Purification and Properties of the Trimethylamine Dehydrogenase of Bacterium 4B6

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1. The trimethylamine dehydrogenase of bacterium 4B6 was purified to homogeneity as judged by analytical polyacrylamide-gel electrophoresis. The specific activity of the purified enzyme is 30-fold higher than that of crude sonic extracts. 2. The molecular weight of the enzyme is 161 000. 3. The kinetic properties of the purified enzyme were studied by using an anaerobic spectrophotometric assay method allowing the determination of trimethylamine dehydrogenase activity at pH 8.5, the optimum pH. The apparent K_m for trimethylamine is $2.0 \pm 0.3 \mu\text{M}$ and the apparent K_m for the primary hydrogen acceptor, phenazine methosulphate, is 1.25 mM. 4. Of 13 hydrogen acceptors tested, only Brilliant Cresyl Blue and Methylene Blue replace phenazine methosulphate. 5. A number of secondary and tertiary amines with *N*-methyl and/or *N*-ethyl groups are oxidized by the purified enzyme; primary amines and quaternary ammonium salts are not oxidized. Of the compounds that are oxidized by the purified enzyme, only trimethylamine and ethyldimethylamine support the growth of bacterium 4B6. 6. Trimethylamine dehydrogenase catalyses the anaerobic oxidative *N*-demethylation of trimethylamine with the formation of stoichiometric amounts of dimethylamine and formaldehyde. Ethyldimethylamine is also oxidatively *N*-demethylated yielding ethylmethylamine and formaldehyde; diethylamine is oxidatively *N*-de-ethylated. 7. The activity of the purified enzyme is unaffected by chelating agents and carbonyl reagents, but is inhibited by some thiol-binding reagents and by Cu^{2+} , Co^{3+} , Ni^{2+} , Ag^+ and Hg^{2+} . Trimethylamine dehydrogenase activity is potently inhibited by trimethylsulphonium chloride, by tetramethylammonium chloride and other quaternary ammonium salts, and by monoamine oxidase inhibitors of the substituted hydrazine and the non-hydrazine types. 8. Inhibition by the substituted hydrazines is time-dependent, is prevented by the presence of trimethylamine or trimethylamine analogues and in some cases requires the presence of the hydrogen acceptor phenazine methosulphate. The inhibition was irreversible with the four substituted hydrazines that were tested.

The isolation and characterization of bacterium 4B6 has been described by Colby & Zatman (1972, 1973). Crude sonic extracts prepared from trimethylamine-grown organisms contain a trimethylamine dehydrogenase that is apparently important for the growth of bacterium 4B6 on trimethylamine. The presence of the enzyme has been demonstrated in another obligate methylotroph, bacterium C2A1 (Colby & Zatman, 1973), but it was not detected in the seven facultative methylotrophs examined. These results suggested that a correlation might exist among trimethylamine-utilizing bacteria between obligate methylotrophy on the one hand and trimethylamine dehydrogenase on the other.

The present paper describes the purification of trimethylamine dehydrogenase and the investigation of some of its properties. An account of part of this work has been presented (Colby & Zatman, 1971).

Materials and Methods

Materials

Trimethylamine hydrochloride, hexamethylene-bis-trimethylammonium chloride, dimethylacetamide, 2-dimethylaminopropan-2-ol, dimethylaminoacetonitrile, dimethylaminobenzoic acid, ethyldimethylamine, diethylmethylamine, 2-hydroxyethyldimethylamine and 2-aminoethyldimethylamine were obtained from Fluka Chemische Fabrik, Buchs, Switzerland. Dimethylformamide, dimethylglycine hydrochloride, 1,4-diaminobutane dihydrochloride, 2-chloroethyldimethylamine hydrochloride, 2-phenylethylamine and betaine hydrochloride were purchased from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Trimethylsulphonium chloride and trimethylhydrazonium iodide were from K & K Laboratories Inc., Plainview, N.Y., U.S.A. Ethyl-

methylamine was obtained from Eastman Organic Chemicals, Rochester, N.Y., U.S.A., and *ON*-dimethylhydroxylamine from Aldrich Chemical Co., Milwaukee, Wis., U.S.A. Spermine and spermidine were from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other potential enzyme substrates were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Fumarate hydratase (pig heart), lactate dehydrogenase (rabbit muscle), malate dehydrogenase (pig heart), catalase (ox liver) and the nicotinamide nucleotide coenzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K. Ferritin was obtained from Calbiochem Ltd., London W.1, U.K.

Benzylhydrazine sulphate, 2-phenylethylhydrazine sulphate, 1-phenylethylhydrazine oxalate, isocarb-oxazid and nialamid were gifts from Fisons Industrial Chemicals (Loughborough, Leics., U.K.), William R. Warner and Co. Ltd. (Eastleigh, Hants., U.K.), Imperial Chemical Industries Ltd. (Alderley Park, Macclesfield, Cheshire, U.K.), Roche Products Ltd. (Welwyn Garden City, Herts., U.K.) and Pfizer Ltd. (Sandwich, Kent, U.K.) respectively. Iproniazid phosphate was purchased from the Sigma (London) Chemical Co., and isopropylhydrazine oxalate was from K & K Laboratories Inc.

Methods

Spectrophotometry. Enzyme assays were performed by using either a Hitachi Perkin-Elmer 124 double-beam grating spectrophotometer or a Unicam SP. 800 spectrophotometer. Both instruments incorporated constant-temperature cuvette housings and were linked to Servoscribe chart recorders (Smiths Industries Ltd., Wembley, Middx, U.K.).

Gas-liquid chromatography. This was performed with a Perkin-Elmer F11 Gas Chromatograph fitted with a flame ionization detector and coupled to a Vitatron type UR 402/M recorder (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.). The carrier gas was N_2 used at flow rates of 10–15 cm³/min. Packed glass columns (3 mm internal diam.) were used throughout.

Buffer solutions. Sodium pyrophosphate buffers were prepared by dissolving the solid in glass-distilled water, adjusting the pH to the required value with 2M-HCl and making up to the correct volume. Other buffer solutions were prepared as described by Dawson *et al.* (1969) or by Good *et al.* (1966).

Analytical methods. (a) Protein. This was measured by the method of Lowry *et al.* (1951) as modified by Kennedy & Fewson (1968).

(b) Chloride. This was determined by titration with mercuric nitrate by a modification of the method of Schales & Schales (1941).

(c) Amines. The methylamines, ethyldimethylamine and ethylmethylamine were identified and determined

by g.l.c. as described by Colby & Zatman (1973). Ethylamine and diethylamine were not resolved on the column used for the methylamines, and the products of diethylamine oxidation were determined by using a glass column (3.66 m × 3 mm internal diam.) packed with Chromosorb W (60–80 mesh) coated with 4% (w/w) tetraethylenepentamine and 12.5% (w/w) diglycerol (Sze *et al.*, 1963). A soda-lime pre-column was housed in the injection block at a temperature of 200°C and the oven temperature was 70°C.

