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Oxidation of C_1 Compounds by *Pseudomonas* Sp. MS

BY HSIANG-FU KUNG* AND CONRAD WAGNER
Department of Biochemistry, Vanderbilt University, and V.A. Hospital, Nashville, Tenn. 37203, *U.S.A.*

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Pseudomonas sp. MS is capable of growth on a number of compounds containing only C_1 groups. They include trimethylsulphonium salts, methylamine, dimethylamine and trimethylamine. Although formaldehyde and formate will not support growth they are rapidly oxidized by intact cells. Methanol neither supports growth nor is oxidized. A particulate fraction of the cell oxidizes methylamine to carbon dioxide in the absence of any external electron acceptor. Formaldehyde and formate are more slowly oxidized to carbon dioxide by the particulate fraction, although they do not appear to be free intermediates in the oxidation of methylamine. Soluble NAD-linked formaldehyde dehydrogenase and formate dehydrogenase are also present. The particulate methylamine oxidase is induced by growth on methylamine, dimethylamine and trimethylamine, whereas the soluble formaldehyde dehydrogenase and formate dehydrogenase are induced by trimethylsulphonium nitrate as well as the aforementioned amines.

In an attempt to learn more about the mechanism whereby certain micro-organisms can grow at the expense of C_1 compounds we have isolated a species of *Pseudomonas* from soil by virtue of its ability to grow on TMS† chloride (Wagner, 1964). Because of the known reactivity of sulphonium compounds (Cantoni, 1960) it was believed that the methyl groups of this compound could be easily metabolized as C_1 units. This organism, designated *Pseudomonas* sp. MS, metabolizes TMS chloride by transfer of a methyl group to H_4folate. This is catalysed by an enzyme which carries out the following reaction:

$$CH_3 \cdot S^+(CH_3) \cdot CH_3 + H_4\text{folate} \rightarrow CH_3 \cdot S \cdot CH_3 + 5\text{-}CH_3\text{-}H_4\text{folate} + H^+$$

The dimethylsulphide thus formed is liberated into the medium, and the 5-CH_3-H_4folate is presumably incorporated into cell material (Wagner, Lusty, Kung & Rogers, 1966, 1967).

Pseudomonas sp. MS can also grow at the expense of methylamine as its sole carbon source. Only a relatively small number of micro-organisms have been reported to grow on methylamine. They include *Pseudomonas aminovorans* (den Dooren de Jong, 1927; Leadbetter & Gottlieb, 1964), *Protaminobacter ruber* (den Dooren de Jong, 1927), *Diplococcus* sp. (Leadbetter & Gottlieb, 1967), *Pseudomonas* sp. M27 (Anthony & Zatman, 1964) and the *Pseudomonas* sp. described by Shaw, Tsai & Stadtman (1966). A comprehensive review of the metabolism of C_1 compounds by micro-organisms was published by Quayle (1961). The present paper describes some aspects of the growth and oxidative metabolism of *Pseudomonas* sp. MS on methylamine and related compounds.

* Present address: Laboratory of Biochemistry, National Heart Institute, National Institutes of Health, Bethesda, Md. 20014, U.S.A.

† Abbreviation: TMS, trimethylsulphonium.

MATERIALS AND METHODS

Organism. Pseudomonas sp. MS was isolated from soil by the enrichment culture technique by virtue of its ability to grow on TMS chloride as the sole carbon source. It is an aerobic, Gram-negative, motile rod measuring 0.8 μm × 1.5 μm and bears two flagella located in a subpolar position. Colonies are small, circular and buff-coloured. When they reach a size of 2–3 mm they take on a yellow appearance. No spores are found. No soluble pigments are formed in liquid culture. The organism has been deposited with the American Type Culture Collection (A.T.C.C. 25262).

Media and growth conditions. Cells were grown in liquid media of the following composition (g/l): $(NH_4)_2SO_4$, 2.0; $MgCl_2,6H_2O$, 0.2; $FeCl_3,6H_2O$, 0.01; $CaCl_2$, 0.01; Na_2EDTA, 0.10; and 40 mM-potassium phosphate buffer, pH 7.0. The carbon source was one of TMS nitrate, methylamine, dimethylamine, trimethylamine, glucose or Bacto-peptone, each of which was added at a concentration of 3 g/l. The media were made to volume with tap water.

