Purification and Properties of an Amine Dehydrogenase from *Pseudomonas* AM1 and its Role in Growth on Methylamine

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1. Whole cells of *Pseudomonas* AM1 grown on methylamine oxidize methylamine, formaldehyde and formate. Crude extracts oxidize methylamine only if supplemented with phenazine methosulphate. 2. By using a spectrophotometric assay, the methylamine-oxidizing enzyme has been purified 20-fold in 31% yield. 3. The enzyme is a dehydrogenase, unable to utilize oxygen, NAD, NADP, flavines or menadione as electron acceptors, but able to utilize phenazine methosulphate, ferricyanide, cytochrome *c* or brilliant cresyl blue. 4. The enzyme is non-specific, readily oxidizing aliphatic monoamines and diamines, histamine and ethanolamine. Secondary and tertiary amines, quaternary ammonium salts and aromatic amines are not oxidized. 5. The pH optima for methylamine, *n*-pentylamine and putrescine are respectively 7.6, 8.0 and 8.5. 6. The K_m value for methylamine is $5.2\mu\text{M}$ and that for phenazine methosulphate $56\mu\text{M}$. 7. The enzyme will withstand heating for 15 min. at 80° without loss of activity, but is inactivated at higher temperatures. It is not inactivated by any pH value between 2.6 and 10.6. 8. The dehydrogenase is inhibited by semicarbazide (K_i $3.35\mu\text{M}$), isoniazid (K_i $1.17\mu\text{M}$), cuprizone (K_i $0.49\mu\text{M}$), *p*-chloromercuribenzoate (K_i 0.45mM) and quinacrine (K_i 12.1mM). 9. The enzyme is absent from succinate-grown cells, and, during adaptation from succinate to methylamine, activity appears before growth on methylamine begins.

Pseudomonas AM1 is a pink Gram-negative bacterium isolated by Peel & Quayle (1961), which will grow on various substrates, including the C_1 compounds methanol, methylamine and formate, as sole source of carbon and energy. The pathway of carbon assimilation by this organism during growth on methanol and formate was investigated by Large, Peel & Quayle (1961). *Pseudomonas* AM1 grown on methanol was shown by Johnson & Quayle (1964) to contain a methanol-oxidizing enzyme resembling that described by Anthony & Zatman (1964*a,b*, 1965) in a similar pink organism, *Pseudomonas* M27. Although the pathway of carbon assimilation during growth on methylamine has been investigated in other organisms (Leadbetter & Gottlieb, 1964; Shaw, Tsai & Stadtman, 1966), no enzyme has been described that catalyses the first essential oxidative step of methylamine metabolism in any organism growing on methylamine as sole carbon source. This paper describes the purification and properties of the enzyme responsible for the oxidation of methylamine by *Pseudomonas* AM1 when it is growing on this compound as sole source of carbon and energy. A preliminary report of part of this work has been

presented to The Biochemical Society (Eady & Large, 1966).

MATERIALS AND METHODS

Maintenance and growth of the organism. Stock cultures were maintained on methylamine-agar slopes as described by Peel & Quayle (1961). Cells were grown in liquid medium with mineral salt composition the same as that used by Jayasuriya (1955) except that, instead of oxalate, the carbon source was 0.5% (w/v) (74 mM) methylamine hydrochloride, and the concn. of MgSO_4 was raised from 20 to 80 mg./100 ml. Bacteria from slopes were transferred to 10 ml. of this medium and incubated at 30° for 24 hr. before being used to inoculate 2 l. of medium in a wide-necked 4 l. conical flask. Cultures were grown at 30° on a Gyrotory shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The cells were harvested after 3 days by centrifugation, washed three times with distilled water, and, if not required immediately, stored at -15° . Large batches of cells were obtained by inoculating 18 l. of sterile medium in a Hoover washing machine with 6 l. of fully-grown suspension, and aerating and agitating vigorously for 24 hr. at 30° . The cells were harvested in a Sharples Super centrifuge and stored at -15° .

Measurement of bacterial growth. Growth was followed by measurement of turbidity at $570\text{m}\mu$ in a Unicam SP. 600

spectrophotometer previously calibrated in terms of dry wt. of cells/ml. of suspension.

Manometric methods. Oxygen uptake by washed cell suspensions or crude cell extracts was measured in conventional Warburg manometers at 30° with air as the gas phase.

Preparation of cell-free extracts. Extracts were prepared either by suspending 1 vol. of cells in 1 vol. of 67 mM-phosphate buffer, pH 7.5, and crushing the frozen suspension in a Hughes (1951) press, followed by thawing the extract and treating it with deoxyribonuclease, or alternatively by cooling the cells to 4° and treating them in a 60 W MSE ultrasonic disintegrator for half-minute periods for a total exposure of up to 8 min. The release of activity by ultrasonic treatment followed closely the release of total protein, and after 8 min. no further release of activity was observed. The resulting extracts were centrifuged at 30000g for 10 min. at 2° to remove unbroken cells and debris. The clear pink supernatant at this stage is referred to as the crude extract.

Chemical determinations. (a) Protein. This was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystalline bovine serum albumin as standard.