(d) Acetaldehyde. This was measured by g.l.c. on a column (1.83 m × 3 mm internal diam.) of Chromosorb W (80–100 mesh) coated with 10% (w/w) Carbowax 20M. The injection temperature was 170°C and the oven temperature was 40°C.

(e) Formaldehyde. A colorimetric method based on that of Schryver (1910) was used to measure formaldehyde. Samples (0.5 ml) containing up to 15 µg of formaldehyde were diluted to 1 ml with propan-2-ol. Phenylhydrazine hydrochloride (0.5 ml of a 7.5%, w/v, solution) was added to each followed, after leaving the solution for 10 min, by 0.3 ml of 5% (w/v) potassium ferricyanide. The samples were left for 5 min and then 2 ml of 10% (w/v) NaOH was added to each. Samples were left for a further 10 min and then the E_{520} was measured. All the operations were done at room temperature. Reaction mixtures containing the components of the oxygen-linked assay were diluted tenfold before the measurement of formaldehyde, and after this dilution phenazine methosulphate (now at 0.2 mM) does not interfere; the standards were also prepared in tenfold-diluted assay mixture.

Determination of trimethylamine dehydrogenase activity. The progress of the purification was monitored by using the spectrophotometric enzyme assay at pH 7.7 as described by Colby & Zatman (1973). This enzyme assay was also used to measure trimethylamine dehydrogenase activities in ethyldimethylamine-grown cells. Subsequent kinetic studies with the purified enzyme were carried out by using the following assays in which pH and the concentration of the reactants are optimum.

(a) Anaerobic spectrophotometric assay. Teflon-capped cuvettes (10 mm light-path) contained in 1 ml: 75 µmol of sodium pyrophosphate buffer, pH 8.5; 2 µmol of phenazine methosulphate; 90 nmol of 2,6-dichlorophenol-indophenol; purified enzyme; 330 nmol of trimethylamine hydrochloride. Before the addition of the trimethylamine hydrochloride solution, each reaction mixture was made anaerobic by bubbling with O_2 -free N_2 by means of a stainless-steel syringe needle inserted through the cap. The substrate solution was subsequently injected through the cap to start the reaction. Reactions were carried out at 30°C and the change in E_{600} was measured. All initial velocities were corrected for the slow reduction of the dyes observed in the absence of both enzyme

and substrate. Specific activities obtained by this assay were approximately twice those obtained with the assay described by Colby & Zatman (1973).

(b) Oxygen-linked assay. O_2 uptakes were measured at 30°C in conventional Warburg manometers with air as the gas phase or in a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Reaction mixtures contained in 2 ml: 150 μ mol of sodium pyrophosphate buffer, pH 8.5; 4 μ mol of phenazine methosulphate; purified enzyme; 20 μ mol of trimethylamine hydrochloride. The output from the oxygen electrode was calibrated by using gas mixtures of known O_2 concentrations.

(c) Enzyme units. One unit of trimethylamine dehydrogenase activity as measured in the anaerobic spectrophotometric assay is defined as the quantity of enzyme that catalyses the reduction of 1 μ mol of 2,6-dichlorophenol-indophenol/min under the stated assay conditions. The molar extinction coefficient of 2,6-dichlorophenol-indophenol was taken to be $21.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at pH 8.5 (Armstrong, 1964). Measured in the oxygen-linked assay, 1 unit of enzyme is the quantity of enzyme that catalyses the uptake of 1 μ mol of O_2 /min. These units should be identical and good agreement between them was observed in practice. These values also agreed well with values obtained by measuring the disappearance of trimethylamine.

Determination of other enzyme activities. Dimethylamine mono-oxygenase, formaldehyde dehydrogenase (NAD^+) and formate dehydrogenase (NAD^+) were assayed as described by Colby & Zatman (1973). Fumarate hydratase, catalase, lactate dehydrogenase and malate dehydrogenase were assayed by the methods of Racker (1950), Bergmeyer (1955), Kornberg (1955) and Ochoa (1955) respectively.

Bulk growth of bacterium 4B6. Organisms were grown in mineral base E (Owens & Keddie, 1969) supplemented with vitamin B_{12} (2.5 $\mu\text{g/litre}$) and trimethylamine hydrochloride (2 g/litre), prepared as described by Colby & Zatman (1973). Large quantities of cells for the purification of trimethylamine dehydrogenase were grown in 8-litre batches of medium contained in 10-litre flat-bottomed boiling flasks. These were inoculated with 500 ml of exponential-phase culture and incubated at 30°C under conditions of vigorous aeration. The flasks contained polypropylene-coated magnetic stirring bars and were incubated in a 30°C hot-room on two-place magnetic stirrers similar to those described by Harvey *et al.* (1968). Cultures were harvested in mid-exponential phase after about 16 h of growth by using Griffin-Christ Junior 15000 continuous-flow centrifuges (Griffin and George Ltd., Wembley, Middx., U.K.). The cells were washed twice with 0.1 M-sodium phosphate buffer, pH 7.5, and the cell pellets were stored at -20°C.

Purification of trimethylamine dehydrogenase. All

procedures were carried out at 2°C unless stated otherwise.

Step 1. Preparation of crude sonic extract. Trimethylamine-grown cells were resuspended in ice-cold 0.1 M-sodium pyrophosphate buffer, pH 7.5, at a concentration of 100 mg wet wt./ml. Portions (50 ml) of the suspension were disrupted in a 75 ml Rosett cooling cell (Rosett, 1965) by using a MSE 100 W ultrasonic disintegrator (MSE Ltd., London, U.K.) fitted with a 190 mm diam. titanium probe. The vessel was surrounded by ice-water mixture and sonication was interrupted for 1 min in every 2 min to prevent local warming of the suspension; the total exposure of the suspension to ultrasonic radiation was 16 min. Cell debris was removed by centrifugation at 38 000 g for 30 min.

Step 2. Heat treatment. The crude sonic extract was heated to 60°C in a water bath and maintained at that temperature for 20 min. The extract was then rapidly cooled in ice-water mixture and the heavy precipitate centrifuged off and discarded.

Step 3. Salmine sulphate precipitation. Salmine sulphate solution (15 mg/ml) was added dropwise, with stirring, to the heat-treated extract until 3 mg had been added per ml of extract. The precipitated nucleic acid was removed by centrifugation and discarded.

