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Microbial Oxidation of Amines

SPECTRAL AND KINETIC PROPERTIES OF THE PRIMARY AMINE DEHYDROGENASE OF *PSEUDOMONAS* AM1

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1. An improved procedure is reported for purification of the amine dehydrogenase from methylamine-grown *Pseudomonas* AM1 which yielded a product homogeneous by sedimentation and disc-electrophoretic analysis, with molecular weight of 133000. 2. The purified enzyme had absorption maxima at 280 and 430nm. On aging, a third peak appeared at 325nm, and the 430nm peak decreased in intensity. This spectrum was independent of pH. 3. Addition of 2.5mM-semicarbazide, phenylhydrazine, hydrazine or hydroxylamine produced modified spectra with maxima respectively at 400, 440, 395 and 425nm. 4. Aerobic addition of methylamine resulted in a bleaching of the 430nm peak and the appearance of a new one at 325nm. This spectral change was retained after removal of the methylamine by dialysis. The original spectrum could be restored on addition of phenazine methosulphate. 5. Addition of borohydride partially inactivated the enzyme and produced spectral changes similar to those observed with methylamine. Pre-treatment with methylamine prevented the inactivation by borohydride. The degree of inactivation could be increased by alternate phenazine methosulphate and borohydride treatments. 6. The addition of methylamine or borohydride each caused shifts in the fluorescence emission maximum from 348 to 380nm. 7. Lineweaver–Burk plots of reciprocal activity against reciprocal concentration of either of the substrates *n*-butylamine or phenazine methosulphate were consistent with a mechanism that involves interconversion of two free forms of the enzyme by the two substrates. 8. The enzyme, although spectrally modified, was not inactivated by dialysis against diethyldithiocarbamate, and contained about 0.27 g-atom of copper/mol, with small traces of cobalt, iron and zinc. 9. Conventional methods of resolution did not release the prosthetic group. Heat denaturation after treatment of the enzyme with methylamine liberated a yellow chromophore which did not reactivate resolved aspartate aminotransferase, and whose spectral, electrophoretic and fluorescence properties did not agree with any recognizable pyridoxal derivatives. 10. Despite the inconclusive results with the isolated chromophore, the observations on the enzyme suggest that it may contain a pyridoxal derivative bound as a Schiff's base which is converted into the pyridoxamine form on aerobic treatment with methylamine and reconverted into the pyridoxal form with phenazine methosulphate. 11. The copper detected is probably not involved in the enzyme mechanism, since most copper-chelating agents are not inhibitory, and since the enzyme does not react with oxygen.

When *Pseudomonas* AM1 grows on methylamine hydrochloride as sole carbon source, it contains an amine dehydrogenase [amine: (acceptor) oxidoreductase (deaminating)] which oxidizes methylamine to formaldehyde and ammonia by using a variety of dyes as electron acceptor, of which the best is *N*-methylphenazonium methosulphate. The dehydrogenase will not react with oxygen. It was partially purified by Eady & Large (1968) and some of its properties were described. Except for its extreme heat stability and its lack of reactivity

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with oxygen, the enzyme resembled in many ways the group of enzymes known as diamine oxidases (EC 1.4.3.6), which contain copper and pyridoxal phosphate as their prosthetic groups.

The present paper describes an improved purification procedure for the enzyme and presents indirect evidence which suggests that it may contain a pyridoxal derivative as its prosthetic group, but it was not possible to demonstrate this conclusively. Although copper has been detected in the purified enzyme, the amount present (0.2–0.3 g-atom/mol of enzyme) is probably insufficient for it to be functionally significant. A preliminary report of part of this work has appeared (Eady & Large, 1969).

MATERIALS AND METHODS

Maintenance and growth of the organism. These have been described previously (Eady & Large, 1968).

Enzyme assay. The amine dehydrogenase was assayed spectrophotometrically by measuring the phenazine methosulphate-dependent reduction of 2,6-dichlorophenol-indophenol as described previously (Eady & Large, 1968).

Purification of the enzyme. Considerable modifications have been made to the earlier procedure, which have improved both the yield and the purity obtained. Because of its extreme heat stability, low temperatures during the purification of the enzyme are not necessary. The enzyme will withstand 15 min at 80°C without loss of activity (Eady & Large, 1968). The heat stability of the enzyme was not affected by protein concentration over the range 2–27 mg of protein/ml, but at a concentration of 0.4 mg/ml, the activity was decreased by 37% by 20 min treatment at 80°C.

Step 1. Preparation of crude extract. Methylamine-grown cells (82 g wet wt.) were suspended in 200 ml of ice-cold 0.1 M-phosphate buffer, pH 7.5, and disrupted by treatment for 4 min with a Dawe Soniprobe 100 W disintegrator (type 1130A, Dawe Instruments Ltd., London W.3, U.K.) operating at full output. During this time a salt–ice mixture was used to keep the temperature below 8°C. The resulting suspension was centrifuged at 14000*g* for 40 min at 2°C. The supernatant was decanted and the pellet resuspended in 200 ml of ice-cold 0.1 M-phosphate buffer, pH 7.5, and treated as before. The supernatants were combined.

Step 2. Heat treatment. The combined supernatant from step 1 was heated rapidly to 75°C in an aluminium container, and maintained at this temperature for exactly 20 min. During this time the suspension was stirred with a mechanical stirrer, but was not allowed to froth. The suspension was then cooled to 2°C and the denatured protein sedimented at 30000*g* for 10 min. The pellet was washed by resuspending in 60 ml of 0.1 M-phosphate buffer, pH 7.5, and recentrifugation. The supernatants from these centrifugations were combined.

Step 3. $(NH_4)_2SO_4$ fractionation. The supernatant from step 2 was made 55% saturated with $(NH_4)_2SO_4$ by the slow addition of solid $(NH_4)_2SO_4$. During the addition the suspension was maintained at 5°C and stirred without frothing with a mechanical stirrer. After standing for 1 h, the precipitated protein was centrifuged off and discarded. The supernatant was then made 85% saturated by addition of further $(NH_4)_2SO_4$. The resulting precipitate, after equilibration as before, was centrifuged, redissolved in 5 mM-phosphate buffer, pH 7.5, and dialysed overnight against 5 litres of the same buffer before assay for activity and protein.

Step 4. Chromatography on DEAE-cellulose. The dialysed precipitate from step 3 was equilibrated with 7.5 mM-phosphate buffer, pH 6.5, by dialysis and then applied to a column (2.8 cm × 30 cm) of microgranular DEAE-cellulose (Whatman DE 32) which had been equilibrated with the same buffer. The column was then eluted at 22°C with a linear gradient of sodium–potassium phosphate buffer, pH 6.5. The gradient used was 7.5–500 mM-phosphate and the total volume was 400 ml. The activity was completely eluted between 10 mM- and 40 mM-phosphate. An activity and protein elution profile for a typical column is shown in Fig. 1(*a*).

Step 5. Chromatography on carboxymethyl-Bio-Gel. The active fractions from step 4 were combined and dialysed against 10 litres of 5 mM-acetate buffer, pH 4.6. The dialysed material was applied to a column (2.8 cm × 32 cm) of carboxymethylated polyacrylamide, Bio-Gel CM-30 (low capacity), equilibrated with the same buffer. The column was eluted at 22°C with a linear gradient of KCl from 0 to 200 mM in 5 mM-acetate buffer, pH 4.6: the gradient volume was 400 ml. The active fractions were associated with a dark-green band. A typical elution profile is shown in Fig. 1(*b*).

Step 6. Gel filtration on Sephadex G-200. The active fractions from step 5 were combined and concentrated by rotary evaporation at 55°C under reduced pressure until the volume was decreased to about 25 ml. The concentrated material was applied to a column (2.8 cm × 60 cm) of Sephadex G-200 equilibrated with 50 mM-phosphate buffer, pH 7.5. The column was developed with this buffer, a typical elution profile being shown in Fig. 1(*c*). At this stage, the purity of the preparation was investigated by disc electrophoresis at pH 9.5. It was occasionally necessary to repeat this step to obtain a homogeneous preparation. In this case the enzyme was reconcentrated by rotary evaporation at 55°C and reapplied to the column. A typical purification achieved by this procedure is summarized in Table 1.

Columns were eluted with buffers propelled by an LKB ReCyChrom peristaltic pump (type 4912A) (LKB Produkter A.B., Stockholm-Bromma 1, Sweden) and fractions collected with a Gilson Mini Escargot (model MTDC) fraction collector operating on a drop-counting basis, with protein concentration in the eluate monitored at 254 nm with an LKB Uvicord and recorder as described previously (Eady & Large, 1968). In later experiments the eluates were monitored at 265 nm with a Gilson 265 IF u.v. absorption meter (W. G. Flaig and Sons, Broadstairs, Kent, U.K.) and recorded on a Rustrak recorder [model (88) 146, Gulton Industries (Britain) Ltd., Sheffield 1, U.K.].

