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Kinetics of the Diamine Oxidase Reaction

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1. The oxidation of *p*-dimethylaminomethylbenzylamine was followed spectrophotometrically by measuring the change in E_{250} caused by the *p*-dimethylaminomethylbenzaldehyde produced under a wide variety of experimental conditions. 2. The effect of variations in concentrations of both substrates (amine and oxygen) and all products (aminoaldehyde, hydrogen peroxide and ammonia) on this reaction was studied and the results used to develop a formal mechanism. 3. The nature of the rate-limiting step was elucidated by studying the effects of alterations in ionic strength, dielectric constant and deuterium substitution on the velocity of the forward reaction. 4. Thermodynamic activation energy parameters were obtained at several pH values from the effects of temperature on the reaction.

Although a considerable amount of information is available concerning the stoicheiometry of the pig kidney diamine oxidase reaction and the nature of the enzyme–substrate interaction (Zeller, 1963), there has been little work relating to the physical chemistry of this particular enzyme.

Preliminary kinetic investigations have suggested the production of superoxide anion radicals in the catalytic sequence (Rotilio *et al.*, 1970) and stopped-flow experiments show that anaerobic addition of substrate causes first a rapid decrease in absorbance at 500nm followed by a slow phase, which may indicate reduction of the enzyme (Mondovì *et al.*, 1969). Isolation of the equivalent of 1mol of aminoaldehyde under anaerobic conditions and the fact that double-reciprocal plots in the presence of various concentrations of the second substrate (oxygen) were parallel suggested a reaction sequence involving only binary complexes (Finazzi-Agrò *et al.*, 1969). It is thought that pyridoxal phosphate is a cofactor for the enzyme, since carbonyl group reagents are potent inhibitors (Zeller, 1963), and this conclusion is strengthened by both spectrophotometric (Mondovì *et al.*, 1967*a*) and chemical evidence (Kumagai *et al.*, 1969).

After a reinvestigation of the structural features necessary for optimum enzyme–substrate interaction (Bardsley *et al.*, 1970, 1971), we have been able to design a substrate (*p*-dimethylaminomethylbenzylamine) that is readily oxidized by diamine oxidase and is useful for the spectrophotometric assay of enzyme activity (Bardsley *et al.*, 1972). This method is based upon the appearance of *p*-dimethylaminomethylbenzaldehyde measured at 250nm and it has proved useful in studying the inhibition of diamine oxidase (Bardsley & Ashford, 1972) and also in the purification and kinetic investigation of the amine oxidase of human placenta (W. G. Bardsley & M. J. C. Crabbe, unpublished work). The related compound *p*-dimethylaminobenzylamine is not significantly oxidized by purified pig kidney diamine oxidase.

Recently we have prepared *p*-dimethylaminomethyl-αα-bisdeuterobenzylamine and studied its oxidation by diamine oxidase under conditions where the ionic strength, temperature and pH were varied. This paper presents and discusses the results of this and other related experiments.

Experimental

Materials

Preparation of p-dimethylaminomethyl-αα-bisdeuterobenzylamine. A solution of *p*-dimethylaminomethylbenzonitrile (4g) in anhydrous tetrahydrofuran (30ml, distilled off sodium hydride) was added dropwise over 1h to a refluxing suspension of $LiAl^2H_4$ (1.2g) in tetrahydrofuran (25ml). After 3 days refluxing under anhydrous conditions, the solution was evaporated to dryness under reduced pressure, decomposed by cautious addition of methanol and partitioned between a solution of Rochelle salt (5g) in water (20ml) and diethyl ether (20ml). The aqueous layer was extracted with chloroform (20ml) and removal of solvent from the combined ether and chloroform extracts left an oil (3.8g), which was distilled (72–76°C, 6.55–13.1 N/m^2)

to give *p*-dimethylaminomethyl-αα-bisdeutero-benzylamine (3.7g, 78% yield). The spectroscopic properties were consistent with the structure assigned.

The infrared spectra of *p*-dimethylaminomethylbenzylamine and deutero analogue as liquid films were almost identical except that the deutero compound had C–^{2}H stretching frequencies at 2080, 2130 and 2190cm^{-1}.

The proton-magnetic-resonance spectra were closely similar (Varian H A 100, in C–$^2HCl_3$ solution) showing an aromatic C–H signal at 2.76τ (4 protons), $-CH_2-$ adjacent to $-N(CH_3)_2$, 6.62τ (2 protons) and $-N(CH_3)_2$, 7.78τ (6 protons). In addition the deutero compound had an $-NH_2$ singlet at 8.55τ (2 protons, removed by adding ^2H_2O) and *p*-dimethylaminomethylbenzylamine had an $-NH_2$ singlet at 8.34τ (2 protons) and $-CH_2-$ adjacent to amino group at 6.18 (2 protons).

The mass spectrum of *p*-dimethylaminomethylbenzylamine (AEI MS 12) had ions of *m/e* 164 (relative abundance 100%), 148 (8.4), 134 (16.5), 120 (42), 104 (23.4), 91 (25), 77 (11.7), 65 (5.8), 58 (70), 44 (6.7), 42 (10), 30 (8.4) and 28 (32%).

The deutero compound gave ions of 166* (100%), 150* (62), 136* (8.8), 122* (31.6), 106* (18.6), 91 (10.5), 77 (5.3), 65 (1.76), 58 (49), 44 (3.5), 42 (7.1), 32* (5.0) and 28 (7.1) (where the ions marked with an asterisk were two mass numbers higher because $-C^2H_2-$ replaced $-CH_2-$).

Preparation of enzyme. The method of Bardsley *et al.* (1971) was used. The average specific activity of the enzyme was 1.50 units/mg at 37°C.

Methods

Spectrophotometric method of assy. Change in E_{250} was measured as described by Bardsley *et al.* (1972). Unless otherwise stated, the reaction was done in air at 20°C in a final volume of 3ml containing enzyme (0.02 unit) in 0.05M-potassium phosphate buffer, pH 7.0. Where reaction conditions were to be varied, preliminary experiments were done to make sure that the enzyme was stable and that the change in E_{250} with time was linear. The initial rate of change in E_{250} was proportional to the amount of enzyme used.

