Biochem. J. (1972) 127, 875–879
Printed in Great Britain

Oxidation of *p*-Dimethylaminomethylbenzylamine by Pig Kidney Diamine Oxidase

A NEW METHOD FOR SPECTROPHOTOMETRIC ASSAY

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(*Received* 3 *December* 1971)

1. The oxidation of some *para*-substituted benzylamines by diamine oxidase produces the corresponding aldehydes. This was studied to develop a spectrophotometric method for following the enzyme reaction, as the aldehydes produced absorb strongly at 250nm where the substrates are almost optically transparent. 2. *p*-Dimethylaminomethylbenzylamine was the most useful substrate and full details of its preparation are given. The synthesis of its related oxidation product, *p*-dimethylaminomethylbenzaldehyde, is also described. 3. The effects of variations in pH, ionic strength, temperature and oxygenation on the reaction are described and the usefulness of the method is illustrated by several applications and assessed by comparison with the standard spectrophotometric assay.

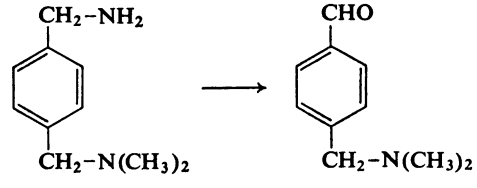
Diamine oxidase (EC 1.4.3.6) oxidizes diamines, producing an aminoaldehyde, ammonia and hydrogen peroxide as follows:

$$H_2N\text{-}[CH_2]_n\text{-}NH_2 + O_2 + H_2O = H_2N\text{-}[CH_2]_{n-1}\text{-}CHO + H_2O_2 + NH_3$$

The reaction has been followed by uptake of oxygen or production of ammonia or hydrogen peroxide (Zeller, 1963). The appearance of the aminoaldehyde can also be measured with putrescine, as the 4-aminobutyraldehyde produced cyclizes spontaneously to give Δ^1-pyrroline, which can be condensed with *o*-aminobenzaldehyde to give 2,3-trimethylene-1,2-dihydroquinazolinium cation with an absorption maximum at 430nm (Holmstedt *et al.*, 1961). If [^{14}C]cadaverine is used, the Δ^1-piperidine can be extracted with toluene and its radioactivity counted (Okuyama & Kobayashi, 1961).

All these methods are time-consuming, of low sensitivity or require expensive materials and we have developed a method of assay that is more convenient than any at present used. This is a development of the standard method for measuring monoamine oxidase activity with benzylamine by measuring the increase in E_{250} caused by the benzaldehyde produced (Tabor *et al.*, 1954).

Diamine oxidase from pig kidney does not oxidize benzylamine (Bardsley *et al.*, 1970), but various substituted benzylamine derivatives have been shown to be oxidized (Bardsley *et al.*, 1971). With *p*-dimethylaminomethylbenzylamine the reaction concerned is:



A linear increase in E_{250} occurs until the substrate is consumed, and then the absorption spectrum of the solution shows a distinct maximum at 250nm typical of aromatic aldehydes.

Materials and Methods

Unless otherwise stated, all materials and methods were as described by Bardsley *et al.* (1971).

Improved method for the synthesis of p-dimethylaminomethylbenzylamine (Bardsley et al., 1971)

Anhydrous dimethylamine (20ml) was added dropwise to a stirred solution of *p*-cyanobenzyl bromide (20g) in anhydrous ether (700ml). The solution was left for 24h at room temperature and then the ether was evaporated off and the residual oil was partitioned between dilute HCl and ether. Basic material (obtained by neutralizing the acid extract with K_2CO_3

