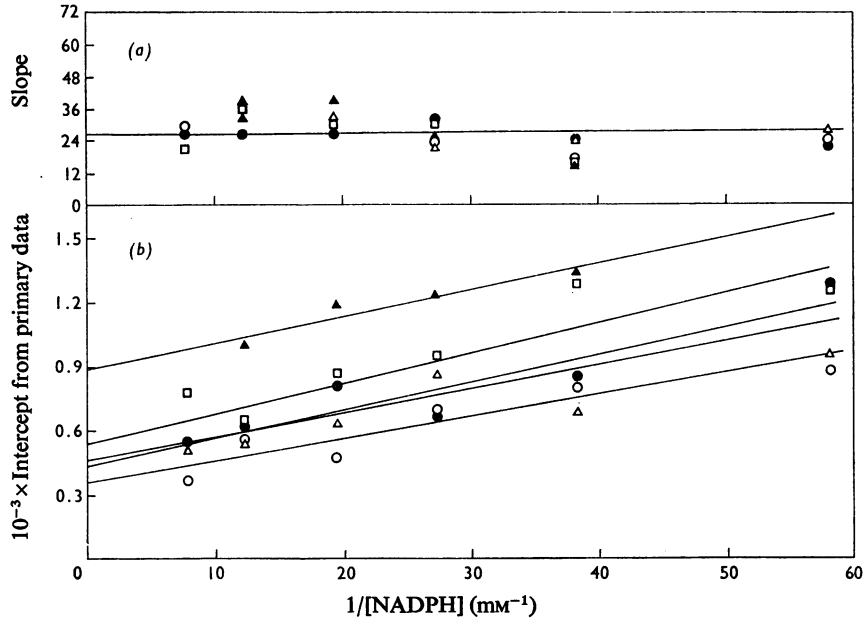


Fig. 1. *Secondary plots of slope and intercept data from Table 2 plotted against reciprocal NADPH concentration*

(*a*) Slopes and (*b*) intercepts from the data of Table 2. The oxygen concentrations used were: ▲, 0.03mM; □, 0.042mM; ●, 0.054mM; △, 0.078mM; ○, 0.126mM.

are kept constant. In Fig. 1 the data are plotted against 1/[NADPH] and in Fig. 2 against $1/[O_2]$. The replotted slopes (Figs. 1*a* and 2*a*) are clearly independent of NADPH and O_2 concentrations respectively. The line in Fig. 1(*a*) has its slope given by eqn. (5):

$$S_2 = \phi_{DM\cdot NADPH} + \phi_{DM\cdot NADPH\cdot O_2}/[O_2] \quad (5)$$

but $S_2 = 0$, so that $\phi_{DM\cdot NADPH}$ and $\phi_{DM\cdot NADPH\cdot O_2}$ are either zero or relatively small. The intercept of the replotted primary slope is given by eqn. (6):

$$I_2 = \phi_{DM} + \phi_{DM\cdot O_2}/[O_2] \quad (6)$$

whence $\phi_{DM\cdot O_2}$ is either zero or very small and $I_2 = \phi_{DM}$.

Similarly, for Fig. 2(*a*), the slope (eqn. 7) is

$$S_4 = \phi_{DM\cdot O_2} + \phi_{DM\cdot NADPH\cdot O_2}/[NADPH] \quad (7)$$

and, since the slope of this line is very small, then $S_4 = 0$ and $\phi_{DM\cdot O_2}$ is either zero or relatively small. The intercept of Fig. 2(*a*) is given by eqn. (8):

$$I_4 = \phi_{DM} + \phi_{DM\cdot NADPH}/[NADPH] \quad (8)$$

and since the line is independent of NADPH concentration, then $I_4 = \phi_{DM}$ and I_2 and I_4 should be equal (which they are approximately; Table 3).