Variation in oxygen concentration. These experiments were performed in cuvettes fitted with Thunberg-type stoppers. Buffer containing substrate (3.5ml) was placed in the cuvette and enzyme solution (0.5ml) in the side arm. The assembly was evacuated by water pump, and then filled and flushed at least three times with the appropriate gas mixture before the final filling and adjustment to atmospheric pressure. At least 5min equilibration was allowed before the contents of the side arm were added, and the cuvettes were inverted to maintain equilibration every 2min during the initial-rate measurement. Gas mixtures were supplied by the British Oxygen Company Ltd., London SW19 3UF, U.K.

Variation in temperature. Cuvettes were immersed in a constant-temperature water bath and rapidly removed at intervals of 2min for absorbance measurement. The enzyme solution was stable up to 50°C but irreversible loss of activity was taking place at 60°C.

Variation in ionic strength. Stock potassium phosphate buffer (0.05M) was raised to high ionic strength (1.5, defined as $I = \frac{1}{2}\sum Mz^2$ where M is the molarity of ion of charge z) by addition of solid KCl. A range of solutions of various ionic strengths was then obtained by making suitable dilutions of this solution and the pH adjusted to 7.0 where necessary.

Variation in dielectric constant. Methanol was added to give the required dielectric constant, the pH being adjusted to 7.0 where necessary.

Graphical methods and equations. Initial rates were obtained over a period of 10–15min with five or more substrate concentrations and double-reciprocal plots were used to obtain kinetic constants. By using the King & Altman (1956) algorithm, a full steady-state rate-equation was calculated and transformed into the forms:

$$1/v = \frac{K_a}{V}\cdot 1/A + 1/V\left(1+\frac{K_b}{B}\right)$$

for A as variable substrate, and:

$$1/v = \frac{K_b}{V}\cdot 1/B + 1/V\left(1+\frac{K_a}{A}\right)$$

for B as variable substrate, with no products present, by using the methods of Cleland (1963*a*). The kinetic constants K_a and K_b were then obtained by a replot of intercepts as a function of reciprocal substrate concentration from: Intercept $= 1/V[1+(K_b/B)]$ etc. Activation energy parameters were calculated from:

$$\frac{\mathrm{d}\ln k}{\mathrm{d}T} = \frac{\Delta H^{0*} + RT}{RT^2}, \quad \Delta S^{0*} = \frac{\Delta H^{0*} - \Delta G^{0*}}{T}$$

and

$$k = \frac{k_B T}{h}\,\mathrm{e}^{\frac{-\Delta G^{0*}}{RT}}$$

assuming a molecular weight of 185000 with two active subunits.

Results

Fig. 1 shows the behaviour of double-reciprocal plots at various concentrations of fixed substrates. The slopes in both cases were constant and the intercept re-plot (Fig. 2*a*) gives $K_a = 3.57$mM as the kinetic constant for amine and $K_b = 0.043$mM for oxygen. Typical product-inhibition patterns are shown in Fig. 3. Ammonia acts as a competitive inhibitor against *p*-dimethylaminomethylbenzylamine with O_2 fixed and as an uncompetitive inhibitor against

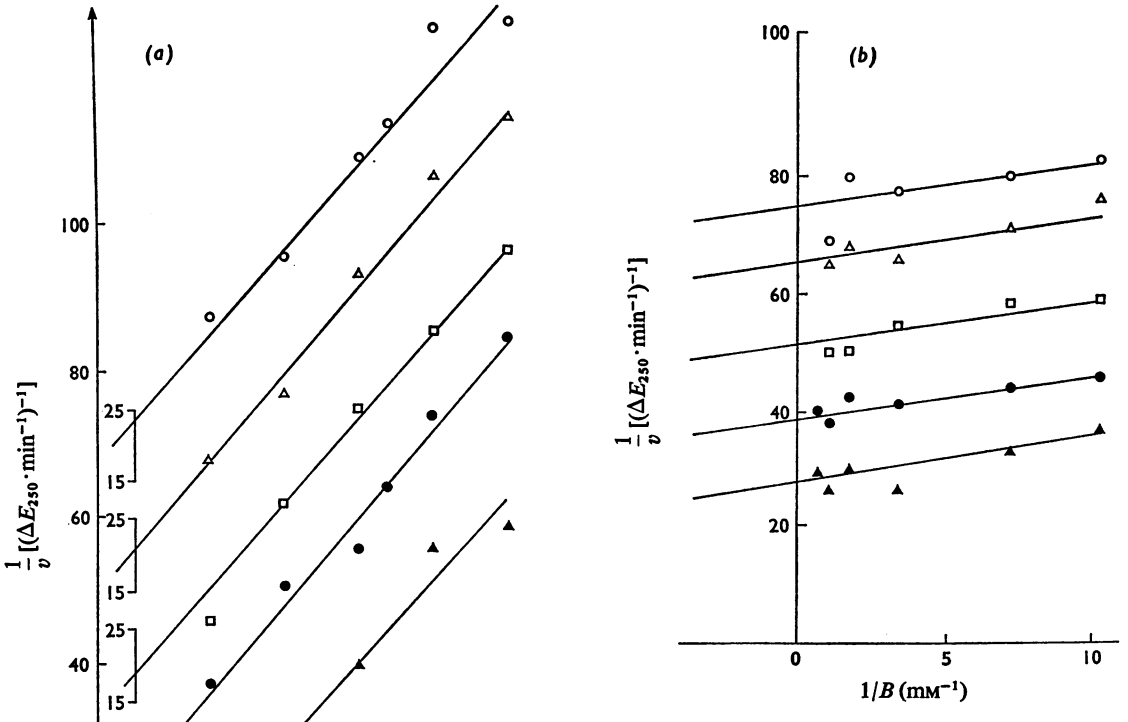


Fig. 1. *Effect of varying the concentration of both substrates on initial rates*

(*a*) *p*-Dimethylaminomethylbenzylamine as the varied substrate (A) against fixed O_2 concentrations: ○, 0.097mM; △, 0.14mM; □, 0.29mM; ●, 0.55mM; ▲, 0.97mM. The ordinate intercepts are displaced vertically for clarity. (*b*) Oxygen as the varied substrate (B) against fixed *p*-dimethylaminomethylbenzylamine concentrations: ○, 1.0mM; △, 1.25mM; □, 1.67mM; ●, 2.5mM; ▲, 5.0mM.