Stock cultures were maintained on slants of the above medium containing TMS nitrate or methylamine and 2% agar. They were grown at 30°C and transferred at weekly

intervals. For the preparation of bacterial cells on a large scale, 10ml of liquid medium was inoculated by wire loop from a slant and grown on a rotary action shaker. This culture was used to inoculate a 1 litre culture, which after 35h of growth was used to inoculate 15l of medium contained in a carboy. The cultures were aerated by shaking or by forced aeration.

Cells were harvested while in the exponential growth phase. They were washed twice with cold 50mM-potassium phosphate buffer, pH7.0, before use.

Growth studies. Growth was measured turbidimetrically in a Bausch and Lamb Spectronic 20 colorimeter at 540nm. The cells used for a particular study were grown on either 0.3% TMS nitrate or TMS chloride growth medium or on 0.3% methylamine growth medium by inoculating with bacteria from an agar slant containing TMS nitrate or TMS chloride or methylamine respectively as the carbon source. When growth reached the stage where the medium had E_{540} 0.6 (midway in the exponential phase), 0.1ml was used to inoculate each of the tubes for the growth studies. These studies were carried out in test tubes (1.7cm×20cm) filled with 10ml of medium and shaken rapidly with a circular motion to provide a vortex for aeration. The temperature was 30°C. All studies were done in triplicate and the values were averaged.

Manometric methods. Oxygen uptake by fresh washed-cell suspensions was measured in a Gilson respirometer with conventional Warburg flasks at 32°C with air as the gas phase.

Preparation of cell fractions. These were prepared immediately after the cells had been harvested. Wet packed cells were suspended in 2 vol. of cold 50mM-potassium phosphate buffer, pH7.0, and passed twice through a French pressure cell at a pressure of 16000–18000lb/in^2. About 1mg of crystalline deoxyribonuclease was added for each 50ml of broken-cell suspension and the mixture was kept in an ice bath for 15min to decrease the viscosity of the preparation. The unbroken intact cells were removed by centrifugation at 4000*g* for 20min at 4°C. Centrifugation of the supernatant fluid at 320000*g* for 30min yielded a clear brown extract. This was considered to be the soluble enzyme fraction. The particulate fraction was obtained by suspending the pellet that sedimented at 32000*g* in a volume of cold 50mM-potassium phosphate buffer, pH7.0, equal to the volume of the original cell suspension.

Extracts were treated in two ways to remove small-molecular-weight material from the enzymes. The particulate fraction was dialysed against 1000 vol. of 50mM-potassium phosphate buffer, pH7.0, for 30h at 4°C. The soluble fraction was passed over a small volume of Sephadex G-25 (Pharmacia Fine Chemicals Inc., Piscatawau, N.J., U.S.A.).

Measurement of radioactivity of oxidative products. Radioactive CO_2 formed during oxidation of ^{14}C-labelled substrates was measured by carrying out the reaction in stoppered Warburg flasks without attachment to manometers. A filter-paper wick laden with 50μl of 20% NaOH was used to trap the CO_2 liberated on termination of the oxidation reaction by tipping in 0.1 vol. of 50% (w/v) trichloroacetic acid from the side arm. Shaking was continued for 10min. The filter-paper wick was dropped into a scintillation vial containing 10ml of Bray's (1960) solution and counted in a Packard Tri-Carb scintillation counter. After removal of the protein by centrifugation, the supernatant fluid from reaction mixtures containing [^{14}C]methylamine was pipetted on to a small column (5mm×20mm) of Dowex 50 (H^+ form; 100–200 mesh). Suitable columns for routine analyses were fashioned from Pasteur pipettes and were discarded after each assay. The anionic and neutral products (predominantly [^{14}C]formic acid) were eluted with a total of 2.0ml of water, at which point the effluent pH approached neutrality. This water eluate was added to 10ml of Bray's solution and counted. The presence of radioactive formaldehyde and formic acid in the water eluate was suggested by the loss in radioactivity that occurred when this eluate was evaporated to dryness in the presence of unlabelled formaldehyde or formic acid.

The radioactivity present in formic acid was determined in the presence of labelled formaldehyde by the specific oxidation of formic acid to CO_2 (Wood & Gest, 1957). The 2ml water eluate obtained from the Dowex 50 column was treated with 0.3g of HgO and 1ml of 2.5% (w/v) H_3PO_4 in a Warburg flask for 16h at 37°C. The $^{14}CO_2$ derived from the [^{14}C]formic acid was trapped by NaOH contained in the centre well of the Warburg flask and the radioactivity measured as described above. All experimental results were corrected for blank incubations, which were performed with enzyme that had been heated for 5min at 100°C.

