Aerobic and Anaerobic Metabolism of Trimethylamine, Dimethylamine and Methylamine in *Hyphomicrobium* x

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Hyphomicrobium strain x was grown on trimethylamine and dimethylamine as the sole sources of carbon and energy under both aerobic and anaerobic conditions and the enzymes involved in the metabolism of these compounds were investigated. During aerobic growth of the organism on trimethylamine, accumulation and subsequent utilization of dimethylamine was observed. When the organism was grown on trimethylamine under anaerobic conditions in the presence of nitrate, a sequential accumulation and utilization of dimethylamine and methylamine was found. In cell-free extracts of *Hyphomicrobium* x grown on trimethylamine or dimethylamine under both aerobic and anaerobic conditions the following enzyme activities were detected: trimethylamine dehydrogenase, dimethylamine dehydrogenase, γ -glutamylmethylamide synthetase, *N*-methylglutamate dehydrogenase, methanol dehydrogenase, formaldehyde dehydrogenase, formate dehydrogenase and hydroxypyruvate reductase. Under neither growth condition were any of the following enzyme activities detected: trimethylamine mono-oxygenase, dimethylamine mono-oxygenase, trimethylamine-*N*-oxide aldolase (demethylase) and primary-amine dehydrogenase. Trimethylamine dehydrogenase and dimethylamine dehydrogenase were partially purified from bacteria grown on dimethylamine and the results suggest that in *Hyphomicrobium* x a novel enzyme, namely dimethylamine dehydrogenase, participates in the oxidation of dimethylamine.

INTRODUCTION

Various micro-organisms can grow aerobically on trimethylamine, dimethylamine and methylamine as the sole source of carbon and energy, and the metabolism of these compounds has been studied extensively (Colby & Zatman, 1973; Anthony, 1975; Boulton & Large, 1977). The initial catabolism of trimethylamine in methylotrophic bacteria may proceed by one of two different pathways. The first route involves the direct demethylation to dimethylamine and formaldehyde, catalysed by trimethylamine dehydrogenase (Colby & Zatman, 1971), while in the other pathway the initial attack is an oxygenation of trimethylamine to trimethylamine *N*-oxide mediated by trimethylamine mono-oxygenase (Large, Boulton & Crabbe, 1972). The *N*-oxide is subsequently demethylated by trimethylamine *N*-oxide aldolase (demethylase) to dimethylamine and formaldehyde (Myers & Zatman, 1971; Large, 1971).

The oxidation of dimethylamine to methylamine and formaldehyde in *Pseudomonas aminovorans* involves a secondary-amine mono-oxygenase (Eady & Large, 1969; Eady, Jarman & Large, 1971) and this enzyme was also reported to function in *Bacterium* 4B6 and some other organisms (Colby & Zatman, 1973). Until recently (Meiberg & Harder, 1976) no other enzyme involved in the oxidation of dimethylamine had been reported, except that

the purified trimethylamine dehydrogenase from *Bacterium* 4B6 (Colby & Zatman, 1974) and a partially purified trimethylamine dehydrogenase from *Hyphomicrobium vulgare* NQ-521 (Large & McDougall, 1975) displayed some activity towards dimethylamine. Large & McDougall (1975) also reported a dimethylamine dehydrogenase activity in an enzyme preparation obtained from *H. vulgare* NQ-521, but they attributed this activity to a contaminating organism. The product of the oxidation of dimethylamine, namely methylamine, may be oxidized directly (Eady & Large, 1968, 1971) or via *N*-methylglutamate (Hersh, Peterson & Thompson, 1971; Loginova, Shishkina & Trotsenko, 1976; Bamforth & Large, 1977).

We recently found that hyphomicrobia can be enriched and isolated by an anaerobic technique (Attwood & Harder, 1972) using trimethylamine or dimethylamine as the substrate and nitrate as terminal electron acceptor (J. B. M. Meiberg, unpublished results). In fact, pure cultures of several *Hyphomicrobium* isolates, previously obtained from enrichments with methanol or methylamine (Attwood & Harder, 1972), could grow on trimethylamine and dimethylamine, both aerobically and anaerobically with nitrate. Under the latter conditions it appeared to be highly improbable that dimethylamine mono-oxygenase was involved in the catabolism of trimethylamine and dimethylamine and we therefore decided to investigate the metabolism of these compounds in one of the isolates available. Since the physiology and biochemistry of *Hyphomicrobium* x is well documented (Harder & Attwood, 1978), this organism was chosen for the present study which describes some aspects of its growth on trimethylamine and dimethylamine under both aerobic and anaerobic conditions and implicates a novel enzyme, namely dimethylamine dehydrogenase, in the oxidation of dimethylamine.

A preliminary report of part of this work has been published (Meiberg & Harder, 1976).

METHODS

Maintenance and growth of the organism. *Hyphomicrobium* strain x (Attwood & Harder, 1972) was grown in mineral salts medium containing (per litre of deionized water): $(\text{NH}_4)_2\text{SO}_4$, 1.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.5 g; K_2HPO_4 , 1.55 g; and trace element solution (Vishniac & Santer, 1957), 0.2 ml. The pH of the medium was 7.2. After it had been heat-sterilized and cooled, filter-sterilized solutions of the various methylated amines were added to a final concentration of 10 mM (aerobic experiments) or 20 mM (anaerobic experiments).