Spectrophotometric measurements. Enzyme assays were performed at 20°C in a Unicam SP. 800 recording spectrophotometer with SP. 820 constant-wavelength scan unit. Absorption spectra were determined on the same instrument by using the slow scan speed or manually on a Unicam SP. 500 instrument by using silica microcuvettes (1 cm light-path length, 0.4 ml capacity).

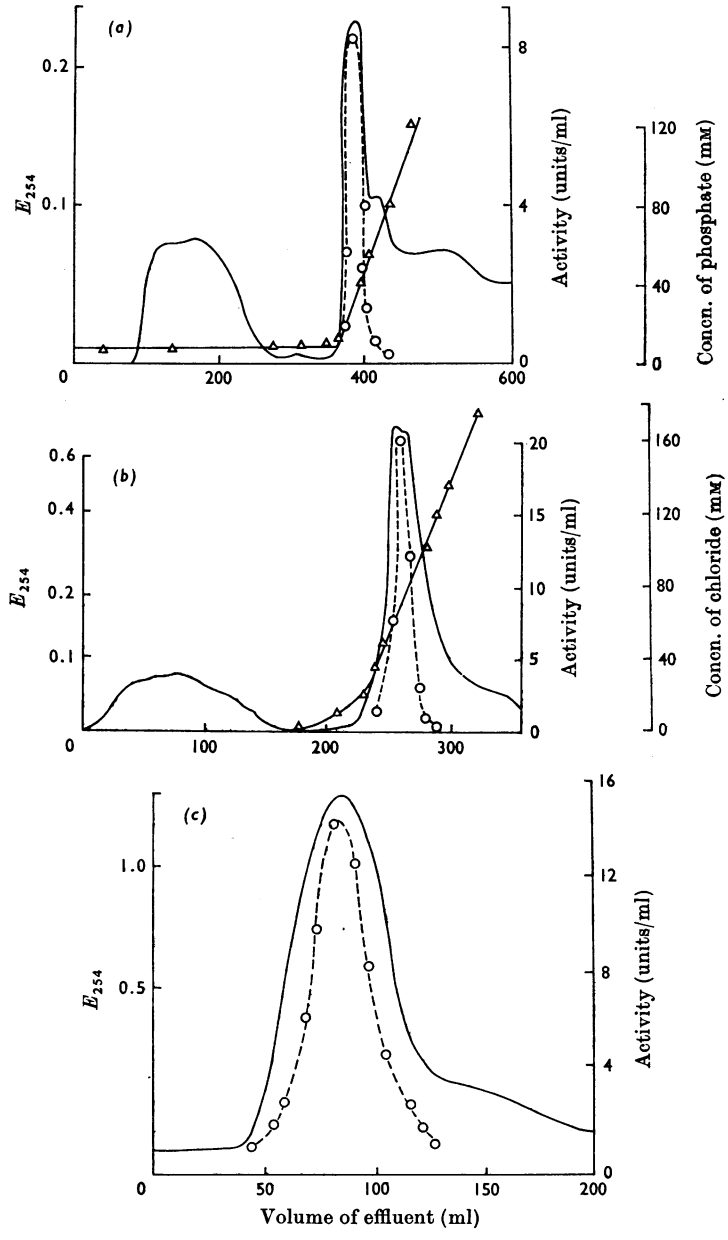


Fig. 1. Purification of *Pseudomonas* AM1 amine dehydrogenase. In all three graphs, the continuous line (——) is a trace from an LKB Uvicord recorder showing transmission at 254nm. The left-hand ordinate scales have been corrected to show extinction for a 1cm light-path. ----, Enzyme activity. (*a*) Chromatography on DEAE-cellulose: △, gradient of phosphate concentration; (*b*) chromatography on Bio-Gel CM-30: △, gradient of chloride concentration. (*c*) Gel filtration on Sephadex G-200. For further details, see the Materials and Methods section.

Fluorescence spectra were measured on an Aminco–Bowman spectrophotofluorimeter fitted with a 100W xenon lamp and an *X–Y* recorder type 1620-827. Spectra, which are uncorrected, were determined at room temperature (20°C) by using slit arrangement no. 3.

Ultracentrifuge measurements. The molecular weight was determined by the method of Yphantis (1964), by using an MSE Analytical Ultracentrifuge. Enzyme (0.1mg of protein/ml in 5mM-sodium–potassium phosphate buffer containing 0.5% NaCl) was centrifuged at

Table 1. *Purification of the amine dehydrogenase from* Pseudomonas *AM*1

Step no.	Fraction	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Purification factor	Yield (%)
1	Supernatant obtained from crude extract after centrifugation	590	845	7560	0.112	1	100
2	Supernatant after removal of heat-denatured protein	590	845	3900	0.216	1.93	100
3	Fraction precipitated by 55–85% satd. $(NH_4)_2SO_4$, redissolved and dialysed	87	695	740	0.94	8.4	82
4	DEAE-cellulose effluent (fractions combined and concentrated)	83	665	464	1.43	12.8	79
5	CM-Bio-Gel effluent (fractions combined and concentrated)	27	615	226	2.72	24.3	73
6	Sephadex G-200 effluent (fractions combined and concentrated)	12.5	537	153	3.51	31.3	63.5
7	Step 6 repeated	8.8	460	119	3.86	34.5	54.5

12000rev./min at 20°C. Measurements were made with an interference optical system.

Chemical determinations. (*a*) Protein. This was measured by the Folin–Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951) by using crystalline bovine serum albumin as standard. An extinction at 280nm of 1.0 (1cm light-path) with the homogeneous amine dehydrogenase was equivalent to 1.18mg of protein/ml expressed on this basis.

(*b*) Phosphate. This was determined by the method of Allen (1940).

(*c*) Chloride. This was determined by titration of samples with 10mM-$AgNO_3$ with dichlorofluorescein as indicator.

(*d*) Copper. This was determined on the digested enzyme (see below) by using 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline (bathocuproine) disulphonate in the form of the Boehringer test combination TC-CU for the determination of serum copper [Boehringer Corp. (London) Ltd., London W.5, U.K.]. The kit was used as a source of standard copper solution containing 2μg of copper/ml and copper reagent containing 0.53mM-bathocuproine disulphonate, 3.3M-acetate, 0.1M-$Na_2S_2O_5$ and 16mM-*p*-methylaminophenol. Extinctions were read at 450nm.

Neutron-activation analysis. Samples were passed through a Sephadex G-15 column equilibrated with 5mM-ammonium acetate, pH7.5, and dried under vacuum. Metal analysis by neutron activation and γ-spectrometry was performed by Mr P. J. Hewitt, School of Nuclear Physics, University of Bradford, by an unpublished method using germanium–lithium solid-state detectors, which avoids chemical separation of the radioactive species.

Disc electrophoresis. This was performed in a Shandon apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by using the pH9.5 buffer system of Davis (1964) or the pH4.3 buffer system of Reisfeld, Lewis & Williams (1962). Gels were stained with 1% (w/v) Naphthalene Black 10B in 7% (v/v) acetic acid, and destained electrolytically. Enzyme activity was located by incubating unfixed gels at 40°C in a solution containing 0.05% *p*-Nitro Blue Tetrazolium, 10mM-methylamine hydrochloride and 50mM-phosphate buffer, pH7.5. A deep-blue band of formazan denoted enzyme activity.

Digestion of enzyme for copper determination. Glass-distilled water was deionized by using an Elgastat B102 deionizer (Elga Products Ltd., Lane End, Bucks., U.K.) and this water was used to prepare all solutions and to rinse glassware. Glassware was cleaned by boiling in 40% (v/v) HNO_3 A.R. and then rinsed exhaustively in deionized water. The micro-Kjeldahl digestion flasks were first cleaned by a dummy digestion procedure, and thereafter by boiling in 40% (v/v) HNO_3. The enzyme solution (1ml) was passed through a small (1cm×10cm) column of Sephadex G-15 equilibrated with 5mM-phosphate buffer, pH7.5, to remove any contaminating copper ions. A sample was then removed for protein determination, and the rest digested with 0.1ml of conc. H_2SO_4. These were heated together in a micro-Kjeldahl flask until charring occurred. The flask was then cooled and two drops of 100vol. H_2O_2 were added and the flask was heated until fumes were evolved. The flask was then cooled and 1ml of water added. It was then treated once more until fumes were observed, and if charring was noted, more H_2O_2 was added and the procedure repeated until the solution was clear at this stage. When the digest was clear, the flask was cooled, 0.5ml of 0.1M-potassium metabisulphite, 0.6ml of water, 0.8ml of 5M-sodium acetate solution and 1.0ml of the copper reagent (see above) was added, giving a final volume of 3ml. Samples were read against a blank consisting of non-protein-containing column eluate which had been put through the digestion procedure. Standards containing 2μg of copper were simultaneously put through the procedure. Recovery experiments showed that 2μg of copper could be recovered in 95% yield after digestion with 10mg of bovine serum albumin.

