Enzymological Aspects of the Pathways for Trimethylamine Oxidation and C₁ Assimilation in Obligate Methylotrophs and Restricted Facultative Methylotrophs

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1. Extracts of trimethylamine-grown W6A and W3A1 (type M restricted facultative methylotrophs) contain trimethylamine dehydrogenase whereas similar extracts of *Bacillus* PM6 and *Bacillus* S2A1 (type L restricted facultative methylotrophs) contain trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase but no trimethylamine dehydrogenase. 2. Extracts of the restricted facultatives and of the obligate methylotroph C2A1 contain hexulose phosphate synthase–hexulose phosphate isomerase activity; hydroxypyruvate reductase was not detected. 3. Neither the restricted facultatives nor the obligates 4B6 and C2A1 contain all the enzymes of the hexulose phosphate cycle of formaldehyde assimilation as originally proposed by Kemp & Quayle (1967). 4. Organisms PM6 and S2A1 lack transaldolase and use a modified cycle involving sedoheptulose 1,7-diphosphate and sedoheptulose diphosphatase. 5. The obligates 4B6 and C2A1, and the type M organisms W6A and W3A1, use a different modification of the assimilatory hexulose phosphate cycle involving the Entner–Doudoroff-pathway enzymes phosphogluconate dehydratase and phospho-2-keto-3-deoxygluconate aldolase. The lack of fructose diphosphate aldolase and hexose diphosphatase in these organisms may be a partial explanation of their restricted growth-substrate range. 6. Enzymological evidence suggests that all the obligates and the restricted facultatives use a dissimilatory hexulose phosphate cycle to accomplish the complete oxidation of formaldehyde to CO₂ and water.

Colby & Zatman (1975) described four pure bacterial cultures each of which grows on trimethylamine and on a restricted range of other compounds; such organisms were designated restricted facultative methylotrophs. Thus the type M isolates W6A and W3A1 grow only on glucose out of 50 non-C₁ compounds tested, whereas the type L isolates *Bacillus* PM6 and *Bacillus* S2A1 grow on betaine, glucose, gluconate, citrate, glutamate, alanine and nutrient agar, but not on any of the other 56 non-C₁ compounds tested. The present paper describes investigations of the mechanisms used by these organisms for the oxidation of trimethylamine and for the assimilation of trimethylamine carbon. The results are compared with those obtained from two previously described obligate methylotrophs, 4B6 and C2A1 (Colby & Zatman, 1972, 1973).

Materials and Methods

Materials

Dimethylamine hydrochloride, methylamine hydrochloride, trimethylamine *N*-oxide hydro-

chloride, paraformaldehyde, methanol, sodium formate and 2,6-dichlorophenol-indophenol were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; trimethylamine hydrochloride was obtained from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K. Phenazine methosulphate, GSH, D-ribose 5-phosphate (disodium salt), sedoheptulose 1,7-diphosphate (sodium salt), lithium hydroxypyruvate, thiamine pyrophosphate chloride, cysteine hydrochloride, ribose phosphate isomerase (spinach) (EC 5.3.1.6), glucose phosphate isomerase (rabbit muscle) (EC 5.3.1.9), transketolase (yeast, type IV) (EC 2.2.1.1) and ribulose phosphate 3-epimerase (yeast) (EC 5.1.3.1) were purchased from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. The Boehringer Corporation (London) Ltd., London W.5, U.K. supplied NAD⁺, NADP⁺, NADPH, NADH, ATP, fructose 6-phosphate (disodium salt), fructose 1,6-diphosphate (trisodium salt), glucose 6-phosphate (disodium salt), gluconate 6-phosphate (trisodium salt), glucose 6-phosphate dehydrogenase (yeast, grade I) (EC 1.1.1.49), aldolase (rabbit muscle) (EC 4.1.2.13), lactate dehydrogenase (pig heart) (EC 1.1.1.27) and glycerol 3-phosphate dehydrogenase–triose phos-

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phate isomerase mixture (rabbit muscle) (EC 1.1.1.8 and EC 5.3.1.1. respectively). Dithiothreitol was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. Cultures of *Hyphomicrobium* strains X and G (Attwood & Harder, 1972) were kindly supplied by Dr. Margaret Attwood.