(b) Formaldehyde. This was determined either by the acetylacetone method of Nash (1953) or by the chromotropic acid method (MacFadyen, 1945) after microdiffusion in a Conway (1962) unit. Both methods were calibrated with a solution of formaldehyde previously standardized gravimetrically with dimedone (Frisell & Mackenzie, 1958).

(c) Methylamine. This was estimated by the 1-fluoro-2,4-dinitrobenzene method of Dubin (1960) after microdiffusion at 40° for 12 hr. in a Conway unit. The interference of ammonia in this method was negligible under the conditions used.

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

(e) Ammonia. This was estimated by the indophenol method (Chaney & Marbach, 1962) after microdiffusion at 30° for 4 hr. in a Conway unit.

Enzyme assays. The activity of the methylamine-oxidizing enzyme was measured with PMS* as electron acceptor by following its reoxidation either with oxygen manometrically or with DCPIP spectrophotometrically at 600 mμ.

(a) Standard spectrophotometric assay. Glass cuvettes (1 cm. light-path) contained in a total volume of 3 ml.: 200 μmoles of phosphate buffer, pH 7.5, 1.0 μmole of PMS, 0.149 μmole of DCPIP and enzyme. With the crude extract, 3 μmoles of KCN were also added. (The purified enzyme was slightly inhibited by this concentration of cyanide.) The reaction was started by the addition of 20 μmoles of methylamine hydrochloride, and the rate of decrease in extinction at 600 mμ consequent on the reduction of DCPIP was followed at 28° on a Beckman DB or Unicam SP.800 recording spectrophotometer against a blank containing all reactants except DCPIP. The assays were carried out in aerobic cuvettes, since the rate of reduction of DCPIP by reduced PMS was found to be the same as under anaerobic conditions. This indicates that reduced PMS reacts with DCPIP much faster than it does with oxygen (Redfearn

& Dixon, 1961); it has been shown by Massey (1959) that this is true for the oxidation of reduced PMS by cytochrome c. One unit is defined as the amount of enzyme required to reduce 1 μmole of DCPIP/min. under these conditions. The molar extinction coefficient of DCPIP at pH 7.5 is 21.5×10^3 (Armstrong, 1964). In inhibitor studies, the inhibitor was preincubated in the dark with the complete reaction mixture (including enzyme, but excluding DCPIP and methylamine), and the reaction was started by adding DCPIP followed by substrate.

(b) Standard manometric assay. Warburg manometer flasks contained, in a total fluid volume of 3 ml.: 0.2 ml. of 40% KOH in the centre well, 200 μmoles of phosphate buffer, pH 7.5, 6 μmoles of PMS and enzyme in the main compartment, and 20 μmoles of methylamine hydrochloride in the side arm. The reaction was initiated by tipping the substrate from the side arm. Oxygen uptake was only linear for 20 min. at 30° and measurements were made during this time.

Disc electrophoresis. Disc electrophoresis on polyacrylamide gel was performed at pH 9.5 by the method of Davis (1964) or at pH 4.3 by the method of Reisfeld, Lewis & Williams (1962) in a Shandon apparatus (Shandon Scientific Co. Ltd., London, N.W. 10). Protein was detected by staining the gel with 1% (w/v) Naphthalene Black 10B (George T. Gurr Ltd., London, S.W. 6) in 7% (v/v) acetic acid, and activity by pouring over the unstained gel the standard spectrophotometric assay mixture solidified by the addition of 2% (w/v) agar. A colourless band denoted reduction of DCPIP wherever enzyme activity was found.

Special chemicals. PMS, *n*-heptylamine, *n*-octylamine, *n*-nonylamine, spermine tetrahydrochloride, spermidine trihydrochloride, putrescine and cadaverine dihydrochlorides, protamine sulphate (grade I, essentially histone-free), crystalline catalase, brilliant cresyl blue, methylene blue and menadione bisulphite were obtained from Sigma (London) Chemical Co., London, S.W. 6. Cytochrome *c* (horse heart, 65% pure) was from the Boehringer Corp. (London) Ltd., London, W. 5; DCPIP, *n*-hexylamine, *n*-decylamine (pract.) and *n*-dodecylamine (pract.) were obtained from Kodak Ltd., Kirkby, Lancs., hexamethylenediamine and *trans*-2-phenylcyclopropylamine were from Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. (obtained through Ralph N. Emanuel Ltd., London, S.E. 1), DL-quinacrine dihydrochloride was from K & K Laboratories Inc., Plainview, N.Y., U.S.A. (obtained through Kodak Ltd.), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid and DEAE-cellulose were from Calbiochem, Los Angeles, Calif., U.S.A., and the remaining chemicals were from British Drug Houses Ltd., Poole, Dorset. All the amines were reagent grade, but analytical grades of the other chemicals were used. Menadione was prepared from the bisulphite compound by treatment with 2*N*-NaOH; the resulting crystals were washed with water and dried at 40°.

Purity of substrates. Purity of the amines was tested by gas chromatography at 120° on a column containing 70% Carbowax on Chromosorb W (Johns-Manville Co., New York, N.Y., U.S.A.) by using an Aerograph A-600C gas chromatograph (Wilkens Instruments, Walnut Creek, Calif., U.S.A.), or by paper chromatography in butan-1-ol-acetic acid-water (40:10:50, by vol.; Brenner & Kenten, 1951). Amines were detected by a ninhydrin spray. When contaminants were detected, the amines were redistilled.