Preparation of the chromophore. Purified enzyme (0.5ml) containing 6.5mg of protein in 50mM-phosphate buffer, pH6.5, was incubated for 30min with 1.6mM-methylamine hydrochloride. It was then dialysed against three changes of 1 litre of 5mM-phosphate buffer, pH6.8, to remove excess of methylamine. At this stage the enzyme had an absorption spectrum typical of the substrate-modified enzyme (Fig. 4*a*). It was then heat-denatured in a boiling-water bath for 5min in a tube covered with aluminium foil to protect the contents from light. The precipitate of denatured protein was centrifuged and discarded. To remove any unprecipitated protein, the preparation was applied to a column (1cm×20cm) of Sephadex G-15, equilibrated with 5mM-phosphate buffer, pH6.8, and the column washed with the same buffer. The effluent was monitored at 280nm, and after a small wash-through of high-molecular-weight material, the yellow material was eluted. The yellow fractions were concentrated by evaporation under a stream of N_2.

Reactivation of resolved aspartate aminotransferase. Aspartate aminotransferase (EC 2.6.1.1) [Boehringer Corporation (London) Ltd.] was assayed as described by Bergmeyer & Bernt (1963), except that phosphate buffer was replaced by *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonate, since phosphate inhibits the combination of the apoaminotransferase with pyridoxal phosphate (Banks, Lawrence, Vernon & Wootton, 1963). The aminotransferase was resolved as described by Scardi, Scotto, Iaccarino & Scarano (1963), and the cofactor activity of the isolated chromophore tested by measuring the reactivation of the resolved material: 2nmol of authentic pyridoxal phosphate was active in this assay.

Buffers. Sodium–potassium phosphate, tris–HCl, sodium acetate–acetic acid and glycine–NaOH buffers were prepared as described by Dawson, Elliott, Elliott & Jones (1969). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid was adjusted to pH7.4 with 2M-NaOH.

Special chemicals. Phenazine methosulphate was obtained from Sigma (London) Chemical Co., London S.W.6, U.K.; 2,6-dichlorophenol-indophenol was an Eastman product from Kodak Ltd., Kirkby, Lancs., U.K.; Bio-Gel CM-30 was from Calbiochem Ltd., London W1H 1AS, U.K.; DEAE-cellulose was from W. & R. Balston Ltd., Maidstone, Kent, U.K. and Sephadex G-200 was from Pharmacia (G.B.) Ltd., London W.5, U.K. All other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.

RESULTS

Purity of the enzyme preparation. If the purified enzyme is 100% pure, then the results in Table 1 indicate that the amine dehydrogenase constitutes 2.9% of the total soluble protein of the organism. The enzyme was considered to be homogeneous on the basis of the following criteria.

(*a*) Sedimentation behaviour. A 0.55% (w/v) solution of the amine dehydrogenase sedimented at 55000rev./min as a single boundary in the analytical ultracentrifuge with a symmetrical schlieren peak at three different pH values: 4.2 (0.1M-acetate buffer), 7.5 (0.1M-phosphate buffer) and 10.6 (50mM-glycine–NaOH buffer and 50mM-tris–HCl buffer).

(*b*) Disc electrophoresis. Purified enzyme (100μg) gave a single band of protein when subjected to electrophoresis at pH4.3, and this band coincided with the activity. At pH9.5, a variable number of active bands (up to 5) could be distinguished, but no inactive protein was detected. The cause of this multiple-band formation is probably not dissociation into subunits, since the results of sedimentation experiments over the pH range 4.2–10.6 suggest that large-scale dissociation into species with lower molecular weights in the buffers used in the ultracentrifuge does not occur.

Molecular weight. This was determined in the ultracentrifuge by the Yphantis (1964) method. By assuming a partial specific volume of 0.72cm^{3}g^{-1}, a value of 133000 was obtained.

Absorption spectrum of the enzyme in relation to its activity

Solutions of the enzyme were greenish yellow in colour, having an absorption maximum in the visible range at 430nm. The ratio of extinction at 430nm to that at 280nm was 0.1 in the freshly purified enzyme (Fig. 2, curve *a*). Aging of the enzyme preparation and repeated freezing and thawing resulted in the appearance of a new peak in the absorption spectrum at 325nm, in addition to the 430nm peak (Fig. 2, curve *b*). The aged enzyme preparation retained full activity and could be reconverted into the original spectral form by treatment first with methylamine and then

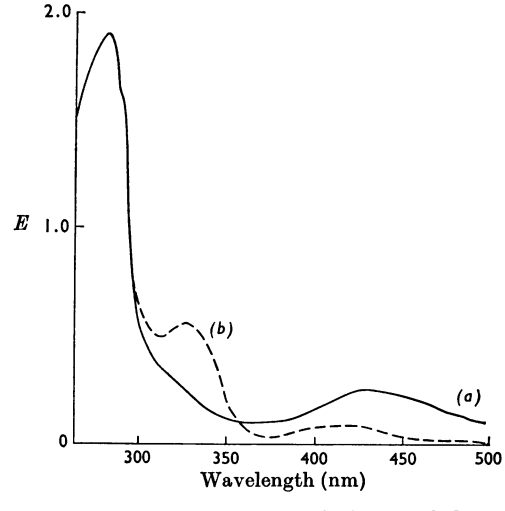


Fig. 2. Absorption spectrum of the purified amine dehydrogenase (2mg of protein/ml in 50mM-phosphate buffer, pH7.5). (*a*) Freshly purified enzyme. (*b*) Aged enzyme.

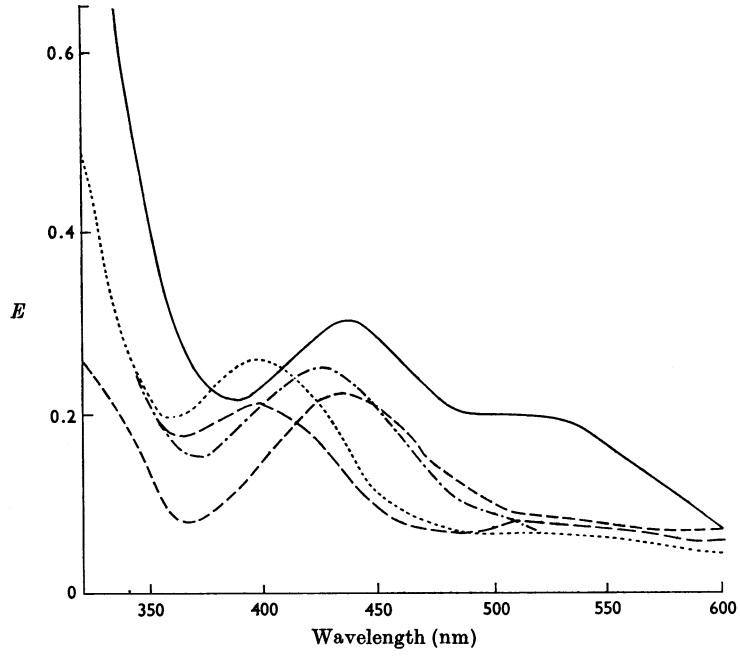


Fig. 3. Absorption spectra of derivatives of the purified amine dehydrogenase (1.28mg of protein/ml), after addition of carbonyl reagents to a final concentration of 2.5mM. The spectra were determined after 30min treatment with reagent, and without dialysis, against the appropriate reagent blank. ——, Phenylhydrazine hydrochloride; ····, semicarbazide hydrochloride; -·-·-, hydroxylamine hydrochloride; ———, hydrazine sulphate; ----, untreated enzyme.

with phenazine methosulphate (see below). Alterations in the spectrum could be produced by the addition of substrate, by carbonyl reagents which inhibit the enzyme, or by reduction of the enzyme with borohydride. These effects (see below) suggest that the chromophore has a role in the enzyme activity.

Effect of pH. Adjustment of the pH of the enzyme (in 50mM-phosphate buffer) with 0.1M-hydrochloric acid or 0.1M-sodium hydroxide to various values in the range 2.5–9.7 resulted in only negligible change in the spectrum. In tris–glycine buffer, pH9.5, however, there developed a broad double peak with maxima at 430 and 470nm. On dialysis against phosphate buffer, pH7.5, the spectrum reverted to normal.