Methods

Preparation of crude sonic extracts. Organisms were grown, harvested in mid-exponential phase and crude sonic homogenates prepared from the washed suspensions as described by Colby & Zatman (1972, 1975). Enzyme assays were done on crude sonic extracts prepared by centrifuging the homogenates at 10000g for 20 min. Extracts for hexulose phosphate synthase-hexulose phosphate isomerase, hexose diphosphatase (EC 3.1.3.11) and sedoheptulose diphosphatase assays were prepared from cells washed twice with 50 mM-Tris-HCl buffer, pH 7.0, and resuspended in 50 mM-triethanolamine-HCl buffer, pH 7.5, containing 5 mM-MgCl₂.

Protein estimations. The concentrations of protein in crude sonic extracts were determined with the Folin-phenol reagent (Kennedy & Fewson, 1968) with crystalline bovine plasma albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as the standard.

Buffer solutions. These were prepared as described by Dawson *et al.* (1969).

Spectrophotometric estimation of pyruvate, triose phosphate and glycerol 3-phosphate in reaction mixtures. Samples (0.5 ml) were first deproteinized by the addition of 0.5 ml of 1 M-HClO₄, and the denatured protein was removed by centrifugation. Tripotassium phosphate (0.7 M, 0.25 ml) was then added to 0.5 ml samples of the supernatants at 0°C and, after allowing the KClO₄ to sediment, the resulting solutions were used for the estimations. Pyruvate was estimated in assay mixtures containing in 1 ml total volume: 50 µmol of sodium phosphate buffer, pH 7.0; 0.3 µmol of NADH; lactate dehydrogenase (5 units); test solution (0.1 ml, 0–0.2 µmol of pyruvate). Triose phosphate was estimated in assay mixtures (1 ml) containing: 75 µmol of glycylglycine-NaOH buffer, pH 7.6; 0.3 µmol of NADH; glycerol 3-phosphate dehydrogenase-triose phosphate isomerase mixture (5 and 30 units respectively); test solution (0.1 ml, 0–0.2 µmol of triose phosphate). In both cases the decrease in E_{340} on addition of the test solution was measured at 30°C. Glycerol 3-phosphate was estimated by measuring the increase in E_{340} at 30°C on addition of a test solution (0.1 ml, 0–0.2 µmol of glycerol 3-phosphate) to an assay mixture (0.9 ml) containing: 200 µmol of glycine-NaOH buffer, pH 9.8; 500 µmol of hydrazine hydrate; 20 µmol of NAD⁺; glycerol 3-phosphate dehydrogenase (5 units).

Spectrophotometry. All spectrophotometric measurements were made in a Hitachi Perkin-Elmer 124 double-beam grating instrument (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.) fitted with a constant-temperature cuvette housing and coupled to a Servoscribe chart recorder (Smiths Industries Ltd., Wembley, Middx., U.K.).