O_2 with a fixed concentration of *p*-dimethylaminomethylbenzylamine. Hydrogen peroxide behaves as an uncompetitive inhibitor against *p*-dimethylaminomethylbenzylamine with O_2 fixed and as a non-competitive inhibitor against O_2 with *p*-dimethylaminomethylbenzylamine fixed. Also substrate inhibition by O_2 is promoted in the presence of H_2O_2. Slope/intercept re-plots (Fig. 4) are linear, giving K_i values: NH_3 against *p*-dimethylaminomethylbenzylamine, K_i (slope) = 33.8mM; NH_3 against O_2, K_i (intercept) = 36.0mM; H_2O_2 against *p*-dimethylaminomethylbenzylamine, K_i (intercept) = 0.3mM; H_2O_2 against O_2, K_i (intercept) = 0.5mM, K_i (slope) = 0.75mM.

In Fig. 5 the similar behaviour of both *p*-dimethylaminomethylbenzylamine and the deuterio-analogue can be seen as ionic strength is varied. For both compounds the substrate inhibition at high amine concentrations, which is apparent at ionic strengths around 0.1, can be seen to be abolished at higher ionic strengths. Fig. 6 further dissects the effect of ionic strength into two separate functions, namely a negative primary kinetic salt effect overcome by increasing substrate concentrations and a larger negative salt effect that is less strongly affected by substrate concentration. Points from the steeper slopes of the Brönsted plots are re-plotted for K^+ as a competitive inhibitor in Fig. 7.

Up to methanol concentrations of 30%, the linear slopes of a plot of log V against reciprocal dielectric constant given by both *p*-dimethylaminomethylbenzylamine and the deutero analogue were constant and not appreciably dependent on substrate concentration (Fig. 8).

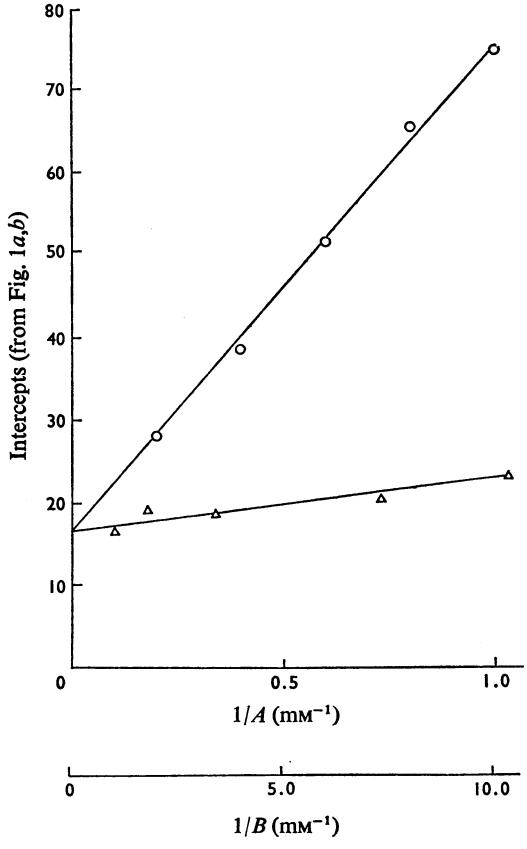


Fig. 2. *Data from Fig.* 1 *re-plotted for a Ping Pong mechanism*

Intercepts as a function of fixed substrate concentration for ○, amine (A) fixed, O_2 varied (Fig. 1*b*), and △, O_2 (B) fixed, amine varied (Fig. 1*a*).

Table 1 records the standard enthalpies, entropies and free energies of activation for *p*-dimethylaminomethylbenzylamine and the deutero analogue obtained from Arrhenius plots at several pH values as in Fig. 9. The deuterium isotope effect is recorded in Table 2 as an effect on k_H/k_D, $V_{max.}$ and K_m at several temperatures, pH values and ionic strengths.

Discussion

A mechanistic scheme for the diamine oxidase reaction

The family of parallel lines produced when reciprocal velocity is plotted as a function of reciprocal substrate concentration varied in the presence of fixed amounts of the non-varied substrate (Fig. 1) obviously suggests a mechanism involving only binary enzyme–