The organism was maintained on the above mineral salts medium supplemented with 0.34% (w/v) methylammonium chloride and 1.5% (w/v) Bacto-agar, and was transferred bimonthly; slopes were incubated at 30 °C for 7 d and then stored at 4 °C.

Bacteria were grown in a 4 l fermenter described by Harder, Visser & Kuenen (1974), filled with 3 l of medium. During growth, the pH was maintained at 7.0 by periodic addition of sterile alkali and the culture was stirred and aerated at 30 l h⁻¹. The temperature was maintained at 30 °C. During anaerobic growth, KNO_3 was added in portions as indicated in the individual experiments. In these experiments air was replaced by nitrogen gas from which traces of oxygen had been removed by passing it through a solution of dihydriboflavin (Strauss & Nickerson, 1961). For both aerobic and anaerobic experiments, a 200 ml culture of the organism, grown on the same substrate in 500 ml conical flasks on a shaker at 30 °C, was used as an inoculum.

Growth was monitored by measuring the absorbance of the culture at 433 nm; one absorbance unit corresponded to 0.22 mg dry wt ml⁻¹.

For enzyme assays in crude cell-free extracts, bacteria from different growth phases, as indicated in the individual experiments, were harvested by centrifugation at 15000 g for 10 min at 4 °C, washed once with 50 mM-potassium phosphate buffer pH 7.0 and used immediately or stored at -20 °C. Trimethylamine and dimethylamine dehydrogenases were purified from bacteria harvested at the end of the active growth phase.

Bacterium 5H2 was obtained from Dr L. J. Zatman, University of Reading. It was grown in the above mineral salts medium in conical flasks on a shaker at 30 °C with 0.3% (w/v) trimethylamine as the carbon and energy source. Bacteria were harvested at the end of the exponential growth phase.

Preparation of cell-free extracts. Bacteria (0.5 g wet wt) were suspended in 4 ml of 50 mM-potassium phosphate buffer pH 7.0 and 0.5 g of acid-washed glass beads (diam. 0.11 mm) was added to the suspension.

The bacteria were disrupted at 20 kHz in a 100 W ultrasonic disintegrator for 2.5 min at 0 °C. Whole cells, debris and glass beads were removed by centrifuging at 25000 *g* for 20 min. The resulting supernatant was used as the crude cell-free extract.

Dry weight determination. Duplicate samples of 200 ml were removed from the culture, centrifuged, washed once with deionized water and dried to constant weight at 110 °C.

Chemical estimations. Protein was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Methylated amines were measured as follows: 10 ml samples were withdrawn from the cultures at appropriate intervals, and bacteria were removed by membrane filtration (pore size 0.2 µm). The concentration of the methylated amines in the filtrates was determined by gas chromatography using a Pye Unicam series 104 chromatograph with a heated dual flame ionization detector. A glass column (1.9 m × 2 mm i.d.) was used, packed with Carbowax B, coated with GP 4 % Carbowax 20 M plus 0.8 % KOH. The column oven temperature was 120 °C, and the detector oven, 150 °C. Nitrogen was used as the carrier gas at a flow rate of 10 ml min⁻¹. Before injection, 1.6 % (w/v) KOH was added to the samples to a final concentration of 0.8 % (w/v). Standards were prepared from mono-, di- and trimethylammonium chlorides dried with Mg(ClO₄)₂.

Nitrite was measured by a modification of the method of Egami & Taniguchi (1963) as follows: to 4 ml of appropriately diluted culture filtrate were added 0.5 ml of 1 % (w/v) sulphanilamide in 1.2 M-HCl and 0.5 ml of 0.02 % (w/v) *N*-1-naphthylethylenediamine dihydrochloride. After 10 min *A*₅₄₀ was measured and the nitrite concentration was read from a calibration curve made with known solutions of NaNO₂ in water.

Nitrate was measured using the spectrophotometric method of Goldsmith *et al.* (1973). Nitrite absorbs light at 210 nm whereas methylated amines do not. The procedure for estimating nitrate in the presence of nitrite was to determine nitrite, as described above, and then subtract the contribution of the nitrite to the total *A*₂₁₀ of the sample (as observed with standard nitrite solutions) from the measured *A*₂₁₀ of the sample containing both nitrate and nitrite. A standard solution of 1.25 mM-KNO₃ was used for calibration purposes.

Formaldehyde was prepared by heating aqueous solutions of paraformaldehyde in a sealed glass vial at 110 °C overnight, and was determined by the method of Nash (1953).

Enzyme assays. All spectrophotometric assays were performed using a Hitachi Perkin-Elmer 124 double-beam recording spectrophotometer equipped with a constant temperature cuvette housing (Charles Goffin, De Bilt, The Netherlands). The incubation temperature was 30 °C in all assays.

Trimethylamine dehydrogenase and **dimethylamine dehydrogenase** were assayed by the method of Colby & Zatman (1973), except that KCN was omitted and assays were done anaerobically. The reaction mixture contained (in 1 ml): 100 µmol sodium pyrophosphate buffer pH 7.7; 0.08 µmol 2,6-dichlorophenolindophenol (DCPIP); 2 µmol phenazine methosulphate (PMS); extract; and 3 µmol trimethylamine or dimethylamine. The reaction was started by adding the substrate, and the initial rate of decrease in *A*₆₀₀ was taken as a measure of enzyme activity.

DCPIP-dependent formaldehyde dehydrogenase was assayed by the method of Johnson & Quayle (1964), except that 0.08 µmol DCPIP was used.