and then extracting with ether) was then distilled [b.p. 78–82°C, 6.55–13.1 N/m² (0.05–0.1 mmHg)] to give *p*-dimethylaminomethylbenzonitrile as a clear oil (14.1 g; 86% yield). This was dissolved in sodium-dried benzene (25 ml) and added dropwise over 1 h to 60 ml of a well-stirred and ice-cooled 70% (w/v) solution of sodium bis(2-methoxyethoxy)-aluminium hydride in benzene (Red-al supplied by R. N. Emanuel Ltd., Wembley, Middx., U.K.). After the vigorous reaction had subsided the solution was refluxed for 1 h, then it was cooled in ice and decomposed by cautious addition of 50 ml of 2 M-NaOH. The benzene layer was separated, dried with anhydrous $CaSO_4$ and distilled to give *p*-dimethylaminomethylbenzylamine as a clear oil [b.p. 92–96°C, 6.55–13.1 N/m² (0.05–0.1 mmHg); 12.7 g; 88% yield], which was converted quantitatively into the dihydrochloride (recrystallized from methanol–ethyl acetate, 1:1 by vol.; m.p. 242–244°C).

Synthesis of p-dimethylaminomethylbenzaldehyde

p-Dimethylaminomethylbenzonitrile (34.3 g) was added slowly to an ice-cooled solution of conc. H_2SO_4 (50 ml) in ethanol (200 ml). The solution was refluxed for 5 days and then i.r. spectroscopy of a sample, taken from the reaction mixture and processed, showed that conversion of nitrile into ester was completed, and the solution was concentrated under reduced pressure to a syrup and diluted with water (200 ml). Neutralization and extraction with chloroform gave *ethyl p-dimethylaminomethylbenzoate* as an oil [34.8 g; 88% yield; b.p. 120–124°C, 26.2 N/m² (0.2 mmHg)]. An *oxalate* was prepared (m.p. 147–149°C. Found: C, 56.7; H, 6.6; N, 4.5; $C_{14}H_{19}NO_6$ requires C, 56.6; H, 6.4; N, 4.7%). A solution of ethyl *p*-dimethylaminomethylbenzoate (34 g) in anhydrous ether (100 ml) was added dropwise to lithium aluminium hydride (10 g) in anhydrous ether (300 ml) over 2 h. The solution was refluxed for 1 h, ethyl acetate was added to decompose any excess of reagent, and then a solution of potassium sodium tartarate (100 g) in water (200 ml) was added. The ether layer was evaporated and the residue distilled to give *p-dimethylaminomethylbenzyl alcohol* [23.4 g; 87% yield; b.p. 130–132°C, 39.3 N/m² (0.3 mmHg)]. t-Butyl chromate was prepared by slowly adding CrO_3 (4.0 g) to 2-methylpropan-2-ol (8.0 g) and then adding sodium-dried benzene (75 ml). After being dried with anhydrous Na_2SO_4, the solution was filtered and added dropwise to an ice-cooled solution of *p*-dimethylaminomethylbenzyl alcohol (10 g) in sodium-dried benzene (100 ml). After being left at room temperature for 1 h, the solution was shaken with oxalic acid (5.0 g) in water (50 ml) and then neutralized by adding saturated K_2CO_3 solution, then it was extracted with ether. The ether solution was dried with anhydrous $CaSO_4$ and then was evaporated under reduced pressure, leaving a dark oil (8.7 g), which was dissolved in ethanol (25 ml) and treated with oxalic acid (7.0 g) in ethanol and then ether (25 ml), to give *p-dimethylaminomethylbenzaldehyde hydrogen oxalate*, which was recrystallized from ethanol–ether (1:1, by vol.), (m.p. 106–108°C; 9.9 g; 82% overall yield. Found: C, 56.5; H, 6.2; N, 5.8; $C_{12}H_{15}N_2O_5$ requires C, 56.9; H, 6.0; N, 5.5%). A sample (5 g) of the basic oil, recovered from this oxalate by neutralizing and extracting with benzene, was distilled to give *p-dimethylaminomethylbenzaldehyde* as a clear oil [4.2 g; b.p. 80–82°C, 6.55–13.1 N/m² (0.05–0.1 mmHg)]. This had the i.r. and proton-magnetic-resonance spectra expected of *p*-dimethylaminomethylbenzaldehyde (Found: C, 73.1; H, 8.1; N, 8.9; $C_{10}H_{13}NO$ requires C, 73.6; H, 8.0; N, 8.6%) and had a molar extinction coefficient of 11000 at 250 nm in 0.1 M-potassium phosphate buffer, pH 7.0. It formed a *hydrobromide* that was recrystallized from propan-2-ol–ether (1:1, by vol.) and had m.p. 140–142°C.