Step 4. Acetone fractionation. The salmine sulphate supernatant was cooled to 0°C in an ice-salt mixture and acetone (precooled to -20°C) was added dropwise, with stirring, until 0.4 vol. had been added. After stirring for 10 min the precipitated protein was removed by centrifugation at -10°C and discarded. A further 0.3 vol. of acetone (calculated from the original volume of salmine sulphate supernatant) was added to the supernatant and the brown precipitate was collected by centrifugation at -10°C and redissolved in 5 ml of 20 mM-sodium phosphate buffer, pH 7.5. The redissolved acetone precipitate was dialysed against 500 vol. of the same buffer for 2 h.

Step 5. DEAE-cellulose chromatography. The dialysed acetone fraction was placed on a column (1.5 cm $\times$ 25 cm) of DEAE-cellulose (Cellex D, standard capacity; Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and eluted with a linear NaCl gradient of 0-0.5 M in 20 mM-sodium phosphate buffer, pH 7.5; the enzyme was eluted at a concentration of 0.2 M-NaCl. The active fractions were combined and concentrated by bringing to 85% saturation with solid $(\text{NH}_4)_2\text{SO}_4$; the precipitated protein was dissolved in 1 ml of 20 mM-sodium phosphate buffer, pH 7.5, containing 0.1 M-NaCl.

Step 6. Gel-filtration. The concentrated DEAE-cellulose eluate was placed on a column (2.5 cm $\times$ 60 cm) of Sephadex G-200 and eluted with 20 mM-sodium phosphate buffer, pH 7.5, containing 0.1 M-NaCl. The active fractions from the column were combined.

Step 7. $(\text{NH}_4)_2\text{SO}_4$ fractionation. The pool from step 6 was brought to 70% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and the precipitate centrifuged off and discarded. More $(\text{NH}_4)_2\text{SO}_4$ was added until the supernatant was 80% saturated and then the orange-brown precipitate was collected by centrifugation and redissolved in 20 mM-sodium phosphate buffer, pH 7.5. Solutions of the purified enzyme were stored at -20°C .

Analytical polyacrylamide-gel electrophoresis. Electrophoresis was done at room temperature in a Shandon disc-electrophoresis apparatus (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by using 7.5% (w/v) acrylamide gels at pH 8.5 (Davis, 1964) and 5% (w/v) acrylamide gels at pH 4.3 (Reisfeld *et al.*, 1962). Electrophoresis was also done in 7.5% (w/v) gels at pH 7.0 by using 0.375 M-Tris-HCl buffer, pH 7.0, as gel buffer and 10 mM-sodium phosphate buffer, pH 7.0, containing 13.7 g of glycine/l as reservoir buffer, and in 5% (w/v) gels at pH 5.6 by using 0.25 M-sodium acetate-acetic acid buffer, pH 5.6, as gel-buffer and 20 mM-sodium phosphate buffer, pH 5.8, as reservoir buffer. Gels were polymerized with 0.14% ammonium persulphate and 0.12% tetramethylethylenediamine. Samples (15–60 μl) were prepared in 5% (w/v) sucrose containing 0.001% Bromophenol Blue marker dye and layered directly on to the gel beneath the reservoir buffer; large-pore stacking gels were not used. Samples were run into the gel at 2 mA/tube and then, when the marker dye had penetrated the gel by about 1 cm, the current was increased to 4 mA/tube. Gels were either stained for protein with Coomassie Brilliant Blue R (Chrambach *et al.*, 1967) or surrounded by molten 1% agar containing 0.48 mM-MTT-tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], 2 mM-phenazine methosulphate, 1 mM-trimethylamine hydrochloride and 50 mM-sodium pyrophosphate buffer, pH 7.5, to detect trimethylamine dehydrogenase activity. In the latter case, the position of the

enzyme was indicated by the development of a purple band within a few minutes.

Experimental and Results

Preparation and homogeneity of the purified enzyme

Data obtained from a typical purification are presented in Table 1. The enzyme was purified some 30-fold with a 21% yield of enzyme units. Electrophoretograms prepared in 7.5% (w/v) acrylamide gels at pH 8.5 and 7.0, and in 5% (w/v) acrylamide gels at pH 5.6 and 4.3, showed that the purified enzyme contained a single protein component. In each case, the mobility of the protein band relative to that of the Bromophenol Blue marker dye (m_{BPB}) was identical with that of the purple band obtained when trimethylamine dehydrogenase was detected on the gel. The m_{BPB} values obtained in gels prepared at pH 8.5, 7.0, 5.6 and 4.3 were 0.360, 0.210, 0.310 and 0 respectively.

Stability of the purified enzyme

Solutions of the purified trimethylamine dehydrogenase (400 μg of protein/ml) in 20 mM-sodium phosphate buffer, pH 7.5, were stored at -20°C as a routine. About 5% of their activity was lost on each freezing and thawing cycle, but no decrease in activity was observed on uninterrupted storage at -20°C for 3 months. Solutions of the enzyme lost about 10% of their activity on storage at 2°C for 14 days. Although the heat-treatment step in the purification procedure described above (60°C for 20 min) caused the loss of 11% of the activity of the crude sonic extract (Table 1), experiments on the heat stability of dilute solutions of the purified enzyme (40 μg of protein/ml) showed that 50% of the activity of such solutions was lost in 20 min at 60°C , whereas 90% of the activity was lost in 10 min at 70°C . The purified enzyme is thus not unusually stable to heating.

Table 1. *Purification of trimethylamine dehydrogenase from crude sonic extracts of bacterium 4B6*

Trimethylamine dehydrogenase activities were determined at pH 7.7 by using the spectrophotometric method of Colby & Zatman (1973) and are therefore only 50% of the values observed by using the anaerobic spectrophotometric assay (see the Materials and Methods section).

	Volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (unit/mg of protein)	Recovery (%)	Purification factor
Crude sonic extract	80	35.7	1190	0.030	100	1.0
Heat-treated extract	71	31.8	270	0.120	89	4.0
Salmine sulphate supernatant	90	29.9	270	0.119	84	4.0
Dialysed acetone fraction	10	27.7	108	0.257	77	8.6
DEAE-cellulose eluate	30	17.9	24	0.750	50	25.0
Sephadex G-200 eluate	16	11.8	12.8	0.918	33	30.6
$(\text{NH}_4)_2\text{SO}_4$ fraction	0.6	7.5	7.5	1.000	21	33.2

Fig. 1. Effect of the pH value and the nature of the buffer on the activity of purified trimethylamine dehydrogenase

Trimethylamine dehydrogenase activities were measured by using the anaerobic spectrophotometric assay. Reaction mixtures (1 ml) contained 75 μ mol of buffer; 2 μ mol of phenazine methosulphate; 90 nmol of 2,6-dichlorophenol-indophenol; purified enzyme (4 μ g of protein); 330 nmol of trimethylamine hydrochloride. The slow spontaneous reduction of the dyes, the rate of which increased with increasing pH value, was taken into account by preparing control reaction mixtures lacking enzyme at each pH value; the rates of 2,6-dichlorophenol-indophenol reduction observed with these were subtracted from those obtained with the corresponding experimental reaction mixture. After the enzyme activity had been measured, the pH value of each reaction mixture was determined by using a glass electrode. ●, Sodium pyrophosphate buffer; ▲, Tricine-[N-tris(hydroxymethyl)methylglycine] buffer; ○, triethanolamine buffer; □, Bicine [N-bis-(2-hydroxymethyl)-glycine] buffer; △, diethanolamine buffer.

