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Trimethylamine Metabolism in Obligate and Facultative Methylotrophs

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1. Twelve bacterial isolates that grow with trimethylamine as sole source of carbon and energy were obtained in pure culture. All the isolates grow on methylamine, dimethylamine and trimethylamine. One isolate, bacterium 4B6, grows only on these methylamines whereas another isolate, bacterium C2A1, also grows on methanol but neither grows on methane; these two organisms are obligate methylotrophs. The other ten isolates grow on a variety of C_1 and other organic compounds and are therefore facultative methylotrophs. 2. Washed suspensions of the obligate methylotrophs bacteria 4B6 and C2A1, and of the facultative methylotrophs bacterium 5B1 and *Pseudomonas* 3A2, all grown on trimethylamine, oxidize trimethylamine, dimethylamine, formaldehyde and formate; only bacterium 5B1 and *Ps.* 3A2 oxidize trimethylamine *N*-oxide; only bacterium 4B6 does not oxidize methylamine. 3. Cell-free extracts of trimethylamine-grown bacteria 4B6 and C2A1 contain a trimethylamine dehydrogenase that requires phenazine methosulphate as primary hydrogen acceptor, and evidence is presented that this enzyme is important for the growth of bacterium 4B6 on trimethylamine. 4. Cell-free extracts of eight facultative methylotrophs, including bacterium 5B1 and *Ps.* 3A2, do not contain trimethylamine dehydrogenase but contain instead a trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase. It is concluded that two different pathways for the oxidation of trimethylamine occur amongst the isolates.

Methylotrophs are micro-organisms that can grow non-autotrophically at the expense of carbon compounds containing one or more carbon atoms but containing no carbon–carbon bonds (Colby & Zatman, 1972); such micro-organisms must be able to synthesize all their cell material from C_1 units derived from their growth substrates (for recent reviews of microbial C_1 metabolism see Quayle, 1969, 1972; Ribbons *et al.*, 1970). In the present paper we describe the isolation of, and some physiological and biochemical studies with, twelve pure cultures of methylotrophic bacteria, each of which grows with trimethylamine as its sole source of carbon and energy.

Two routes are known for the non-enzymic oxidation of tertiary alkylamines; either or both of these routes might be found in biological systems. The first non-enzymic route involves an initial oxygen incorporation step with the formation of trimethylamine *N*-oxide (Mueller, 1958). Evidence for the occurrence of this route in trimethylamine-grown *Pseudomonas aminovorans* and *Hyphomicrobium vulgare* NQ has been reported (Large *et al.*, 1972). Extracts of these organisms contain a trimethylamine mono-oxygenase that catalyses reaction (1):

$$(CH_3)_3N + O_2 + NAD(P)H + H^+ \rightarrow (CH_3)_3NO + NAD(P)^+ + H_2O \quad (1)$$

a similar enzyme is found in pig liver microsomal material (Baker & Chaykin, 1962; Baker *et al.*, 1963; Ziegler *et al.*, 1969). Microbial enzymes catalysing the demethylation of trimethylamine *N*-oxide yielding dimethylamine and formaldehyde have been observed in *Bacillus* PM6 (Myers & Zatman, 1971) and in *Ps. aminovorans* (Large, 1971). These two enzymes, trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase, therefore constitute one possible route for the initial stages in the microbial oxidation of trimethylamine. The second non-enzymic route involves an oxidative *N*-demethylation yielding dimethylamine and formaldehyde directly (Horner & Kirmse, 1955; Cullis & Waddington, 1958; De La Mare, 1960; Smith & Loeppky, 1967; Hull *et al.*, 1969); the occurrence of this route in one of the present isolates, bacterium 4B6, was reported briefly by Colby & Zatman (1971). Evidence for the occurrence of both of these routes amongst our trimethylamine-utilizing isolates is given in the present paper.

Materials and Methods

Materials

Trimethylamine hydrochloride was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K. or from Fluka A.G., Buchs, Switzerland. Trimethylamine *N*-oxide hydrochloride, dimethylamine hydrochloride and methylamine hydrochloride were supplied by British Drug Houses Ltd., Poole, Dorset,

U.K. Phenazine methosulphate was obtained from the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.; solutions were kept in the dark at all times and stored at −20°C. 2,6-Dichlorophenol-indophenol was obtained from British Drug Houses Ltd.; solutions were standardized spectrophotometrically at 600nm in 0.1 M-sodium phosphate buffer, pH 7.0, and the molar extinction coefficient was taken to be $20.6 \times 10^3 M^{-1} \cdot cm^{-1}$ (Armstrong, 1964). Nicotinamide nucleotides, both reduced and oxidized, were obtained from the Boehringer Corporation (London) Ltd., London W.5, U.K.

Methods

Growth medium and isolation methods. For most of this work the mineral medium contained the following in 1 litre of solution made up with glass-distilled water: K_2HPO_4, 1.20g; KH_2PO_4, 0.62g; $CaCl_2,6H_2O$, 0.05g; $MgSO_4,7H_2O$, 0.20g; NaCl, 0.10g; $FeCl_3,6H_2O$, 1.0mg; $(NH_4)_2SO_4$, 0.50g; $CuSO_4,5H_2O$, 5.0μg; $MnSO_4,5H_2O$, 10.0μg; $Na_2MoO_4,2H_2O$, 10.0μg; H_3BO_3, 10.0μg; $ZnSO_4,7H_2O$, 70.0μg; $CoCl_2,6H_2O$, 5.0μg. For some experiments in the later stages of the work the chelated medium (mineral base E) of Owens & Keddie (1969) was used. The mineral media were sterilized by autoclaving at 121°C for 20min, cooled, then filter-sterilized aqueous solutions of vitamins were added to give a basal medium containing in 1 litre: thiamin hydrochloride, 0.5mg; calcium pantothenate, 0.5mg; nicotinic acid, 0.5mg; biotin, 1μg; riboflavin, 0.5mg; vitamin B_{12}, 2.5μg; *p*-aminobenzoic acid, 10.0μg; folic acid, 10.0μg; pyridoxal hydrochloride, 2.0mg. Filter-sterilized solutions of trimethylamine hydrochloride were added to the cool basal medium to a final concentration of 0.1%. The final pH of the medium was 7.0. Solidified media were prepared by the addition of 1% Oxoid Ionagar no. 2 before autoclaving.