Measurement of enzyme activities in crude sonic extracts. All assays were done at 30°C. The assay methods for trimethylamine dehydrogenase, trimethylamine mono-oxygenase (spectrophotometric method), dimethylamine mono-oxygenase, primary amine dehydrogenase, methanol dehydrogenase (EC 1.1.99.8), formaldehyde dehydrogenase (NAD⁺) (EC 1.2.1.1), formaldehyde dehydrogenase (2,6-dichlorophenol-indophenol), formate dehydrogenase (EC 1.2.1.2) and hydroxypyruvate reductase (EC 1.1.1.29) were those described by Colby & Zatman (1972, 1973). Hexulose phosphate synthase and hexulose phosphate isomerase (see Cox & Zatman, 1974) were assayed together spectrophotometrically as described by Dahl *et al.* (1972), except that assays were done in 50 mM-triethanolamine-HCl-NaOH buffer, pH 7.5, and contained 1.5 units of ribose-phosphate isomerase. These modifications ensure respectively that interference by glyceraldehyde phosphate dehydrogenase which might be present in the crude extracts would be minimized by excluding phosphate, and that the production of ribulose 5-phosphate (see Kemp, 1972) from ribose 5-phosphate would not be rate-limiting. The activities of the following enzymes were determined by the methods quoted: glucose phosphate isomerase (Wu & Racker, 1959); 6-phosphofructokinase (EC 2.7.1.11; Ling *et al.*, 1966); ribose phosphate isomerase (Axelrod & Jang, 1954); glucokinase (EC 2.7.1.2; Anderson & Kamel, 1966); glucose 6-phosphate dehydrogenase (Kornberg & Horecker, 1955); phosphogluconate dehydrogenase (EC 1.1.1.44; Horecker & Smyrniotis, 1955).

Trimethylamine N-oxide demethylase. This enzyme was assayed by the colorimetric method B of Myers (1971). Reaction mixtures (1.5 ml total volume) contained: 100 µmol of triethanolamine-HCl-NaOH buffer, pH 8.0; 10 µmol of GSH; 100 nmol of FeSO₄; 25 µmol of sodium L-ascorbate; crude sonic extract; 50 µmol of trimethylamine N-oxide hydrochloride (adjusted to pH 8). The reaction was started by the addition of substrate and samples were then removed at intervals for the determination of formaldehyde as described by Colby & Zatman (1973).

Transketolase. Reaction mixtures contained in 1 ml: 50 µmol of glycylglycine-NaOH buffer, pH 7.6; 1 µmol of MgCl₂; 0.1 µmol of thiamine pyrophosphate; 0.17 µmol of NADH; glycerol 3-phosphate dehydrogenase-triose phosphate isomerase mixture (0.5 and 3 units respectively); ribose phosphate isomerase (1.5 units); ribulose phosphate 3-epimerase

(1 unit); crude sonic extract; 2.5 μ mol of ribose 5-phosphate. The reaction was started by the addition of the ribose 5-phosphate and the rate of decrease in E_{340} was measured. It was appreciated that false positives could be obtained with this assay if extracts contained phosphoketolase (EC 4.1.2.9). Extracts were therefore examined for their ability to catalyse the production of acetyl phosphate from ribose 5-phosphate under the conditions of the transketolase assay by assay method B of Goldberg *et al.* (1966). None of the extracts catalysed the production of acetyl phosphate. Similarly these extracts catalysed only very slow rates of disappearance of acetyl phosphate (5–10 nmol/min per mg of protein) under the conditions of the assay.

Transaldolase (EC 2.2.1.2). Reaction mixtures contained in 1 ml: 50 μ mol of glycylglycine–NaOH buffer, pH 7.6; 1 μ mol of $MgCl_2$; 0.1 μ mol of thiamine pyrophosphate; 0.5 μ mol of NADP⁺; glucose 6-phosphate dehydrogenase (0.2 unit); ribose phosphate isomerase (1.5 units); transketolase (1 unit); crude sonic extract; 2.5 μ mol of ribose 5-phosphate. It was not necessary to add ribulose phosphate epimerase or glucose phosphate isomerase as these are present in the crude extracts. The reaction was started by the addition of ribose 5-phosphate and the rate of increase in E_{340} recorded.