Variables in the anaerobic spectrophotometric assay

The spectrophotometric assay described by Colby & Zatman (1973) was not performed at the optimum pH value for trimethylamine dehydrogenase activity. Two phenomena made the measurement of initial velocities at pH 8.5, the optimum, difficult: (1) the reoxidation of the reduced dyes by molecular O_2 , although insignificant below pH 8.0, increases rapidly at more alkaline pH values; (2) the initial velocities measured at pH 8.5 depend on the concentration of the terminal hydrogen acceptor, 2,6-dichlorophenol-indophenol. These problems were overcome by performing the assay at pH 8.5 anaerobically and in the presence of a higher concentration of the terminal hydrogen acceptor than used by Colby & Zatman (1973). A correction was made for the slow reduction of the dyes observed in the absence of both enzyme and substrate.

(a) *pH value and the nature of the buffer.* The pH-activity curve for the purified enzyme shows the optimum pH to be 8.5 (Fig. 1). The variation in the molar extinction coefficient of 2,6-dichlorophenol-indophenol with pH value was taken into account when calculating the results (Armstrong, 1964). The nature of the buffering agent did not affect the pH optimum nor, in most cases, the initial velocity observed at that pH value. However, the initial velocities observed in 75 mM-Tris-HCl buffers, pH 7.5–9.0, and in boric acid (50 mM)-KCl (50 mM)-NaOH buffers, pH 8.0–10.0, were about 40% lower than the values obtained with other buffers at the same pH values (Fig. 1); imidazole buffer (75 mM) inhibited the enzyme completely. The initial velocity was independent of the concentration of sodium pyrophosphate buffer, pH 8.5, between 10 and 100 mM.

(b) *Phenazine methosulphate concentration.* The apparent K_m value for phenazine methosulphate calculated from double-reciprocal plots (Lineweaver & Burk, 1934) was 1.25 mM at pH 8.5. The highest initial velocity was observed at 2 mM, higher concentrations being inhibitory.

(c) *2,6-Dichlorophenol-indophenol concentration.* The initial velocity was dependent on the concentration of the terminal hydrogen acceptor (Fig. 2). However, the concentration used in the assay, 90 μ M, is almost saturating and no difficulty was experienced in measuring initial rates.

(d) *Substrate concentration.* The apparent K_m value for trimethylamine is very low, which made accurate

Fig. 2. Effect of 2,6-dichlorophenol-indophenol concentration on the activity of the purified trimethylamine dehydrogenase

Trimethylamine dehydrogenase activities were measured by using the anaerobic spectrophotometric assay. Eight reaction mixtures, each with a different concentration of 2,6-dichlorophenol-indophenol, were used and the initial rate of decrease in E_{600} after the addition of trimethylamine hydrochloride was measured in each case.

Table 2. *Specificity of the purified trimethylamine dehydrogenase for primary hydrogen acceptors*

Autoxidizable acceptors were tested in the oxygen electrode by using the oxygen-linked assay (*). The reduction of other acceptors was followed spectrophotometrically under N₂. In addition to the acceptors at the concentrations shown, reaction mixtures (1 ml) contained 75 μ mol of sodium pyrophosphate buffer, pH 8.5; purified enzyme (4–80 μ g of protein); 330 nmol of trimethylamine hydrochloride. Specific activities are given as μ mol of trimethylamine oxidized/min per mg of protein calculated from the rate of reduction of the terminal acceptor. The following molar extinction coefficients were used: 2,6-dichlorophenol-indophenol at 600 nm ($21.95 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$), potassium ferricyanide at 400 nm ($1.02 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$), cytochrome *c* at 550 nm ($21.0 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$), NAD⁺ and NADP at 340 nm ($6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Acceptor (mM)	Specific activity (units/mg of protein)	E'_0 (pH 7, 30°C)
*O ₂ (0.25)	0	+0.815
*Menadione (0.1–10.0)	0	+0.408
*Phenazine methosulphate (2.0)	1.82	+0.080
*Brilliant Cresyl Blue (1.0)	0.37	+0.047
*Methylene Blue (10.0)	0.69	+0.011
*Pyocyanine (1.0)	0.06	–0.034
*FAD (0.1–10.0)	0	–0.219
*FMN (0.1–10.0)	0	–0.219
*Benzyl viologen (0.1–10.0)	0	–0.359
Potassium ferricyanide (0.1–1.0)	0	+0.360
Cytochrome <i>c</i> (horse heart) (0.1)	0	+0.220
2,6-Dichlorophenol-indophenol (0.1)	0	+0.217
Phenazine methosulphate (+0.1 mM- 2,6-dichlorophenol-indophenol) (2.0)	2.03	+0.080
Phenazine methosulphate (+0.1 mM- cytochrome <i>c</i>) (2.0)	2.07	+0.080
NAD ⁺ (0.1–1.0)	0	–0.320
NADP ⁺ (0.1–1.0)	0	–0.320

determination difficult. By taking many initial-velocity measurements at each substrate concentration a value of $2 \mu\text{M} \pm 0.3 \mu\text{M}$ (s.d.) was obtained from double-reciprocal plots (Lineweaver & Burk, 1934). Concentrations of trimethylamine higher than 3.3 mM were inhibitory.

(e) *Enzyme concentration.* The initial velocity of reduction of 2,6-dichlorophenol-indophenol increased linearly with increasing enzyme concentration up to 20 μ g of protein/ml. The specific activity of the purified enzyme at pH 8.5 and 30°C was 2.0–2.1 units/mg of protein.

Molecular weight of the purified enzyme

The molecular weight of the purified enzyme was estimated by gel filtration through Sephadex G-200 (Andrews, 1965, 1970) by using, as markers, ferritin (mol.wt. 550 000), catalase (mol.wt. 240 000), fumarate hydratase (mol.wt. 185 000), lactate dehydrogenase (mol.wt. 135 000) and malate dehydrogenase (mol.wt. 50 000). The location of trimethylamine dehydrogenase activity in the eluate fractions in three such experiments indicated a molecular weight of $161\,000 \pm 3000$.

Alternative primary hydrogen acceptors

Autoxidizable acceptors were tested in the oxygen electrode by using the oxygen-linked assay; others

were tested spectrophotometrically. Only Brilliant Cresyl Blue and Methylene Blue replaced phenazine methosulphate as primary hydrogen acceptor (Table 2), and the maximum rates obtained with these two compounds were 20 and 38%, respectively, of the rate with phenazine methosulphate.