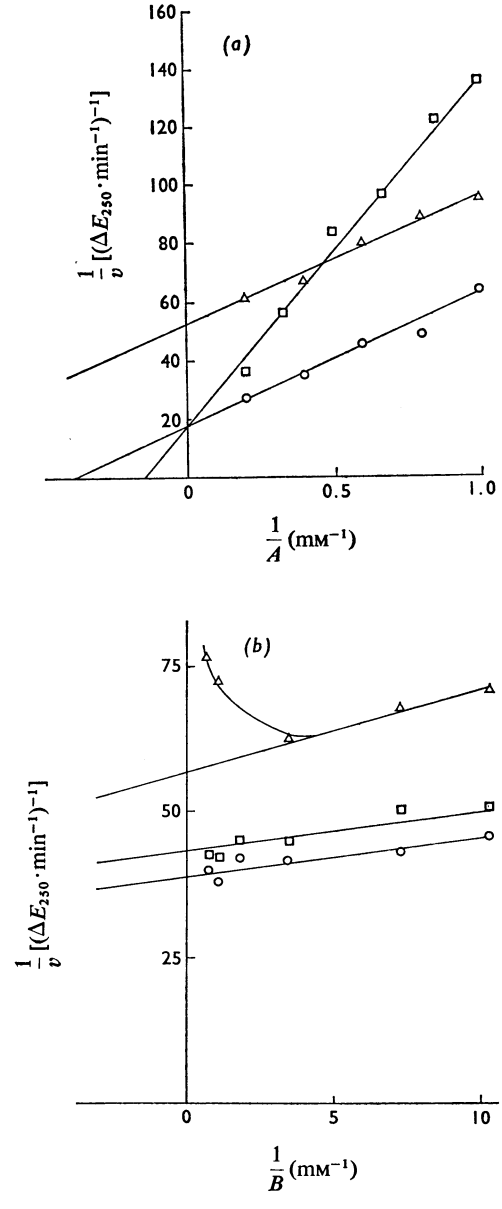


Fig. 3. *Inhibition of initial velocities in the presence of products*

(*a*) Products against *p*-dimethylaminomethylbenzylamine (A) with air as the gaseous phase. ○, No products present; △, 1.0mM-H_2O_2 present; □, 30mM-NH_3 present. (*b*) Products against O_2 (B) with *p*-dimethylaminomethylbenzylamine fixed at 3.0mM. ○, No products present; △, 2.0mM-H_2O_2 present; □, 20mM-NH_3 present.

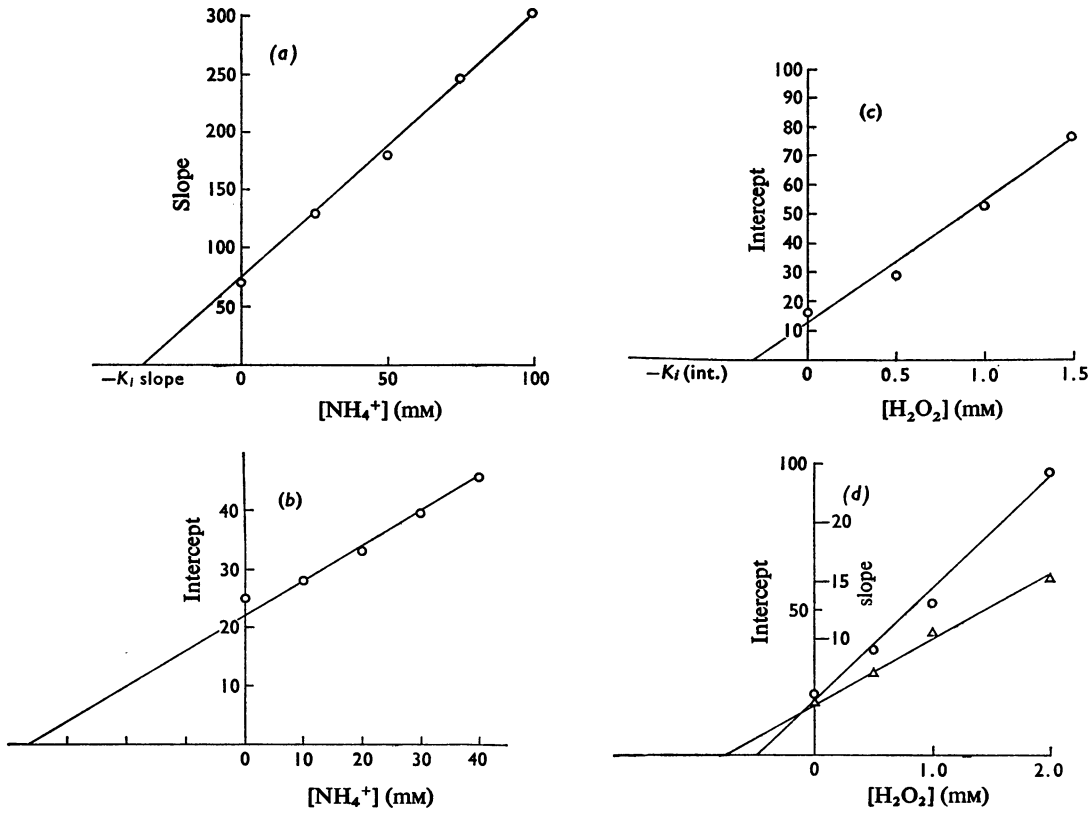


Fig. 4. *Re-plots of slope and intercept effects produced by product inhibitors*

(*a*) Ammonia against *p*-dimethylaminomethylbenzylamine with O_2 fixed (air) ($K_i = 33.8$mM); (*b*) NH_3 against O_2 with *p*-dimethylaminomethylbenzylamine fixed at 3.0mM ($K_i = 36$mM); (*c*) H_2O_2 against *p*-dimethylaminomethylbenzylamine with O_2 fixed (air) ($K_i = 0.3$mM); (*d*) H_2O_2 against O_2 with *p*-dimethylaminomethylbenzylamine fixed at 3.0mM [K_i int. (○) = 0.5mM; K_i slope (△) = 0.75mM].

substrate complexes. The rate of oxidation of *p*-dimethylaminomethylbenzylamine is only slightly increased as the concentration of O_2 is raised from 0.014mM (1% O_2 in the gaseous phase) to 0.553mM (40% in the gaseous phase) and some slight substrate inhibition by O_2 occurs at 0.970mM (70% O_2 as gaseous phase). At fixed concentrations of O_2, however, the velocity of the forward reaction is strongly dependent on amine concentration, being rapidly increased as the concentration of amine is raised until at high concentrations (7.5mM-*p*-dimethylaminomethylbenzylamine) substrate inhibition by *p*-dimethylaminomethylbenzylamine becomes apparent. It can be concluded that because of the high affinity for O_2 the enzyme is almost fully saturated with 1% O_2 in the gaseous phase and that throughout the concentration range used, the supply of O_2 is never likely to be rate-limiting.