Potential sources of bacteria were screened for trimethylamine utilizers by direct streaking on trimethylamine basal agar plates and also by liquid enrichment in shaken trimethylamine basal medium cultures. When visible growth occurred in the liquid enrichments, subcultures were made into fresh medium and incubated under the same conditions. After two subcultures in liquid medium the isolates were streaked on to trimethylamine basal agar plates. The isolates were purified by streaking isolated colonies on trimethylamine basal agar plates. The temperature for all incubations was 30°C.

Bacterial characterization methods. Bacteria 4B6 and C2A1 grew only on 'one-carbon' substrates and all characterization tests were done on organisms grown on trimethylamine basal medium; the other ten isolates grew well on complex media such as nutrient broth (0.5% Evans peptone, 0.5% Lab-Lemco, 0.5% NaCl). The morphology of organisms taken from young and old trimethylamine basal medium or nutrient broth cultures was determined from wet mounts by phase-contrast microscopy; hanging-drop preparations from the same cultures were examined for motility. Organisms taken from moist trimethylamine basal agar or nutrient agar slopes were stained for flagella by using the methods of Rhodes (1958) and Leifson (1951). The Gram, oxidase and catalase reactions of the isolates were tested on material taken from trimethylamine basal agar or nutrient agar slope-cultures. Pigmentation was determined on trimethylamine basal agar and, where growth occurred, on King's agar A and King's agar B (King *et al.*, 1954). All cultures were incubated at 30°C.

The results of physiological tests were recorded after 2, 4 and 7 days. The oxygen requirements of the isolates were determined in shake cultures by using trimethylamine basal agar, nutrient agar, nutrient agar containing 1% (w/v) glucose+Bromocresol Purple indicator, and Hugh & Leifson's (1953) medium containing 1% (w/v) glucose. The glucose nutrient agar and Hugh & Leifson's (1953) medium shake cultures were also used to test for the aerobic and/or anaerobic production of acid from glucose. The ability to reduce nitrate was tested in nitrate–peptone water [1% (w/v) peptone; 0.5% NaCl; 0.1% KNO_3].

Determination of the growth substrate specificity of the isolates. The range of potential carbon and energy sources that would support the growth of the isolates was determined by streaking each organism on a number of basal agar plates each incorporating a different growth substrate. Solutions of the growth substrates were neutralized, sterilized by filtration and mixed with melted basal agar at about 50°C, to a final concentration of 0.1%. After pouring and drying, each plate was divided into quadrants then each quadrant was streaked with a different isolate. Inocula were taken from exponential-phase trimethylamine basal medium cultures. Each isolate was also streaked on a basal agar control plate with no added growth substrate. Where growth occurred, isolates were subcultured twice on the same medium.

The ability of isolates 4B6, C2A1, 5B1 and 3A2 to grow on methane was tested in 250ml screw-capped medical-flat bottles fitted with silicone rubber Subaseal caps and containing 50ml of basal medium (cf. Whittenbury *et al.*, 1970). Filter-sterilized methane (100ml) was added, followed by 0.5ml of an exponential-phase trimethylamine basal medium culture, then the bottles were incubated horizontally on a reciprocal shaker at 30°C.

Bulk growth of the isolates for physiological studies. The isolates were grown in 2-litre conical flasks containing 1 litre of growth medium incubated at 30°C on an orbital shaker. Mineral medium was used for

the growth of *Pseudomonas* 3A2 and bacterium C2A1, vitamin B_{12}-supplemented (2.5μg/l) mineral medium for bacterium 4B6, thiamin-supplemented (0.5mg/l) mineral medium for bacterium 5B1 and basal medium for the other isolates; all media contained 0.2% of growth substrate. Before use as an inoculum (usually 1%, v/v) organisms were subcultured three times in the presence of the growth substrate to be used in the main experiment. Organisms were harvested in mid-exponential phase (after 16–36h of incubation, depending on the organism and the growth substrate) after rapid cooling of the culture in an ice–water mixture. The cells were washed twice with ice-cold 0.1M-sodium phosphate buffer, pH7, and resuspended in the same buffer. Washed suspensions for whole-cell experiments were kept at 0°C and used within 12h of their preparation. Cells to be used for the preparation of cell-free extracts were sometimes stored as pellets at −20°C until required.

Oxygen electrode experiments. The ability of organisms to oxidize a wide range of compounds was tested by using a Clarke-type oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.) connected to a Servoscribe chart recorder (Smith's Industries Ltd., Wembley, Middx., U.K.). For the calibration of the electrode the sample compartment contained glass-distilled water through which gases of known composition containing from 0% to 100% oxygen were bubbled and the output from the electrode was measured in each case. For the measurement of oxygen uptakes the sample compartment contained in 3ml: 0.3mmol of sodium phosphate buffer, pH7; 2–4mg dry weight of cells; 10μmol of substrate (formaldehyde, 5μmol). The temperature of the sample compartment was maintained at 30°C by a water jacket.

Manometry. Oxygen uptakes were measured by using conventional Warburg manometric techniques (Umbreit *et al.*, 1964); the experimental details are given in the legend to Table 4.

Preparation of cell-free extracts. Cell-free extracts of the isolates were prepared by sonication. Washed suspensions (100–200mg wet wt./ml) of bacterium 4B6 in 0.1M-sodium phosphate buffer, pH7, were sonicated in 12ml portions at 20kHz by using an MSE 100 W ultrasonic disintegrator (Measuring & Scientific Equipment Ltd., London S.W.1, U.K.). The suspension was contained in a 25ml Rosett cooling-cell (Rosett, 1965) surrounded by an ice–water mixture and sonication was interrupted for 1min in every 2min to prevent local warming of the suspension; the total time of exposure of the suspension to ultrasonic radiation was 4min. These conditions resulted in about 85% cell breakage as judged by total cell counts before and after sonication. The product, crude sonicate, was centrifuged at 35000*g* for 30min and the supernatant, crude sonic extract, was retained. Similar conditions were used to prepare crude sonic extracts of the other isolates.