Tertiary-plot data. The intercepts of the primary plots, on the other hand, do vary with the reciprocal concentration of NADPH and O_2 (Figs. 1*b* and 2*b*), and give a series of parallel lines. The slopes and intercepts of the lines in Fig. 1(*b*) are given by

$$S_1 = \phi_{NADPH} + \phi_{NADPH\cdot O_2}/[O_2] \quad (9)$$

and

$$I_1 = \phi_0 + \phi_{O_2}/[O_2] \quad (10)$$

and a re-plot of these slopes and intercepts from Fig. 1(*b*) as a function of $1/[O_2]$ is given as a tertiary plot in Fig. 3. Clearly the slope of the replotted slopes S_1 is again independent of O_2 concentration (Fig. 3*a*), so that $S_1 = \phi_{NADPH} = 13.2$ (Table 3). The replotted intercepts (Fig. 3*b*) give us ϕ_0 from the intercept on the ordinate and ϕ_{O_2} from the slope (Dalziel, 1969). These results are summarized in Table 3. The slopes and intercepts from Fig. 2(*b*) are replotted as a function of 1/[NADPH] in Fig. 4. In this case the slopes are once more independent of concentration, so since

$$S_3 = \phi_{O_2} + \phi_{NADPH\cdot O_2}/[NADPH] \quad (11)$$

then $\phi_{NADPH\cdot O_2}$ is either zero or relatively small and $S_3 = \phi_{O_2}$. The replotted intercepts give a line represented by eqn. (12):

$$I_3 = \phi_0 + \phi_{NADPH}/[NADPH] \quad (12)$$

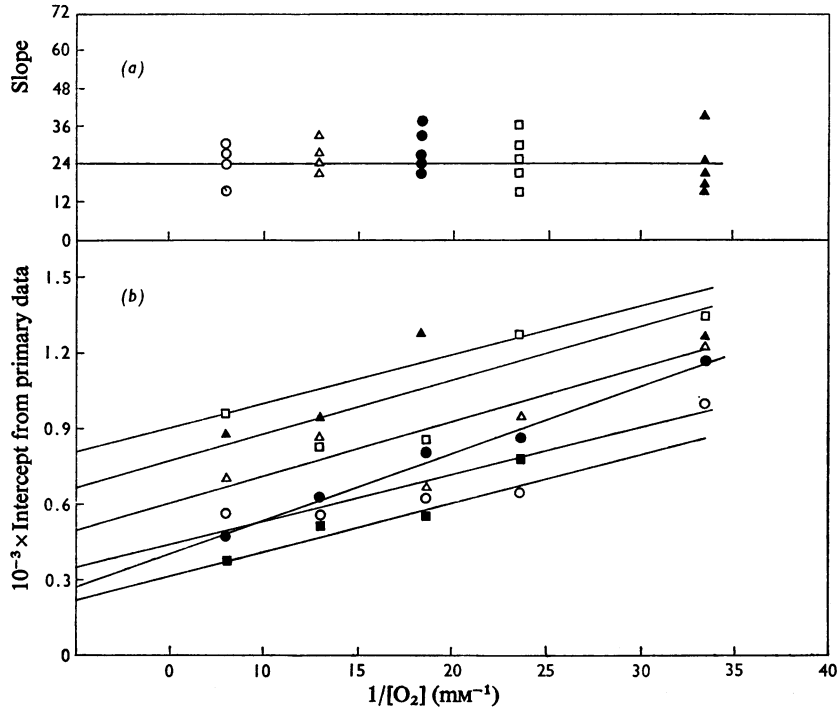


Fig. 2. *Secondary plots of slope and intercept data from Table 2 against reciprocal oxygen concentration*

(*a*) Slopes and (*b*) intercepts from the data of Table 2. The NADPH concentrations used were: □, 0.017mM; ▲, 0.026mM; △, 0.037mM; ●, 0.052mM; ○, 0.082mM; ■, 0.128mM.

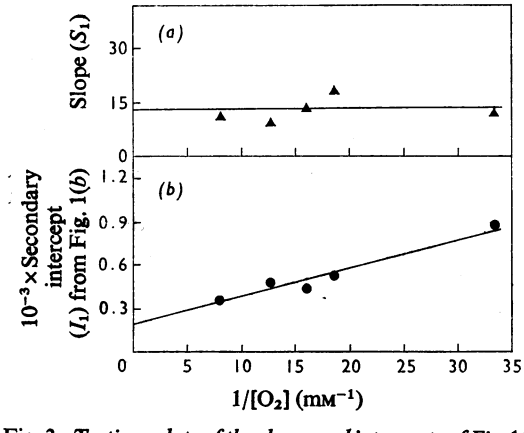


Fig. 3. *Tertiary plots of the slopes and intercepts of Fig. 1(b) against reciprocal oxygen concentration*

