Oxidation of \( p \)-Dimethylaminomethylbenzylamine by Pig Kidney Diamine Oxidase

A NEW METHOD FOR SPECTROPHOTOMETRIC ASSAY

By W. G. BARD SLEY, M. J. C. CRABBE and J. S. SHINDLER

Department of Biochemistry, University of Manchester Institute of Science and Technology, Manchester M60 1QD, U.K.

and J. S. ASHFORD

Department of Obstetrics and Gynaecology, University of Manchester, St. Mary's Hospital, Manchester M13 0JH, U.K.

(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 250 nm 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 aminoa ldehyde, ammonia and hydrogen peroxide as follows:

\[
\begin{align*}
H_2N-[\text{CH}_2]_n-\text{NH}_2 + O_2 + H_2O &= \rightarrow \\
H_2N-[\text{CH}_2]_n-\text{CHO} + H_2O_2 + NH_3
\end{align*}
\]

The reaction has been followed by uptake of oxygen or production of ammonia or hydrogen peroxide (Zeller, 1963). The appearance of the amino aldehyde can also be measured with putrescine, as the 4-aminobutyraldehyde produced cyclizes spontaneously to give \( \Delta^1 \)-pyrroline, which can be condensed with o-aminobenzaldehyde to give 2,3-trimethylene-1,2-dihydroquinazolinium cation with an absorption maximum at 430 nm (Holmstedt et al., 1961). If \( [^{14}C] \) cadaverine is used, the \( \Delta^1 \)-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:

\[
\text{CH}_2-N(\text{CH}_3)_2 + \text{CHO} \rightarrow \text{CH}_2-N(\text{CH}_3)_2\text{CHO}
\]

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 250 nm 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 (20 ml) was added drop-wise to a stirred solution of \( p \)-cyanobenzyl bromide (20 g) in anhydrous ether (700 ml). The solution was left for 24 h 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 \( \text{K}_2\text{CO}_3 \)
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 78–82°C, and verted quantitatively hydride in amine crystallized (0.05–0.1 mmHg). After refluxing was processed, showed added 78–82°C, chloroform was added under reduced water as anate sample, H₂SO₄ (m.p. 147–149°C. (0.2 mmHg)]. Ether (300 ml) was added to anhydrous aluminium hydride with ether. The residue was collected and then recrystallized from water-ether (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. H₂SO₄ (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-dimethylaminomethylenzoate 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; C₁₂H₁₅NO₄ 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 tartrate (100 g) in water (200 ml) was added. The ether layer was evaporated and the residue distilled to give p-dimethylaminomethylbenzal 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 CrO₃ (4.0 g) to 2-methylpropan-2-ol (8.0 g) and then adding sodium-dried benzene (75 ml). After being dried with anhydrous Na₂SO₄, 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 K₂CO₃ solution, then it was extracted with ether. The ether solution was dried with anhydrous CaSO₄ 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; C₁₂H₁₅N₂O₅ 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; C₁₀H₁₃NO 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](image)

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.
Oxidation was measured as the change in $E_{250}/\text{min}$ (o); the assay mixture contained 0.005 unit of enzyme in 3.0 ml of 0.1 M potassium phosphate buffer, pH 7.0, at 20°C. Double-reciprocal plots of $1/v$ ($\Delta E_{250}/\text{min}$) against $1/[S]$ (mM$^{-1}$) were plotted. Δ, p-dimethylaminomethylbenzylamine; ○, p-bis(aminomethyl)benzene (p-xylylenediamine); ⊘ p-aminomethylbenzylidimethylsulphonium bromide.

Distinct maximum at 250 nm ($\varepsilon = 11000$). 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 ($\Delta E$) at 250 nm.

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 2.3 mM, that for p-aminomethylbenzylamine (p-xylylenediamine) was 0.14 mM.

Vol. 127
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 E250/min (v) with alterations in ionic strength (I) produced by (○) and that for p-aminomethylbenzylidimethylsulphonium bromide was 1.5mM. By using O2 uptake measured in a Warburg apparatus at 37°C the corresponding values were p-dimethylaminomethylbenzylamine (Km = 2.0mM), p-aminomethylbenzylamine (Km = 0.3mM) and p-aminomethylbenzylidimethylsulphonium bromide (Km = 1.2mM).

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. 3b).

Fig. 4 shows that at low ionic strength (below I = 0.06M where I = ZΣciz; cI is the concentration in mol/litre of ion charge, zi) 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 (K2HPO4–KH2PO4, Na2SO4, KCl and sodium acetate) the decrease in rate was not as marked as for bivalent cations (MgSO4 and CaCl2) (Fig. 4a). For reactions between ions in solution the usual Brønsted 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 (k0) by the expression

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

where za is the charge on ion a and zb 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(v0/v0) against the effective ionic strength \(\sqrt{I}/(1+1.56\sqrt{I})\), where v0 is the rate at zero ionic strength and v1 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

mixed potassium phosphates, (●) Na2SO4 and (△) CaCl2. (b) illustrates the alteration in log(v0/v0) with effective ionic strength, \(\sqrt{I}/(1+1.56\sqrt{I})\), where v1 is the initial rate for oxidation of substrate (5mM) at ionic strength I and v0 is the initial rate at zero ionic strength obtained by extrapolation of a graph of log(v0) against effective ionic strength. ○, Mixed potassium phosphates; ●, Na2SO4; △, CaCl2.
DIAMINE OXIDASE OXIDATIONS

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

Change of $E_{250}$ with time in 3 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.005 enzyme unit and a final concentration of 3.0 mM p-dimethylaminomethylbenzylamine at 37°C (○) and 20°C (□). The o-aminobenzaldehyde method under the same conditions was measured at 430 nm (∆) and 37°C with 3.0 mM putrescine and 3.0 mM o-aminobenzaldehyde.

over a limiting range the Brønsted theory applies and the interaction between negatively charged enzyme and positively charged substrate is substantiated, but that at high ionic strength (above 0.3 M) 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, 3.0 mM) are incubated at 37°C and 20°C and when the enzyme reacts with putrescine (3.0 mM) and o-aminobenzaldehyde (3.0 mM). 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 pH 7.0 (Fig. 2) and gives, on oxidation, an aldehyde with known molar extinction coefficient at 250 nm 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 pH 6.8–7.4 and ionic strength 0.04–0.35 M, with optimum values pH 7.2 and ionic strength 0.06 M. Under these conditions a unit of enzyme is defined as that amount of enzyme causing a change in $E_{250}$ of 3.67 in 1 min when dissolved in 3.0 ml of buffer containing a suitable excess of substrate at 37°C (i.e. 1 μmol 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, Cheshire, 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.

References