We have reviewed the kinetic evidence available for this enzyme (Bardsley & Ashford, 1972) and concluded that the most reasonable scheme is a Ping Pong Bi Ter reaction sequence in which amine (A) adds to the oxidized form of the enzyme (E) to give a complex (EA–FP) that dissociates into reduced enzyme (F) and aminoaldehyde (P). Oxygen (B) then gives a complex (FB–EQR) that releases H_2O_2 (Q) and NH_3 (R). Such a scheme could be tested by Cleland's rules (Cleland, 1963*a*,*b*,*c*), since P should be non-competitive against A, competitive against B; Q should be uncompetitive against A, non-competitive against B and R should be competitive against A but uncompetitive against B. Saturation with B should abolish inhibition by P against A and saturation with A should abolish inhibition by R against B. In practice, experimental difficulties have prevented a satisfactory completion of all these experiments but

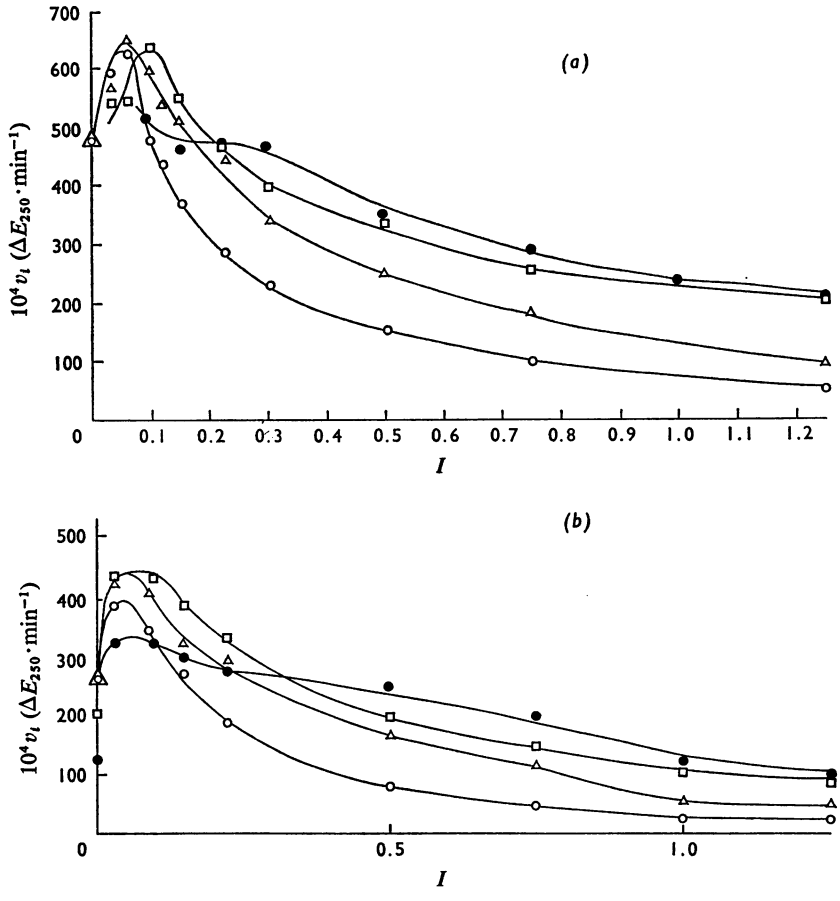


Fig. 5. *Plot of initial velocity against ionic strength*

(*a*) *p*-Dimethylaminomethylbenzylamine; ○, 1.0mM; △, 2.5mM; □, 5.0mM; ●, 7.5mM. (*b*) *p*-Dimethylaminomethyl-αα-bisdeuterobenzylamine: ○, 1.0mM; △, 2.5mM; □, 5.0mM; ●, 10.0mM.

sufficient information is available to support the scheme outlined above.

p-Dimethylaminomethylbenzaldehyde as inhibitor. It is possible to use *p*-dimethylaminomethylbenzaldehyde as a product inhibitor only up to a concentration of 0.15mM, since high absorbance above this concentration prevents accurate determination of change in E_{250}. Even at this concentration there is little marked effect on the velocity of the reaction, the only effect noticed being an increase in substrate inhibition by *p*-dimethylaminomethylbenzylamine. Since the enzyme is probably saturated with O_2, there should be no inhibition against *p*-dimethylaminomethylbenzylamine but there should be some inhibition against O_2 and we must conclude that the aminoaldehyde has a high K_i against O_2 and that the solution is not sufficiently concentrated at 0.15mM to give appreciable inhibition.

The production of 1mol of aminoaldehyde under anaerobic conditions (Finazzi-Agrò *et al.*, 1969) does, however, identify the first product, P, as an aminoaldehyde. Amine oxidases from ox plasma (Oi *et al.*, 1970), ox liver (Oi *et al.*, 1971), pea seedlings (Yamasaki *et al.*, 1970) and pig brain (Tipton, 1968) also produce 1mol of aldehyde anaerobically, but pig plasma amine oxidase gives NH_3 as the first product (Taylor & Knowles, 1971).

Hydrogen peroxide as inhibitor. Hydrogen peroxide destroys this enzyme (Mondovì *et al.*, 1967*b*) but this does not occur appreciably at concentrations below 3.0mM. It gives uncompetitive inhibition against *p*-dimethylaminomethylbenzylamine but it