Buffers. Sodium-potassium phosphate, tris hydro-

* Abbreviations: PMS, *N*-methylphenazonium methosulphate (phenazine methosulphate); DCPIP, 2,6-dichlorophenol-indophenol.

chloride, sodium acetate-acetic acid, glycine-HCl, and glycine-NaOH buffers were prepared as described by Dawson, Elliott, Elliott & Jones (1959). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Good *et al.* 1966) was adjusted to pH 7.5 with 2*N*-NaOH.

Adaptation of succinate-grown *Pseudomonas* AM1 to growth on methylamine. *Pseudomonas* AM1 was taken through five serial subcultures at 30° on 19mm-sodium succinate [0.5% (w/v) Na₂C₄H₄O₄·6H₂O] as sole carbon and energy source. When the last culture (400 ml.) was well grown it was divided into two equal volumes and the cells from each harvested aseptically by centrifugation at 20°. After being washed in sterile 50mm-phosphate, pH 7.5, the pellets were resuspended in 500 ml. of mineral salt medium containing either 19mm-sodium succinate or 74mm-methylamine hydrochloride as sole carbon source. These suspensions were used to inoculate 1600 ml. of the same medium contained in 4 l. flasks (fitted with an outlet for aseptic removal of samples), which were then shaken at 30°. The initial cell density was 75 µg. dry wt./ml. Samples were withdrawn at intervals from the two cultures for measurement of growth and specific activity. Cells for specific-activity determinations were centrifuged, washed in 5 ml. of cold 50mm-phosphate, pH 7.5, and stored at -15° until assayed.

Purification of the methylamine-oxidizing enzyme. Step 1: preparation of crude extract. Methylamine-grown cells (20g. wet wt.) were mixed with 40 ml. of ice-cold 0.1M-phosphate buffer, pH 7.5, and divided into five portions. Each of these was disrupted by sonic disintegration, as described above. The portions were combined and centrifuged at 30000g for 10 min. at 2°.

Step 2: protamine sulphate treatment. The crude extract was treated with 2% (w/v) protamine sulphate by dropwise addition until no more precipitate was formed. The precipitate was centrifuged off and discarded.

Step 3: heat treatment. The supernatant from step 2 was heated rapidly to 75° in an aluminium container, and maintained at this temperature for exactly 20 min. During this time the suspension was constantly stirred by a mechanical stirrer but was not allowed to froth. After 20 min., it was chilled to 2° and the denatured protein centrifuged off.

Step 4: (NH₄)₂SO₄ fractionation. The supernatant from step 3 was made 50% saturated with respect to (NH₄)₂SO₄ by the slow addition of solid (NH₄)₂SO₄, and allowed to equilibrate at 2° for 1 hr. The precipitate formed was centrifuged off and discarded. The supernatant was made 85% saturated by the addition of more solid (NH₄)₂SO₄. The resulting precipitate, after equilibration as before, was centrifuged off and redissolved in 7 ml. of 0.1M-phosphate buffer, pH 7.5.

Step 5: gel filtration. The redissolved (NH₄)₂SO₄ precipitate was applied to a column (1.5 cm. × 30 cm.) of Sephadex G-75. The column was eluted at 22° with 50mm-phosphate buffer, pH 7.5, by using an LKB ReCyChrom peristaltic pump (type 4912A) (LKB Produkter A.B., Stockholm-Bromma 1, Sweden), and 5.2 ml. fractions were collected in an LKB RadiRac fraction collector operating on a time basis (controller type 3403B). The eluate was monitored for u.v.-absorbing material by measurement of extinction at 254 mµ in an LKB Uvicord (type 4701A) with recorder (type 6520A). The protein and activity profile of the eluate is shown in Fig. 1. The enzyme was excluded from the gel and was the first protein to emerge from the column as a

Fig. 1. Molecular exclusion of amine dehydrogenase by Sephadex G-75. Conditions are given in the text. The continuous line is a trace from an LKB Uvicord recorder that showed transmission at 254 mµ. The left-hand ordinate scale has been corrected to show extinction for a 1 cm. light-path. ●, Enzyme activity (units/ml.).

yellow peak. The succeeding peak was red in colour and was followed by a second yellow peak.

Step 6: dialysis and ion-exchange chromatography. The most active Sephadex fractions were combined and dialysed overnight at 4° against 10 l. of 5mm-sodium-potassium phosphate buffer, pH 6. The combined dialysed fractions were applied to a column (1.5 cm. × 22 cm.) of DEAE-cellulose (Bio-Rad Cellex-D, high capacity) equilibrated with the same buffer. The protein was eluted at 22° with a linear gradient of sodium-potassium phosphate, pH 6, from an initial concentration of 5 mM to a final concentration of 0.5M. The gradient was produced by a simple linear gradient device (Morris & Morris, 1964), the total volume being 400 ml.