(*a*) Slopes (S_1) and (*b*) intercepts (I_1) of lines in Fig. 1(*b*).

so the intercept of Fig. 4(*b*) gives ϕ_0 and the slope ϕ_{NADPH} (Table 3). The value of ϕ_0 should agree with the value from Fig. 3(*b*), which it does reasonably well (Table 3). Since the primary data are plots of $1/v$ rather than e/v, the ϕ values in Table 3 are all functions of enzyme concentration, but since this is identical in all cases, the values are directly comparable. By using the mean values of ϕ_{DM}/e, ϕ_{NADPH}/e and ϕ_{O_2}/e from Table 3, the Michaelis constants can be calculated from the relationship $K_A = \phi_A/\phi_0$, etc. (Cleland, 1963; Dalziel, 1969). This gives values of $K_{DM} = 0.160$mM, $K_{NADPH} = 0.086$mM and $K_{O_2} = 0.121$ mM respectively.

Comparison of Table 3 with Table 2 of Dalziel (1969) suggests that over the range of concentrations studied, the secondary-amine mono-oxygenase reaction mechanism involves two intermediate forms of the enzyme with successive addition of a single substrate and departure of product, i.e. the triple-transfer mechanism II(a) of Dalziel (1969). Cleland (1963) calls this mechanism 'Hexa Uni Ping Pong'. For such a system, the generalized rate eqn. (2) reduces to eqn. (13):

$$\frac{e}{v} = \phi_0 + \frac{\phi_{DM}}{[DM]} + \frac{\phi_{NADPH}}{[NADPH]} + \frac{\phi_{O_2}}{[O_2]} \qquad (13)$$

with no terms involving ternary or quaternary complexes.

Table 3. *Values of the kinetic parameters (Dalziel, 1969) for the secondary-amine mono-oxygenase system*

All values were measured at an enzyme concentration (e) of 4 μg/ml.

Parameter	Units	Value obtained from Fig. ... 1(*a*)	2(*a*)	3(*a*)	3(*b*)	4(*a*)	4(*b*)	Mean value
ϕ_0/e	min				195.0		120.0	157.0
ϕ_{DM}/e	mM/min	26.4	24.0					25.2
ϕ_{NADPH}/e	mM/min			13.2			13.5	13.5
ϕ_{O_2}/e	mM/min				19.5	18.6		19.0
$\phi_{DM\cdot NADPH}/e$	mM^2/min	0	0					0
$\phi_{NADPH\cdot O_2}/e$	mM^2/min			0		0		0
$\phi_{DM\cdot O_2}/e$	mM^2/min	0	0					0
$\phi_{DM\cdot NADPH\cdot O_2}/e$	mM^3/min	0	0					0

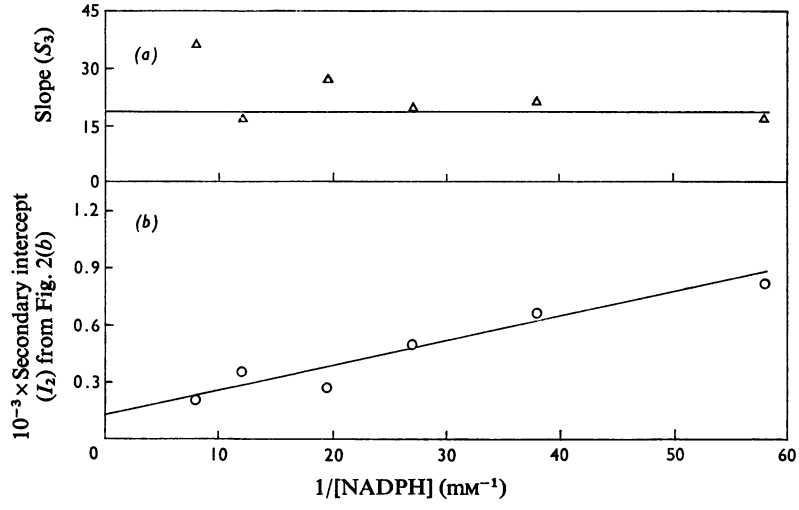


Fig. 4. *Tertiary plots of the slopes and intercepts of Fig. 2(b) against reciprocal NADPH concentration*

(*a*) Slopes (S_3) and (*b*) intercepts (I_3) of lines in Fig. 2(*b*).