Effect of carbonyl reagents. Carbonyl reagents, which are potent inhibitors of the enzyme (Eady & Large, 1968), produced significant changes in the absorption spectrum (Fig. 3). The spectra of the derivatives formed when the enzyme was treated with carbonyl reagents (at final concentrations of 2.5mM) had the following maxima: semicarbazide 400nm, phenylhydrazine 440nm, hydrazine 395nm and hydroxylamine 425nm. The enzyme treated with 2.5mM-semicarbazide hydrochloride had lost 50% of its activity when compared with untreated enzyme. Treatment with an excess of methylamine hydrochloride (30mM) did not alter

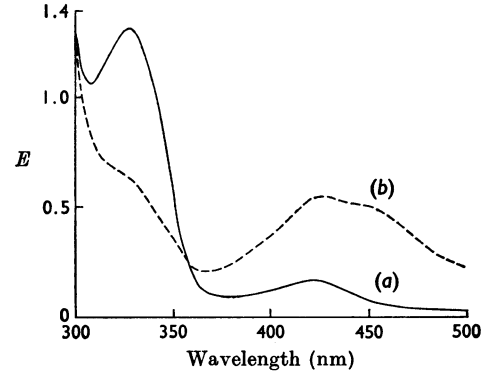


Fig. 4. Spectral changes of the methylamine-treated amine dehydrogenase on the addition of phenazine methosulphate. (*a*) Methylamine-treated enzyme dialysed against 5mM-phosphate buffer, pH7.5 (6mg of protein/ml). (*b*) Sample *a* 18min after addition of phenazine methosulphate to a final concentration of 19μM in both test and blank curvettes.

the spectral change produced by semicarbazide. Dialysis of the semicarbazide-treated enzyme against 10mM-methylamine did not cause any alteration in the spectrum, which retained its 400nm maximum, nor did dialysis reverse the inhibition. Pre-treatment of the enzyme with methylamine (followed by dialysis) led to slightly less inactivation when semicarbazide was added.

Effect of methylamine and phenazine methosulphate. The aerobic addition of methylamine at a final concentration of 25μM to the enzyme resulted in a bleaching of the 430nm peak and the appearance of a new one at 325nm (Fig. 4, curve *a*). These changes were reflected in alterations in the fluorescence excitation and emission spectra (see below). The spectral change produced by addition of methylamine was retained after dialysis against 50mM-phosphate buffer, pH 7.5, to remove excess of methylamine, so that addition of substrate had produced a stable modified form of the enzyme. The addition of phenazine methosulphate to this modified enzyme resulted in a rapid alteration of the spectrum, complete in 18min, in which the 325nm peak disappeared and the 430nm peak was restored (Fig. 4, curve *b*). The spectrum of the reoxidized enzyme was very similar to that of the freshly purified enzyme, even though a preparation with the altered spectrum due to aging (see above) was the starting material in this experiment. In the reoxidized enzyme, a shoulder at 455nm was apparent. No loss of activity could be detected in enzyme treated with methylamine or phenazine methosulphate.

Effect of borohydride. Three samples of enzyme were used to determine the effect of borohydride. One sample was pre-treated with methylamine, one was pre-treated with phenazine methosulphate, and both were then dialysed overnight. The third sample was dialysed without pre-treatment. All had identical specific activities at this stage. The colourless form of the enzyme produced by methylamine pre-treatment was not inactivated by addition of 2mg of solid sodium borohydride followed by dialysis, nor was the spectrum in any way altered. The pale-yellow untreated enzyme, or deep-yellow enzyme pre-treated with phenazine methosulphate were both 10–20% inactivated by borohydride treatment, and there was a partial bleaching of the 430nm peak and an enhancement of the 325nm peak. Further reduction produced little change in either spectrum or activity unless it was preceded by treatment with phenazine methosulphate. Phenazine methosulphate treatment (followed by dialysis) did not affect the activity of any of the samples, but it increased the size of the 430nm peak in all cases, particularly with the colourless enzyme pre-treated with methylamine. After phenazine methosulphate treatment, borohydride reduction caused further bleaching of the 430nm peak and decreased the activity of the methylamine-pre-treated sample. Two cycles of alternating phenazine methosulphate and borohydride treatment produced 90% inactivation of the methylamine-pre-treated sample. The phenazine methosulphate-pre-treated sample and the untreated control, however, although they had lost virtually all their absorption at 430nm, had only lost 60–70% of their initial activity at this stage. The spectrum of the borohydride-reduced enzyme was very similar to that produced by addition of methylamine to the enzyme (Fig. 4, curve *a*).

Fluorescence excitation and emission spectra of the enzyme

The enzyme was fluorescent, having an excitation maximum at 282nm and an emission maximum at 348nm (Fig. 5). The effects of the addition of methylamine and of reduction with borohydride on the excitation and emission maxima are shown in Table 2. Treatment with methylamine produced a fivefold enhancement of the maximum fluorescence of the enzyme, but the intensity of the emission peak at 380nm thereby produced diminished rapidly with time on irradiation at 328nm. This effect was accompanied by the appearance of a brown colour which was not discharged on the addition of more methylamine.

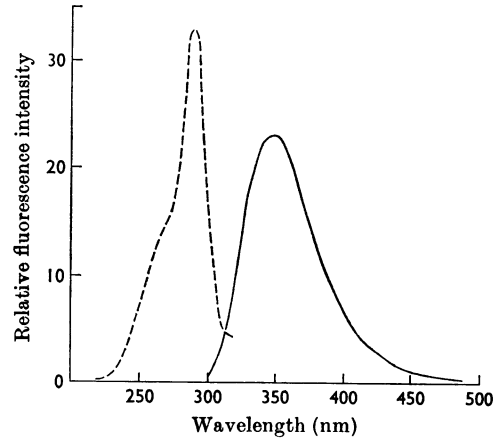


Fig. 5. Fluorescence excitation and emission spectra of the amine dehydrogenase at pH 7.5. ----, Excitation spectrum (fluorescence measured at 348nm). ——, Fluorescence emission spectrum (excitation wavelength 282nm). Spectra are uncorrected and were determined in an Aminco–Bowman spectrophotofluorimeter with slit arrangement no. 3. Meter multiplier setting was 0.1 and sensitivity 37. Enzyme concentration was 1.94mg of protein/ml.

Table 2. *Fluorescence spectral data for the amine dehydrogenase, the reduced enzyme and the substrate-treated enzyme*

Fluorescence spectra, which are uncorrected, were measured by using an Aminco–Bowman spectrophotofluorimeter with slit arrangement no. 3.

Sample	Excitation maximum (nm)	Emission maximum (nm)
Amine dehydrogenase, pH 7.0 1.56 mg of protein/ml (11.7 μM assuming a mol. wt. of 1.33×10^5)	282	348
Amine dehydrogenase, pH 7.0, plus methylamine at a final concentration of 25 μM	328	380
Amine dehydrogenase, pH 7.0, reduced with $NaBH_4$	280 330	380

This probably reflects the photosensitivity of the substrate-treated enzyme when irradiated at its absorption maximum by a high-intensity light-source; this is observed with the pyridoxamine form of several pyridoxal phosphate-containing enzymes (Churchich, 1969).

Kinetic experiments on the binding of n-*butylamine and phenazine methosulphate to the enzyme*

Velick & Vavra (1962) derived the following equation for a bimolecular Ping Pong reaction (nomenclature of Cleland, 1963) for two substrates A and B where v is the reaction velocity and square brackets denote concentrations:

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

When $1/v$ is plotted against 1/[A] at various fixed concentrations of B, there is observed a family of parallel straight lines whose intercept on the $1/v$ axis bears a linear relationship with 1/[B]. This method of analysis was used to investigate the nature of the substrate binding to the amine dehydrogenase by using *n*-butylamine as a substrate. *n*-Butylamine was chosen because its K_m is large enough (about 350 μM) to enable accurate Lineweaver–Burk (1934) plots to be obtained. With substrates of a lower K_m such as methylamine, this is not possible (Eady & Large, 1968). The results of several Lineweaver–Burk plots of the initial reaction rate in the standard assay against *n*-butylamine concentration at various fixed phenazine methosulphate concentrations are shown in Fig. 6(*a*). A family of straight lines was obtained which on replotting the value of the intercept on the ordinate against the reciprocal of the phenazine methosulphate concentration gave a straight line (Fig. 6*b*). A family of straight lines was also obtained (Fig. 6*c*) when phenazine methosulphate was the variable substrate and *n*-butylamine the fixed substrate, and again a replot of the ordinate intercept values against reciprocal *n*-butylamine concentration gave a straight line (Fig. 6*d*). The K_m values obtained from the secondary plots were 21.7 μM for phenazine methosulphate and 420 μM for *n*-butylamine (cf. values of 56 μM and 350 μM obtained previously; Eady & Large, 1968; Large, Eady & Murden, 1969). The form of the primary and secondary plots is consistent with the mechanism of the reaction catalysed by the amine dehydrogenase being of the Ping Pong type.

To check the internal consistency of the data, a Lineweaver–Burk plot was carried out in which both substrates were varied simultaneously at a constant ratio. This should give a straight line with an intercept on the abscissa of $1/K'$ where $K'=K_m^A/x+K_m^B$ and $x=[A]/[B]$ (Henson & Cleland, 1964). When the *n*-butylamine/phenazine methosulphate concentration ratio was kept constant at 76:1 and the reciprocal initial reaction velocity at different substrate concentrations plotted against 1/[phenazine methosulphate], a straight line was obtained giving K' 28 μM. This compares with a value of 27.2 μM calculated from the values obtained for K_m^{PMS} and K_m^{But} from Fig. 6.