In some cases crystalline material was plated on steel planchets and counted in an end-window gas-flow counter. Counts were corrected for self-absorption.

Assays. Ammonia was determined by the diffusion method of Seligson & Seligson (1951), followed by colorimetric determination with Nessler's reagent (Ballentine, 1957). Under the conditions of this procedure the small amounts of methylamine liberated failed to interfere with the colour reaction between ammonia and Nessler's reagent.

Protein was determined by the methods of Lowry, Rosebrough, Farr & Randall (1951) or Gornall, Bardawill & David (1949). Crystalline bovine serum albumin (Sigma Chemical Co., St Louis, Mo., U.S.A.) was used as the standard. The preparation and crystallization of the 2,4-dinitrophenylhydrazone of formaldehyde was carried out from methanol–water as described by Cheronis & Entrikin (1947).

Special chemicals. [^{14}C]Methylamine, [^{14}C]formaldehyde, [^{14}C]paraformaldehyde and sodium [^{14}C]formate were obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The [^{14}C]paraformaldehyde was converted into [^{14}C]formaldehyde by heating with 5ml of water in a sealed tube at 100°C for 1h. Snake-venom phospholipase, lysozyme, deoxyribonuclease, sodium deoxycholate, digitonin, NAD^+, $NADP^+$, FAD, phenazine methosulphate, 2,6-dichlorophenol-indophenol, menadione and 2,3,5-triphenyltetrazolium chloride were obtained from Sigma Chemical Co.

TMS iodide was purchased from the Aldrich Chemical Co. Inc., Milwaukee, Wis., U.S.A. It was converted into the chloride salt as described by Wagner *et al.* (1967). TMS nitrate was prepared from the iodide by treating a solution of the iodide with $Pb(NO_3)_2$. The lead was removed as the sulphide by bubbling H_2S through the solution and filtering off the precipitate.

Table 1. *Growth of* Pseudomonas *sp. MS and related organisms on various substrates*

Growth was determined as described in the Materials and Methods section. Tubes containing the indicated media were inoculated from a liquid culture grown on TMS chloride as substrate. +, Growth; −, no growth; N.T., not tested.

Reference ...		Peel & Quayle (1961)	Anthony & Zatman (1964)	Shaw *et al.* (1966)	L. E. den Dooren de Jong, cited by Peel & Quayle (1961)	
Carbon source	*Pseudomonas* sp. MS	*Pseudomonas* AM1	*Pseudomonas* sp. M27	*Pseudomonas* sp.	*Pseudomonas aminovorans*	*Protamino-bacter ruber*
Methylamine	+	+	+	+	+	+
Dimethylamine	+	+	−	−	+	+
Trimethylamine	+	+	N.T.	−	+	+
TMS chloride	+	N.T.	N.T.	−	N.T.	N.T.
Methane	−	−	−	−	N.T.	N.T.
Methanol	−	+	+	−	+	+
Formaldehyde	−	−	−	−	N.T.	N.T.
Formate	−	+	+	−	+	+
Glucose	+	+	+	+	+	+
Fructose	+	+	N.T.	+	+	N.T.
Acetate	+	−	+	N.T.	+	+
Propionate	N.T.	−	+	N.T.	+	N.T.
Citrate	−	+	+	N.T.	+	+
Succinate	N.T.	N.T.	+	−	+	+
Lactate	+	+	+	+	+	+
Pyruvate	N.T.	N.T.	N.T.	+	N.T.	N.T.
Ethanol	−	+	+	−	+	−
Glycine	−	−	−	−	−	N.T.
Alanine	+	−	N.T.	N.T.	−	N.T.
Glutamate	+	+	N.T.	+	+	−
Serine	+	−	−	−	N.T.	N.T.

RESULTS

Growth studies. Table 1 compares the range of compounds that support the growth of *Pseudomonas* sp. MS with other bacteria that have been reported to grow on methylamine as the sole carbon source. Although growth of *Pseudomonas* sp. MS takes place with a large number of multi-carbon compounds, the only C_1 sources that supported growth were compounds containing methyl groups. Not all compounds containing methyl groups can support growth, however, since tetramethylammonium chloride, dimethyl sulphoxide and methanol were ineffective. The results presented in Table 1 for growth of the related bacteria are taken from the references cited, with the exception of the inability of the *Pseudomonas* sp. described by Shaw *et al.* (1966) to grow on TMS chloride. This organism was kindly sent to us by Dr E. R. Stadtman and this study was performed in our laboratory.