NAD/glutathione-linked formaldehyde dehydrogenase was assayed by the method of Johnson & Quayle (1964), modified by using 0.4 µmol NAD⁺ and 3 µmol glutathione (GSH).

Methanol dehydrogenase and **formaldehyde dehydrogenase** were measured by a modification of the polarographic method of Harder & Attwood (1975). The reaction mixture contained (in 3 ml): 300 µmol Tris/HCl buffer pH 9.0; 4 µmol PMS; 45 µmol NH₄Cl; extract; and 16 µmol methanol or 30 µmol formaldehyde. The reaction was started by adding substrate.

Hydroxypyruvate reductase was assayed by the method of Blackmore & Quayle (1970), except that 50 mM-potassium phosphate pH 7.0 was used as a buffer.

γ-Glutamylmethylamide synthetase was measured by a modification of the method of Loginova *et al.* (1976). The complete reaction mixture contained (in 1 ml): 16 µmol Tris/HCl buffer pH 7.6; 5 µmol [¹⁴C]methylamine (1.0 µCi); 10 µmol 2-mercaptoethanol; 5 µmol ATP; 5 µmol MnCl₂; 10 µmol L-glutamate; and extract. The reaction was started by adding [¹⁴C]methylamine and was allowed to continue for various periods of time (up to 30 min). Controls without ATP or Mn²⁺ or with boiled extract were included. The reaction was stopped by the addition of 0.1 ml of 30 % (w/v) trichloroacetic acid. Denatured protein was removed by centrifugation and 25 µl of the supernatant was applied to a cellulose thin-layer chromatogram and developed in two dimensions with 2-methylpropan-2-ol/ethyl methyl ketone/water/aq. NH₄OH (sp.gr. 0.91) (40:30:20:10, by vol.) followed by phenol/formic acid/water (500:13:167, by vol.). The plates were dried overnight and scanned to locate the radioactive spot of γ-glutamylmethylamide, using a Berthold LB 2723 scanner (Charles Goffin, De Bilt, The Netherlands). The radioactive areas were scraped from the plate and counted by liquid scintillation using a mixture of Triton/toluene (1:2, v/v) containing 1 % (w/v) 2,5-diphenyloxazole.

The following enzyme activities were assayed by unmodified published methods: trimethylamine mono-

oxygenase, dimethylamine mono-oxygenase and trimethylamine-*N*-oxide demethylase (Colby & Zatman, 1973); primary-amine dehydrogenase (Eady & Large, 1968); NAD-dependent formate dehydrogenase (Johnson & Quayle, 1964); *N*-methylglutamate dehydrogenase (Bamforth & Large, 1977).

Purification of trimethylamine and dimethylamine dehydrogenases. Bacteria were grown anaerobically with dimethylamine as the substrate, and were harvested by centrifugation as described above. A bacterial suspension (1 g wet wt per 4 ml of 50 mM-potassium phosphate buffer pH 7.0) was disrupted ultrasonically for 7.5 min at 0 °C as described above. To the crude cell-free extract (7.5 mg protein ml⁻¹), solid ammonium sulphate was added to give 50 % saturation, while maintaining the pH at 7.5 with 10 % (v/v) NH₄OH. After 1 h at 0 °C the precipitate was removed by centrifuging at 13000 g for 20 min. More solid ammonium sulphate was added to bring the clear supernatant to 70 % saturation. After 1 h at 0 °C the precipitate was collected by centrifugation and dissolved in 4 ml of 20 mM-sodium phosphate buffer pH 7.5. This fraction, which contained most of the enzyme activity, was dialysed for 16 h against 3 l of 20 mM-sodium phosphate buffer pH 7.5, with one change of buffer. The dialysed fraction was then applied to a column (1.5 × 27 cm) of DEAE-cellulose (Whatman, DE32), previously equilibrated with 20 mM-sodium phosphate buffer pH 7.5. Protein was eluted with a linear gradient of 0 to 0.5 M-NaCl in 20 mM-sodium phosphate buffer pH 7.5; fractions of 3.3 ml were collected. Trimethylamine dehydrogenase activity was eluted between 0.16 and 0.23 M-NaCl and dimethylamine dehydrogenase activity between 0.20 and 0.29 M-NaCl. The fractions containing trimethylamine dehydrogenase (nos 38 to 49) and those containing dimethylamine dehydrogenase (nos 50 to 70) (see Fig. 5) were pooled separately and each concentrated to 2.3 ml by ultrafiltration using an Amicon PM10 membrane. The purification procedure is summarized in Table 2.

Chemicals. Methylammonium chloride, dimethylammonium chloride and trimethylammonium chloride were obtained from Merck. Bovine serum albumin was from Poviet Producten, Amsterdam, The Netherlands. Carbopack B was from Supelco, Bellefonte, Pennsylvania, U.S.A. Other chemicals and reagents were from Sigma or Fluka, Buchs, Switzerland. Biochemicals were from Boehringer. [¹⁴C]Methylamine was from The Radiochemical Centre, Amersham. γ -Glutamylmethylamide was synthesized as described by Lichtenstein (1942).

RESULTS

Aerobic growth of Hyphomicrobium x on trimethylamine and dimethylamine

With trimethylamine as the sole carbon and energy source, *Hyphomicrobium x* grew at a specific growth rate of 0.10 h⁻¹ (Fig. 1), which is similar to that observed in media containing methanol (Harder, Attwood & Quayle, 1973).