Heat-treatment of crude sonic extracts of bacterium 4B6. Test tubes containing 3ml of crude sonic extract were heated to 50°C in a water bath and maintained at that temperature for 15min. The tubes were then rapidly cooled in ice–water mixture and the heavy precipitate was centrifuged down, leaving a clear yellow liquid, the heat-treated extract.

Protein determinations. The concentration of protein in cell-free extracts was determined with the Folin–phenol reagent (Kennedy & Fewson, 1968). Standard curves were prepared by using crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.).

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

Spectrophotometry. All spectrophotometric enzyme assays were done in a Hitachi Perkin–Elmer 124 double-beam grating spectrophotometer (Perkin–Elmer Ltd., Beaconsfield, Bucks., U.K.) or a Unicam SP.800 spectrophotometer (Pye–Unicam Ltd., Cambridge, U.K.). Both instruments were fitted with constant-temperature cuvette housings and were coupled to Servoscribe chart recorders.

Assay of methylamines by g.l.c. G.l.c. was done by using a Perkin–Elmer F11 gas chromatograph fitted with a flame-ionization detector and coupled to a Vitatron type UR 402 chart recorder (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.). The concentration of methylamines in culture filtrates and in assay mixtures for trimethylamine mono-oxygenase was determined on a 3.66m glass column (internal diameter 3mm) packed with Chromosorb W (60–80 mesh) coated with 15% (w/v) tetrahydroxyethylenediamine and 5% (w/v) tetraethylenepentamine (Sze *et al.*, 1963). A pre-column of soda lime, housed in the injection heating block at 180°C, released the free amines from their salts. The oven temperature was 65°C and the carrier gas was nitrogen at a flow rate of 10cm^3/min. Standard solutions of amine hydrochlorides for calibration purposes were prepared from material that had been dried over phosphorus pentoxide for at least 7 days.

Enzyme assays. Spectrophotometric assays were performed at 30°C in 10mm light-path cuvettes with water in the reference cuvette. Initial rates were measured, except in trimethylamine *N*-oxide demethylase assays when the reaction was terminated after a fixed time and the concentration of the product, formaldehyde, was determined by the method of Nash (1953).

Formaldehyde dehydrogenase (NAD-dependent)

and formaldehyde dehydrogenase (2,6-dichlorophenol-indophenol-dependent) were assayed by the methods of Johnson & Quayle (1964), formate dehydrogenase (NAD-linked) by the method of Johnson *et al.* (1964), methanol dehydrogenase by the method of Anthony & Zatman (1964*b*) with 3μmol of phenazine methosulphate, and primary amine dehydrogenase by the method of Eady & Large (1968).

Trimethylamine dehydrogenase. Reaction mixtures contained in 3ml: 0.3mmol of sodium pyrophosphate buffer, pH7.7; 0.12μmol of 2,6-dichlorophenol-indophenol; 6μmol of phenazine methosulphate; 3μmol of KCN; crude sonic extract; 10μmol of trimethylamine hydrochloride. The reaction was started by the addition of substrate and the initial rate of decrease in E_{600} measured.

Trimethylamine mono-oxygenase. (i) Spectrophotometric method. Reaction mixtures contained in 3ml: 0.3mmol of sodium phosphate buffer, pH7.0; 0.6μmol of NADPH; crude sonic extract; 10μmol of trimethylamine hydrochloride. The reaction was started by the addition of substrate and the initial rate of decrease in E_{340} was measured.

(ii) G.l.c. method. Extracts of the facultative methylotrophs bacterium 5B1 and *Pseudomonas* 3A2, when grown on trimethylamine, contain high trimethylamine *N*-oxide demethylase and dimethylamine mono-oxygenase activities (see Table 6). It might be expected therefore that dimethylamine, arising from the oxidation of trimethylamine by trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase, might itself undergo an NADPH-dependent reaction, thus contributing to the trimethylamine mono-oxygenase activity as measured by the spectrophotometric method. All quantitative determinations of trimethylamine mono-oxygenase activities in extracts of these facultative methylotrophs were therefore done by the following method, in which trimethylamine disappearance is measured directly by g.l.c.

Conical flasks (25ml) contained in 2ml: 0.1mmol of sodium phosphate buffer, pH7.0; 2μmol of NADP; 1 unit of yeast glucose 6-phosphate dehydrogenase [grade II; Boehringer Corporation (London) Ltd.]; 40μmol of glucose 6-phosphate; 10μmol of $MgCl_2$; crude sonic extract; 10μmol of trimethylamine hydrochloride. Control flasks lacked extract or NADP. The flasks were incubated at 30°C on a reciprocal shaker and at intervals 1μl samples were removed and injected into the g.l.c. apparatus for measurement of trimethylamine. A control complete reaction mixture was also incubated anaerobically at 30°C under nitrogen in a Thunberg tube.

Trimethylamine N-oxide demethylase. The colorimetric method B of Myers (1971) was modified. Test tubes contained the following in 1.5ml: 75μmol of triethanolamine buffer, pH7.5; crude sonic extract; 50μmol of trimethylamine *N*-oxide hydrochloride (adjusted to pH7.5). Controls lacked extract or substrate. The reaction was started by the addition of substrate and was terminated after static incubation for 5min at 30°C by the addition of an equal volume of 1M-trichloroacetic acid. The denatured protein was removed by centrifugation and the concentration of formaldehyde in the supernatant was determined by the method of Nash (1953). Calibration curves were prepared with A.R. formaldehyde which had been standardized by oxidation to formic acid with iodine in alkaline solution, the unchanged iodine being titrated against standard thiosulphate (Treadwell & Hall, 1935).

Dimethylamine mono-oxygenase (*cf. Eady et al.*, 1971). Reaction mixtures contained in 3ml: 0.3mmol of sodium phosphate buffer, pH7.0; 0.6μmol of NADPH; crude sonic extract; 10μmol of dimethylamine hydrochloride. The reaction was started by the addition of substrate and the rate of decrease in E_{340} measured.