The eluate was monitored for protein with the Uvicord as before. Fractions (4.7 ml.) were collected, and the protein concentration in these was determined from the extinction at 280 mµ measured on a Zeiss PMQ II spectrophotometer. Fig. 2 shows the protein and activity pattern of the eluate. The activity was completely eluted between 23 mM- and 38 mM-phosphate. The active fractions, which were yellow in colour, were stored at -15°. The purification procedure is summarized in Table 1.

RESULTS

Oxidation of C₁ substrates by whole cells and crude extracts

Washed suspensions of methylamine-grown *Pseudomonas* AM1 readily oxidized methylamine hydrochloride and formaldehyde. Formate was oxidized at a low rate after a lag of 10 min. (Table 2). Methanol was slightly oxidized. Crude Hughespress extracts showed slight oxidative activity

Fig. 2. Elution of amine dehydrogenase from a DEAE-cellulose column. Conditions are given in the text. $\circ$, E_{280} ; $\bullet$, activity of the enzyme; Δ , gradient of phosphate concentration.

Table 1. *Purification of the amine-oxidizing enzyme from Pseudomonas AM1*

Step no.	Fraction	Volume (ml.)	Activity (units/ml.)	Concn. of protein (mg./ml.)	Total activity (units)	Total protein (mg.)	Sp. activity (units/mg. or protein)	Purification factor	Yield (%)
1	Supernatant obtained from crude extract after centrifugation	44.4	3.62	21.2	161	940	0.171	1	100
2	Supernatant after removal of protamine sulphate precipitate	49.3	3.21	15.6	158	769	0.206	1.20	98
3	Supernatant after removal of heat-denatured protein (20 min. at 75°)	41.8	3.5	7.0	146.5	293	0.500	2.92	91
4	Fraction precipitated by 50–85% satd. $(\text{NH}_4)_2\text{SO}_4$	7	7.9	11	55.2	77.0	0.715	4.18	34.4
5	Sephadex G-75 effluent (combined fractions)	15	4.3	2.86	64.5	43	1.52	8.9	40.1
6	DEAE-cellulose effluent (combined fractions)	19	2.63	0.75	50	14.3	3.50	20.5	31.1

with formaldehyde. On the addition of 0.35 mM-PMS and 1 mM-potassium cyanide, however, there was a rapid oxidation of methylamine, and formaldehyde was also oxidized. Methanol and formate were only slightly oxidized. The enzymes oxidizing formaldehyde and formate are presumably the same as those present in methanol-grown cells

(Johnson & Quayle, 1964), and were not further investigated.

Methylamine oxidation by extracts could also be followed spectrophotometrically by replacing oxygen with DCPIP. The addition of 1 mM-cyanide was necessary with crude extracts to inhibit cytochrome oxidase activity.

The methylamine-oxidizing activity did not appear to be particle-bound, since over 90% of the activity of Hughes-press extracts was not sedimentable in 90 min. at 150 000g in an MSE Super-speed 50 ultracentrifuge.

Changes in the specific activity of the methylamine-oxidizing enzyme during adaptation of succinate-grown Pseudomonas AM1 to growth on methylamine

To investigate the role of the enzyme in growth on methylamine, the appearance of enzyme activity during adaptation of the cells from growth on succinate to growth on methylamine was followed. After five serial subcultures on 17 mM-succinate, no methylamine-oxidizing activity was detectable spectrophotometrically in the cell extracts. Such succinate-grown cells were resuspended in either succinate or methylamine medium (approximately equal in g.-atoms of carbon/l.), and samples were taken at intervals to measure growth and enzyme activity. The results of this experiment are shown in Fig. 3. Cells transferred from succinate to growth continued to grow after a lag of 1 hr. Cells transferred from succinate to methylamine grew after a lag period of 5 hr. During this period before growth began there was a rapid increase in the specific activity of the methylamine-oxidizing enzyme from a specific activity of 0 to one of 40 mμmoles/min./mg. of protein, which was about half the final specific activity of the cells in this culture. The enzyme was undetectable throughout the cycle of growth on succinate.

Purity of the methylamine-oxidizing enzyme

The purification procedure described in the Materials and Methods section gave a preparation that was 20-fold more active than the crude extract. Crude extracts of methylamine-grown cells on disc electrophoresis in polyacrylamide gel at pH 9.5 showed a band of enzyme activity at R_F 0.15–0.2 (bromophenol blue = 1.0; Chang, Srb & Steward,

Table 2. Oxidation of C_1 substrates by methylamine-grown *Pseudomonas* AM1

Manometer flasks contained in a total fluid volume of 3 ml.: in the main compartment, 200 μmoles of phosphate buffer, pH 7.5, and whole cells (2 mg. dry wt.) or extracts (8 mg. of protein); in the centre well, 0.2 ml. of 20% (w/v) KOH; and in the side arm 20 μmoles of substrate. In addition, flasks with extracts contained 1 μmole of PMS and 3 μmoles of KCN in the main compartment. The gas phase was air and the temperature 30°.

Substrate	Rate of oxidation	
	Whole cells (μl. of O ₂ /hr./ mg. dry wt.)	Crude extracts (μl. of O ₂ /hr./ mg. of protein)
None	2	3
Methylamine	190	268
Formaldehyde	110	14
Sodium formate	10*	4
Methanol	43	6

* After 10 min. lag.