Sensitivity of growth to amethopterin. Since previous work (Wagner, 1964; Wagner *et al.* 1966, 1967) has shown that one methyl group of TMS nitrate is metabolized as a folate derivative, we attempted to evaluate the role played by folate carriers in the metabolism of other C_1 substrates by measuring the sensitivity of cells grown on various substrates to the folate antagonist, amethopterin. Cells were grown on glucose or on C_1 substrates in the absence and presence of amethopterin. The sensitivity of the cells to amethopterin is expressed as the concentration resulting in half-maximal growth. The results are presented in Table 2. They indicate that cells grown on TMS nitrate were about twice as sensitive to amethopterin as cells grown on glucose. This is consistent with the enzymic role played by H_4folate in the metabolism of TMS chloride. When grown on the three methylamines, however, the cells showed differing degrees of sensitivity to amethopterin. The cells grown on methylamine appeared to be no more sensitive than cells grown on glucose, and those grown on trimethylamine were most sensitive of all. This also suggests that growth of *Pseudomonas* sp. MS on each of these substrates that serve as sources of methyl groups may involve different mechanisms. If each of the substrates served simply to supply methyl groups that were then metabolized via a common pathway, the sensitivity of the cells to amethopterin should be the same in every case.

Oxidation by washed cells. Intact cells oxidize

Table 2. *Sensitivity of* Pseudomonas *sp. MS to amethopterin*

Growth was measured as described in the Materials and Methods section. The inoculum for each medium was obtained from cells grown on the corresponding medium. The amount of growth in the presence of amethopterin was measured at the time maximal growth was reached in the absence of amethopterin. These times were: for glucose, 28h; for methylamine, 52h; for TMS nitrate, 56h; for trimethylamine, 62h; for dimethylamine, 65h.

Growth substrate	Concn. of amethopterin allowing half-maximal growth (mg/ml)
Glucose (0.2%)	4.0
TMS nitrate (0.3%)	2.1
Methylamine (0.3%)	4.5
Dimethylamine (0.3%)	3.0
Trimethylamine (0.3%)	1.6

Table 3. *Oxidation of* C_1 *compounds by TMS chloride-grown cells*

Oxygen uptake was measured as described in the Materials and Methods section. Each flask contained 200μmol of potassium phosphate buffer, pH7.0, and 35mg wet wt. of cells in a total volume of 2.8ml. Each centre well contained 0.2ml of 20% (w/v) KOH to absorb respired CO_2. One side arm of each flask contained 0.2ml of 3% (w/v) $HgCl_2$ to absorb dimethyl sulphide formed when TMS chloride was the substrate. Values for endogenous O_2 uptake have been subtracted. Incubations were carried out in air at 30°C for 3h.

Substrate	O_2 uptake (μmol) Observed	Calculated
TMS chloride (5μmol)	5.7	7.5
TMS chloride (10μmol)	9.2	15.0
Formaldehyde (5μmol)	4.6	5.0
Formaldehyde (10μmol)	8.5	10.0
Formate (10μmol)	5.5	5.0
Methanol (10μmol)	0.0	15.0

formaldehyde and formate in addition to all of the C_1 substrates that will support growth. Methanol was not oxidized. Table 3 shows the amount of oxygen uptake when TMS nitrate-grown cells were incubated with various substrates for 3h. The oxidation of TMS chloride, formaldehyde and formate began immediately without any lag period. Table 4 shows that cells grown on methylamine oxidized methylamine, formaldehyde and formate without any lag period. The oxidation of di- and tri-methylamine by cells grown on methylamine was preceded by a lag period of about 20min. Similarly, when cells were grown on dimethylamine, oxidation of methylamine and trimethylamine was preceded by a lag period, and when cells were grown on trimethylamine oxidation of methylamine and dimethylamine was preceded by a lag. This behaviour suggests that the lag period is required for induction of enzymes required for transport and/or oxidation of the amines other than those on which the cells had been grown.