Substrate specificity of the purified enzyme

Trimethylamine dehydrogenase was tested in the anaerobic spectrophotometric assay with a large number of primary, secondary, tertiary and quaternary amines (Table 3). Also some compounds were tried, which, although not amines, share some common structural features with trimethylamine. The purity of those compounds that elicited a positive response was estimated by g.l.c. All contained less than 0.1 mol of trimethylamine/100 mol and most contained less than 0.01 mol/100 mol. In every case the degree of contamination with trimethylamine or with other known enzyme substrates was insufficient to account for the amount of dye reduced in the spectrophotometric assay.

Only secondary or tertiary amines with *N*-methyl and/or *N*-ethyl groups were oxidized; quaternary ammonium salts were not oxidized, neither were primary amines nor diamines. The sulphur, tin and carbon analogues of trimethylamine were not substrates. Except for diethylamine, the best substrates were

Table 3. *Substrate specificity of the purified trimethylamine dehydrogenase and apparent K_m and V_{max} values for some substrates*

Each potential substrate was tested in the anaerobic spectrophotometric assay at two concentrations, 1 and 10 mM, and with two enzyme concentrations, 4 μ g and 40 μ g of protein/ml. The highest specific activities observed are recorded in the table as a percentage of the specific activity obtained with 330 μ M-trimethylamine hydrochloride. The numbers in parentheses are the concentrations (mM) of substrate used that gave the rates recorded. The apparent K_m and V_{max} values were calculated from double-reciprocal plots (Lineweaver & Burk, 1934) of the activity of the purified enzyme (by using the anaerobic spectrophotometric assay) as a function of substrate concentration. N.D., not determined.

Compounds not oxidized			Compounds oxidized		
			Enzyme activity (% of rate with trimethylamine)	Apparent K_m (μ M)	V_{max} (units/mg of protein)
Trimethylamine analogues	Secondary amines				
Trimethyltin chloride	<i>N</i> -Methylglutamate				
2-Methylpropane	Di- <i>n</i> -propylamine				
Trimethylsulphonium chloride	Primary amines				
Quaternary ammonium salts	Methylamine				
Trimethylamine <i>N</i> -oxide	Ethylamine				
Hexamethylene bis-trimethylammonium chloride	<i>n</i> -Propylamine				
Tetramethylammonium chloride	<i>n</i> -Butylamine				
<i>NNN</i> -Trimethyl- <i>N</i> -phenylammonium iodide	Isopropylamine				
<i>NNN</i> -Trimethylhydrazonium iodide	<i>t</i> -Butylamine				
Choline chloride	Benzylamine				
Betaine hydrochloride	Aniline				
Tertiary amines	1-Phenylethylamine				
Dimethylformamide	2-Phenylethylamine				
Dimethylacetamide	Diamines				
<i>NN</i> -Dimethyl- <i>p</i> -aminobenzoate	1,2-Diaminoethane				
Dimethylglycine	1,3-Diaminopropane				
Dimethylaniline	1,4-Diaminobutane				
Tri- <i>n</i> -propylamine	1,5-Diaminopentane				
	Polyamines				
	Spermine				
	Spermidine				
	Tertiary amines				
	Trimethylamine (0.33)	100		2	2.3
	2-Dimethylamino-2-methylpropanol (10)	3		N.D.	N.D.
	Dimethylaminoacetone (10)	3		N.D.	N.D.
	Triethylamine (1)	3		130	0.1
	Diethylmethylamine (1)	11		8	0.3
	Ethylmethylamine (1)	16		2	0.4
	2-Aminoethylmethylamine (1)	10		67	0.5
	2-Hydroxyethylmethylamine (1)	70		6	1.4
	2-Chloroethylmethylamine (1)	40		2	0.7
	Secondary amines				
	Dimethylamine (1)	5		N.D.	N.D.
	Ethylmethylamine (1)	5		N.D.	N.D.
	<i>ON</i> -Dimethylhydroxylamine (10)	2		N.D.	N.D.
	Diethylamine (10)	47		1700	1.9

tertiary alkylamines bearing at least one *N*-methyl group and with ethyl or substituted ethyl residues comprising the remaining alkyl group(s). Diethylamine was oxidized rapidly, although the high apparent K_m value for this substrate (Table 3) suggests that it has a very low affinity for the enzyme.

Trimethylamine, ethyldimethylamine, diethylmethylamine and triethylamine form a continuous series of tertiary amines differing only in the relative proportions of methyl and ethyl groups comprising their alkyl residues. All were oxidized by the purified enzyme. Apparent K_m and V_{max} values for these substrates calculated from double-reciprocal plots (Lineweaver & Burk, 1934) are shown in Table 3 together with similar data for diethylamine.

The six substrates that were oxidized most rapidly by trimethylamine dehydrogenase, i.e. ethyldimethylamine, diethylmethylamine, 2-chloroethyldimethylamine, 2-hydroxyethyldimethylamine, 2-aminoethyl-

Fig. 3. Disappearance of reactants and appearance of products during the oxidation of trimethylamine by trimethylamine dehydrogenase

Reaction mixtures contained the following in 2 ml: 150 μmol of sodium pyrophosphate buffer, pH 8.5; 4 μmol of phenazine methosulphate; purified enzyme (160 μg of protein); 20 μmol of trimethylamine hydrochloride. Control reaction mixtures lacked enzyme, phenazine methosulphate or trimethylamine hydrochloride. In one experiment the reaction mixtures were contained in 25 ml conical flasks and incubated on a reciprocating water bath at 30°C. At intervals after the addition of substrate, 1 μl samples were removed and injected into the gas chromatograph for the determination of the methylamines. Samples (0.1 ml) were also removed for formaldehyde measurements. In a second experiment O₂ uptake was measured by using standard Warburg manometric techniques at 30°C with air as gas phase and with 0.1 ml of 40% (w/v) KOH in the centre well. No disappearance of trimethylamine or appearance of products was observed in the absence of phenazine methosulphate or enzyme. No O₂ uptake was observed in the absence of phenazine methosulphate, enzyme or trimethylamine hydrochloride. ○, Trimethylamine; □, dimethylamine; △, formaldehyde; ●, O₂ uptake.

dimethylamine and diethylamine, were also tested as potential growth substrates for bacterium 4B6 as described by Colby & Zatman (1972). Only ethyldimethylamine supported growth and the mean generation time in shaken liquid culture was 34 h compared with 2.5 h for growth on trimethylamine. Crude sonic extracts of ethyldimethylamine-grown bacterium 4B6 contained specific activities of trimethylamine dehydrogenase, dimethylamine monooxygenase, formaldehyde dehydrogenase (NAD⁺) and formate dehydrogenase (NAD⁺) similar to those found in trimethylamine-grown cells (Colby & Zatman, 1973).