Enzyme preparation

This was as described by Bardsley *et al.* (1971) except that the activity was followed by using the spectrophotometric method now described. The specific activity of the purest fraction from the Sephadex G-200 column was 1.73, which is the highest yet recorded for this enzyme. The specific activity of combined material was 1.48 i.u./mg of protein.

Results

Fig. 1 shows the u.v.-absorption spectrum of *p*-dimethylaminomethylbenzaldehyde. It shows a dis-

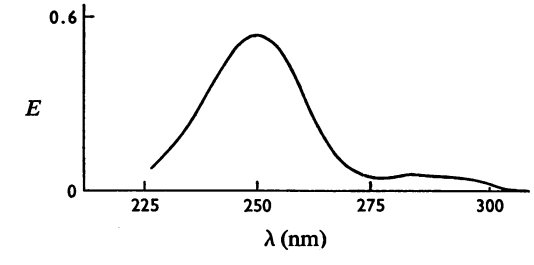


Fig. 1. *Ultraviolet spectrum of p-dimethylaminomethylbenzaldehyde*

The spectrum was taken on a 0.04 mM solution in 0.1 M-potassium phosphate buffer, pH 7.0, in a 1 cm light-path silica cuvette at 20°C. The spectrum showed no appreciable alterations within the range of pH values and ionic strengths used in this study. At this concentration, *p*-dimethylaminomethylbenzylamine gives no detectable absorption spectrum.

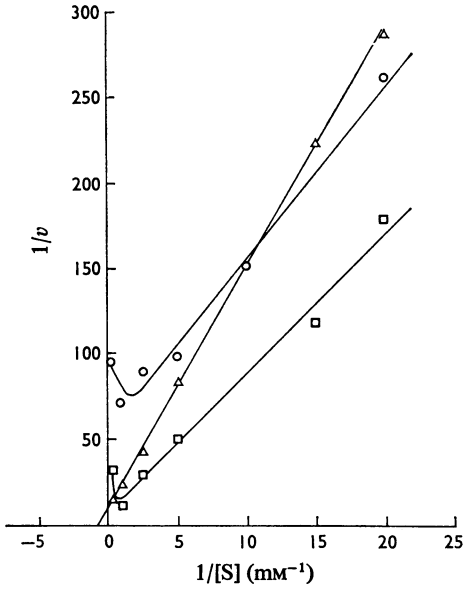


Fig. 2. *Oxidation of benzylamine derivatives*

Oxidation was measured as the change in E_{250}/min (v); the assay mixture contained 0.005 unit of enzyme in 3.0ml of 0.1M-potassium phosphate buffer, pH7.0, at 20°C. Double-reciprocal plots of $1/v$ (ΔE_{250}/min) against 1/[S] (mM^{-1}) were plotted. △, *p*-dimethylaminomethylbenzylamine; ○, *p*-bis(aminomethyl)-benzene (*p*-xylylenediamine); □ *p*-aminomethylbenzyldimethylsulphonium bromide.

tinct maximum at 250nm ($\epsilon = 11000$). Spectra were also obtained by mixing the aldehyde and its corresponding amine, *p*-dimethylaminomethylbenzylamine, and these were directly additive, i.e. no reaction such as Schiff-base formation occurred under these conditions to alter the determination of aldehyde in the presence of amine by simply measuring the change in absorbance (ΔE) at 250nm.

Fig. 2 illustrates the use of several benzylamine derivatives as substrates for spectrophotometric assay procedures. The *p*-aminomethyl- and *p*-dimethyl-sulphonium derivatives of benzylamine show substrate inhibition more strongly than the *p*-dimethylaminomethyl derivative and are therefore less useful. The *S*-(*p*-aminomethylbenzyl)isothiuronium bromide is also oxidized by the enzyme, but does not give a linear change in E_{250} as the reaction proceeds. The 2-, 3- and 4-aminomethylpyridine are too slowly oxidized to be useful. At 20°C and with 0.005 unit of enzyme, the K_m for *p*-dimethylaminomethylbenzylamine was 2.3mM, that for *p*-aminomethylbenzylamine(*p*-xylylenediamine) was 0.14mM,