Product-inhibition studies. Although the kinetic data presented above indicate that the reaction is a triple-transfer mechanism, they do not distinguish the sequence in which the substrates bind to the enzyme. By using product-inhibition studies, however, it is possible to obtain evidence of the order of binding. Figs. 5(*a*), 5(*b*) and 5(*c*) respectively show the effect of formaldehyde and $NADP^+$ on the reciprocal reaction rate when plotted against the reciprocal NADPH, oxygen or dimethylamine concentration. The other reaction product, methylamine, could not be tested as an inhibitor because it behaves as a pseudo-substrate (non-substrate effector) (Jarman & Large, 1972*b*). Inhibition by formaldehyde is competitive with O_2 but non-competitive with NADPH. $NADP^+$, by contrast, is competitive with dimethylamine. These observations suggest that $NADP^+$ and dimethylamine bind to one form of the enzyme, and formaldehyde and O_2 to a different form, whereas formaldehyde and NADPH do not react with a common form.

Discussion

Taken in conjunction with the proposed Hexa Uni Ping Pong nature of the reaction, a mechanism consistent with the above observations over the six-fold concentration range investigated is shown in Scheme 1.

Further confirmation of this mechanism could be obtained if kinetic studies could be made on the reverse reaction, but this is not possible with the present system. If the triple-transfer mechanism of Scheme 1 is correct, it might be possible, under suitable conditions, to isolate or identify the modified

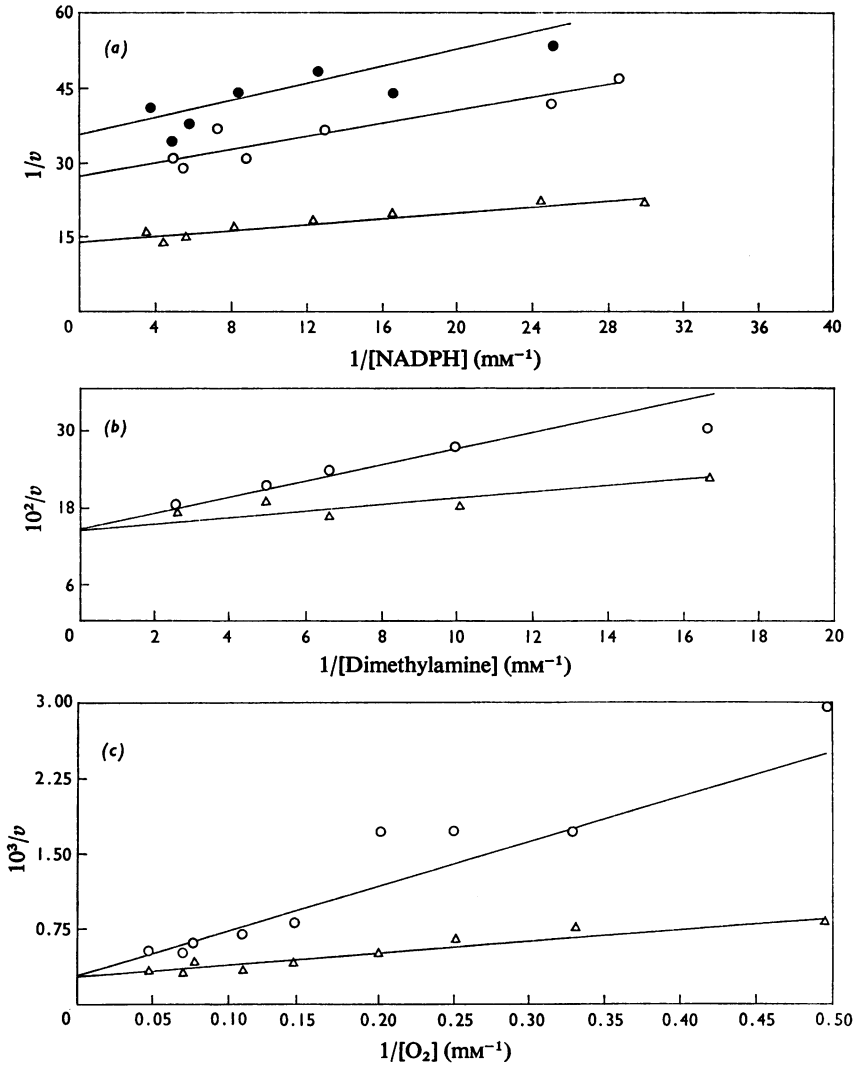
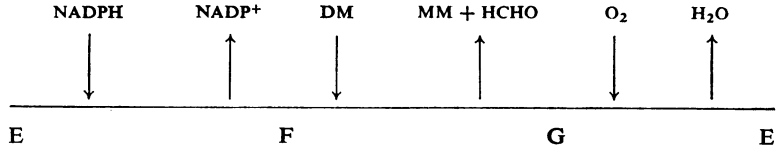