During aerobic growth on trimethylamine, accumulation and subsequent utilization of dimethylamine were observed (Fig. 1). The kinetics of the utilization of trimethylamine and of accumulation of dimethylamine indicated that an almost stoichiometric conversion of trimethylamine into dimethylamine occurred during part of the exponential growth phase (from 17 to 24 h). During this period the organism apparently was unable to metabolize dimethylamine to any appreciable extent and growth was exclusively due to the degradation of trimethylamine to dimethylamine. After the trimethylamine concentration had dropped to below 1.5 mM, the apparent inhibition of dimethylamine catabolism was relieved and the concentration of dimethylamine in the medium decreased. At the end of exponential growth, when trimethylamine was undetectable, growth was exclusively at the expense of dimethylamine. These results suggest that dimethylamine is an intermediate in the metabolism of trimethylamine by *Hyphomicrobium x*.

When the organism was grown on 10 mM-dimethylamine as the initial substrate, accumulation and subsequent utilization of methylamine were observed (Fig. 2). The kinetics of the accumulation and disappearance of these compounds were such that concomitant utilization of both dimethylamine and methylamine by the organism must be postulated over the whole period of exponential growth. Initially (5 to 8 h after the start of the experiment), when the concentration of dimethylamine in the culture was above 4 mM, the rate of methylamine production from dimethylamine was higher than the rate of its utilization, whereas at later stages of growth methylamine was utilized faster than it was formed. These results indicate that methylamine may be an intermediate in the metabolism of dimethylamine by *Hyphomicrobium x*. Methylamine was not excreted during growth on trimethylamine (Fig. 1), possibly due to the low concentration (5 mM) of the accumulated dimethylamine.

Fig. 1

Fig. 2

Fig. 1. Aerobic growth of *Hyphomicrobium x* on trimethylamine: ○, growth; ●, trimethylamine and, ■, dimethylamine concentrations in the culture. Enzyme activities were measured in cell-free extracts prepared from culture samples taken at two different stages of growth, as indicated by the arrows I and II.

Fig. 2. Aerobic growth of *Hyphomicrobium x* on dimethylamine: ○, growth; ■, dimethylamine and, ▲, methylamine concentrations in the culture.

Anaerobic growth of Hyphomicrobium x on trimethylamine and dimethylamine in the presence of nitrate

Growth of *Hyphomicrobium x* under denitrifying conditions on trimethylamine and dimethylamine was much slower than that observed in the presence of oxygen. Initially the experiments were carried out in media in which 50 mM-nitrate was used as the electron acceptor. However, nitrite rapidly accumulated under these conditions resulting in a complete inhibition of growth [although Sperl & Hoare (1971) reported that the major end-product of nitrate respiration in hyphomicrobia is nitrogen]. Therefore, in subsequent experiments, nitrate was added to the culture in three separate portions and although nitrite accumulation still occurred, the concentration did not become high enough to inhibit growth completely; however, some growth inhibition was still apparent (Figs 3, 4).

Anaerobic growth of *Hyphomicrobium x* on trimethylamine revealed a complicated pattern of substrate utilization (Fig. 3). As was observed during aerobic growth (Fig. 1), the initial growth was almost exclusively due to the oxidation of trimethylamine to dimethylamine. Very little, if any, of the dimethylamine produced was metabolized until the concentration of trimethylamine in the culture was very low. During the subsequent oxidation of dimethylamine, accumulation of methylamine was observed. However, as had been observed during aerobic growth on dimethylamine, the data indicated a concomitant utilization of dimethylamine and methylamine, although the rate of production of methylamine was initially higher than its rate of utilization. In these experiments exponential growth (specific growth rate 0.025 h^{-1}) of the organism was only observed in the early stages of the batch culture when the nitrite concentration was low. Growth of *Hyphomicrobium x* stopped completely when the electron acceptor was exhausted from the culture, although the concentration of the carbon source was still sufficient to support growth.

In contrast to the results obtained for aerobic growth of *Hyphomicrobium x* on dimethylamine, anaerobic growth on this substrate did not lead to any accumulation of methylamine (Fig. 4). Growth of the organism was only exponential in the early stages of the batch culture,

Fig. 3

Fig. 4

Fig. 3. Anaerobic growth of *Hyphomicrobium x* on trimethylamine in the presence of nitrate: ○, growth; ●, trimethylamine, ■, dimethylamine, ▲, methylamine, □, nitrate and △, nitrite concentrations in the medium. Enzyme activities were measured in cell-free extracts prepared from culture samples taken at different stages of growth, as indicated by the arrows I and II. Nitrate was supplied to the culture in three separate portions.

Fig. 4. Anaerobic growth of *Hyphomicrobium x* on dimethylamine in the presence of nitrate: ○, growth; ■, dimethylamine, □, nitrate and △, nitrite concentrations in the medium. Nitrate was supplied to the culture in three separate portions.

when the nitrite concentration was very low. The specific growth rate of 0.05 h^{-1} observed initially gradually declined as the nitrite concentration increased to approximately 15 mM and continued to decline until the dimethylamine was exhausted.