Oxidation by cell-free extracts. Washed cell preparations were disrupted by passage through a French pressure cell and the mixture was separated into a particulate and a soluble fraction as described in the Materials and Methods section. Each fraction was tested for its ability to oxidize methylamine, formaldehyde and formate by measuring the formation of radioactive CO_2 formed from ^{14}C-labelled substrates. The results are presented in Table 5. They show that the ability to oxidize methylamine to CO_2 is located exclusively in the particulate fraction of the cell, whereas the ability to oxidize formaldehyde and formate to CO_2 is located in both particulate and soluble fractions. No methanol-oxidizing activity was found in either fraction. Only negligible enzymic activity towards methylamine was found in the soluble fraction, which was tested at pH values from 6.0 to 8.8. Since the enzymic activity resides in the particulate

Table 4. *Oxidation of various substrates by cells grown on different methylamines*

Cells were grown on 0.3% methylamine, dimethylamine or trimethylamine and the rates of O_2 uptake with various substrates were measured by using the techniques described in Table 3, except that $HgCl_2$ was omitted from the side arm. Endogenous values have been subtracted. The values in *italics* were rates obtained after a 15min lag period during which no O_2 uptake was seen. The amount of substrate used in each oxidation flask was 5μmol except as indicated.

Substrate for oxidation / Growth substrate ...	Rate of O_2 uptake (μmol/min) Methylamine	Dimethylamine	Trimethylamine
Methylamine	0.25	*0.26*	*0.19*
Dimethylamine	*0.16**	0.38	*0.18**
Trimethylamine	*0.02*†	*0.13*†	0.44
Formaldehyde	0.06	—	—
Formate	0.02	—	—

* 3μmol of dimethylamine used as substrate.
† 2μmol of trimethylamine used as substrate.

Table 5. *Cellular localization of oxidative enzymes*

The formation of $^{14}CO_2$ was measured as described in the text. Each flask contained 100 μmol of potassium phosphate buffer, pH 7.0, substrate and enzyme fraction containing 4.2 mg of protein in a total volume of 1.0 ml. The centre well contained 0.1 ml of 20% (w/v) NaOH plus a filter-paper wick. The amounts of substrate used were: methylamine, 2 μmol (52 nCi); formaldehyde, 2 μmol (32 nCi); formate, 5 μmol (240 nCi). At the end of the reaction 0.1 ml of 50% (w/v) trichloroacetic acid was tipped into the main compartment from the side arm and incubation continued for 10 min. The temperature of incubation was 34°C. Control values from incubations in which boiled enzyme fractions were used have been subtracted.

			$^{14}CO_2$ formed (nmol)	
Substrate	Atmosphere	Time (h)	Particulate	Soluble
Methylamine	N_2	0.5	71	—
Methylamine	Air	0.5	386	—
Methylamine	Air	1.0	724	—
Methylamine	Air	2.0	775	—
Methylamine	Air	4.0	800	1
Formaldehyde	Air	1.0	112	50
Formate	Air	1.0	83	324

Table 6. *Cofactor requirements for the oxidation of* C_1 *substrates*

Experimental details are as described in Table 5 except that the particulate fraction was dialysed before use and the soluble fraction was treated with Sephadex G-25 to remove small molecules. The amounts of ^{14}C-labelled methylamine, formaldehyde and formate are the same as in Table 5. The particulate fraction was incubated for 30 min and the soluble fraction was incubated for 60 min.

	$^{14}CO_2$ formed (nmol)		
	Particulate fraction	Soluble fraction	
Addition ...	Methylamine	Formaldehyde	Formate
None	252	0	51
NAD^+ (2 μmol)	302	309	2500
$NADP^+$ (2 μmol)	—	15	61
FAD (1 μmol)	218	17	92

fraction, it was possible that this activity was due to contamination of the particulate fraction with intact cells that had not been removed during the initial centrifugation at 4000*g* for 20 min. A viable cell count of this fraction showed a negligible contamination by intact cells.

All attempts to solubilize the methylamine-oxidizing activity were unsuccessful. These included sonication, snake-venom phospholipase (Ringler & Singer, 1962), lysozyme (in the absence and presence of EDTA) (Repaske, 1958), sodium deoxycholate (Morton, 1955), digitonin (Wadkins & Lehninger, 1963) and cold acetone (Gunsalus, 1955). Addition of electron acceptors such as FAD, NAD^+, $NADP^+$, menadione, phenazine methosulphate, tetrazazolium dyes, 2,6-dichlorophenolindophenol and potassium ferricyanide did not stimulate the production of $^{14}CO_2$ from methylamine by the soluble fraction. No evidence was obtained for a soluble phenazine methosulphate-linked methylamine dehydrogenase as described by Eady & Large (1968).