Enzyme units. One munit of trimethylamine dehydrogenase, primary amine dehydrogenase, formaldehyde dehydrogenase, methanol dehydrogenase or formate dehydrogenase is defined as that quantity of enzyme which catalyses the reduction of 1nmol of the hydrogen acceptor per min at 30°C in the assays described above. The molar extinction coefficient of 2,6-dichlorophenol-indophenol at 600nm was taken to be $20.6\times10^3 M^{-1}\cdot cm^{-1}$ at pH7.0, $21.69\times10^3 M^{-1}\cdot cm^{-1}$ at pH7.5–7.7 and $22.0\times10^3 M^{-1}\cdot cm^{-1}$ at pH9 (Armstrong, 1964). The molar extinction coefficient of NADH at 340nm was taken to be $6.22\times10^3 M^{-1}\cdot cm^{-1}$ (Dawson *et al.*, 1969). One munit of dimethylamine mono-oxygenase is the quantity of enzyme that catalyses the oxidation of 1nmol of NADPH per min at 30°C. One munit of trimethylamine mono-oxygenase activity is that quantity of enzyme which catalyses the NADP- and oxygen-dependent disappearance of 1nmol of trimethylamine per min at 30°C. One munit of trimethylamine *N*-oxide demethylase is the quantity of enzyme that catalyses the formation of 1nmol of formaldehyde per min at 30°C.

Results and Discussion

Organisms isolated

Twelve isolates were obtained in pure culture by direct streaking or liquid enrichment by using trimethylamine basal media. Some properties of the isolates are recorded in Table 1 together with the source from which each was obtained and, where possible from the data, the genus to which each isolate has been tentatively assigned. Six isolates are strictly aerobic, Gram-negative, oxidase-positive, regular rods which are motile by polar flagella, properties which suggest that they should be assigned to the

Table 1. *Some morphological and physiological properties of twelve trimethylamine-utilizing bacterial isolates*

Details of the methods used to characterize the isolates are given in the Materials and Methods section. All the isolates were strict aerobes, catalase-positive and oxidase-positive. The following symbols are used: A, cocci in 2–3 days trimethylamine basal medium cultures giving rise to irregular rods on subculture; B, short regular rods; C, long slender regular rods; D, coccal rods; NO_2^-, nitrate reduced to nitrite; *, non-diffusible pigment; NG, no growth; ?, the taxonomic position of these organisms is discussed in the Results and Discussion section.

Source ...	Soil				Ditch water		Air	River	Fish			
Isolate ...	1A3	1B1	1A1	1A2	2B2	2A3	3A2	4B6	C2A1	5B1	7B1	8B1
Morphology	B	B	A	A	A	B	C	B	B	D	B	B
Gram reaction	–	–	–	–	–	–	–	–	–	–	–	–
Rods			+	–	+							
Cocci												
Motility	+	+	None	None	None	+	+	None	+	None	+	+
Flagella	Polar	Polar	None	None	None	Polar	Polar	None	Polar	None	Polar	Polar
Slime produced	Much	Little	None	None	None	None	None	None	None	None	None	None
Pigmentation	None	None	None	None	Yellow*	None	Red*	None	None	None	None	None
Nitrate reduction	NO_2^-	NO_2^-	NO_2^-	None	None	None	None	NG	NG	NO_2^-	None	None
Acid produced from glucose aerobically	None	None	None	Little	Little	None	None	NG	NG	None	None	None
Probable genus	*Pseudomonas*		*Arthrobacter*			*Pseudomonas*			?		*Pseudomonas*	

genus *Pseudomonas* (cf. Stanier *et al.*, 1966). A further three isolates (1A1, 1A2 and 2B2) were provisionally allocated to the genus *Arthrobacter* almost entirely on morphological evidence; thus, after incubation for 2–3 days, trimethylamine basal medium cultures consist entirely of coccal forms that become irregular rods on transfer to fresh medium. Isolate 5B1 is a strictly aerobic, Gram-negative, non-motile, oxidase-positive, non-pigmented, rod-shaped bacterium; its ability to grow on glucose excludes it from the genus *Moraxella* as described by Baumann *et al.* (1968) and bacteria of this type have often been allotted to the *Achromobacter–Alcaligenes* group (see Thornley, 1967). Isolates C2A1 and 4B6 do not grow in complex laboratory media and this eliminates the use of most routine physiological and biochemical tests as an aid to identification; these isolates were therefore not even tentatively assigned to genera.

Growth substrate specificity of the isolates

The ability of each isolate to grow on a variety of carbon and energy sources, tested as described in the Materials and Methods section, is shown in Table 2. Bacterium 4B6 grows only on trimethylamine, dimethylamine and methylamine whereas bacterium C2A1 grows only on these compounds and on methanol; these two organisms are therefore considered to be obligate methylotrophs (Colby & Zatman, 1972). The remaining isolates grow on a variety of C_1-substrates as well as on a wide range of other carbon compounds; these are therefore facultative methylotrophs.

Vitamin requirements of isolates C2A1, 4B6, 3A2 and 5B1

The basal medium used for the isolation and purification of the bacterial isolates contained a mixture of vitamins. Further investigation of the vitamin requirements of isolates C2A1, 4B6, 3A2 and 5B1 showed that C2A1 and 3A2 do not require added vitamins, 4B6 requires only vitamin B_{12}, and 5B1 requires only thiamin hydrochloride.

Oxidation of various substrates by washed suspensions of trimethylamine-grown bacterium C2A1, bacterium 4B6, bacterium 5B1 and Pseudomonas 3A2

The ability of these four isolates to oxidize a range of organic compounds was tested in the Clarke-type oxygen electrode (Table 3). All the trimethylamine-grown isolates oxidized trimethylamine, dimethylamine, diethylamine, formaldehyde, formate and, where tested, *N*-methylglutamate; all except 4B6 oxidized methylamine and ethylamine. These results

Table 2. *Growth of the trimethylamine-utilizing isolates on other substrates*

Experimental details are given in the Materials and Methods section. The results recorded in the Table were obtained after 14 days of incubation at 30°C. ++, Good growth; +, poor growth; −, no growth; NT, not tested.