Activities of enzymes involved in methylated amine metabolism in Hyphomicrobium x

Cell-free extracts of *Hyphomicrobium x*, grown under aerobic or anaerobic conditions on trimethylamine, dimethylamine or methylamine, were analysed for enzymes previously implicated in the microbial metabolism of methylated amines (see Anthony, 1975). The results (Table 1) indicated that, in *Hyphomicrobium x*, trimethylamine dehydrogenase (EC 1.5.99.7) was the only enzyme present for the oxidation of trimethylamine. This enzyme was also found in bacteria grown on dimethylamine, but was absent from extracts prepared from methylamine-grown bacteria, indicating that it was induced by both trimethylamine and dimethylamine. Neither trimethylamine mono-oxygenase nor trimethylamine-*N*-oxide aldolase (demethylase) (EC 4.1.2.-) was detected, although both enzymes were present in cell-free extracts of trimethylamine-grown *Bacterium 5H2* (Table 1), indicating that the failure to demonstrate these enzymes in extracts of *Hyphomicrobium x* was not due to inadequacies of the assay system used. The oxidation of dimethylamine in *Hyphomicrobium x* is also mediated by a dehydrogenase and in this respect the organism is different from a number of other methylotrophs in which dimethylamine mono-oxygenase has been implicated in the metabolism of trimethylamine. As was found for the tri-

Table 1. *Specific activities of various enzymes in crude extracts of Hyphomicrobium x grown on trimethylamine, dimethylamine or methylamine and of Bacterium 5H2 grown on trimethylamine*

Samples of trimethylamine-grown *Hyphomicrobium x* were taken at two stages of growth, as indicated by the arrows (I and II) in Figs 1 and 3; organisms grown on dimethylamine and methylamine were taken at the end of the active growth phase. Specific activities are expressed as nmol substrate transformed min⁻¹ (mg protein)⁻¹.

Abbreviations: TMA, trimethylamine-grown; DMA, dimethylamine-grown; MA, methylamine-grown; aer, anaer, cultivation under aerobic and anaerobic conditions, respectively; ND, not determined.

Enzyme	<i>Hyphomicrobium x</i>								<i>Bacterium 5H2</i>
	TMA aer		DMA aer	MA aer	TMA anaer		DMA anaer	MA anaer	TMA aer
	I	II			I	II			
Trimethylamine dehydrogenase (PMS)	21	18	7	0	37	36	22	0	0
Trimethylamine mono-oxygenase (NADPH)	0	0	0	0	0	0	0	0	22
Trimethylamine- <i>N</i> -oxide aldolase (demethylase)	0	0	0	ND	0	0	0	ND	168
Dimethylamine dehydrogenase (PMS)	6	10	8	0	24	36	43	0	0
Dimethylamine mono-oxygenase (NADPH)	0	0	0	0	0	0	0	0	7
Primary-amine dehydrogenase (PMS)	ND	0	0	0	ND	0	0	0	0
γ -Glutamylmethylamide synthetase	ND	2	11	1	ND	1	2	5	ND
<i>N</i> -Methylglutamate dehydrogenase (DCPIP)	ND	4	5	6	ND	8	6	8	ND
Methanol dehydrogenase (PMS)	ND	342	554	357	ND	140	92	236	0
Formaldehyde dehydrogenase (PMS)	ND	348	641	192	ND	106	56	205	0
Formaldehyde dehydrogenase (DCPIP)	ND	26	29	32	ND	25	31	113	6
Formaldehyde dehydrogenase (NAD/GSH)	ND	0	0	0	ND	0	0	0	175
Formate dehydrogenase (NAD)	ND	129	183	177	ND	191	180	763	21
Hydroxypyruvate reductase (NADH)	ND	1429	2459	2984	ND	2280	2096	2380	ND

methylamine dehydrogenase, the dimethylamine dehydrogenase displayed its highest activity in cells grown anaerobically on dimethylamine, whereas the enzyme was absent from cell-free extracts of methylamine-grown cells. Activity of dimethylamine mono-oxygenase (EC 1.14.99.-) was not detected in extracts of *Hyphomicrobium x*, although it was detected in trimethylamine-grown *Bacterium 5H2*.

The metabolism of methylamine, a product of the action of dimethylamine dehydrogenase, is likely to proceed via γ -glutamylmethylamide and *N*-methylglutamate to formaldehyde. Activities of γ -glutamylmethylamide synthetase (EC 6.3.4.12) and *N*-methylglutamate dehydrogenase (EC 1.5.99.5) were detected in extracts of *Hyphomicrobium x* grown on all methylated amines tested, whereas the primary-amine dehydrogenase was not present (Table 1). The formaldehyde generated in the successive oxidations may be utilized for either of two purposes. It can be oxidized to formate and CO₂ for the generation of energy by the action of primary-alcohol dehydrogenase (Harder & Attwood, 1975) or dye-linked formaldehyde dehydrogenase (Johnson & Quayle, 1964) and formate dehydrogenase, respectively. Alternatively it may be utilized for the assimilation of one-carbon units via the icl⁻ serine pathway (Harder *et al.*, 1973; Attwood & Harder, 1977); the high levels of hydroxypyruvate reductase activity (Table 1) are consistent with the operation of this latter pathway during growth on methylated amines.

In order to investigate whether the observed accumulation of dimethylamine in the

Fig. 5. DEAE-cellulose chromatography of trimethylamine dehydrogenase and dimethylamine dehydrogenase from *Hyphomicrobium x*, grown under anaerobic conditions with dimethylamine as the growth substrate. The 50 to 70 % ammonium sulphate fraction (containing 59 mg protein) was chromatographed using a DEAE-cellulose column, previously equilibrated with 20 mM-sodium phosphate buffer pH 7.5. Protein was eluted with a gradient of 0 to 0.5 M-NaCl (not completely shown) in the same buffer and 3.3 ml fractions were collected; the flow rate was 24 ml h⁻¹. ●, A_{280} ; ▲, trimethylamine dehydrogenase activity; ○, dimethylamine dehydrogenase activity; ---, NaCl gradient.