Fig. 3. Growth and changes in specific activity of the methylamine-oxidizing enzyme during adaptation of succinate-grown cells to growth on methylamine. Cells of succinate-grown *Pseudomonas* AM1 were washed and resuspended in growth medium containing either 19 mM-sodium succinate or 74 mM-methylamine hydrochloride as sole carbon source. Subsequent growth on both substrates was followed turbidimetrically and cells were removed at intervals for determination of the specific activity of the methylamine-oxidizing enzyme. Methylamine medium: □, growth; ■, specific activity of enzyme. Succinate medium: ○, growth; ●, specific activity of enzyme.

Fig. 4. pH optimum of the purified amine dehydrogenase. Glass cuvettes (1cm. light-path) contained, in a total volume of 3ml.: 20 μ moles of substrate, 1 μ mole of PMS; 0.146 μ mole of DCPIP, 200 μ moles of buffer and 8.3 μ g. of enzyme. The rate of DCPIP reduction was measured at 600m μ . The temperature was 30°. □, Methylamine; ○, *n*-pentylamine; Δ, putrescine. Buffers used were: acetate below pH 6.0; phosphate, pH 6.0–8.0; glycine–NaOH above pH 8.0.

1962). This band was absent from succinate-grown cells. The purified enzyme showed a single band of activity in the same position, and when 100 μ g. of purified enzyme were applied to the gel, although six minor contaminating bands were detected at pH 9.5 and one contaminant at pH 4.3, the major protein band coincided with the activity. The contaminants were estimated (by comparison with known amounts of protein) to comprise some 3 μ g. of protein, which suggested that the purity of the purified enzyme was greater than 95%.

Variables in the spectrophotometric assay system

Effect of pH on enzyme activity. The pH optimum of the enzyme was dependent on the substrate that was being oxidized. The pH–activity curves for methylamine, *n*-pentylamine and putrescine are shown in Fig. 4. The pH optima for these substrates are 7.6, 8.0 and 8.5 respectively. In determining the activity, the variation of the molar extinction coefficient of DCPIP with pH (Armstrong, 1964) has been taken into account. Tris hydrochloride buffer could not be used, since formaldehyde in the presence of tris was found to reduce DCPIP non-enzymically.

Substrate concentration. The substrate concentration in the standard assay system was 6.67mM. The K_m for methylamine, calculated from Dixon (1953) plots in the presence of various inhibitors

(see Table 6 below), gave an average figure of 5.2 μ M. This value is so small that the total change in extinction did not allow reliable initial rates to be determined in the micromolar concentration range, which precluded the possibility of measuring the K_m directly by a conventional Lineweaver & Burk (1934) plot. The standard concentration was judged to be saturating but not inhibitory.

PMS concentration. The rate of reduction of DCPIP by the methylamine-oxidizing enzyme at saturating methylamine concentration was found to be dependent upon the PMS concentration. A Lineweaver–Burk plot of velocity of DCPIP reduction at different PMS concentrations gave a K_m for PMS of 56 μ M.

DCPIP concentration. The rate of DCPIP reduction in this system was not dependent on DCPIP concentration. Under the conditions of the standard assay system the rate of reduction remained linear for a change in extinction of 0.6.

Enzyme concentration. The rate of reduction of DCPIP remained linear with increasing enzyme concentrations up to 300 μ g. of protein for the purified enzyme, when methylamine was being oxidized, which corresponds to a rate of change in extinction of 0.3/min.

Properties of the purified amine dehydrogenase

Stoichiometry and products of the oxidation of methylamine by the purified enzyme. Methylamine oxidation was followed manometrically in the presence of PMS (Table 3). The identity of formaldehyde is shown by the reaction of the product in the specific chromotropic acid assay (MacFadyen, 1945). The non-quantitative recovery of formaldehyde was thought to be due to its oxidation by the hydrogen peroxide formed in the reaction. When 8×10^4 units of crystalline catalase were included in the reaction mixture, the oxygen uptake was decreased by 50% and reasonably satisfactory yields of formaldehyde were obtained. The data obtained are consistent with the following reactions occurring:

Specificity for electron acceptors. Oxygen is not active as a primary acceptor for the enzyme, there being an obligatory requirement for the presence of an artificial electron acceptor. Table 4 shows the relative rates of reaction of the dehydrogenase with a variety of acceptors. Apart from PMS,

Table 3. *Stoichiometry of methylamine oxidation by the purified amine dehydrogenase*

Manometer flasks contained in a total fluid volume of 3 ml.: 150 μ moles of phosphate buffer, pH 7.5, 3 μ moles of PMS, 390 μ g. of purified enzyme (and in one flask, 8×10^4 units of crystalline catalase) in the main compartment; and 0.1 ml. of 40% (w/v) KOH in the centre well. After equilibration for 10 min. at 30°, the reaction was started by tipping 10 μ moles of methylamine hydrochloride from the side arm. Oxygen uptake was followed for 25 min., and the reaction was then stopped by adding 0.5 ml. of 10% (v/v) HClO₄. Samples were removed and methylamine, formaldehyde and ammonia were determined as described in the Materials and Methods section.