Substrate Isolate ...	1A1	1A2	1A3	1B1	2A3	2B2	3A2	4B6	C2A1	5B1	7B1	8B1
Methane	NT	NT	NT	NT	NT	NT	−	−	−	−	NT	NT
Methanol	++	+	+	++	−	++	++	−	+	++	+	+
Formate	+	++	−	++	+	++	+	−	−	++	−	++
Methylamine	++	++	++	++	++	++	++	++	+	++	++	++
Dimethylamine	++	++	++	++	++	++	+	++	++	++	++	++
Trimethylamine	++	++	++	++	++	++	++	++	++	++	++	++
Tetramethylammonium chloride	NT	NT	NT	NT	NT	NT	NT	−	−	NT	NT	NT
Trimethylamine *N*-oxide	++	++	+	++	++	++	+	−	−	++	−	++
Trimethylsulphonium chloride	NT	NT	NT	NT	NT	NT	NT	−	−	NT	NT	NT
Ethylamine	++	++	−	+	+	++	++	−	−	+	+	++
Diethylamine	++	++	−	+	+	++	+	−	−	+	+	++
Triethylamine	++	++	−	+	+	++	++	−	−	+	+	++
Ethanol	++	++	++	++	+	++	++	−	−	++	+	++
Acetate	++	++	++	++	+	++	++	−	−	++	++	++
Glycollate	+	+	++	++	−	−	−	−	−	−	+	+
Glyoxylate	++	++	++	++	−	+	+	−	−	+	−	−
Oxalate	−	−	−	++	−	−	+	−	−	−	+	−
Pyruvate	++	++	++	++	++	++	++	−	−	++	++	++
Lactate	++	++	++	++	++	++	++	−	−	++	++	++
Citrate	++	++	−	++	++	++	−	−	−	+	++	++
Succinate	++	++	−	++	++	++	++	−	−	++	++	++
Fumarate	++	++	−	++	++	++	++	−	−	++	++	++
Malate	++	++	−	++	++	+	++	−	−	++	++	++
Glycine	++	++	+	++	−	++	−	−	−	−	+	++
Serine	++	++	++	++	−	++	+	−	−	++	++	+
Glutamate	++	++	++	++	++	++	+	−	−	++	+	++
Aspartate	++	++	++	++	++	++	+	−	−	+	++	++
Glucose	++	++	++	−	++	++	++	−	−	++	++	+
Fructose	NT	NT	NT	NT	NT	NT	NT	−	−	NT	NT	NT

are consistent with the stepwise oxidative demethylation of trimethylamine via dimethylamine and, except perhaps for isolate 4B6, methylamine, yielding formaldehyde and eventually formate and CO_2; *N*-methylglutamate might be involved as an intermediate in methylamine oxidation (cf. Hersh *et al.*, 1971). The oxidation of the ethylamines and, in some cases, of *n*-propylamine, probably reflects a lack of specificity in the systems for the oxidation of the homologous methylamines [e.g. the primary amine dehydrogenase of Eady & Large (1968, 1971)].

Two patterns were observed with regard to the ability of the isolates to oxidize trimethylamine *N*-oxide. The facultative methylotrophs *Ps.* 3A2 and bacterium 5B1, grown on trimethylamine, oxidize trimethylamine *N*-oxide rapidly whereas the obligate methylotrophs bacteria 4B6 and C2A1 do not. Disregarding possible permeability problems for the present, these results suggest that trimethylamine *N*-oxide is not an intermediate in the oxidation of trimethylamine by bacteria C2A1 and 4B6 but may well be involved in trimethylamine oxidation in bacterium 5B1 and *Ps.* 3A2.

Bacterium C2A1, bacterium 5B1 and *Ps.* 3A2 all grow on methanol (Table 2). Trimethylamine-grown C2A1 and 3A2 oxidize methanol, suggesting that the methanol-oxidizing system in these organisms is constitutive as in *Ps.* M27 (Anthony & Zatman, 1964*a*). Bacterium 5B1 probably has an inducible system for methanol oxidation.

Oxidation of various substrates by washed suspensions of trimethylamine-, dimethylamine- and methylamine-grown bacterium 4B6

The results of the oxygen electrode experiments suggested that dimethylamine, formaldehyde, form-

Table 3. *Oxidation of various substrates by washed suspensions of trimethylamine-grown C2A1, 4B6, 3A2 and 5B1*

The experiments were done at 30°C with an oxygen electrode; experimental details are described in the Materials and Methods section. All values are rates of oxygen uptake expressed as nmol of oxygen consumed/min per mg dry wt. of trimethylamine-grown cells and are corrected for the endogenous rate. None of the suspensions oxidized *n*-butylamine, choline, betaine, glucose, malate, fumarate, L-aspartate, L-glutamate, L-lysine or glycine. NT, not tested.

Substrate	Isolate ...	Oxygen uptake rate C2A1	4B6	3A2	5B1
Trimethylamine		135	134	161	290
Trimethylamine *N*-oxide		0	0	196	109
Dimethylamine		120	106	208	239
Methylamine		18	0	80	89
N-Methylglutamate		27	8	89	NT
Triethylamine		0	1	9	17
Diethylamine		11	8	46	49
Ethylamine		8	0	9	21
n-Propylamine		3	0	0	15
Methanol		32	0	10	0
Formaldehyde		47	110	109	75
Formate		6	25	41	27
None		0	3	5	2

Table 4. *Oxidation of various substrates by washed suspensions of trimethylamine-, dimethylamine- and methylamine-grown bacterium 4B6*

The experiments were done at 30°C with air as the gas phase by using conventional manometric techniques. The main compartment of the Warburg flasks contained 300 μmol of sodium phosphate buffer, pH 7, and 4 mg dry wt. of cells in a total volume of 2.9 ml. Test substrates (0.1 ml of a 100 mM solution) were added from the side arm and the centre-well contained 0.2 ml of 20% (w/v) KOH and a filter-paper wick to absorb CO_2. All rates of oxygen uptake are expressed as nmol of oxygen consumed/min per mg dry wt. and have been corrected for the endogenous rate.