Products of the reactions catalysed by trimethylamine dehydrogenase

Products of trimethylamine oxidation. Purified trimethylamine dehydrogenase was incubated aerobically at 30°C with trimethylamine and the other components of the oxygen-linked assay system. Samples were removed at intervals for the measurement of the methylamines by g.l.c. and for the colorimetric determination of formaldehyde. In another experiment, performed under as near identical conditions as possible, O₂ consumption was determined manometrically.

The purified enzyme catalyses the oxidative *N*-demethylation of trimethylamine with the formation of stoichiometric quantities of dimethylamine and formaldehyde (Fig. 3). The results obtained are consistent with the following reactions occurring:

where PMS is phenazine methosulphate. As would be predicted from the second equation, the addition of 8×10^4 units of catalase decreased the total O₂ uptake by half.

Products of the oxidation of ethyldimethylamine and diethylmethylamine. Purified enzyme (1.2 mg of protein) was incubated with ethyldimethylamine (18.0 μmol) in reaction mixtures which were otherwise similar to those described in the previous section and samples were removed at intervals for the measurement of acetaldehyde, formaldehyde, ethyldimethylamine, ethylmethylamine and the methylamines. The disappearance of 18.0 μmol of ethyldimethylamine was associated with the appearance of 18.5 μmol of ethylmethylamine and 18.2 μmol of formaldehyde, and with the uptake of 18.0 μmol of O₂. The results are consistent with the following reactions occurring:

Neither dimethylamine nor acetaldehyde were detected during the oxidation of ethyldimethylamine,

despite the ability of trimethylamine dehydrogenase to de-ethylate substrates such as diethylamine (see below). When purified enzyme was similarly incubated with diethylmethanamine, two amine products were formed, i.e. diethylamine and ethylmethanamine. This experiment was necessarily qualitative because one product of the reaction, diethylamine, is also rapidly oxidized by trimethylamine dehydrogenase. It was estimated that about one-third of the substrate was de-ethylated to ethylmethanamine and acetaldehyde, and two-thirds was demethylated to diethylamine and formaldehyde.

Products of diethylamine oxidation. Of those substrates rapidly oxidized by trimethylamine dehydrogenase only diethylamine is not capable of undergoing *N*-demethylation. In an experiment performed essentially as described in the previous section, purified trimethylamine dehydrogenase (400 μ g of protein) was incubated with 18.5 μ mol of diethylamine hydrochloride. The disappearance of 11.0 μ mol of diethylamine was associated with the formation of 10.5 μ mol of ethylamine and 10.2 μ mol of acetaldehyde, and with the uptake of 10.8 μ mol of O_2 . These results are consistent with the occurrence of the following reactions:

The reaction did not go to completion, probably owing to the marked time-dependent inhibition observed when the purified enzyme was incubated with the products of the reaction, ethylamine and acetaldehyde.

Inhibitors

In the following experiments, purified trimethylamine dehydrogenase was incubated at 30°C with each inhibitor for 15–30 min before the addition of trimethylamine to start the reaction. Chelating agents and cations were tested in a modified assay system incorporating 75 mM-triethanolamine buffer, pH 8.5, in place of the usual sodium pyrophosphate buffer. Unless stated otherwise, enzyme activities were measured by using the anaerobic spectrophotometric assay, the reaction mixtures being made anaerobic by bubbling with N_2 for about 1 min immediately before the addition of substrate.

Chelating agents. Trimethylamine dehydrogenase activity was unaffected by the following chelating agents: EDTA, 1 mM; KCN, 1 mM; 1,10-phenanthroline, 1 mM; 8-hydroxyquinoline, 1 mM; 2,2'-bipyridyl, 1 mM; neocuproine, 1 mM; cuprizone, 0.2 mM. The enzyme was also unaffected by dialysis for 24 h against 500 vol. of 100 mM-sodium pyrophosphate buffer, pH 8.5, containing 30 mM-EDTA or against 500 vol. of 50 mM-sodium phosphate buffer, pH 7.5, containing 10 mM-diethyldithiocarbamate.

Cations. Purified trimethylamine dehydrogenase (400 μ g of protein/ml) was dialysed for 24 h at 2°C against 1000 vol. of 2 mM-sodium phosphate buffer, pH 7.5, before determination of the effect of various cations on its activity. A range of univalent, bivalent and trivalent cations were tested at concentrations of 0.1 to 1.0 mM. Cu^{2+} , Co^{2+} and Ni^{2+} caused 31–46% inhibition at 1 mM concentration, whereas Hg^{2+} and Ag^+ caused 48–53% inhibition at 0.1 mM; Fe^{2+} could not be tested because it reduced the dyes. Cu^{2+} , Co^{2+} and Mn^{2+} interfered with the spectrophotometric assay and were tested in the oxygen-linked assay. Enzyme activity was unaffected by K^+ , Mg^{2+} , Ca^{2+} , Ba^{2+} , Mn^{2+} , Fe^{3+} , Zn^{2+} or Cd^{2+} at 1 mM concentration, or by Cr^{3+} or Pb^{2+} at 0.1 mM.

Thiol-binding reagents. Iodoacetamide, *N*-ethylmaleimide and *p*-chloromercuribenzoate were inhibitory, the latter being the most effective (41% inhibition at 1 mM concentration). Alloxan (10 mM), arsenite (10 mM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (2 mM) were without effect. Considered together with the inhibition observed with some heavy metals, these results suggest some thiol-group involvement in enzyme activity.

Carbonyl-binding reagents. No inhibition of trimethylamine dehydrogenase activity was observed in the presence of hydroxylamine (0.1 mM), hydrazine (0.1 mM), semicarbazide (1 mM), isonicotinic acid hydrazide (1 mM) or 2,4-dinitrophenylhydrazine (0.1 mM).

Other potential inhibitors. Those analogues of trimethylamine and related compounds which were not oxidized (see Table 3) were tested as inhibitors of trimethylamine oxidation in the anaerobic spectrophotometric assay. Trimethylsulphonium chloride and the quaternary ammonium salts tetramethylammonium chloride, *NNN*-trimethyl-*N*-phenylammonium chloride, hexamethylene bis-trimethylammonium chloride, *NNN*-trimethylhydrazonium iodide and choline chloride were all potent inhibitors, causing 70–100% inhibition at 1 mM concentration. Betaine hydrochloride caused 37% inhibition at 1 mM, but the remaining compounds had no significant effect.