Evidence for the non-involvement of copper in the enzymic reaction

Effect of dialysis against diethyldithiocarbamate. Dialysis against diethyldithiocarbamate has been shown to inactivate pea seedling diamine oxidase (Hill & Mann, 1962), pig kidney diamine oxidase (Mondovì *et al.* 1967*b*), plasma amine oxidase (Yamada & Yasunobu, 1962*b*) and the *Aspergillus niger* enzyme (Yamada, Adachi & Ogata, 1965*b*). The inactivated enzymes had modified spectra: the pea seedling enzyme was orange–pink (Hill & Mann, 1964) with a maximum at about 480 nm less intense than that of the untreated enzyme at 500 nm; the plasma enzyme was not pink, absorbing maximally at 380 nm (Yamada & Yasunobu, 1962*b*). The enzymes were reactivated by the addition of cupric sulphate at about 0.1 μM (Yamada & Yasunobu, 1962*b*; Hill & Mann, 1964; Mondovì *et al.* 1967*b*). Because of the similarity of the amine dehydrogenase to these enzymes which are inactivated by diethyldithiocarbamate, the effect of this compound on the enzyme was investigated. It could not be tested directly as an inhibitor because it interfered with the assay system, so the following procedure was used. A solution of the enzyme was dialysed for 18 h against 10 mM-sodium diethyldithiocarbamate in 20 mM-sodium–potassium phosphate buffer, pH 7.0, prepared from distilled deionized water.

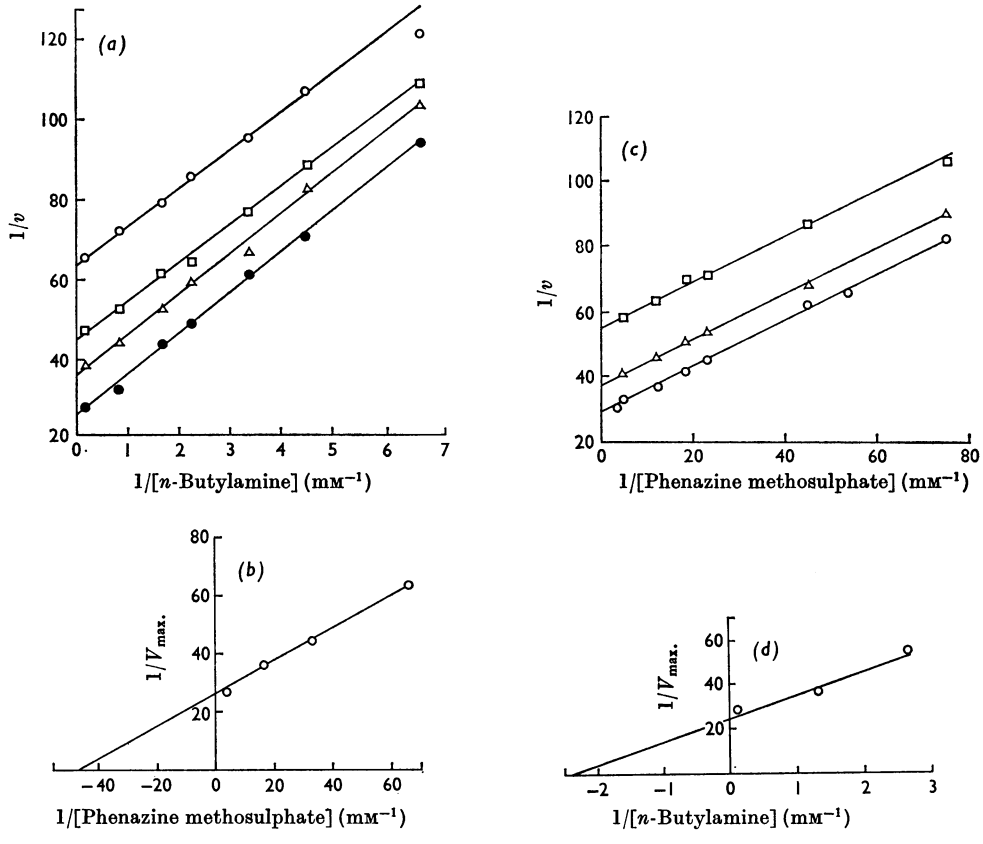


Fig. 6. Effect of varying the concentration of the second substrate on the double-reciprocal plots for *n*-butylamine hydrochloride and phenazine methosulphate. v=μmol of dichlorophenol-indophenol reduced/min. (*a*) Primary plot of $1/v$ against reciprocal *n*-butylamine concentration at different concentrations of phenazine methosulphate: ○, 15.2μM; □, 30.4μM; △, 60.9μM; ●, 253μM. (*b*) Secondary plot of intercept on the ordinate of (*a*) (i.e. $1/V_{max.}$) against reciprocal phenazine methosulphate concentration. (*c*) Primary plot of $1/v$ against reciprocal phenazine methosulphate concentration at different concentrations of *n*-butylamine: □, 0.374mM; △, 0.745mM; ○, 7.45mM. (*d*) Secondary plot of intercept on the ordinate of (*c*) (i.e. $1/V_{max.}$) against reciprocal *n*-butylamine concentration. Other components of the reaction mixture were: 200μmol of phosphate buffer, pH7.5, 0.15μmol of 2,6-dichlorophenol-indophenol, 50μg of enzyme and water in a total volume of 3.0ml. Reduction of dichlorophenol-indophenol was followed spectrophotometrically at 600nm.

After this time excess of reagent was removed by dialysis against phosphate buffer alone for 8h. The treated enzyme, which had developed a modified spectrum with a broadened enhanced peak in the visible range with a shoulder at 510nm, was then assayed and its specific activity compared with a sample which had been dialysed under similar conditions in phosphate buffer not containing diethyldithiocarbamate. There was no decrease in the specific activity of the diethyldithiocarbamate-treated amine dehydrogenase, despite the spectral change which occurred during this time.

Copper content of the enzyme. This was examined by two procedures: a microchemical colorimetric method on digested samples of the enzyme as described in the Materials and Methods section, and by neutron-activation analysis and γ-spectrometry on a sample of enzyme taken to dryness in ammonium acetate buffer. The amount of copper detected was low (Table 3) and averaged about 2ng-atoms of copper/mg of protein, which on the basis of a molecular weight of 1.33×10^5 is about 0.27g-atom/mol. The value for the copper content obtained by neutron-activation analysis was based on the 1.34MeV peak of the γ-emission spectrum. The γ-spectrum also revealed the presence of small amounts of cobalt and zinc and a trace of iron in the enzyme.

Table 3. *Copper content of the amine dehydrogenase*

Before analyses were carried out, the enzyme was passed through a column of Sephadex G-15 to remove any traces of copper from the solution. Colorimetric determinations were made with bathocuproine disulphonate on wet-ashed samples; neutron-activation analysis and γ-spectrometry were done on material taken to dryness in a desiccator.

Enzyme preparation	Analytical method	Amount of enzyme used (mg)	Copper content		$10^{-3} \times$ Minimum mol.wt. based on Cu content
			μg/mg of protein	g-atom/mol*	
1	Colorimetric	5.2	0.24	0.505	264
	Colorimetric	4.0	0.104	0.218	610
2	Colorimetric	6.45	0.105	0.22	605
	Colorimetric	9.2	0.066	0.141	945
	Neutron-activation analysis	5.4	0.13	0.271	490

* Based on mol.wt. 1.33×10^5.

Resolution of the enzyme and properties of the isolated chromophore

The prosthetic group of the amine dehydrogenase was found to be very tightly bound to the enzyme protein. It was not released by treatment with acid (Matsuo & Greenberg, 1958), alkali (Jenkins & Sizer, 1960) or urea. Nor could the enzyme be resolved by cysteine treatment followed by dialysis against cysteine–EDTA–methylamine (Schirch & Mason, 1962), dialysis against penicillamine (Morino & Snell, 1967), dialysis against semicarbazide, or treatment with cysteine in the presence of imidazole–citrate (Shaltiel, Hedrick & Fischer, 1966). Dialysis against tris hydrochloride (Nishimura & Greenberg, 1961) was also ineffective, although it modified the spectrum of the enzyme by the formation of a new peak at 470nm (see above). It was found, however, that heat denaturation of enzyme previously treated with semicarbazide or methylamine and then dialysed released the chromophore from the protein.