	Substrates consumed (μ moles)		Products formed (μ moles)	
	Methylamine	Oxygen	Ammonia	Formaldehyde
No catalase	8.4	8.0	8.3	5.9
Catalase present	8.2	4.25	8.3	7.5

Table 4. *Specificity of the purified amine dehydrogenase for primary electron acceptors*

PMS was tested by coupling with other acceptors or oxygen rather than by measuring PMS reduction directly (Singer & Kearney, 1958); other primary electron acceptors were tested at the wavelength indicated; autoxidizable acceptors (*) were tested in anaerobic cuvettes under N₂. Maximum rates and K_m values were obtained from double-reciprocal plots (Lineweaver & Burk, 1934).

Acceptor	Wavelength (m μ)	10 ⁻³ ϵ	Maximum rate of reduction (μ moles/ min./mg. of protein)	K_m of acceptor (mM)
Oxygen (manometric assay)			0	—
PMS (+ DCPIP)	600	21.5	2.5	0.056
PMS (+ cytochrome <i>c</i>)	550	21.0	3.75	0.063
PMS (+ ferricyanide)	420	1.02	3.32	0.055
PMS (+ O ₂ , manometric assay)			2.86	0.475
Cytochrome <i>c</i>	550	21.0	1.24	10.0
Ferricyanide	420	1.02	0.602	0.77
DCPIP	600	21.5	0.058	—
NAD	340		0	—
NADP	340		0	—
FMN*	450		0	—
FAD*	450		0	—
Methylene blue*	620		0	—
Brilliant cresyl blue (manometric assay)			0.448	185.0
Menadione (manometric assay)			0	—

cytochrome *c* and ferricyanide were the most active; no activity was detectable with nicotinamide nucleotides, flavines or menadione. The rates are expressed as V_{max} , obtained by extrapolation of double-reciprocal plots of velocity and acceptor concentration. The importance of expressing relative rates in this manner, rather than comparing rates at an arbitrary concentration of acceptor, is discussed by Massey & Singer (1957). In addition to the compounds shown in Table 4, resazurin and Wurster's blue were found to act as acceptors when tested at high enzyme concentrations in Thunberg tubes flushed with nitrogen.

Stability of the enzyme. The enzyme was stable to freezing and thawing and to storage for at least 4 months at -15° at a protein concentration of 1 mg./ml. There was no detectable loss in activity on exposure of the enzyme for 75 min. to pH values

within the range 2.6 to 10.6. The enzyme is heat-stable, withstanding 15 min. at 80° without loss of activity. Above 80°, the enzyme is rapidly inactivated (Fig. 5).

Effect of cations on activity. No activation by univalent or bivalent cations could be demonstrated. When tested at 5 mM in the standard spectrophotometric assay at pH 7.5 (hydroxyethyl-piperazineethanesulphonate replacing phosphate as buffer), Mn²⁺, Ca²⁺, Co²⁺ and Na⁺ were slightly inhibitory, and Mg²⁺, K⁺ and NH₄⁺ were not stimulatory. Cu²⁺, Fe²⁺ and Zn²⁺ could not be tested since they interfered with the assay system.

Effect of inhibitors. Table 5 shows the results of testing a wide range of potential inhibitors of the enzyme. The most active inhibitors were quinacrine, biscyclohexanoneoxalyldihydrazide (cuprizone), semicarbazide, isonicotinic acid hydrazide

(isoniazid) and *p*-chloromercuribenzoate. These were further investigated (Table 6) to determine the nature of the inhibition and the K_i , by plotting the reciprocal of the velocity against inhibitor

Fig. 5. Temperature-stability of the purified amine dehydrogenase. Samples (0.5 ml.) of enzyme (1.8 mg. of protein/ml.) were heated in a constant-temperature bath at the temperature indicated. Samples (0.05 ml.) were withdrawn at various time-intervals and diluted 1:20 with phosphate buffer, pH 7.5, before assay in the standard spectrophotometric assay system at 28°. ●, 70°; ○, 80°; ▲, 82°; □, 84°; ■, 88°.

concentration (Dixon, 1953). From the Dixon plots at two methylamine concentrations, the K_m for methylamine was calculated. The non-competitive inhibition produced by semicarbazide, cuprizone and isoniazid was not instantaneous; the inhibitors required preincubation with the enzyme for maximum inhibition to be observed. Fig. 6 shows the time-course of the inhibition produced by these compounds. Hydroxylamine was tested as an inhibitor manometrically because it reduced DCPIP non-enzymically. Phenylhydrazine and diethyldithiocarbamate, which are likely inhibitors of the enzyme, could not be tested because they reacted with PMS.

Substrate specificity. The purified enzyme was found to have a wide specificity, oxidizing many compounds that contain a primary amino group. With both monoamines and diamines, the maximum rate was observed with the C_3 homologue. Table 7 shows the range of amines tested as substrates and the relative rates of oxidation of those compounds that were active. Potential substrates were tested in the spectrophotometric assay system at a final concentration of 6.6 mM. With the relatively insoluble long-chain monoamines the final assay concentration was 0.66 mM. Secondary and tertiary amines and quaternary ammonium salts were not oxidized. The polyamines spermine and spermidine

Table 5. *Effect of chelating agents, thiol reagents, carbonyl reagents and other potential inhibitors on methylamine oxidation by the purified amine dehydrogenase*

Enzyme activity was measured under standard spectrophotometric assay conditions (see the Materials and Methods section). The incubation mixture was equilibrated at 30° in the dark for the time indicated before the reaction was started by the addition of substrate. The absolute rate in the uninhibited system was 2.45 μ moles of methylamine oxidized/min./mg. of protein.