Inhibition of trimethylamine dehydrogenase activity by monoamine oxidase inhibitors

Effect of substituted hydrazines. Trimethylamine dehydrogenase is inhibited by very low concentrations of the monosubstituted hydrazines benzylhydrazine, phenylhydrazine, 1-phenylethylhydrazine, 2-phenylethylhydrazine and isopropylhydrazine, and the *NN'*-disubstituted hydrazines iproniazid (isopropyl-isonicotinic acid hydrazide), nialamid [1-(2-benzylcarbamoyl)ethyl-2-isonicotinylhydrazine] and isocarboxazid [1-benzyl-2-(5-methyliso-oxazol-3-ylcarbonyl)hydrazine]. All are very potent inhibitors (0.1–4 μ M for 50% inhibition within 15 min) when

Table 4. *Inhibition of trimethylamine dehydrogenase activity by substituted hydrazines*

Substituted hydrazines were tested with the purified enzyme (4 μ g of protein) in the anaerobic spectrophotometric assay at a final concentration of 10 μ M. Inhibitors were (i) preincubated with enzyme, buffer and 2,6-dichlorophenol-indophenol at 30°C for 15 min before starting the reaction by the addition of a solution containing both trimethylamine hydrochloride and phenazine methosulphate, (ii) preincubated with enzyme, buffer, phenazine methosulphate and 2,6-dichlorophenol-indophenol at 30°C for 15 min, before starting the reaction by the addition of trimethylamine, or (iii) preincubated with enzyme, 2,6-dichlorophenol-indophenol and trimethylamine for 15 min before starting the reaction with phenazine methosulphate. In all cases the reaction mixtures were made anaerobic by bubbling with N₂ for 1 min immediately before starting the reaction. *I*₅₀, concentration of inhibitor required for 50% inhibition after 15 min preincubation with enzyme, buffer, 2,6-dichlorophenol-indophenol and phenazine methosulphate, determined from similar experiments by using a range of inhibitor concentrations. N.D., not determined.

Inhibitor	Inhibition after pre-incubation with enzyme and 2,6-dichlorophenol-indophenol (%)	Inhibition after pre-incubation with phenazine methosulphate, enzyme and 2,6-dichlorophenol-indophenol (%)	Inhibition after pre-incubation with enzyme, 2,6-dichlorophenol-indophenol and trimethylamine (%)	<i>I</i> ₅₀ (μ M)
Iproniazid phosphate	8	100	N.D.	0.8
Isopropylhydrazine oxalate	10	92	N.D.	0.2
2-Phenylethylhydrazine sulphate	41	91	0	2.0
1-Phenylethylhydrazine oxalate	84	85	0	0.4
Nialamid	0	80	N.D.	4.0
Isocarboxazid	14	100	N.D.	0.3
Benzylhydrazine sulphate	80	88	0	0.1
Phenylhydrazine hydrochloride	78	82	0	3.0

Fig. 4. *Effect of preincubation time on inactivation of trimethylamine dehydrogenase by iproniazid*

Trimethylamine dehydrogenase activities were measured by the anaerobic spectrophotometric assay. Reaction mixtures (1 ml) contained 75 μ mol of sodium pyrophosphate buffer, pH 8.5, 2 μ mol of phenazine methosulphate, purified enzyme (4 μ g of protein), 1 nmol of iproniazid phosphate (where present) and 330 nmol of trimethylamine hydrochloride. Iproniazid was preincubated with enzyme, buffer and dyes for the time indicated before the addition of the trimethylamine hydrochloride to start the reaction. Reaction mixtures were made anaerobic by bubbling with N₂ for 1 min immediately before the addition of the trimethylamine hydrochloride. The zero time-point was obtained by adding a solution containing both iproniazid phosphate and trimethylamine hydrochloride. ●, Iproniazid present; Δ, iproniazid absent.

preincubated with the purified enzyme and phenazine methosulphate, but iproniazid, isopropylhydrazine, nialamid and isocarboxazid caused little or no inhibition when preincubated with the enzyme in the absence of phenazine methosulphate (Table 4). Except for isopropylamine, which is a weak inhibitor at 1 mM concentration, the corresponding amines aniline, benzylamine, isopropylamine, 1-phenylethylamine and 2-phenylethylamine are not inhibitors. Inhibition by the substituted hydrazines is time-dependent (Fig. 4, and similar results were obtained with the other substituted hydrazines) and this inhibition is not relieved by prolonged dialysis of the inactivated enzyme (Table 5) where this has been examined, i.e. for iproniazid, isocarboxazid, isopropylhydrazine and nialamid. None of the substituted hydrazines (100 μ M) inhibited the enzyme when added simultaneously with substrate, i.e. none was an instantaneous competitive inhibitor.

Preincubation of benzylhydrazine, phenylhydrazine, 1-phenylethylhydrazine, or 2-phenylethylhydrazine with enzyme and trimethylamine hydrochloride resulted in no decrease in enzyme activity when the reaction was subsequently started by the addition of phenazine methosulphate (Table 4). The effect of substrate on inhibition by iproniazid, nialamid, isopropylhydrazine and isocarboxazid could not be tested in this way because phenazine methosulphate is required for inhibition. Trimethylsulphonium chloride and tetramethylammonium chloride are reversible inhibitors of trimethylamine dehydrogenase activity,

Table 5. *Effect of the substrate analogues trimethylsulphonium chloride and tetramethylammonium chloride on the inhibition of trimethylamine dehydrogenase activity by iproniazid, isopropylhydrazine, nialamid or isocarboxazid*

Purified trimethylamine dehydrogenase (20 units/ml) was preincubated aerobically at 30°C for 20 min in mixtures (total vol. 2 ml) containing 200 μ mol of sodium pyrophosphate buffer, pH 8.5, phenazine methosulphate (4 μ mol), 2,6-dichlorophenol-indophenol (180 nmol), and various combinations of the following reagents as indicated in the table: iproniazid (20 nmol); nialamid (20 nmol); isopropylhydrazine (20 nmol); isocarboxazid (20 nmol); trimethylsulphonium chloride (200 nmol); tetramethylammonium chloride (200 nmol). Each preincubation mixture was then dialysed at 2°C against 500 vol. of 0.1 M-sodium pyrophosphate buffer, pH 8.5, for 8 h and then against a further 500 vol. of the same buffer for 12 h. Trimethylamine dehydrogenase activity was then determined by using the anaerobic spectrophotometric assay. The dialysed material was incubated with buffer, phenazine methosulphate and 2,6-dichlorophenol-indophenol for 15 min before starting the reaction by the addition of trimethylamine hydrochloride. Reaction mixtures were made anaerobic by bubbling with N₂ for 1 min immediately before the addition of substrate.