Properties of the isolated chromophore. Solutions of the chromophore were deep yellow. At pH 1 the spectrum had an absorption maximum at 430nm with a main peak at 275nm and a shoulder at 290nm. The E_{275}/E_{430} ratio was 6.2. At pH 7 there was an increase in the extinction in the 310–330nm range, and the peak at 430nm increased in magnitude and shifted to 405nm. At pH 10 the 290nm shoulder became a small peak and there was a slight increase in extinction in the 325–350nm range. No change in the spectrum was produced at pH 7 by addition of semicarbazide (final concentration 2.5mM). Addition of phenylhydrazine hydrochloride to the same final concentration caused the disappearance of the 430nm peak with an increase in extinction as a shoulder in the 310–330nm region. The fluorescence excitation maxima were at 310nm, 318nm and 325nm (uncorrected); the emission maximum when excited at these wavelengths was 375nm. Although it was derived from what is thought to be the pyridoxamine form of the enzyme, these results do not agree well with the spectral properties reported in the literature for pyridoxamine (see Discussion). By using high-voltage paper electrophoresis, the electrophoretic properties of the chromophore were compared with various pyridoxal derivatives. At pH 9.5 in 5mM-borate buffer the chromophore migrated towards the anode. Under these conditions pyridoxal phosphate, pyridoxamine phosphate, pyridoxal and 4-pyridoxic acid all migrated towards the cathode. Pyridoxamine was the only derivative which migrated towards the anode, approximately the same distance as the chromophore. When the electrophoresis strip was sprayed with 0.1% ninhydrin in acetone and heated to 70°C for 2min the marker spots of pyridoxamine and pyridoxamine phosphate gave bright red–orange spots, but the chromophore gave no colour reaction. The chromophore gave positive colour reactions typical of a phenolic compound, a blue colour with Folin–Ciocalteu reagent and a yellow–brown colour with ferric chloride. Although these tests are obviously not specific, pyridoxal derivatives do give positive results. The chromophore did not reactivate aspartate apoaminotransferase in an assay which could have detected 2nmol of pyridoxal phosphate, nor did it inhibit the binding of pyridoxal phosphate to the resolved aminotransferase. Further work to identify the components of the chromophore preparation was not possible because of the low yield of chromophore from large quantities of purified enzyme.

DISCUSSION

Spectral properties of the enzyme. It has previously been shown (Eady & Large, 1968) that the substrate specificity and inhibitor pattern of the

primary amine dehydrogenase of *Pseudomonas* AM1 are similar to those of the 'diamine oxidase' ('histaminase') group of enzymes (Buffoni, 1966) which have been shown to contain Cu^{2+}, and in some cases pyridoxal phosphate as their prosthetic groups, for example the ox plasma enzyme (Yamada & Yasunobu, 1962*b*, 1963), the pig plasma enzyme (Blaschko & Buffoni, 1966), the pig kidney intracellular enzyme (Kapeller-Adler & MacFarlane, 1963; Mondovì, Costa, Finazzi-Agrò & Rotilio, 1967*a*), the pea seedling enzyme (Hill & Mann, 1964), and the enzymes from *A. niger* (Yamada *et al.* 1965*b*; Adachi & Yamada, 1969) and *Trichosporon* sp. (Yamada, Kumagai, Uwajima & Ogata, 1966). However, the spectral properties of the present enzyme do not resemble the spectra of these diamine oxidases. The latter are all pink in colour with absorption maxima in the 470–510nm region with significant absorption above 500nm. The *Pseudomonas* AM1 amine dehydrogenase in contrast, is yellow, with an absorption maximum at 325 or 430nm, very little absorption above 500nm and shows a stronger resemblance to enzymes that contain pyridoxal phosphate but not copper. The effect of removing copper from the diamine oxidases is to cause a colour change from pink to yellow and a shift of absorption maximum to lower wavelengths (Hill & Mann, 1964; Yamada & Yasunobu, 1962*b*). This suggests that the *Pseudomonas* AM1 amine dehydrogenase is devoid of functional copper. The spectral characteristics of the amine dehydrogenase are most simply explained by suggesting that as isolated it is a mixture of at least two forms. One of these has properties consistent with it containing a pyridoxal derivative (perhaps pyridoxal phosphate) bound as a protonated Schiff's base ($\lambda_{max.}$ 410–435nm; Snell & di Mari, 1970). The second, colourless, form ($\lambda_{max.}$ 325nm) is also formed when the enzyme is treated with methylamine, and has properties suggesting that it is the pyridoxamine form of the enzyme, e.g. it is resistant to inactivation by borohydride. The first form can be converted into the second by treatment with methylamine, and the second form reconverted into the first by treatment with phenazine methosulphate. Both forms have identical activity. In addition, aging of the enzyme seems to cause a change in the first form of the enzyme to a form spectrally similar to the second form, without any loss of activity. We tentatively suggest that this last derivative might be an adduct formed by addition of some group in the enzyme protein across the azomethine bond of the Schiff's base, analogous to the azomethine adduct postulated by Kent, Krebs & Fischer (1958) for glycogen phosphorylase. It is possible that this third form is present as an additional constituent of the enzyme as isolated, and could account for the residual activity observed after repeated borohydride and phenazine methosulphate treatments. The presence of a variety of active forms could explain the multiplicity of active bands seen when the sample was examined by disc electrophoresis at pH 9.5, although other causes might be possible, e.g. the existence of isoenzymes with slightly different primary structures. The present results are not sufficient to confirm for certain the existence of such an azomethine adduct. Its activity could be explained by postulating a reversible conversion into the Schiff's base form so that addition of methylamine would pull the equilibrium in favour of the Schiff's base.

The bleaching of the spectral peak in the visible range by addition of methylamine is analogous to the bleaching observed when substrate is added to the diamine oxidase group of enzymes (Mann, 1961; Hill & Mann, 1964; Yamada & Yasunobu, 1962*a*; Buffoni & Blaschko, 1965; Yamada, Adachi & Ogata, 1965*a*; Mondovì *et al.* 1967*b*) except that in the present case the effect is apparent under aerobic conditions.

The change in absorption spectrum on reduction with sodium borohydride and the concomitant loss of activity are characteristic of those enzymes which contain pyridoxal phosphate bound as a Schiff's base (Martinez-Carrion & Jenkins, 1965; Klein & Sagers, 1967; Blethen, Boeker & Snell, 1968). Borohydride reduces the azomethine bond of the Schiff's base in these enzymes to a stable secondary amine, peptide-bound ϵ-pyridoxyl-lysine (Fasella, 1967). We suggest that borohydride may cause a similar secondary amine to be formed in the amine dehydrogenase.

The spectra of the derivatives formed by addition of carbonyl reagents have different spectral maxima from those described for the diamine oxidase group of enzymes (including the bovine plasma enzyme when copper has been removed; Yamada & Yasunobu, 1963). Only the peak at 435–440nm formed by addition of phenylhydrazine resembles the spectral change observed with the diamine oxidases (Yamada & Yasunobu, 1963; Blaschko & Buffoni, 1966; Mondovì *et al.* 1967*b*). The effect of the addition of semicarbazide suggests that it binds firmly to the Schiff's base form of the enzyme, thereby inactivating it, and that it cannot be displaced by methylamine treatment, or prolonged dialysis. The active portion of the enzyme after semicarbazide addition is presumably the pyridoxamine form. The potency of carbonyl reagents as inhibitors (Eady & Large, 1968) would occur because under the conditions of assay, phenazine methosulphate is added before methylamine, and presumably converts all the pyridoxamine form present into the pyridoxal Schiff's base.

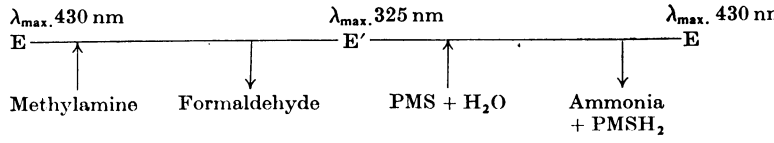
The fluorescence excitation and emission spectra of the enzyme at pH 7.0 suggest the presence in

the protein of both tyrosine (Weber, 1961) and tryptophan (Teale, 1960). Although a number of pyridoxal phosphate-containing enzymes show fluorescence at 500nm when excited at 430nm (Shukuya & Schwert, 1960; Fasella, Turano, Giartosio & Hammady, 1961; Klein & Sagers, 1966, 1967), in other cases this fluorescence has not been observed. Thus Churchich & Harpring (1965) with aspartate aminotransferase and Blaschko & Buffoni (1966) with pig plasma diamine oxidase observed only fluorescence due to protein amino acids, as we have done. This suggests that under certain conditions the fluorescence of the Schiff's base is quenched. The emission maximum at 380nm on addition of substrate or on reduction with borohydride is in the range expected for pyridoxal enzymes (375–400nm), which have an emission maximum at about 380nm in the presence of substrate (Shukuya & Schwert, 1960) or when reduced with borohydride (Churchich & Harpring, 1965; Klein & Sagers, 1967). In the case of the amine dehydrogenase the change of emission maximum from 348nm to 380nm on reduction with borohydride or on substrate addition is presumably due to the modification of the structure of the prosthetic group to a form in which the fluoresence is no longer quenched by the protein, i.e. the pyridoxamine form in the case of substrate, and a stable secondary amine in the case of borohydride reduction, even though these have different activation spectra. The pyridoxamine form of alanine aminotransferase has an absorption maximum at 325nm, a fluorescence emission maximum at 375nm, and is unstable on prolonged irradiation (Churchich, 1969). All these properties are closely similar to the present observations on the methylamine-treated amine dehydrogenase.