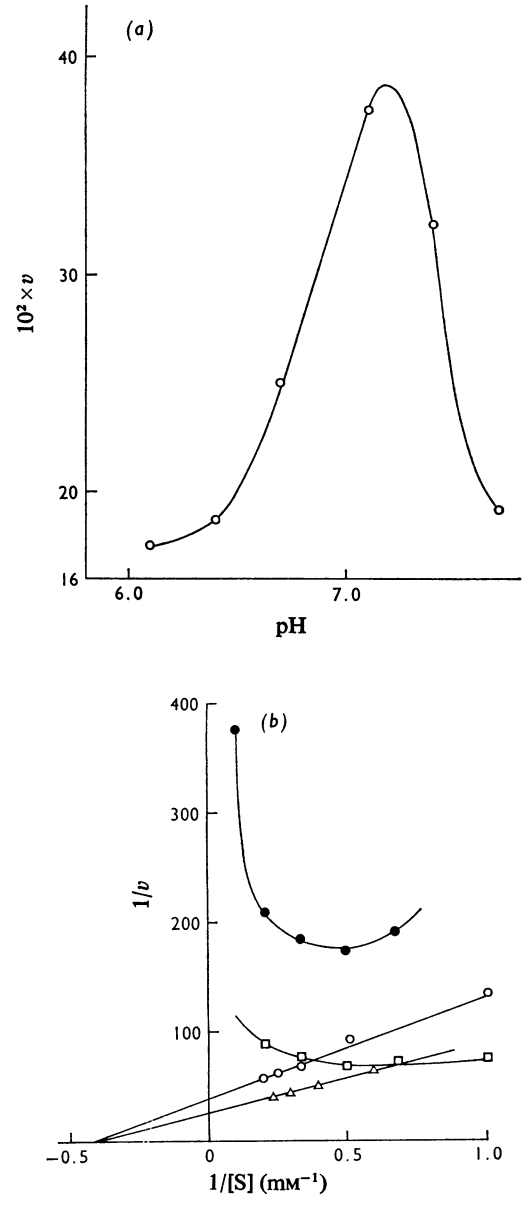


Fig. 3. *Oxidation of p-dimethylaminomethylbenzylamine as a function of pH*

(*a*) Maximum velocity (v) was measured as the change in E_{250}/min for 0.02 unit of enzyme at 20°C. Where substrate inhibition was pronounced the maximum velocity was obtained from plots of $1/v$ against substrate concentration. Otherwise, the values were obtained from double-reciprocal plots, as in (*b*), of $1/v$ (ΔE_{250}^{-1} · min) against 1/[S] (mM^{-1}). Ionic strength remained constant throughout the pH range studied: ○, pH6.7; △, pH7.1; □, pH7.7; ●, pH9.0.