Inhibitors present in the preincubation mixture	Specific activity after dialysis (units/mg of protein)	Inhibition (%)
None	2.05	0
Iproniazid	0.08	96
Iproniazid, trimethylsulphonium chloride	2.10	-2
Isopropylhydrazine	0.05	97
Isopropylhydrazine, tetramethylammonium chloride	2.06	0
Nialamid	0.25	88
Nialamid, tetramethylammonium chloride	2.01	2
Isocarboxazid	0.08	96
Isocarboxazid, tetramethylammonium chloride	2.06	0

which presumably compete with trimethylamine for the substrate binding site. Therefore trimethylsulphonium chloride or tetramethylammonium chloride were included in the preincubation mixture and trimethylamine dehydrogenase activity was determined after removal of the trimethylsulphonium chloride or tetramethylammonium chloride by dialysis (Table 5); in all cases these compounds prevented inhibition. It seems likely therefore that all the substituted hydrazines act at or near the substrate binding site of the enzyme.

Effect of substituted hydrazines on the absorption spectrum of the purified enzyme. As reported by Colby & Zatman (1971) the absorption spectrum of purified trimethylamine dehydrogenase from bacterium 4B6 has a peak at 445 nm, which disappears on the addition of a substrate such as trimethylamine or of a reducing agent such as sodium borohydride or sodium dithionite. Addition of the monosubstituted hydrazines phenylhydrazine, benzylhydrazine, 1-phenylethylhydrazine, 2-phenylethylhydrazine and isopropylhydrazine at 10 μ M concentration also reduced the enzyme. However, the *NN'*-disubstituted hydrazines iproniazid, nialamid and isocarboxazid had no effect on the absorption spectrum of the enzyme at concentrations of 10 μ M–1 mM, nor did they prevent the disappearance of the 445 nm peak on the subsequent addition of trimethylamine (330 μ M).

Non-hydrazine monoamine oxidase inhibitors. trans-

2-Phenylcyclopropylamine, *N*-benzyl-*N*-methylpropargylamine and harmaline all inhibited trimethylamine dehydrogenase activity (82, 27 and 65%, respectively, at 0.1 mM), but harmine (0.1 mM) was without effect.

Discussion

Trimethylamine dehydrogenase comprises a significant proportion, about 3%, of the total soluble protein of trimethylamine-grown bacterium 4B6, although its specific activity (2 units/mg of protein) is very low compared with that of most enzymes. This low specific activity may be due in part to the artificial nature of the assay system used to measure it; if the natural hydrogen acceptor were available higher activities might be expected. The E'_0 values of the three primary hydrogen acceptors that were active with the purified enzyme lie in the range +0.011 V to +0.080 V, which suggests that the natural acceptor has an E'_0 of about or just above zero. Although FAD and FMN (E'_0 = -0.219 V) were inactive as primary hydrogen acceptors it is quite possible that the natural acceptor is a flavoprotein; some flavoproteins have E'_0 values of about zero (Mahler, 1956).

The purified trimethylamine dehydrogenase catalyses the anaerobic oxidative *N*-dealkylation of its secondary and tertiary amine substrates. A possible mechanism involves the following steps:

Support for this mechanism comes from the interaction of the enzyme with quaternary ammonium salts such as tetramethylammonium chloride; although not oxidized as substrates these compounds potentially inhibit trimethylamine oxidation. This distinguishes the quaternary ammonium salts from other non-substrates such as the primary amines methylamine, ethylamine etc., which do not inhibit trimethylamine oxidation, suggesting that they have a poor affinity for the enzyme. Thus it seems that the tetramethylammonium ion readily attaches to the substrate-binding site of trimethylamine dehydrogenase but is not oxidized. This is consistent with the mechanism outlined above because the fully substituted nature of the tetramethylammonium ion precludes the formation of a substituted imine intermediate. Other possible mechanisms, for example one involving the transfer of a methyl group to an acceptor as the initial step, cf. the oxidation of the trimethylsulphonium ion by *Pseudomonas* MS (Wagner *et al.*, 1966, 1967), would not necessarily exclude the oxidation of quaternary methylammonium salts.

In its inhibitor specificity the trimethylamine dehydrogenase of bacterium 4B6 resembles the monoamine oxidases (EC 1.4.3.4) from animal tissues (Blaschko, 1963; Kapeller-Adler, 1970). Like trimethylamine dehydrogenase these enzymes are not inhibited by most chelating agents nor by carbonyl-binding reagents, but they are potentially inhibited by certain substituted hydrazines with the general formula=N-NHR, where R is an alkyl or an aryl group (Zeller *et al.*, 1955; Biel *et al.*, 1964; Juchau & Horita, 1973), and by some non-hydrazine compounds such as *trans*-2-phenylcyclopropylamine, *N*-benzyl-*N*-methylpropargylamine and harmaline (Burger & Nara, 1965; Zirkle & Kaiser, 1964). Also the characteristics of the inhibition of trimethylamine dehydrogenase activity observed with the substituted hydrazines resemble those observed with monoamine oxidase. Thus all these inhibitors caused time-dependent inactivation at very low concentrations (less than 10 μ M) and inhibition was prevented by the presence of substrate or substrate analogues during the preincubation (cf. Davison, 1957; Biel *et al.*, 1964). Inhibition by those inhibitors tested was irreversible (cf. Davison, 1957) and the potentiation by phenazine methosulphate of the inactivation by iproniazid, isopropylhydrazine, nialamid, isocarboxazid and 2-phenylethylhydrazine may be equated with the oxygen-dependent inhibition of monoamine oxidase by some of the substituted hydrazines (Davison, 1957; Green, 1964; Schwartz, 1962; Tipton, 1971, 1972; Tipton & Spire, 1971, 1972).

The mechanism by which the substituted hydrazines inhibit trimethylamine dehydrogenase is not clear. Although the evidence suggests that the inhibitors bind at or near the substrate-binding site, it is unlikely that they inhibit by virtue of any close structural simi-

larity to the normal amine substrates. None is a competitive inhibitor when added simultaneously with substrate and the corresponding amines are neither substrates nor inhibitors, with the exception of isopropylamine, which is a weak inhibitor. There is evidence that suggests that some of the substituted hydrazine inhibitors are oxidized by monoamine oxidase and that their oxidation is involved in the inhibition process (Davison, 1957; McEwan *et al.*, 1969; Tipton, 1971, 1972; Tipton & Spire, 1971, 1972; Clineschmidt & Horita, 1969*a,b*). It seems likely that a similar mechanism operates during the inhibition of trimethylamine dehydrogenase by those substituted hydrazines for which inhibition is potentiated by phenazine methosulphate, i.e. iproniazid, isopropylhydrazine, nialamid, isocarboxazid and 2-phenylethylhydrazine. However, the failure of iproniazid, nialamid and isocarboxazid to reduce the enzyme prosthetic group suggests that a different mechanism operates during the oxidation of these compounds by trimethylamine dehydrogenase from that which operates during trimethylamine oxidation (cf. Tipton, 1971; Tipton & Spire, 1971).

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