Cofactor requirements for oxidation of C_1 *compounds.* The particulate fraction was dialysed against 1000 vol. of 50 mM-potassium phosphate buffer, pH 7.0, at 4°C for 30 h and the soluble enzyme fraction was passed over a Sephadex G-25 column to remove small molecules. Table 6 shows that the ability of the particulate fraction to oxidize methylamine to CO_2 was not lost on dialysis, whereas the ability of the soluble fraction to oxidize formaldehyde and formate was lost. These latter activities were restored specifically by the addition of NAD^+. Other common electron acceptors were ineffective in restoring the oxidation of formaldehyde and formate. These results indicate the presence of both formaldehyde dehydrogenase and formate dehydrogenase in *Pseudomonas* MS.

Intermediates in the oxidation of methylamine to

carbon dioxide. Since the particulate enzyme preparations oxidized methylamine all the way to CO_2 and this activity was not lost on dialysis, it appeared that a tightly coupled enzyme complex was involved. The results depicted in Table 5 indicated that, although both formaldehyde and formate were oxidized to CO_2 by the particulate complex, they were not oxidized as rapidly as methylamine itself. This suggests that formaldehyde and formate might not themselves be intermediates in the oxidation. An experiment was carried out in which unlabelled formate and formaldehyde were added to the particulate enzyme oxidizing [^{14}C]methylamine in an attempt to trap these compounds as intermediates. In addition to collection of the liberated CO_2, the contents of the reaction flasks were passed through small columns containing Dowex 50 (H^+ form) resin as described in the Materials and Methods section. The unchanged methylamine was adsorbed on the resin. The anionic and neutral radioactive products were collected by washing the columns with water. The radioactive products appearing in this fraction were considered to be predominantly formaldehyde and formate for the following reasons. The radioactivity is volatile and is lost when a portion of the fraction is evaporated to dryness in the presence of formate and formaldehyde. A portion of the radioactivity in this fraction is converted into CO_2 by oxidation with mercuric oxide by the method of Wood & Gest (1957). This method is specific for the oxidation of formic acid to CO_2. Radioactive formaldehyde can be isolated from this fraction by adding carrier formaldehyde and 2,4-dinitrophenylhydrazine (Table 7). The results depicted in Table 8 show that both unlabelled formaldehyde (Expt. 1) and unlabelled formate (Expt. 2) decreased the amount of $^{14}CO_2$ formed and increased the amount of radioactive material found in the neutral and anionic fraction. In Expt. 2 the amount of [^{14}C]-formate in the neutral and anionic fraction was also determined by oxidation to CO_2 with mercuric oxide. This indicates that most of the radioactivity trapped by unlabelled formate is indeed present as [^{14}C]formate.

Table 6 shows that the oxidation of both formaldehyde and formate to CO_2 by the soluble fraction is dependent on the presence of NAD^+. However, it is not known whether the oxidation of formaldehyde to formate is NAD^+-dependent or whether the NAD^+ requirement for formaldehyde oxidation is

Table 7. *Isolation of formaldehyde 2,4-dinitrophenylhydrazone from the oxidation of methylamine by the particulate fraction*

The particulate enzyme fraction containing 21 mg of protein was incubated in the manner described in Table 5. The main compartment contained, in a total volume of 5.0 ml: potassium phosphate buffer, pH 7.0, 500 μmol; [^{14}C]methylamine, 10 μmol (260 nCi); formaldehyde, 1.85 μmol. After 30 min incubation 0.5 ml of 50% (w/v) trichloroacetic acid was tipped into the main compartment from the centre well. The liberated CO_2 was trapped in the centre well. The anionic and neutral products were isolated from the reaction mixture as described in the Materials and Methods section. A small amount of unlabelled formaldehyde was added to this fraction followed by a solution of 2,4-dinitrophenylhydrazine. The formaldehyde 2,4-dinitrophenylhydrazone was recrystallized as described in the Materials and Methods section and the radioactivity of the crystals was measured as described in the text.

Crystallization step	Total radioactivity (c.p.m.)	Specific radioactivity (c.p.m./mg)
1	246	49
2	212	48

Table 8. *Effect of unlabelled formaldehyde and formate on production of $^{14}CO_2$ from [^{14}C]methylamine*

Experimental conditions are the same as for Table 5 except for the use of the particulate fraction and the various additions as indicated. The amount of [^{14}C]methylamine substrate used in Expt. 1 was 2 μmol (52 nCi) and in Expt. 2 was 5 μmol (90 nCi). The reaction mixture was incubated for 30 min. Radioactive CO_2, the non-cationic fraction and formate were determined as described in the Materials and Methods section.