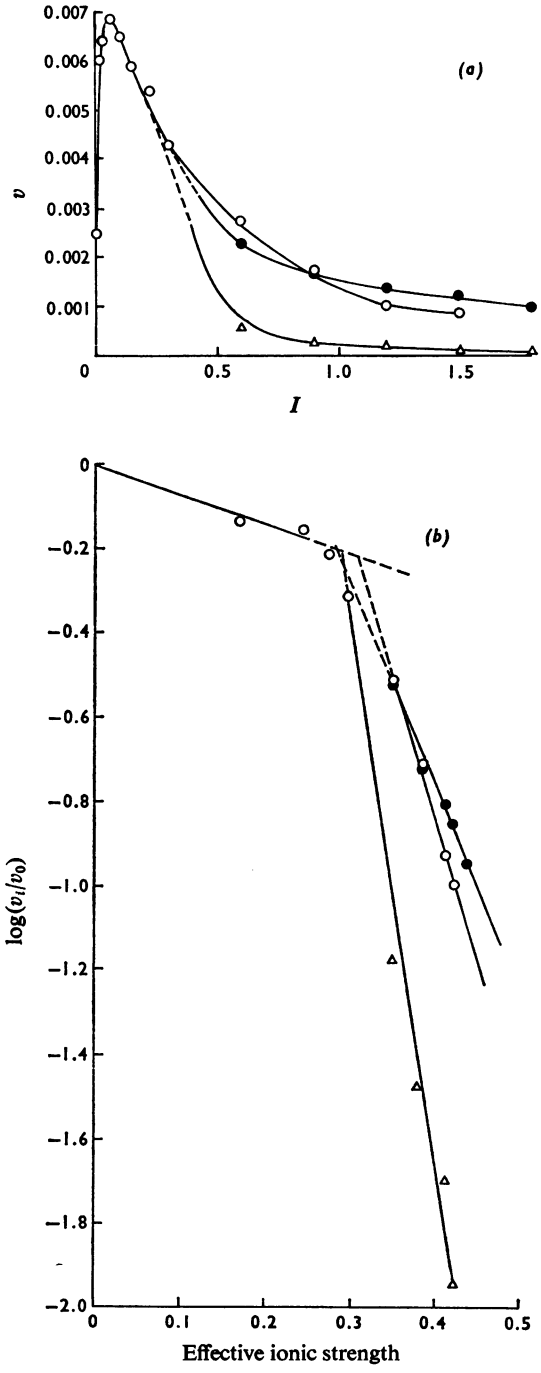


Fig. 4. *Effect of alterations in ionic strength (I) on the oxidation of p-dimethylaminomethylbenzylamine (5mM) by pig kidney diamine oxidase (0.02 unit in a final volume of 3ml) at pH7.4 and 20°C*

(*a*) shows the rate of change of E_{250}/min (v) with alterations in ionic strength (I) produced by (o) mixed potassium phosphates, (●) Na_2SO_4 and (△) $CaCl_2$. (*b*) illustrates the alteration in $\log(v_i/v_0)$ with effective ionic strength, $\sqrt{I}/(1+1.56\sqrt{I})$, where v_i is the initial rate for oxidation of substrate (5mM) at ionic strength I and v_0 is the initial rate at zero ionic strength obtained by extrapolation of a graph of $\log v_i$ against effective ionic strength. o, Mixed potassium phosphates; ●, Na_2SO_4; △, $CaCl_2$.

and that for *p*-aminomethylbenzyldimethylsulphonium bromide was 1.5mM. By using O_2 uptake measured in a Warburg apparatus at 37°C the corresponding values were *p*-dimethylaminomethylbenzylamine ($K_m = 2.0$mM), *p*-aminomethylbenzylamine ($K_m = 0.3$mM) and *p*-aminomethylbenzyldimethylsulphonium bromide ($K_m = 1.2$mM).

Fig. 3(*a*) illustrates the effect of pH on this reaction. There is a distinct optimum pH at pH7.2. Up to pH7.0 there is little evidence of substrate inhibition, but from pH7.4 to 9.0 substrate inhibition becomes progressively more pronounced whether the buffer is borate, glycine or tris. (Fig. 3*b*).

Fig. 4 shows that at low ionic strength (below $I = 0.06$M where $I = \frac{1}{2}\Sigma c_i z_i^2$; c_i is the concentration in mol/litre of ion charge, z_i) the rate of oxidation increases with increase of ionic strength. However, over a large range of ionic strength (0.06–1.8M) the rate of reaction is decreased by increased ionic strength. For univalent cations (K_2HPO_4–KH_2PO_4, Na_2SO_4, KCl and sodium acetate) the decrease in rate was not as marked as for bivalent cations ($MgSO_4$ and $CaCl_2$) (Fig. 4*a*). For reactions between ions in solution the usual Brönsted primary kinetic salt-effect theory predicts that the rate constant (k) for the reaction is related to the rate constant for the same reaction at zero ionic strength (k_0) by the expression

$$\log k = \log k_0 + 1.02\, z_a z_b \frac{\sqrt{I}}{1+1.56\sqrt{I}}$$

where z_a is the charge on ion a and z_b the charge of ion b. As it has been suggested that the diamine oxidase reaction involves the interaction between a positively charged substrate and a negatively charged enzyme (Bardsley *et al.*, 1971) and as the decrease of the reaction with increasing ionic strength is of an exponential form, it seemed reasonable to plot $\log(v_i/v_0)$ against the effective ionic strength $\sqrt{I}/(1+1.56\sqrt{I})$, where v_0 is the rate at zero ionic strength and v_i is the rate at ionic strength I. For a simple reaction between a positive and negative ion the slope of this graph would be −1. Fig. 4(*b*) illustrates that at an effective ionic strength of 0.3 the slope of the line suddenly increases (from −0.7 to −6.8 for potassium phosphate). This seems to indicate that