Inhibitor	Preincubation period (min.)	Final concentration (mM)	Inhibition (%)
Thiol reagents			
<i>p</i> -Chloromercuribenzoate (sodium salt)	30	6.05	78
Iodoacetic acid	30	35.4	0
<i>N</i> -Ethylmaleimide	30	35.4	0
Chelating agents			
Cuprizone	15	0.0014	25
Neocuproine	15	0.6	0
8-Hydroxyquinoline	15	11.0	(5% stimulation)
$\alpha\alpha'$ -Bipyridyl	15	0.6	0
EDTA	15	12.0	(10% stimulation)
Potassium cyanide	15	11.2	7.5
Carbonyl reagents			
Semicarbazide	15	0.0018	70
Hydroxylamine (manometric assay)	15	0.049	100
Isoniazid	15	0.0365	55
Other potential inhibitors			
Quinacrine	0	0.66	25
Methylene blue (manometric assay)	15	1.06	0
<i>trans</i> -2-Phenylcyclopropylamine	10	0.2	

Table 6. Summary of kinetic data of the inhibition of the amine dehydrogenase by selected inhibitors

Enzyme activity was measured under the standard spectrophotometric assay conditions (see the Materials and Methods section), except that either the methylamine concentration was lowered to $6.67\mu\text{M}$ or the PMS concentration was lowered to $33.3\mu\text{M}$, in order to obtain data from Dixon (1953) plots of the inhibition produced by these compounds. The mixture was incubated at 30° in the dark for the time indicated before the reaction was started by the addition of substrate.

Inhibitor	Preincubation period (min.)	Type of inhibition	K_i'	K_m calculated for methylamine (μM)	K_m calculated for PMS (μM)
<i>p</i> -Chloromercuribenzoate	15	Competitive	0.45 mM	3.37	—
Isoniazid	15	Uncompetitive	$1.17\mu\text{M}$	8.86	—
Semicarbazide	15	Non-competitive	$3.35\mu\text{M}$	3.97	—
Quinacrine	0	Competitive	12.1 mM	—	39.8
Cuprizone	15	Non-competitive	$0.49\mu\text{M}$	4.53	—
Cuprizone	15	Non-competitive	$0.2\mu\text{M}$	—	38.6

Fig. 6. Time-course of the inhibition of the amine dehydrogenase produced by cuprizone, semicarbazide and isoniazid. Purified enzyme ($57\mu\text{g.}$) was incubated at 30° in a system containing: inhibitor at the concentration indicated below, $10\mu\text{moles}$ of PMS, 1.5 m-moles of phosphate buffer, pH 7.5, and water, to give a final volume of 27.5 ml. Samples (2.75 ml.) were removed at intervals, and $0.149\mu\text{mole}$ of DCPIP and $20\mu\text{moles}$ of methylamine were added in a volume of 0.25 ml. to start the reaction. ●, No inhibitor; ○, cuprizone ($1.45\mu\text{M}$); □, isoniazid ($36.5\mu\text{M}$); Δ, semicarbazide ($1.82\mu\text{M}$).

were oxidized, as were certain substituted primary amines, notably ethanolamine and histamine.

DISCUSSION

The data with whole cells and crude extracts suggest that in *Pseudomonas* AM1 the oxidation of methylamine proceeds via formaldehyde and formate to carbon dioxide, and that methanol is not an intermediate. This agrees with the observations on methylamine oxidation made by Iyer & Kallio (1958) with *Bacillus sphaericus*. It seems possible, however, that the *Pseudomonas* sp. of

Shaw *et al.* (1966) uses a different pathway for methylamine oxidation, since, although their organism oxidized methylamine with formate as an intermediate, neither cells nor crude extracts oxidized formaldehyde or methanol. Similar observations have been made (J. M. Cook & P. J. Large, unpublished work) on methylamine oxidation by *Pseudomonas aminovorans*. The methanol dehydrogenase (Anthony & Zatman, 1964b, 1965) present in methanol-grown *Pseudomonas* AM1 (Johnson & Quayle, 1964) appears to be absent from methylamine-grown cells.