Test substrate	Growth substrate ...	Oxygen uptake rate Trimethylamine	Dimethylamine	Methylamine
Trimethylamine		89	103	0
Dimethylamine		94	135	0
Methylamine		0	15	36
N-Methylglutamate		0	25	58
Formaldehyde		55	27	33
Formate		19	18	18
None		2	2	2

ate and possibly *N*-methylglutamate might be intermediates in the oxidation of trimethylamine by bacterium 4B6. Although methylamine is not oxidized by trimethylamine-grown cells (Table 3), it would be expected to arise as a product of dimethylamine oxidation (Eady *et al.*, 1971). The results of experiments in which these five compounds were tested manometrically with washed suspensions of bacterium 4B6 grown on trimethylamine, dimethylamine or methylamine are presented in Table 4.

Trimethylamine oxidation and dimethylamine oxidation are apparently dependent on inducible systems that are present in trimethylamine- and dimethylamine-grown organisms but not in methylamine-grown organisms. Similarly the systems responsible for the oxidation of methylamine and of *N*-methylglutamate are apparently inducible and are present in methylamine- and dimethylamine-grown organisms but not in trimethylamine-grown organisms. *N*-Methylglutamate is thus implicated as a

possible intermediate in methylamine oxidation. The total oxygen uptake observed on the addition of *N*-methylglutamate to washed suspensions of methylamine-grown organisms was always very similar to that observed with methylamine; glutamate itself was not oxidized. These results suggest that only the aminomethyl group of *N*-methylglutamate is being oxidized. It is possible that dimethylamine- and methylamine-grown bacterium 4B6, like methylamine-grown *Pseudomonas* MA and *Ps.* MS, oxidize methylamine by first converting it into *N*-methylglutamate, which is then oxidized to formaldehyde and glutamate by an *N*-methylglutamate dehydrogenase (Hersh *et al.*, 1971).

The failure of trimethylamine-grown cells to oxidize exogenous methylamine or *N*-methylglutamate might be caused by the inability of these compounds to enter trimethylamine-grown cells. If, however, organisms grown on trimethylamine lack a methylamine-oxidizing system this would suggest that the methylamine, which is presumably formed inside the cell during growth on trimethylamine, is either assimilated by some unknown pathway or excreted into the medium. G.l.c. analysis of culture filtrates did not detect an accumulation of methylamine in the medium during growth on trimethylamine.

Enzyme activities present in extracts of bacterium 4B6

Oxidation of trimethylamine by crude sonic extracts. The addition of trimethylamine to extracts of trimethylamine-grown cells did not result in the reduction of added NAD^+ or $NADP^+$. No trimethylamine-dependent oxidation of NADH or NADPH occurred as would be expected with a mono-oxygenase-catalysed reaction and the addition of trimethylamine to extracts failed to stimulate an uptake of oxygen in the oxygen electrode as would be expected if an amine oxidase were involved.

A trimethylamine-dependent and phenazine methosulphate-dependent decolorization of 2,6-dichlorophenol-indophenol was observed when crude sonic extracts were incubated with these components anaerobically or aerobically in Thunberg tubes. Only insignificant rates of decolorization of the dye were observed in the absence of substrate, phenazine methosulphate or extract, and boiling the extract destroyed its activity. The decolorization time was inversely proportional to the amount of extract added and dialysis of the extract against 1000vol. of 50mM-sodium phosphate buffer, pH7, for up to 12h did not significantly decrease its activity.

It was concluded that crude sonic extracts of trimethylamine-grown bacterium 4B6 contain a trimethylamine dehydrogenase that requires phenazine methosulphate as primary hydrogen acceptor. The Thunberg tube assay provided the basis for a convenient and rapid assay method in which the rate of decolorization of 2,6-dichlorophenol-indophenol is measured spectrophotometrically at 600nm.

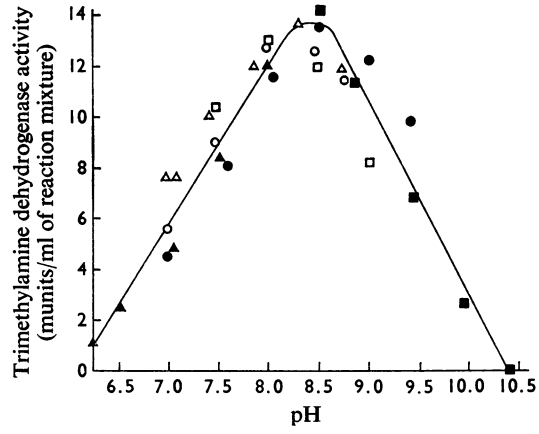


Fig. 1. *Effect of pH on the trimethylamine dehydrogenase activity in heat-treated extracts of trimethylamine-grown bacterium 4B6*

Trimethylamine dehydrogenase activity was assayed by the spectrophotometric method described in the Materials and Methods section, except that a variety of buffers of different pH values was used. Heat-treated extract (0.2mg of protein) of trimethylamine-grown bacterium 4B6 (see the Materials and Methods section) was used for each assay. After the initial rate had been recorded, the pH value of the reaction mixture was measured by using a glass electrode. The buffers used were: ●, sodium pyrophosphate; ○, triethanolamine; □, diethanolamine; ▲, sodium phosphate; △, tricene; ■, carbonate–bicarbonate.

Variables in the spectrophotometric assay for trimethylamine dehydrogenase. In exploratory experiments the stability of trimethylamine dehydrogenase in crude sonic extracts towards heating was noted; a threefold purification could be readily achieved by heating extracts at 50°C for 15min, cooling them, and removing the denatured protein by centrifugation. The experiments described below were done with such a heat-treated extract of trimethylamine-grown bacterium 4B6 prepared as described in the Materials and Methods section.

(i) pH value and the nature of the buffer. The variation of the initial velocity with pH value was determined by using six buffers covering the pH range 6.0–10.5 (Fig. 1). The highest activities were recorded at pH values between 8.3 and 8.5. The pH optimum in sodium pyrophosphate buffer, the buffer finally adopted for the assay, was 8.5. In calculating the results the variation of the extinction coefficient

of 2,6-dichlorophenol-indophenol with pH has been taken into account (Armstrong, 1964).