Table 2. *Partial purification of trimethylamine and dimethylamine dehydrogenases from Hyphomicrobium x*

Total activities are expressed as nmol substrate transformed min⁻¹ and specific activities as nmol substrate transformed min⁻¹ (mg protein)⁻¹.

Abbreviations: TMA, trimethylamine dehydrogenase activity; DMA, dimethylamine dehydrogenase activity.

Fraction	Vol. (ml)	Total protein (mg)	Total activity		Specific activity		Recovery (%)		Purification factor	
			TMA	DMA	TMA	DMA	TMA	DMA	TMA	DMA
Crude cell-free extract	40	300	5105	6313	17.0	21.0	100	100	1.0	1.0
Ammonium sulphate fraction 50 to 70 % saturation	5	73.5	4030	3887	54.8	52.9	78.9	61.6	3.2	2.5
Pooled fractions nos 38 to 49 DEAE-cellulose eluate*	2.3	3.6	653	88	180.4	24.3	12.8	1.4	10.6	1.2
Pooled fractions nos 50 to 70 DEAE-cellulose eluate*	2.3	7.2	349	1919	48.6	267.6	6.8	30.4	2.9	12.7

* Assayed after concentration by ultrafiltration.

dissimilation of trimethylamine during growth of *Hyphomicrobium x* on this compound (Figs 1, 3) was due to repression of the synthesis of dimethylamine dehydrogenase, cell-free extracts were prepared at different stages of growth (arrows I and II in Figs 1 and 3). The results (Table 1) indicated that the failure of the cells to metabolize dimethylamine sufficiently during part of the exponential phase of growth on trimethylamine cannot be attributed to the absence of dimethylamine dehydrogenase, although the activity of the enzyme is somewhat lower during the initial stages of growth.

Fig. 6. Proposed scheme for trimethylamine, dimethylamine and methylamine metabolism in *Hyphomicrobium x*. The enzymes of the pathway are: (1), trimethylamine dehydrogenase (EC 1.5.99.7); (2), dimethylamine dehydrogenase; (3), γ -glutamylmethylamide synthetase (EC 6.3.4.12); (4), enzyme not yet characterized (see Loginova *et al.*, 1976); (5), *N*-methylglutamate dehydrogenase (EC 1.5.99.5); (6), formaldehyde dehydrogenase; (7), formate dehydrogenase (EC 1.2.1.2).

Partial purification of trimethylamine and dimethylamine dehydrogenase activities from Hyphomicrobium x

The data of Table 1 suggest that the oxidation of dimethylamine in *Hyphomicrobium x* is mediated by a novel enzyme. However, an alternative explanation could be that the observed oxidation of dimethylamine by the dye-linked dehydrogenase was due to a dual substrate specificity of the trimethylamine dehydrogenase, although the ratio of the two activities observed in cell-free extracts of bacteria grown under different conditions suggested otherwise. Furthermore, preliminary experiments on the stability of the two enzyme activities indicated a different behaviour towards heat treatment. For instance, the ratio of trimethylamine dehydrogenase activity to dimethylamine dehydrogenase activity in cell-free extracts changed from 1.7 to 5.3 upon heating the extract for 20 min at 60 °C. To substantiate further the probability that the two enzyme activities were associated with different proteins, both enzymes were partially purified from cells grown anaerobically on dimethylamine (Table 2, Fig. 5). Although the activities of trimethylamine dehydrogenase and dimethylamine dehydrogenase were not completely separated after DEAE-cellulose column chromatography (Fig. 5), the results indicated that they are indeed associated with different proteins. A small peak of dimethylamine-oxidizing activity which co-chromatographed with the trimethylamine dehydrogenase was probably due to a minor activity of the trimethylamine dehydrogenase towards dimethylamine.

DISCUSSION

On the basis of the results obtained in the present study, we propose a pathway for the metabolism of methylated amines in *Hyphomicrobium x* as shown in Fig. 6. Trimethylamine is oxidized by a dye-linked dehydrogenase which also displays some activity (approximately

15 % of the activity with trimethylamine as the substrate) towards dimethylamine. A similar dimethylamine-oxidizing activity was found for the purified trimethylamine dehydrogenase of *Bacterium* 4b6 (Colby & Zatman, 1974) and also for a partially purified trimethylamine dehydrogenase of *Hyphomicrobium vulgare* NQ-521 (Large & McDougall, 1975). However, such a substrate specificity is not necessarily an intrinsic property of all trimethylamine dehydrogenases since the enzyme purified from *Bacterium* w3A1 did not show any activity with dimethylamine as the substrate (Steenkamp & Mallinson, 1976). The dimethylamine is further oxidized by another dye-linked dehydrogenase which most probably is specific for dimethylamine and does not show activity towards trimethylamine (Fig. 5). Both enzymes are present during growth on dimethylamine and trimethylamine (Table 1). Similar to the situation reported for *H. vulgare* 3 (Trotsenko, Loginova & Shishkina, 1974) and *H. vulgare* zV (Loginova *et al.*, 1976), in *Hyphomicrobium* x methylamine is oxidized via γ -glutamylmethylamide and *N*-methylglutamate. The formaldehyde generated in the above reactions can either be oxidized to CO₂ via formate or assimilated via the icl⁻ serine pathway (Fig. 6; Harder *et al.*, 1973; Harder & Attwood, 1975; Attwood & Harder, 1977).