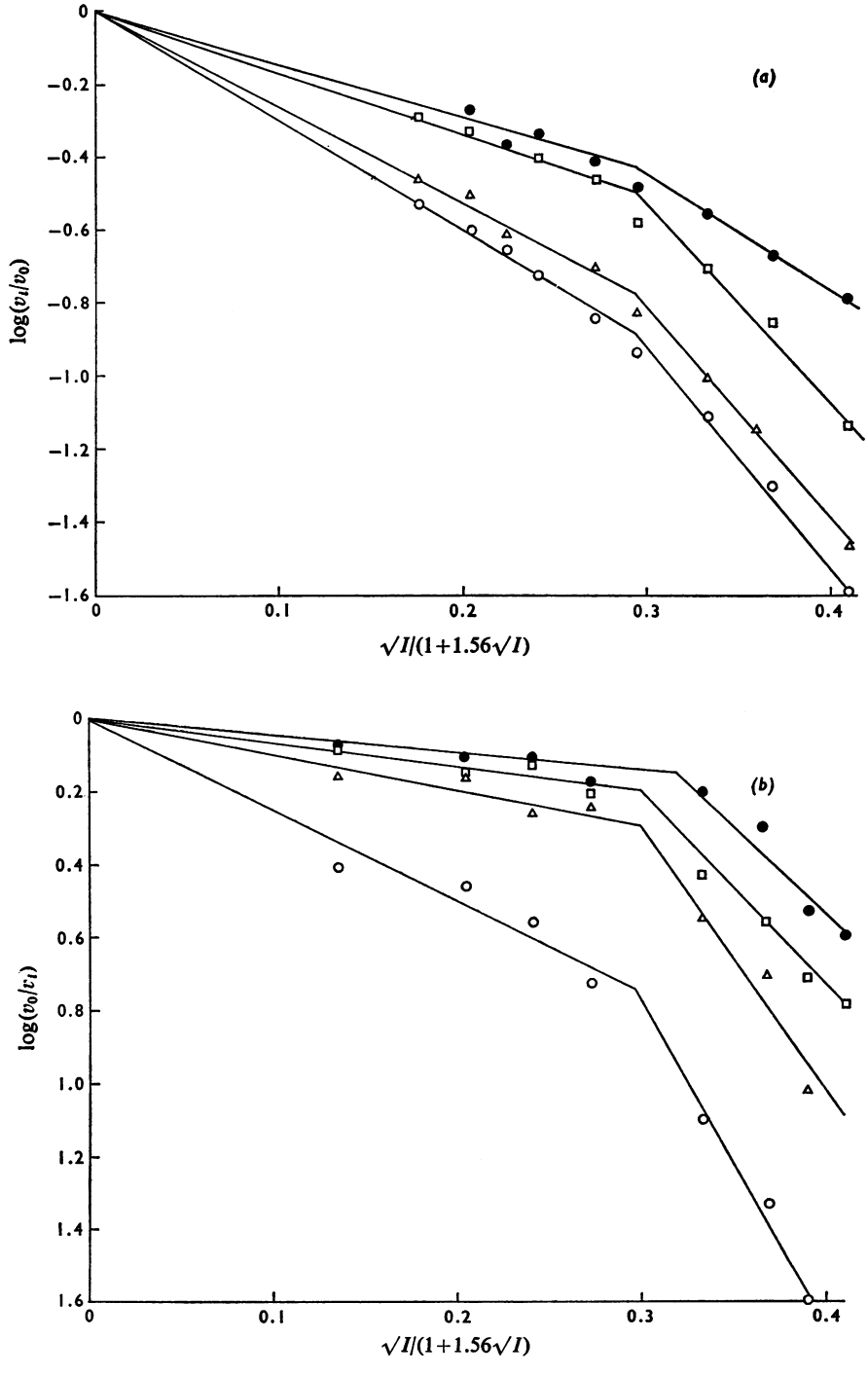


Fig. 6. *Brönsted plots for the effect of ionic strength on initial rates*

(*a*) *p*-Dimethylaminomethylbenzylamine: ○, 1.0mM; △, 1.67mM; □, 2.5mM; ●, 5.0mM. (*b*) *p*-Dimethylaminomethyl-αα-bisdeuterobenzylamine: ○, 1.0mM; △, 2.5mM; □, 5.0mM; ●, 10.0mM.

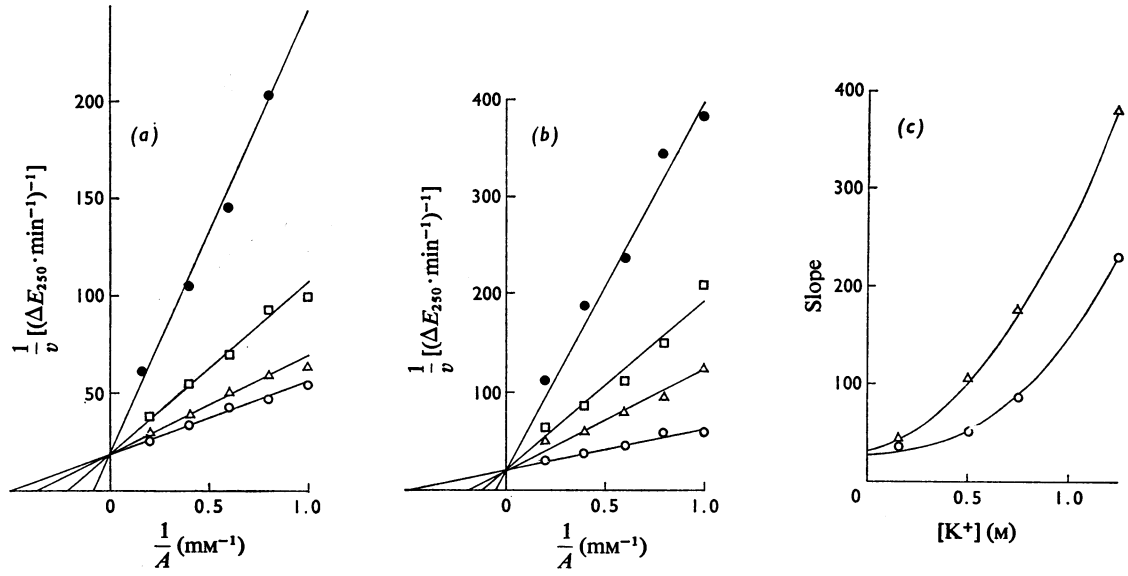
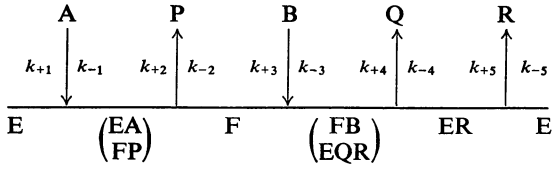


Fig. 7. *K^+ as a competitive inhibitor at high concentrations against amine with fixed O_2 (air) concentration*