Kinetics of the enzyme. The kinetic experiments on the binding of *n*-butylamine and phenazine methosulphate to the enzyme suggest that the amine dehydrogenase of *Pseudomonas* AM1 catalyses the oxidation of the amine substrate by a Ping Pong mechanism. In such a mechanism the combination of the two substrates with the enzyme is separated by product release. One substrate combines with the enzyme to produce a modified enzyme, and one product is released. The second substrate then combines with the enzyme to give the second product and the enzyme in its original form. The observation that addition of methylamine to the amine dehydrogenase produces a spectrally-modified complex, which is stable to dialysis and which is restored to the original spectral form of the enzyme on the addition of phenazine methosulphate (Fig. 4), strongly supports the kinetic evidence for a Ping Pong mechanism. Scheme 1 indicates a postulated reaction sequence for the enzyme in which the modified form of the enzyme E′ is the pyridoxamine form.

It has been shown for the pea seedling enzyme (Yamasaki, Swindell & Reed, 1970), for pig kidney diamine oxidase (Finazzi-Agrò, Rotilio, Costa & Mondovì, 1969) and for the bovine plasma enzyme (Oi, Inamasu & Yasunobu, 1970) that incubation of the enzyme with ^{14}C-labelled substrate under rigorously anaerobic conditions results in the stoicheiometric release of [^{14}C]aldehyde (1mol/mol of enzyme). In addition, Ping Pong kinetics have been demonstrated for each of these enzymes, although only in the work of Oi *et al.* (1970) was variation of both substrates, amine and oxygen, examined simultaneously.

Role of copper in the enzyme. The low copper content of the amine dehydrogenase (about 2ng-atoms of copper/mg of protein) compared with the diamine oxidases (Table 4), and the fact that it is not inactivated by dialysis against sodium diethyldithiocarbamate, suggest that copper is not involved in the reaction catalysed by the enzyme. The inhibition produced by the copper-chelating agent cuprizone (Eady & Large, 1968) may well be due to some other property of this compound, since other copper-chelating agents are not inhibitory. Erwin & Hellerman (1967) give evidence for a similar suggestion with regard to the inhibition of mitochondrial monoamine oxidase by 8-hydroxyquinoline. The role of copper in the diamine oxidases is not clear, since, although it is essential for activity, no valency change has been observed during reaction of the enzyme–substrate complex with oxygen (Yamada, Yasunobu, Yamano & Mason, 1963; Mondovì *et al.* 1967*b*; Buffoni, Della Corte & Knowles, 1968). Hamilton (1969) suggests that copper is probably required in the diamine oxidases to facilitate the transfer of electrons to oxygen. If copper is involved in the



Scheme 1. Postulated reaction sequence for the enzyme. PMS and $PMSH_2$, oxidized and reduced forms of *N*-methylphenazonium methosulphate.

Table 4. *Copper content of various highly purified amine oxidases*

Enzyme	Copper content: μg/mg of protein	Copper content: g-atoms/mol	Reference
1. Diamine oxidases			
Ox plasma amine oxidase	0.9	4	Yamada & Yasunobu (1962*b*)
Pea seedling amine oxidase	0.8–0.9	2	Mann (1961)
Pig plasma amine oxidase	0.99	3.04	Buffoni & Blaschko (1965)
Pig kidney diamine oxidase	0.75	2.17	Yamada, Kumagai, Kawasaki, Matsui & Ogata (1967)
	0.74	1.5	Mondovì *et al.* (1967*b*)
A. niger amine oxidase	0.77	3.0	Yamada *et al.* (1965*b*)
Trichosporon amine oxidase	0.40	1	Yamada *et al.* (1966)
2. Monoamine oxidases			
Sarcina lutea tyramine oxidase	0.014	0.028	Kumagai, Matsui, Ogata & Yamada (1969)
Ox liver mitochondrial monoamine oxidase	0.15	0.73	Yasunobu, Igaue & Gomes (1968)
Ox kidney mitochondrial monoamine oxidase	0.15	0.23	Erwin & Hellerman (1967)
Rat liver mitochondrial monoamine oxidase	0.34	1.63	Youdim & Sourkes (1966)
3. Amine dehydrogenase			
Pseudomonas AM1 amine dehydrogenase	0.13	0.27	Present work (see Table 3)

movement of electrons through the π and d orbital system of a complex (of which the metal ion forms a part) to oxygen, as suggested by Hamilton (1968), then the low amounts of copper in the amine dehydrogenase of *Pseudomonas* AM1 would be understandable, since the enzyme does not react with oxygen. The evidence as to the presence of copper in the monoamine oxidase group of enzymes (EC 1.4.3.4) (which contain FAD) is conflicting (see Table 4). Erwin & Hellerman (1967) concluded that monoamine oxidase does not require copper for activity. It should be noted that the amount of copper which Erwin & Hellerman (1967) regarded as insignificant is of the same order as has been detected in the present enzyme, where the same conclusion has been drawn.

Further investigation of the present enzyme is needed to establish more definitely the sequence of product release postulated in Scheme 1 and to determine the nature of the interaction between the enzyme and the electron acceptor. Although phenazine methosulphate is the best artificial acceptor (Eady & Large, 1968), the natural acceptor is probably a cytochrome (Anthony, 1970).

Identity of the prosthetic group. Because of the extremely tight binding of the prosthetic group to the enzyme, it was impossible to obtain unequivocal evidence that it was pyridoxal phosphate. It was not possible to resolve the enzyme to give an inactive apoenzyme and demonstrate reactivation by the addition of pyridoxal phosphate, since denaturation of the protein appeared to be a prerequisite of chromophore release.

The present enzyme once again resembles the diamine oxidases in the difficulty of resolving it: great difficulties were encountered in doing this with the plasma enzymes (Yamada & Yasunobu, 1963; Blaschko & Buffoni, 1966), the pig kidney enzyme (Mondovì *et al.* 1967*a*) and the *Aspergillus* enzyme (Adachi & Yamada, 1969). All these workers had to resort to proteolytic techniques to release the chromophore. Only Mondovì *et al.* (1967*a*) and Blaschko & Buffoni (1966) were able to characterize the hydrolysis product as a recognizable pyridoxal derivative, although the chromophore of Yamada & Yasunobu (1963) had similar spectral properties to pyridoxal phosphate. Hill (1967) was unable to identify pyridoxal phosphate conclusively in the pea seedling enzyme.

The spectral properties of the isolated chromophore preparation are not typical of pyridoxal or related compounds, which have characteristic absorption peaks in the 300–400nm region (Peterson & Sober, 1954) and fluorescence emission maxima at or above 400nm (with the exception of pyridoxal which has an emission maximum at 382nm at pH 6; Bridges, Davies & Williams, 1966). The absorption peak at 430nm is more typical of a pyridoxal Schiff's base than it is of the free forms of the coenzyme. However, such an aldimine would be expected to fluoresce at 500nm (Fasella *et al.* 1961; Klein & Sagers, 1966). The chromophore preparation behaved differently on electrophoresis from the vitamin B_6 derivatives investigated. The electrophoretic behaviour suggests that it is not phosphorylated. It is known that pyridoxal derivatives of hydrolysed phosphorylase readily undergo degradation of the pyridoxal nucleus which

results in a loss of characteristic chemical and spectral properties (Fischer, 1964). This degradation occurs under mild conditions and is accompanied by a dephosphorylation of the pyridoxal nucleus. If this occurred during the isolation of the chromophore from the amine dehydrogenase it would account for the lack of correlation between the properties of the chromophore preparation and pyridoxal derivatives, since free pyridoxal is only 0.1% as effective as pyridoxal phosphate in reactivating aspartate apoaminotransferase (Wada & Snell, 1962). In this context it is noteworthy that the chromophores isolated from ox plasma amine oxidase (Yamada & Yasunobu, 1963) and the *Aspergillus* amine oxidase (Yamada, Adachi & Ogata, 1968), although they had vitamin B_6 activity when assayed microbiologically with *Saccharomyces carlsbergensis*, did not reactivate apotryptophanase, and they differed in their chromatographic and electrophoretic behaviour from pyridoxal derivatives (Adachi & Yamada, 1969). The fluorescence spectrum of the latter authors showed more resemblance to authentic pyridoxal derivatives than does the spectrum observed in the present work. Thus although indirect evidence suggests that the functional prosthetic group in the *Pseudomonas* AM1 amine dehydrogenase is probably a pyridoxal derivative, direct evidence to support this conclusion has not been obtained from the properties of the isolated chromophore. No evidence for the presence of flavin in the amine dehydrogenase has been obtained in the present work, although the presence of a second prosthetic group cannot be ruled out.