The activities of the enzymes implicated in the metabolism of trimethylamine and dimethylamine in aerobically grown *Hyphomicrobium* x were too low to account for the growth rate of the organism. Although the observed growth rates under aerobic conditions were considerably higher than under anaerobic conditions in the presence of nitrate (Figs 1 to 4), the activities of trimethylamine and dimethylamine dehydrogenases were lower (Table 1). Despite several attempts to increase the activities of these enzymes in cell-free extracts by changing both the conditions used for the preparation of extracts or in the assay procedure, they remained low in aerobically grown cells. However, a decrease in oxygen tension in the culture caused an increase in the activity of these enzymes and it was therefore not unexpected to find the highest activities under anaerobic conditions.

During growth of *Hyphomicrobium* x on trimethylamine, accumulation of intermediates of the degradative pathway of this compound was observed in the medium (Figs 1 and 3). A similar phenomenon has been described during growth of *Micrococcus* sp. on trimethylamine (Tate & Alexander, 1976). These workers reported that accumulation of dimethylamine in the medium was affected by the nature of the inorganic nitrogen source and by the initial pH value. It is not known whether similar changes of the medium may affect the accumulation of dimethylamine from trimethylamine by cultures of *Hyphomicrobium* x. In the course of our work we observed that trimethylamine was a potent competitive inhibitor of the partially purified dimethylamine dehydrogenase. Since we also observed that trimethylamine dehydrogenase shows some activity towards dimethylamine, we suggest the following explanation for the accumulation of dimethylamine. During the initial stages of the growth of *Hyphomicrobium* x on trimethylamine, any activity of dimethylamine dehydrogenase will be effectively inhibited by the relatively high concentration of trimethylamine in the medium. And, although the dimethylamine produced by the action of trimethylamine dehydrogenase may be further oxidized by this enzyme, accumulation of dimethylamine in the medium may result from poor kinetics of the enzyme for this substrate. Only when the trimethylamine concentration is decreased to a low level will the inhibition of dimethylamine dehydrogenase be relieved, resulting in the decrease of the concentration of dimethylamine in the culture. Further studies with purified enzymes are required to substantiate this hypothesis.

We suggest that dimethylamine dehydrogenase plays a major role in the metabolism of trimethylamine and dimethylamine in those *Hyphomicrobium* spp. that are able to grow on these compounds under both aerobic and denitrifying conditions. However, it is not peculiar to all representatives of the genus, since *H. vulgare* NQ (Eady *et al.*, 1971) and *H. vulgare* zV (Loginova *et al.*, 1976) contain dimethylamine mono-oxygenase during (aerobic) growth on trimethylamine. Also, the further metabolism of this compound in *H. vulgare* zV differs from that in *Hyphomicrobium* x in that the former organism contains NAD-linked

N-methylglutamate dehydrogenase and formaldehyde dehydrogenase, whereas in the latter organism these enzymes are dye-linked. If *H. vulgare* NQ and ZV are able to grow on trimethylamine under denitrifying conditions, it would be of interest to see if dimethylamine dehydrogenase is involved in the metabolism of these compounds.