(*a*) *p*-Dimethylaminomethylbenzylamine (A) with K^+ concentrations: ○, 0.15M; △, 0.5mM; □, 0.75M; ●, 1.75M. (*b*) *p*-Dimethylaminomethyl-αα-bisdeuterobenzylamine (A) with K^+ concentrations: ○, 0.15M; △, 0.5M; □, 0.75M; ●, 1.25M. (*c*) Re-plot of slope effect with K^+ used as inhibitor for: ○, *p*-dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethyl-αα-bisdeuterobenzylamine.

also promotes substrate inhibition by O_2, so that whereas there is a very definite intercept effect against reciprocal O_2 concentration, it is not easy to see if there is a slope effect because of the curvature due to substrate inhibition. If the inhibition were uncompetitive, it is difficult to see how it could also be uncompetitive against *p*-dimethylaminomethylbenzylamine and we conclude that a slope effect is present, i.e. the inhibition against O_2 is non-competitive and H_2O_2 is Q, the second product, released after addition of B, O_2.

Ammonia as inhibitor. Even at concentrations of 100mM, NH_3 gives only slope effects against variable concentrations of *p*-dimethylaminomethylbenzylamine (air as gaseous phase), a re-plot of the slope as a function of inhibitor concentration being linear. Since there are no intercept effects, NH_3 must compete with *p*-dimethylaminomethylbenzylamine for the same enzyme form, E, therefore NH_3 must be R, the last product released. This is further strengthened by the uncompetitive behaviour towards O_2 and increases confidence in a mechanism of the type:



Primary kinetic isotope effect and elucidation of the rate-limiting step

Where C–H bond breaking constitutes a rate-limiting step, the replacement of C–H by C–^{2}H gives an isotope effect of variable magnitude. Although k_H/k_D values of up to 18 are possible (Collins & Bowman, 1970*a*), values of 6–10 are often taken as indicating involvement in the rate-limiting step of straightforward chemical reactions (Jencks, 1969), and in enzyme systems values of about 2 are commonly taken to implicate rate-determining C–H bond cleavage (Richards, 1970; Collins & Bowman, 1970*b*). A kinetic isotope effect of about 2.0 has been reported for monoamine oxidase (Bellau & Moran, 1963).

The range of k_H/k_D values from 1.14 to 5.5 with mean values usually around 2.0 under a wide variety of experimental conditions must be taken to indicate breaking of the α C–H bond at least as part of the rate-determining step during the oxidation of *p*-dimethylaminomethylbenzylamine and its deuterio-analogue by diamine oxidase. The isotope effect is still present at the lowest O_2 concentrations, which further proves that oxidation by O_2 actually follows the rate-limiting process in the catalytic sequence. Table 1 shows that, throughout most of the pH range studied, the increased free energy of activation for the deutero compound as opposed to *p*-dimethylaminomethylbenzylamine is mainly due to the

Table 1. *Activation energy parameters for pig kidney diamine oxidase with p-dimethylaminomethylbenzylamine (^1H-substrate) and p-dimethylaminomethyl-αα-bisdeuterobenzylamine (^2H-substrate) in air at $I = 0.15$*

	k (s^{-1})		ΔH^{0*} ($kJ \cdot mol^{-1}$)		ΔG^{0*} ($kJ \cdot mol^{-1}$)		ΔS^{0*} ($J \cdot deg^{-1}$)	
pH	^{1}H-substrate	^{2}H-substrate	^{1}H-substrate	^{2}H-substrate	^{1}H-substrate	^{2}H-substrate	^{1}H-substrate	^{2}H-substrate
6.0	7.14×10^{-1}	3.23×10^{-1}	73.3	73.3	72.0	74.0	+ 4.4	− 2.4
6.5	2.93	1.41	59.2	62.4	69.0	71.0	−33.5	−29.4
7.0	4.1	1.94	86.3	63.7	68.0	70.0	+62.5	−21.5
7.5	3.80	2.59	65.3	67.5	68.5	69.0	−11.0	− 5.1
8.0	4.1	2.0	50.0	61.7	68.0	70.0	−61.4	−28.3

Table 2. *Values for k_H/k_D, $V_{max.}$ and K_m under various conditions of ionic strength, pH, temperature and substrate concentration with air as gaseous phase*

Unless otherwise indicated, $I = 0.15$, pH = 7.0 and the temperature was 20°C. ^{1}H-substrate and ^{2}H-substrate are identified in Table 1.

	k_H/k_D				K_m		$V_{max.}$ (E_{250}/min)	
Substrate concn. (mM) ...	1.0	2.5	5.0	10.0	^{1}H-substrate	^{2}H-substrate	^{1}H-substrate	^{2}H-substrate
I								
0.225	1.53	1.31	1.39	1.58	1.25	2.50	0.05	0.025
0.5	1.94	1.52	1.67	1.14	2.78	5.26	0.05	0.025
0.75	2.13	1.52	1.71	1.34	4.76	7.69	0.05	0.025
1.00	2.00	1.79	1.71	1.79	12.5	16.7	0.05	0.025
pH								
6.0	4.82	3.78	3.3	2.73	1.94	4.5	0.009	0.004
6.5	2.67	2.42	2.3	2.19	1.92	2.78	0.038	0.018
7.0	2.5	2.0	1.9	1.8	1.9	3.3	0.05	0.025
7.5	1.77	1.65	1.55	1.5	—	—	0.055	0.033
8.0	1.81	1.63	1.43	1.35	—	—	0.052	0.263
Temp. (°C)								
30	2.1	1.96	1.73	1.33	1.93	2.86	0.077	0.048
40	2.36	2.46	2.5	2.34	2.0	2.0	0.25	0.12
50	2.02	2.28	2.44	2.67	1.95	1.11	0.55	0.2