Expt. no.	Addition	Products formed (c.p.m.)		
		$^{14}CO_2$	Non-cationic	[^{14}C]Formate
1	None	9300	4300	—
	Formaldehyde (0.12 μmol)	7250	4940	—
	Formaldehyde (0.37 μmol)	4500	10400	—
2	None	16475	6200	—
	Formate (5 μmol)	9929	8320	—
	Formate (15 μmol)	4131	15000	13300*

* Measured by the HgO-catalysed oxidation to $^{14}CO_2$.

Table 9. *NAD^+ requirement for [^{14}C]formaldehyde oxidation by the soluble fraction*

The experimental conditions were the same as described for Table 5 except for the use of the soluble enzyme fraction and the additions as indicated. The substrate was 5 μmol (80 nCi) of [^{14}C]formaldehyde. Treatment of the enzyme with Sephadex G-25 and measurement of $^{14}CO_2$ and formate were as described in the text. The incubation time was 1 h.

		Products formed (c.p.m.)	
Enzyme preparation	Additions	$^{14}CO_2$	[^{14}C]Formate
Untreated	None	3000	—
	Formate (5 μmol)	1060	—
Sephadex G-25-treated	None	0	0
	Formate (5 μmol)+NAD^+ (2 μmol)	1304	1050

a consequence of the formate dehydrogenase. The product of the formaldehyde dehydrogenase reaction was measured by trapping the radioactive material formed from [^{14}C]formaldehyde in the presence of unlabelled formate. Oxidation of this material to CO_2 by the mercuric oxide-catalysed reaction showed that this radioactive material was indeed formate. The oxidation of formaldehyde to formate is also NAD^+-dependent as shown in Table 9.

It is also possible to measure the reduction of NAD^+ directly by means of the increase in E_{340}. In experiments not shown here the soluble fraction catalysed the formate- and formaldehyde-dependent reduction of NAD^+. $NADP^+$ was less than 10% as effective as NAD^+.

Formation of ammonia from methylamine. The oxidation of methylamine by the particulate enzyme fraction results in the production of ammonia in amounts equivalent to the sum of the CO_2 and formate plus formaldehyde. When 2 μmol of [^{14}C]methylamine (52 nCi) was incubated with particulate enzyme containing 4.2 mg of protein for 30 min the following products were obtained: CO_2, 0.32 μmol; formaldehyde plus formate, 0.15 μmol; ammonia, 0.48 μmol. The $^{14}CO_2$ was trapped in the centre well of a Warburg reaction flask by sodium hydroxide and the formate and formaldehyde were contained in the non-cationic fraction of the reaction mixture as described in the Materials and Methods section. The number of μmol of CO_2 and of formaldehyde plus formate were calculated on the basis of the specific radioactivity of the methylamine used. The ammonia was determined by reaction with Nessler's reagent as described in the Materials and Methods section.

Specificity of the amine-oxidizing system. The studies with intact cells presented in Table 4 showed that cells grown on methylamine could oxidize dimethylamine and trimethylamine at a rapid rate only after a short lag period. This suggested that during the lag period certain enzymes were induced that were required for the oxidation of the secondary and tertiary amines. Table 10 shows that the particulate enzyme prepared from cells grown on methylamine oxidizes both dimethylamine and trimethylamine in addition to methylamine. This suggests that the lag period before oxidation observed in whole cells (Table 4) is due to the time required for the induction of a permease for compounds not present in the growth medium.

Table 10. *Specificity of particulate enzyme for methylamine oxidation*

The experimental conditions were the same as described for Table 5. The particulate enzyme fraction containing 2.1 mg of protein was used. In each case 5 μmol of substrate was used and the specific radioactivity was 5000 c.p.m./μmol. The incubation time was 40 min. The μmoles of CO_2 formed were calculated on the basis of the specific radioactivity per C atom of the substrate.

	$^{14}CO_2$ formed	
Substrate	(c.p.m.)	(μmol)
[^{14}C]Methylamine	2844	0.57
[^{14}C]Dimethylamine	1249	0.50
[^{14}C]Trimethylamine	534	0.32

Induction of oxidative enzymes. An indication of the relative importance of the various oxidative enzymes for growth on C_1 compounds can be deduced from their induction by various substrates. Table 11 presents results on the ability of different growth substrates to induce the formation of the particulate methylamine-oxidizing system and the two soluble systems that oxidize formaldehyde and formate. Only when one of the three different methylamines was used as a growth substrate was the particulate methylamine-oxidizing system induced. The two soluble systems oxidizing formaldehyde and formate were induced by growth on any one of the C_1 substrates including TMS nitrate, but not by growth on glucose or peptone.