At pH8 and above the E_{600} versus time plot for a given assay rapidly became non-linear, the reaction velocity apparently decreasing with time. This phenomenon is due to two factors: (i) reoxidation of the dyes by molecular oxygen, and (ii) dependence of the reaction velocity on the concentration of the terminal hydrogen acceptor, 2,6-dichlorophenol-indophenol. Neither of these factors operates significantly below pH8. In routine assays these problems were avoided by performing the assays at pH7.7 in 0.1M-sodium pyrophosphate buffer.

(ii) Phenazine methosulphate concentration. The K_m for phenazine methosulphate was calculated from double-reciprocal plots (Lineweaver & Burk, 1934) and a value of 0.4mM obtained.

(iii) 2,6-Dichlorophenol-indophenol concentration. At pH7.7 the reaction velocity is independent of the concentration of the terminal hydrogen acceptor between 4 and 80μM.

(iv) Substrate concentration. The initial velocity was independent of trimethylamine concentration between 16μM and 3.3mM. Higher concentrations were inhibitory.

(v) Concentration of heat-treated extract. Initial velocities measured in the spectrophotometric assay increased linearly with increasing concentration of heat-treated extract between 0 and 0.14mg of protein/ml of reaction mixture. The specific activity of a typical heat-treated extract of trimethylamine-grown organisms was 150munits/mg of protein.

Other enzyme activities present in extracts of bacterium 4B6. Crude sonic extracts of cells grown on trimethylamine, dimethylamine or methylamine were examined for the presence of a number of enzymes; the specific activities of trimethylamine dehydrogenase, dimethylamine mono-oxygenase, NAD-dependent formaldehyde dehydrogenase and NAD-dependent formate dehydrogenase are given in Table 5. The NAD-dependent formaldehyde dehydrogenase did not require glutathione for its activity even after dialysis and neither was it specific for formaldehyde; acetaldehyde was an alternative equally good substrate. Trimethylamine mono-oxygenase, trimethylamine *N*-oxide demethylase, primary amine dehydrogenase, methanol dehydrogenase and 2,6-dichlorophenol-indophenol-dependent formaldehyde dehydrogenase were not detected in extracts of cells grown on any of the three substrates.

Induction of trimethylamine dehydrogenase by trimethylamine in methylamine-grown bacterium 4B6. The details and the results of this experiment are given in Fig. 2. Extracts of cells harvested from the trimethylamine-containing medium during the first 2h showed no trimethylamine dehydrogenase activity; thereafter the specific activity of trimethylamine dehydrogenase increased rapidly but no significant growth was observed until the specific activity had reached about 50% of its maximum value. Growth after the first doubling was not associated with any further increase in the specific activity of trimethylamine dehydrogenase. In the parallel experiment growth in the methylamine-containing medium occurred without a lag and no trimethylamine dehydrogenase was detected at any time. The results of this experiment support the conclusion that trimethylamine dehydrogenase is important for the growth of bacterium 4B6 on trimethylamine.

Enzymes present in crude sonic extracts of other isolates

Extracts of the facultative methylotrophs bacterium 5B1 and *Pseudomonas* 3A2 grown on trimethylamine and on acetate were examined for a number of enzymes; the results are shown in Table 6. It is clear from these results that the oxidation of trimethylamine by these facultative methylotrophs is mediated by trimethylamine mono-oxygenase (yielding trimethylamine *N*-oxide; Large *et al.*, 1972) and by trimethylamine *N*-oxide demethylase. These results are consistent with the ability of these organisms to grow on trimethylamine *N*-oxide (Table 2) and with the ability of trimethylamine-grown organ-

Table 5. *Specific activities of various enzymes in crude sonic extracts of trimethylamine-, dimethylamine- and methylamine-grown bacterium 4B6*

Experimental details are given in the Materials and Methods section. All values are specific activities expressed as nmol/min per mg of protein.

Enzyme	Growth substrate ...	Specific activity		
		Trimethylamine	Dimethylamine	Methylamine
Trimethylamine dehydrogenase		36	40	0
Dimethylamine mono-oxygenase		22	18	0
Formaldehyde dehydrogenase (NAD)		14	13	12
Formate dehydrogenase (NAD)		9	33	0

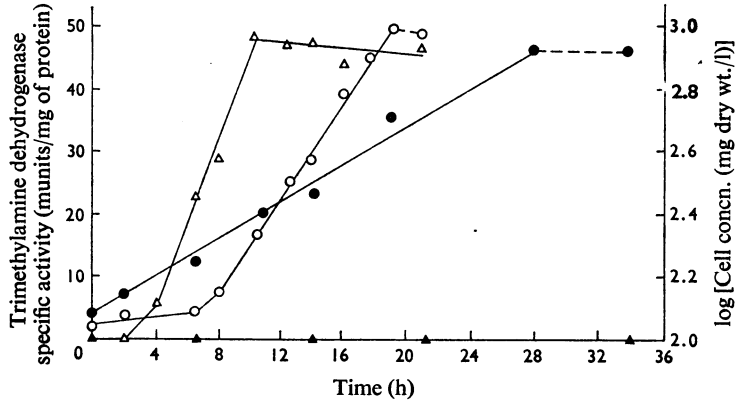


Fig. 2. *Induction of trimethylamine dehydrogenase during growth of a methylamine-grown inoculum of bacterium 4B6 on trimethylamine and on methylamine*