Fig. 5. *Product inhibition of the secondary-amine mono-oxygenase*

The standard assay conditions were used except that one reactant was varied. (*a*) Effect of formaldehyde, with NADPH as the variable substrate. △, No formaldehyde present; ○, 0.67mM-formaldehyde; ●, 6.67mM-formaldehyde. (*b*) Effect of $NADP^+$, with dimethylamine as the variable substrate. △, No $NADP^+$ present; ○, 61mM-$NADP^+$ present. (*c*) Effect of formaldehyde with O_2 as the variable substrate. △, No formaldehyde present; ○, 10mM-formaldehyde present.



Scheme 1. *Postulated reaction sequence for the enzyme*

F and G are intermediate, modified forms of the enzyme, DM is dimethylamine and MM is methylamine. E could, conceivably, be an oxygenated and F a reduced-oxygenated form of the enzyme.

forms of the enzymes F and G. The addition of substrates has been shown to produce changes in the absorption and electron-paramagnetic-resonance spectra of the enzyme (T. R. Jarman, D. F. Brook, R. Cammack & P. J. Large, unpublished work), but it is difficult at present to determine whether such spectrally observed intermediates are of catalytic significance. A Hexa Uni Ping Pong mechanism would predict an exchange of label between [^{14}C]-dimethylamine and unlabelled monomethylamine in the absence of other substrates if enzyme in the appropriate form were used.

Other mono-oxygenase three-substrate systems which have been studied give different kinetics, which is not surprising, since the nature of the electron donor or the chemical nature of the enzyme are, in each case, quite different. The copper enzyme, dopamine (3,4-dihydroxyphenethylamine) β-hydroxylase (EC 1.14.17.1) gives a Uni Uni Bi Bi Ping Pong mechanism (Goldstein *et al.*, 1968). The flavoprotein melilotate hydroxylase (EC 1.14.13.4) gives Bi Uni Uni Bi Ping Pong kinetics (Strickland & Massey, 1973) [concerted substitution mechanism II(c) of Dalziel, 1969]. Phenylalanine 4-hydroxylase (EC 1.14.16.1), which contains iron, was at first thought to show Ping Pong kinetics, but work over a wider range of substrate concentrations (Kaufmann & Fisher, 1974) has shown that it has an ordered Ter Ter mechanism with a complete set of intersecting double-reciprocal plots. McIntyre & Vaughan (1975) have performed a three-substrate kinetic analysis of the phenolase (EC 1.14.18.1)-catalysed hydroxylation of *p*-coumaric acid, and obtained ambiguous results in which a decision between parallel and intersecting double-reciprocal plots was difficult to make, and substrate-inhibition studies were necessary before a mechanism could be proposed. Although not all the lines from the data in Table 2 are parallel, the deviation observed is entirely of a random nature, and does not lead to any kind of intersecting pattern which would imply a non-Ping Pong type of mechanism. We cannot rule out the possibility that converging double-reciprocal plots might be observed outside the concentration range tested, but it was not possible to investigate this experimentally because both NADPH and dimethylamine show inhibition at concentrations higher than those used.

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