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REFERENCES

Adachi, O. & Yamada, H. (1969). *Agric. biol. Chem., Tokyo*, **33**, 1707.

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.

Anthony, C. (1970). *Biochem. J.* **119**, 54P.

Banks, B. E. C., Lawrence, A. J., Vernon, C. A. & Wootton, J. F. (1963). In *Chemical and Biological Aspects of Pyridoxal Catalysis*, p. 197. Ed. by Snell, E. E., Fasella, P. M., Braunstein, A. & Rossi-Fanelli, A. Oxford: Pergamon Press Ltd.

Bergmeyer, H.-U. & Bernt, E. (1963). In *Methods of Enzymatic Analysis*, p. 837. Ed. by Bergmeyer, H.-U. New York and London: Academic Press.

Blaschko, H. & Buffoni, F. (1966). *Proc. R. Soc.* B, **163**, 45.

Blethen, S. L., Boeker, E. A. & Snell, E. E. (1968). *J. biol. Chem.* **243**, 1671.

Bridges, J. W., Davies, D. S. & Williams, R. T. (1966). *Biochem. J.* **98**, 451.

Buffoni, F. (1966). *Pharmac. Rev.* **18**, 1163.

Buffoni, F. & Blaschko, H. (1965). *Proc. R. Soc.* B, **161**, 153.

Buffoni, F., Della Corte, L. & Knowles, P. F. (1968). *Biochem. J.* **106**, 575.

Churchich, J. E. (1969). *Biochim. biophys. Acta*, **178**, 480.

Churchich, J. E. & Harpring, L. (1965). *Biochim. biophys. Acta*, **105**, 575.

Cleland, W.W. (1963). *Biochim. biophys. Acta*, **67**, 104.

Davis, B. J. (1964). *Ann. N.Y. Acad. Sci.* **121**, 404.

Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. (1969). *Data for Biochemical Research*, 2nd ed., p. 483. Oxford: Clarendon Press.

Eady, R. R. & Large, P. J. (1968). *Biochem. J.* **106**, 245.

Eady, R. R. & Large, P. J. (1969). *Abstr. FEBS 6th Meet., Madrid*, p. 149.

Erwin, V. G. & Hellerman, L. (1967). *J. biol. Chem.* **242**, 4230.

Fasella, P. (1967). *A. Rev. Biochem.* **36**, 185.

Fasella, P., Turano, C., Giartosio, A. & Hammady, I. (1961). *G. Biochim.* **10**, 175.

Finazzi-Agrò, A., Rotilio, G., Costa, M. T. & Mondovì, B. (1969). *FEBS Lett.* **4**, 31.

Fischer, E. H. (1964). In *FEBS Symp. no.* 1: *Structure and Activity of Enzymes*, p. 111. Ed. by Goodwin, T. W., Harris, J. I. & Hartley, B. S. London and New York: Academic Press.

Hamilton, G. A. (1968). In *Pyridoxal Catalysis: Enzymes and Model Systems*, p. 375. Ed. by Snell, E. E., Braunstein, A. E., Severin, E. S. & Torchinsky, Y. M. New York: Interscience Publishers Inc.

Hamilton, G. A. (1969). *Adv. Enzymol.* **32**, 55.

Henson, C. P. & Cleland, W. W. (1964). *Biochemistry, Easton*, **3**, 338.

Hill, J. M. (1967). *Biochem. J.* **104**, 1048.

Hill, J. M. & Mann, P. J. G. (1962). *Biochem. J.* **85**, 198.

Hill, J. M. & Mann, P. J. G. (1964). *Biochem. J.* **91**, 171.

Jenkins, W. T. & Sizer, I. W. (1960). *J. biol. Chem.* **235**, 620.

Kapeller-Adler, R. & MacFarlane, H. (1963). *Biochim. biophys. Acta*, **67**, 542.

Kent, A. B., Krebs, E. G. & Fischer, E. H. (1958). *J. biol. Chem.* **232**, 549.

Klein, S. M. & Sagers, R. D. (1966). *J. biol. Chem.* **241**, 206.

Klein, S. M. & Sagers, R. D. (1967). *J. biol. Chem.* **242**, 301.

Kumagai, H., Matsui, H., Ogata, K. & Yamada, H. (1969). *Biochim. biophys. Acta*, **171**, 1.

Large, P. J., Eady, R. R. & Murden, D. J. (1969). *Analyt. Biochem.* **32**, 402.

Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

Mann, P. J. G. (1961). *Biochem. J.* **79**, 623.

Martinez-Carrion, M. & Jenkins, W. T. (1965). *J. biol. Chem.* **240**, 3538.

Matsuo, Y. & Greenberg, D. M. (1958). *J. biol. Chem.* **230**, 561.

Mondovì, B., Costa, M. T., Finazzi-Agrò, A. & Rotilio, G. (1967*a*). *Archs Biochem. Biophys.* **119**, 373.

Mondovì, B., Rotilio, G., Costa, M. T., Finazzi-Agrò, A., Chiancone, E., Hansen, R. E. & Beinert, H. (1967*b*). *J. biol. Chem.* **242**, 1160.

Morino, Y. & Snell, E. E. (1967). *J. biol. Chem.* **242**, 5591.

Nishimura, J. S. & Greenberg, D. M. (1961). *J. biol. Chem.* **236**, 2684.

Oi, S., Inamasu, M. & Yasunobu, K. T. (1970). *Biochemistry, Easton*, **9**, 3378.

Peterson, E. A. & Sober, H. A. (1954). *J. Am. chem. Soc.* **76**, 169.

Reisfeld, R. A., Lewis, U. J. & Williams, D. E. (1962). *Nature, Lond.*, **195**, 281.

Scardi, V., Scotto, P., Iaccarino, M. & Scarano, E. (1963). *Biochem. J.* **88**, 172.

Schirch, L. & Mason, M. (1962). *J. biol. Chem.* **237**, 2578.

Shaltiel, S., Hedrick, J. L. & Fischer, E. H. (1966). *Biochemistry, Easton*, **5**, 2108.

Shukuya, R. & Schwert, G. W. (1960). *J. biol. Chem.* **235**, 1653.

Snell, E. E. & di Mari, S. J. (1970). In *The Enzymes*, 3rd ed., vol. 2, p. 335. Ed. by Boyer, P. D. New York and London: Academic Press.

Teale, F. J. W. (1960). *Biochem. J.* **76**, 381.

Velick, S. F. & Vavra, J. (1962). *J. biol. Chem.* **237**, 2109.

Wada, H. & Snell, E. E. (1962). *J. biol. Chem.* **237**, 127.

Weber, G. (1961). *Nature, Lond.*, **190**, 27.

Yamada, H., Adachi, O. & Ogata, K. (1965*a*). *Agric. biol. Chem., Tokyo*, **29**, 864.

Yamada, H., Adachi, O. & Ogata, K. (1965*b*). *Agric. biol. Chem., Tokyo*, **29**, 912.

Yamada, H., Adachi, O. & Ogata, K. (1968). In *Pyridoxal Catalysis: Enzymes and Model Systems*, p. 347. Ed. by Snell, E. E., Braunstein, A. E., Severin, E. S. & Torchinsky, Y. M. New York: Interscience Publishers Inc.

Yamada, H., Kumagai, H., Kawasaki, H., Matsui, H. & Ogata, K. (1967). *Biochem. biophys. Res. Commun.* **29**, 723.

Yamada, H., Kumagai, H., Uwajima, T. & Ogata, K. (1966). *Mem. Res. Inst. Fd Sci. Kyoto Univ.* **27**, 1.

Yamada, H. & Yasunobu, K. T. (1962*a*). *J. biol. Chem.* **237**, 1511.

Yamada, H. & Yasunobu, K. T. (1962*b*). *J. biol. Chem.* **237**, 3077.

Yamada, H. & Yasunobu, K. T. (1963). *J. biol. Chem.* **238**, 2669.

Yamada, H., Yasunobu, K. T., Yamano, T. & Mason, H. S. (1963). *Nature, Lond.*, **198**, 1092.

Yamasaki, E. F., Swindell, R. & Reed, D. J. (1970). *Biochemistry, Easton*, **9**, 1206.

Yasunobu, K. T., Igaue, I. & Gomes, B. (1968). *Adv. Pharmac.* **6**, 43.

Youdim, M. B. H. & Sourkes, T. L. (1966). *Can. J. Biochem.* **44**, 1397.

Yphantis, D. A. (1964). *Biochemistry, Easton*, **3**, 297.