Table 11. *Induction of oxidative enzymes*

Experimental details are the same as described for Table 5. Enzyme fractions were prepared from cells grown on the indicated carbon source and fractions containing 4.2 mg of protein were used in each case. The period of incubation was 1 h.

	$^{14}CO_2$ formed (nmol)		
	Particulate fraction	Soluble fraction	
Growth substrate ...	Methylamine	Formaldehyde	Formate
Glucose	7	0	0
Peptone	29	0	0
TMS nitrate	23	42	219
Trimethylamine	794	73	253
Dimethylamine	740	37	232
Methylamine	750	36	311

DISCUSSION

The results presented here provide a general picture of the oxidative metabolism of *Pseudomonas* sp. MS. Oxidation of methylamine proceeds via formaldehyde and formate to CO_2. Methanol is apparently not an intermediate. This agrees with observations made by Iyer & Kallio (1958) with *Bacillus sphaericus* and by Eady & Large (1968) with *Pseudomonas* AM 1.

Pseudomonas sp. MS contains soluble formaldehyde dehydrogenase and formate dehydrogenase. In contrast with the situation in other bacteria capable of growing on C_1 compounds, the formaldehyde dehydrogenase from *Pseudomonas* sp. MS is linked directly to NAD and has no requirement for thiols. The formaldehyde dehydrogenases from *Pseudomonas methanica*, *Protaminobacter ruber* and *Pseudomonas extorquens* use NAD as electron acceptor but require the addition of GSH, and the formaldehyde dehydrogenase from *Pseudomonas* AM 1 uses 2,6-dichlorophenol-indophenol as electron acceptor (Johnson & Quayle, 1964).

The soluble formate dehydrogenase from *Pseudomonas* sp. MS was not investigated in detail, since similar enzymes have been found widely distributed in Nature (Iida & Taniguchi, 1959; Nason & Little, 1955; Kaneda & Roxburgh, 1959; Johnson & Quayle, 1964).

Unlike the situation in *Pseudomonas* AM 1, the oxidation of methylamine by *Pseudomonas* sp. MS is carried out by a particulate enzyme preparation that uses oxygen as the terminal electron acceptor. This particulate preparation is capable of oxidizing methylamine to CO_2 with intermediate stages at the level of formaldehyde and formate. Although a small amount of radioactivity can be trapped by unlabelled formaldehyde when [^{14}C]methylamine is oxidized by the particulate fraction, and the amount of $^{14}CO_2$ formed in the presence of unlabelled formaldehyde is decreased, formaldehyde is not oxidized as rapidly as methylamine by the particulate fraction. In experiments not reported here the addition of carbonyl reagents such as semicarbazide to the reaction mixture did not trap any radioactive formaldehyde. These observations suggest that free formaldehyde is not the true intermediate in methylamine oxidation, but that the intermediate is some compound that can give rise to formaldehyde. Formate can readily be isolated from reaction mixtures in which methylamine is oxidized by the particulate fraction, although it, too, is not oxidized as rapidly as methylamine. It is therefore possible that free formate is also not an intermediate in the oxidation of methylamine by the particulate fraction. One possibility is that the oxidation of formaldehyde and formate in the particulate fraction occurs as the H_4folate derivatives, as suggested by Johnson & Quayle (1964). Since no external electron acceptors are required by the particulate fraction it is possible that they are all tightly bound to the enzyme complex.

The particulate methylamine oxidase could not be solubilized by a variety of different procedures. This, plus the slow oxidation of free formaldehyde and formate, suggests that the oxidation of methylamine may occur in some structural complex that carries out the oxidation all the way to CO_2. This might be linked to an energy-generating system. Since methylamine is the sole carbon source, its rapid oxidation to CO_2 must provide a source of energy. Synthesis of cell materials from methylamine must occur at some other location in the cell, because once methylamine reacts with the particulate fraction it appears to be committed to complete oxidation. Formaldehyde and formate produced during oxidation of methylamine are not readily available for synthesis of cell constituents. Indeed, *Pseudomonas* sp. MS cannot grow on C_1 compounds more oxidized than methylamine or

TMS salts. This may be because formaldehyde and formate cannot be effectively oxidized by the particulate fraction and coupled to energy generation.

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