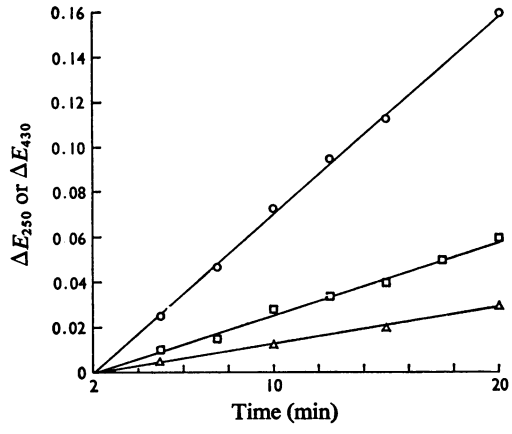


Fig. 5. *Oxidation of p-dimethylaminomethylbenzylamine compared with the o-aminobenzaldehyde method for spectrophotometric assay*

Change of E_{250} with time in 3ml of 0.1M-phosphate buffer, pH7.0, containing 0.005 enzyme unit and a final concentration of 3.0mM-*p*-dimethylaminomethylbenzylamine at 37°C (○) and 20°C (□). The *o*-aminobenzaldehyde method under the same conditions was measured at 430nm (△) and 37°C with 3.0mM putrescine and 3.0mM *o*-aminobenzaldehyde.

over a limiting range the Brönsted theory applies and the interaction between negatively charged enzyme and positively charged substrate is substantiated, but that at high ionic strength (above 0.3M) other factors operate and the result is difficult to interpret.

Fig. 5 shows the linear increase in E_{250} when the enzyme (0.005 unit) and substrate (*p*-dimethylaminomethylbenzylamine, 3.0mM) are incubated at 37°C and 20°C and when the enzyme reacts with putrescine (3.0mM) and *o*-aminobenzylaldehyde (3.0mM). Under these conditions the direct spectrophotometric method is considerably more sensitive than the standard *o*-aminobenzaldehyde method. Shaking the reaction mixture to increase oxygenation had no effect on the rate of the reaction.

Discussion

It seems that *p*-dimethylaminomethylbenzylamine is the best substrate to use for the spectrophotometric assay of diamine oxidase, as it is easy to prepare, shows little substrate inhibition at pH7.0 (Fig. 2) and gives, on oxidation, an aldehyde with known molar extinction coefficient at 250nm that has now been synthesized. The effect of pH is straightforward and the effect of high ionic strength seems to substantiate the conclusion, reached from other considerations, that the reaction between substrate and enzyme involves the interaction between oppositely charged ionic species (Bardsley *et al.*, 1971).

It would appear that a reasonable range of reaction conditions would be pH6.8–7.4 and ionic strength 0.04–0.35M, with optimum values pH7.2 and ionic strength 0.06M. Under these conditions a unit of enzyme is defined as that amount of enzyme causing a change in E_{250} of 3.67 in 1min when dissolved in 3.0ml of buffer containing a suitable excess of substrate at 37°C (i.e. 1μmol of substrate oxidized/min). At 20°C the corresponding change in E_{250} would be 1.36/min.

We thank Professor W. I. C. Morris of the Department of Obstetrics and Gynaecology, Manchester University, for providing laboratory facilities, T. Wall and Sons (Meat and Handy Foods) Ltd., Godley, Hyde, Ches., U.K., for supplying fresh pig kidneys and Mr. M. Hart and the staff of the Analytical Chemistry Section, Department of Chemistry, University of Manchester, for determinations of elementary analyses.

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