The purified amine dehydrogenase of *Pseudomonas* AM1 is similar, both in its substrate specificity and in its inhibitor pattern, to amine oxidases from several sources. The substrate specificity of the purified enzyme is comparable with that of the purified amine oxidases [amine-oxygen oxidoreductases (deaminating)] of pea seedlings (Hill & Mann, 1964) and ox plasma (Tabor, Tabor & Rosenthal, 1954), in that monoamines, diamines and polyamines are all readily oxidized. The inhibitor pattern of the *Pseudomonas* AM1 enzyme is similar to that of those amine oxidases that have been shown to contain Cu^{2+} and in some cases pyridoxal phosphate as their prosthetic group, for example, the pea seedling enzyme (Hill & Mann, 1964), the ox plasma enzyme (Yamada & Yasunobu, 1962, 1963), the pig plasma enzyme (Blaschko & Buffoni, 1966), the enzymes from *Aspergillus niger* (Yamada, Adachi & Ogata, 1965c) and *Trichosporon* sp. (Yamada, Kumagai, Uwajima & Ogata, 1966), and also pig kidney diamine oxidase (Mondovì, Costa, Agrò & Rotilio, 1967) and histaminase (Kapeller-Adler & MacFarlane, 1963). These enzymes are potentially inhibited by semicarbazide, cuprizone and isonicotinic acid hydrazide, compounds that also inhibit the amine dehydrogenase of *Pseudomonas* AM1. It differs from these enzymes in that it will not react with oxygen, but only with an electron acceptor such as cytochrome *c*, PMS, ferricyanide

Table 7. *Substrate specificity of the purified amine dehydrogenase of Pseudomonas AM1*

Enzyme activity was measured under the standard spectrophotometric assay conditions (see the Materials and Methods section). The absolute rate of methylamine oxidation was 2.47 μ moles/min./mg. of protein. All the substrates were added as their hydrochlorides to give a final concentration of 6.6 mM [except in two cases (*), where 0.66 mM was used].

Substrates oxidized				Substrates not oxidized
	Rate (% of rate with methylamine)		Rate (% of rate with methylamine)	
1. Primary aliphatic monamines		2. Primary aliphatic diamines		
Methylamine	100	1,2-Diaminoethane	23.2	Dimethylamine
Ethylamine	98.5	1,3-Diaminopropane	450	Trimethylamine
<i>n</i> -Propylamine	127	1,4-Diaminobutane	168	Tetramethylammonium chloride
Isopropylamine	0.1	1,5-Diaminopentane	96.5	Diethylamine
<i>n</i> -Butylamine	100	1,6-Diaminohexane	256	Triethylamine
Isobutylamine	0.1			Tetraethylammonium chloride
<i>n</i> -Pentylamine	98.5	3. Polyamines		<i>sec.</i> -Butylamine
<i>n</i> -Hexylamine	27.6	Spermine	21.5	<i>tert.</i> -Butylamine
<i>n</i> -Heptylamine	1.6	Spermidine	81	1,2-Diaminopropane
<i>n</i> -Octylamine	6.4			Tryptamine
<i>n</i> -Nonylamine	2.5	4. Substituted primary amines		L-Noradrenaline
<i>n</i> -Decylamine*	0.2	Ethanolamine	150	L-Lysine
<i>n</i> -Dodecylamine*	0.2	Benzylamine	1.9	
		Phenethylamine	8.1	
		Histamine	160	
		Tyramine	0.6	
		L-Ornithine	39.4	

or brilliant cresyl blue, and hence would be classified as an amine dehydrogenase [amine-(acceptor) oxidoreductase (deaminating)]. Enzymes of this type have been described in crude preparations from an *Achromobacter* sp. adapted to putrescine or histamine (Satake & Fujita, 1953). These preparations used DCPIP as electron acceptor, were inhibited by quinacrine and were specific for the amine to which the cells were adapted. A specific spermidine dehydrogenase (attacking the secondary amino group) has been purified from *Serratia marcescens* (Bachrach, 1962; Campbello, Tabor & Tabor, 1965), and requires PMS as electron acceptor. It is assumed that the natural electron acceptors of such enzymes are flavoproteins (Muraoka, Hoshika, Yamasaki, Yamada & Adachi, 1966). These last workers report low activity of their amine oxidase (crystallized from *Aspergillus niger*) with horse heart cytochrome *c*, although the physiological significance of this observation remains to be evaluated. They consider that this enzyme may be similar to xanthine oxidase (EC 1.2.3.2), which can also use other electron acceptors such as dyes, quinones and cytochrome *c*.

The most unusual feature of the enzyme described in the present paper is its heat-stability (Fig. 5), which is much greater than that of the oxidases from *Aspergillus*, *Trichosporon* or beef liver mitochondria, all of which are inactivated within 10–15 min.

at 60° (Yamada, Adachi & Ogata, 1965*b*; Yamada *et al.* 1966; Nara, Gomes & Yasunobu, 1966).

The amine dehydrogenase described here is essential for growth on methylamine by *Pseudomonas* AM1. This is evident from the complete absence of the enzyme from succinate-grown cells and the appearance of enzyme activity before growth begins when succinate-grown cells are transferred to methylamine medium. We suggest that the amine dehydrogenase is important in the energy metabolism of *Pseudomonas* AM1 during growth on methylamine, since the organism is presumed to obtain its energy for growth and biosynthesis by the direct oxidation of its C₁ substrate to carbon dioxide and water. A dehydrogenase coupled to oxidative phosphorylation would be the most efficient mechanism for the cell in terms of energy production, rather than non-energy-yielding oxidases, such as are found in micro-organisms utilizing amines as sole nitrogen but not carbon source (Yamada, Adachi & Ogata, 1965*a*; Yamada *et al.* 1966).

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