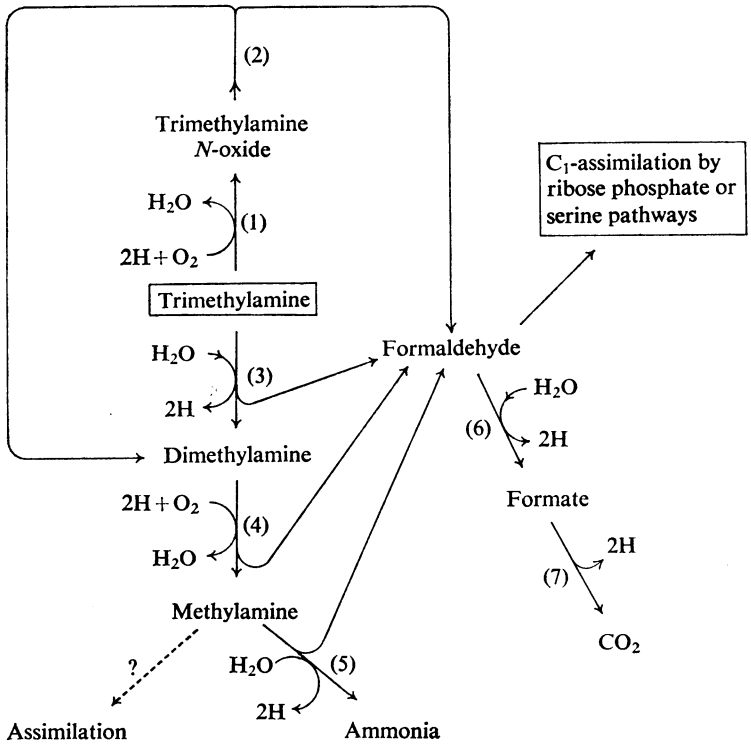
Late-exponential-phase organisms grown on methylamine were washed aseptically in warm mineral medium and dispensed into a series of flasks of vitamin B_{12}-supplemented mineral medium containing either 0.2% methylamine or 0.2% trimethylamine. The cultures were incubated at 30°C with vigorous aeration (achieved with magnetic stirring bars). At suitable intervals samples were removed aseptically for (i) determination of cell concentration determined nephelometrically by using a calibration curve to give mg dry wt. of organism per ml, and (ii) preparation of crude sonic extracts and determination of trimethylamine dehydrogenase activity and protein concentration (see the Materials and Methods section.) The following symbols are used: specific activity of trimethylamine dehydrogenase in crude sonic extracts of cells incubated in trimethylamine medium, △, and of cells incubated in methylamine medium, ▲; log[cell concn. (mg dry wt./l)] of trimethylamine medium culture, ○, and of methylamine medium culture, ●.

Table 6. *Specific activities of various enzymes in crude sonic extracts of bacterium C2A1, bacterium 5B1 and Pseudomonas 3A2*

Experimental details are given in the Materials and Methods section. All values are expressed as nmol/min per mg of protein. NT, not tested.

	Specific activity				
Isolate ...	C2A1	3A2		5B1	
Growth substrate ...	Trimethyl-	Trimethyl-		Trimethyl-	
Enzyme	amine	amine	Acetate	amine	Acetate
Trimethylamine dehydrogenase	72	0	NT	0	NT
Trimethylamine mono-oxygenase	0	60	0	35	0
Trimethylamine *N*-oxide demethylase	0	66	0	130	0
Dimethylamine mono-oxygenase	15	32	0	41	0
Primary amine dehydrogenase	136	0	NT	0	NT
Formaldehyde dehydrogenase (2,6-dichlorophenol-indophenol)	21	10	NT	16	NT
Formaldehyde dehydrogenase (NAD)	0	10	10	60	50
Methanol dehydrogenase	62	272	270	14	0
Formate dehydrogenase (NAD)	58	12	0	21	0

isms to oxidize trimethylamine *N*-oxide (Table 3). The absence from crude sonic extracts of acetate-grown organisms of trimethylamine mono-oxygenase, trimethylamine *N*-oxide demethylase, dimethylamine mono-oxygenase and formate dehydrogenase shows that these enzymes are induced during growth on



Scheme 1. *Oxidation of trimethylamine by obligate and facultative methylotrophs*

The enzymes of the pathways are: (1), trimethylamine mono-oxygenase; (2), trimethylamine *N*-oxide demethylase; (3), trimethylamine dehydrogenase; (4), dimethylamine mono-oxygenase; (5), primary amine dehydrogenase; (6), formaldehyde dehydrogenase; (7), formate dehydrogenase.

trimethylamine. Trimethylamine mono-oxygenase (assayed by the spectrophotometric method) and trimethylamine *N*-oxide demethylase activities were also found to be present in crude sonic extracts of five other trimethylamine-grown facultative methylotrophs, isolates 1A1, 1A2, 1A3, 1B1 and 2B2; no trimethylamine dehydrogenase activity was detected in these extracts.

The results of enzyme assays with extracts of the obligate methylotrophs C2A1 (Table 6) and 4B6 (Table 5) suggest that trimethylamine *N*-oxide is not an intermediate during trimethylamine oxidation by these organisms; these results are consistent with the inability of these organisms to grow on (Table 2) or to oxidize (Table 3) trimethylamine *N*-oxide. The oxidation of trimethylamine by these obligate methylotrophs is mediated by trimethylamine dehydrogenase, which catalyses the oxidation of trimethylamine to dimethylamine and formaldehyde (Colby & Zatman, 1971).

The present results show that two routes of trimethylamine oxidation to dimethylamine and formaldehyde occur amongst our isolates (see Scheme 1); the obligate methylotrophs use a trimethylamine dehydrogenase-dependent route whereas the facultative methylotrophs use a trimethylamine mono-oxygenase-dependent route. Large *et al.* (1972) have reported the presence of trimethylamine mono-oxygenase and the absence of trimethylamine dehydrogenase in two other facultative methylotrophs, *Ps. aminovorans* and *Hyphomicrobium vulgare* NQ. It will be of interest to determine by examination of further trimethylamine-utilizing isolates whether the apparent correlation between trimethylamine dehydrogenase and obligate methylotrophy on the one hand, and trimethylamine mono-oxygenase and facultative methylotrophy on the other, can be confirmed.

An obvious difference between the two pathways in Scheme 1 is that whereas the oxidation of one molecule of trimethylamine to dimethylamine and formaldehyde by the obligate methylotrophs yields

one molecule of reduced cofactor, the corresponding oxidation by the facultative methylotrophs requires one molecule of reduced cofactor. This implies that the obligate methylotrophs use a more efficient pathway with regard to the genesis of reducing power and the harnessing of metabolic energy.

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