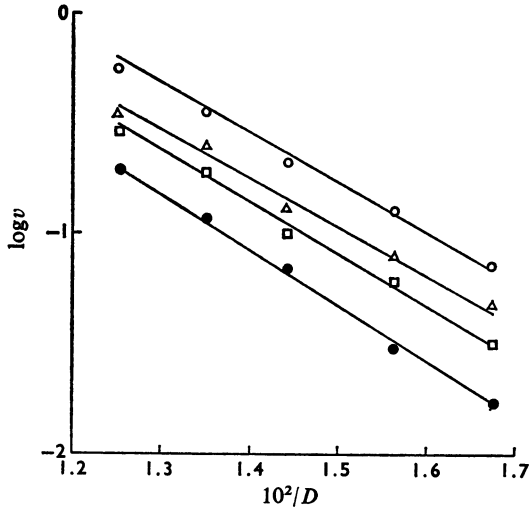


Fig. 8. *Logarithm of initial rate as a function of reciprocal dielectric constant*

○, *p*-Dimethylaminomethylbenzylamine ($V_{max.}$); △, *p*-dimethylaminomethyl-αα-bisdeuterobenzylamine ($V_{max.}$); □, *p*-dimethylaminomethylbenzylamine (4mM); ●, *p*-dimethylaminomethyl-αα-bisdeuterobenzylamine (4mM).

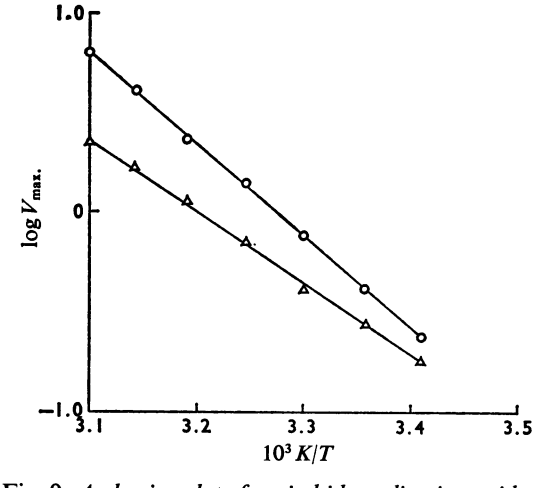


Fig. 9. *Arrhenius plots for pig kidney diamine oxidase at pH7.0, ionic strength* 0.15M *in air*

○, *p*-Dimethylaminomethylbenzylamine; △, *p*-dimethylaminomethyl-αα-bisdeuterobenzylamine.

expected increase in enthalpy of activation, but there is an interesting situation at pH7.0 where an entropy compensation for *p*-dimethylaminomethylbenzylamine occurs.

In this work we have no information regarding the magnitude of any of the rate constants for the reverse reaction, since only initial rates were obtained. The constants k_{+2}, k_{+3}, k_{+4} and k_{+5} must be larger than the composite rate-limiting constant k_{+1}, which describes the association of enzyme and substrate, favourable orientation of the enzyme–substrate complex, Schiff-base formation with pyridoxal phosphate at the active site and then a prototropic shift converting this Schiff base into a Schiff base between amino aldehyde and pyridoxamine. Although the ionic-strength experiments show that the association of enzyme and substrate is partially rate-limiting, the kinetic isotope results indicate that breaking of the αC–H bond in the isomerization step is mainly responsible for the overall rate-determining nature of this particular sequence. At 20°C and pH7.0 a lower limit to the value of k_{+1} would be $4.1\,s^{-1}$ for *p*-dimethylaminomethylbenzylamine and $1.9\,s^{-1}$ for its deutero analogue.

Effects of the medium

p-Dimethylaminomethylbenzylamine and its deutero analogues behave similarly with increasing ionic strength, the isotope effect being present throughout the ionic-strength range investigated. Up to $I = 0.06$M the velocity increases with increasing ionic strength and this is readily explained as being due to progressive loss of enzyme activity at low ionic strength, a property that has been utilized as a precipitation step in purifying this enzyme (Mondovì *et al.*, 1967*c*). Fig. 5 shows that, at substrate concentrations in excess of 5.0mM, substrate inhibition is observed up to $I = 0.25$M, but that this inhibition disappears as the ionic strength is increased further. Obviously substrate inhibition involves the interaction between a positively charged substrate and negatively charged enzyme and this is simply the operation of a primary kinetic salt effect. Since substrate inhibition by A is enhanced by P, and inhibition by B is enhanced by Q, this suggests that the inhibition is uncompetitive against the second substrate (point of addition separated by an irreversible step, release of product at zero concentration) but non-competitive against the first substrate, i.e. due to association of the type FP+A = FPA and EQR+B = EQRB. The reason for the decreasing rate of oxidation is clarified by the Brönsted plots in Fig. 6, which show two interesting features: a negative slope that increases from a value of −3.0 for *p*-dimethylaminomethylbenzylamine and −2.5 for its deutero analogue, to approach zero slope for both substrates as their concentration increases, and a large negative slope for both substrates above $I = 0.5$M.

The first feature can be explained as an effect of ionic strength on the activity coefficients of substrate and enzyme since, at low substrate concentration, the

association of enzyme and substrate, being the association between oppositely charged species (Bardsley *et al.*, 1971), is subject to a negative kinetic salt effect. The magnitude of the slope suggests that the substrate is fully protonated (di-positively charged) in this association step. However, at high substrate concentrations, the enzyme becomes saturated with substrate and this effect can no longer operate; hence the disappearance of the salt effect as saturating conditions are approached. This supposes a contribution of the association of enzyme and substrate to the overall rate-limiting step, which is further evinced by the linear relationship between the logarithm of the velocity and reciprocal dielectric constant shown in Fig. 8.

The second feature of the Brönsted plot might be due to the inactivation of the enzyme at ionic strength greatly in excess of physiological conditions, but the pronounced slope effect on the double-reciprocal plots (Fig. 7) seems to indicate that K^+ is acting as a weak competitive inhibitor and this effect becomes progressively more noticeable at $I > 0.3$ M. The slope re-plots in Fig.7 illustrate that this competitive inhibition is not a simple linear function of K^+ concentration.

In preliminary experiments we discovered that alterations in ionic strength and dielectric constant do not affect the shape of the pH profile or position of the pH optimum sufficiently to contribute appreciably to the rate effects observed.

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