REFERENCES

- ANTHONY, C. (1975). The biochemistry of methylotrophic micro-organisms. *Science Progress, Oxford* **62**, 167–206.
- ATTWOOD, M. M. & HARDER, W. (1972). A rapid and specific enrichment procedure for *Hyphomicrobium* spp. *Antonie van Leeuwenhoek* **38**, 369–378.
- ATTWOOD, M. M. & HARDER, W. (1977). Isocitrate lyase activity in *Hyphomicrobium* spp.: a critical reappraisal. *FEMS Microbiology Letters* **1**, 25–30.
- BAMFORTH, C. W. & LARGE, P. J. (1977). Solubilization, partial purification and properties of *N*-methylglutamate dehydrogenase from *Pseudomonas aminovorans*. *Biochemical Journal* **161**, 357–370.
- BLACKMORE, M. A. & QUAYLE, J. R. (1970). Microbial growth on oxalate by a route not involving glyoxylate carboligase. *Biochemical Journal* **118**, 53–59.
- BOULTON, C. A. & LARGE, P. J. (1977). Synthesis of certain assimilatory and dissimilatory enzymes during bacterial adaptation to growth on trimethylamine. *Journal of General Microbiology* **101**, 151–156.
- COLBY, J. & ZATMAN, L. J. (1971). The purification and properties of a bacterial trimethylamine dehydrogenase. *Biochemical Journal* **121**, 9p–10p.
- COLBY, J. & ZATMAN, L. J. (1973). Trimethylamine metabolism in obligate and facultative methylotrophs. *Biochemical Journal* **132**, 101–112.
- COLBY, J. & ZATMAN, L. J. (1974). Purification and properties of the trimethylamine dehydrogenase of *Bacterium* 4b6. *Biochemical Journal* **143**, 555–567.
- EADY, R. R. & LARGE, P. J. (1968). Purification and properties of an amine dehydrogenase from *Pseudomonas* AM1 and its role in growth on methylamine. *Biochemical Journal* **106**, 245–255.
- EADY, R. R. & LARGE, P. J. (1969). Bacterial oxidation of dimethylamine, a new mono-oxygenase reaction. *Biochemical Journal* **111**, 37p–38p.
- EADY, R. R. & LARGE, P. J. (1971). Microbial oxidation of amines. Spectral and kinetic properties of the primary amine dehydrogenase of *Pseudomonas* AM1. *Biochemical Journal* **123**, 757–771.
- EADY, R. R., JARMAN, T. R. & LARGE, P. J. (1971). Microbial oxidation of amines. Partial purification of a mixed-function secondary-amine oxidase system from *Pseudomonas aminovorans* that contains an enzymically active cytochrome-*P*-420-type haemoprotein. *Biochemical Journal* **125**, 449–459.
- EGAMI, F. & TANIGUCHI, S. (1963). Nitrate. In *Methods of Enzymatic Analysis*, 1st edn, pp. 636–639. Edited by H. U. Bergmeyer. New York and London: Academic Press.
- GOLDSMITH, J., LIVONI, J. P., NORBERG, C. L. & SEGEL, I. H. (1973). Regulation of nitrate uptake in *Penicillium chrysogenum* by ammonium ion. *Plant Physiology* **52**, 362–367.
- HARDER, W. & ATTWOOD, M. M. (1975). Oxidation of organic C₁ compounds by *Hyphomicrobium* spp. *Antonie van Leeuwenhoek* **41**, 421–429.
- HARDER, W. & ATTWOOD, M. M. (1978). Biology, physiology and biochemistry of *Hyphomicrobia*. *Advances in Microbial Physiology* **17** (in the Press).
- HARDER, W., ATTWOOD, M. M. & QUAYLE, J. R. (1973). Methanol assimilation by *Hyphomicrobium* sp. *Journal of General Microbiology* **78**, 155–163.
- HARDER, W., VISSER, K. & KUENEN, J. G. (1974). Laboratory fermenter with an improved magnetic drive. *Laboratory Practice* **23**, 644–645.
- HERSH, L. B., PETERSON, J. A. & THOMPSON, A. A. (1971). An *N*-methylglutamate dehydrogenase from *Pseudomonas* MA. *Archives of Biochemistry and Biophysics* **145**, 115–120.
- JOHNSON, P. A. & QUAYLE, J. R. (1964). Microbial growth on C₁ compounds. VI. Oxidation of methanol, formaldehyde and formate by methanol-grown *Pseudomonas* AM1. *Biochemical Journal* **93**, 281–290.
- LARGE, P. J. (1971). Non-oxidative demethylation of trimethylamine *N*-oxide by *Pseudomonas aminovorans*. *FEBS Letters* **18**, 297–300.
- LARGE, P. J. & MCDUGALL, H. (1975). An enzymic method for the microestimation of trimethylamine. *Analytical Biochemistry* **64**, 304–310.
- LARGE, P. J., BOULTON, C. A. & CRABBE, M. J. C. (1972). The reduced nicotinamide-adenine dinucleotide phosphate- and oxygen-dependent *N*-oxygenation of trimethylamine by *Pseudomonas aminovorans*. *Biochemical Journal* **128**, 137p–138p.
- LICHTENSTEIN, N. (1942). Preparation of γ -alkylamides of glutamic acid. *Journal of the American Chemical Society* **64**, 1021–1022.
- LOGINOVA, N. V., SHISHKINA, V. N. & TROTSENKO, YU. A. (1976). Primary metabolic pathways of methylated amines in *Hyphomicrobium vulgare*. *Mikrobiologiya* (English translation) **45**, 34–40.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.
- MEIBERG, J. B. M. & HARDER, W. (1976). Aerobic and anaerobic degradation of trimethylamine and dimethylamine by *Hyphomicrobium* x. *Proceedings of the Society for General Microbiology* **4**, 45.
- MYERS, P. A. & ZATMAN, L. J. (1971). The metabolism of trimethylamine *N*-oxide by *Bacillus* PM6. *Biochemical Journal* **121**, 10p.
- NASH, T. (1953). The colorimetric estimation of

- formaldehyde by means of the Hantzsch reaction. *Biochemical Journal* **55**, 416–421.
- SPEERL, G. T. & HOARE, D. S. (1971). Denitrification with methanol: a selective enrichment for *Hyphomicrobium* species. *Journal of Bacteriology* **108**, 733–736.
- STEENKAMP, D. J. & MALLINSON, J. (1976). Trimethylamine dehydrogenase from a methylo-trophic bacterium. I. Isolation and steady-state kinetics. *Biochimica et biophysica acta* **429**, 705–719.
- STRAUSS, G. & NICKERSON, W. J. (1961). Photochemical cleavage of water by riboflavin. II. Role of activators. *Journal of the American Chemical Society* **83**, 3187–3191.
- TATE, III, R. L. & ALEXANDER, M. (1976). Microbial formation and degradation of dimethylamine. *Applied and Environmental Microbiology* **31**, 399–403.
- TROTSSENKO, YU. A., LOGINOVA, N. V. & SHISHKINA, V. N. (1974). Metabolism of methylamine by *Hyphomicrobium*. *Dokladi Akademii nauk SSSR (Proceedings of the Academy of Sciences of the U.S.S.R.)* **216**, 1413–1415.
- VISHNIAC, W. & SANTER, M. (1957). The thiobacilli. *Bacteriological Reviews* **21**, 195–213.