Fructose diphosphate aldolase. Reaction mixtures contained in 1.5 ml: 75 μ mol of Tris–acetate buffer, pH 7.5; 150 μ mol of potassium acetate; 1 μ mol of $CoCl_2$; 0.15 μ mol of L-cysteine hydrochloride; 10 μ mol of NADH; glycerol 3-phosphate dehydrogenase–triose phosphate isomerase mixture (2 and 12 units respectively); crude sonic extract; 10 μ mol of fructose 1,6-diphosphate. Incubations were carried out at 30°C in three Thunberg tubes for each assay in an atmosphere of N_2 to prevent oxidation of NADH by NADH oxidase. Samples, taken 0, 5 and 10 min after starting the reactions in the respective tubes with fructose 1,6-diphosphate, were mixed with an equal volume of 1 M-HClO₄, and the concentration of glycerol 3-phosphate was estimated as described above.

Ribulose phosphate 3-epimerase. Reaction mixtures contained in 1 ml: 50 μ mol of glycylglycine–NaOH buffer, pH 7.6; 1 μ mol of $MgCl_2$; 0.1 μ mol of thiamine pyrophosphate; 0.17 μ mol of NADH; glycerol 3-phosphate dehydrogenase–triose phosphate isomerase mixture (0.5 and 3 units respectively); transketolase (1 unit); ribose phosphate isomerase (1.5 units); crude sonic extract; 2.5 μ mol of ribose 5-phosphate. The reaction was started by the addition of the ribose 5-phosphate and the rate of decrease in E_{340} recorded.

Phosphogluconate dehydratase (EC 4.2.1.12) and *phospho-2-keto-3-deoxygluconate aldolase* (EC 4.1.2.14). These two enzymes were assayed together by incubating crude sonic extracts with 6-phospho-

gluconate and following the appearance of pyruvate and glyceraldehyde 3-phosphate. Reaction mixtures contained the following in 1.5 ml: 50 μ mol of imidazole–HCl buffer, pH 8.0; 1 μ mol of $MnCl_2$; 1.5 μ mol of GSH; crude sonic extract; 10 μ mol of 6-phosphogluconate. The reaction was started by the addition of 6-phosphogluconate and samples were removed after 0, 5 and 10 min incubation; pyruvate and triose phosphate in the samples were estimated as described above. These two enzymes were also assayed by the method of Keele *et al.* (1970); both methods yielded very similar values.

Sedoheptulose diphosphatase and hexose diphosphatase (EC 3.1.3.11). Both diphosphatases were assayed by the P_i liberation method of Pontremoli (1966) with sedoheptulose 1,7-diphosphate or fructose 1,6-diphosphate as substrate; P_i was estimated with the Amidol reagent of Allen (1940).

Enzyme units. One munit of enzyme is defined as that quantity that catalyses the transformation of 1 nmol of substrate or the formation of 1 nmol of product/min at 30°C in the assays described above.

Results and Discussion

Specific activities of enzymes involved in trimethylamine oxidation in extracts of the restricted facultative methylotrophs

Trimethylamine may be oxidized to dimethylamine and formaldehyde either by trimethylamine dehydrogenase or by the sequential action of trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase (Colby & Zatman, 1973, 1974; Boulton *et al.*, 1974). As shown in Table 1, the type L isolates S2A1 and PM6 grown on trimethylamine, but not organisms grown on glucose or citrate, contain trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase whereas trimethylamine dehydrogenase was not detected. In contrast, the type M organisms W3A1 and W6A grown on trimethylamine contain trimethylamine dehydrogenase but lack trimethylamine mono-oxygenase and trimethylamine *N*-oxide demethylase, thereby resembling the obligates 4B6 and C2A1 (Colby & Zatman, 1973); glucose-grown organisms lack trimethylamine dehydrogenase. Boulton *et al.* (1974) have observed trimethylamine dehydrogenase activity but no trimethylamine mono-oxygenase activity in extracts of *Hyphomicrobium* NQ. We have confirmed these findings with the demonstration of low activities of trimethylamine dehydrogenase (11 munits/mg of protein), but no detectable trimethylamine mono-oxygenase or trimethylamine *N*-oxide demethylase, in crude sonic extracts of *Hyphomicrobium* strains X and G grown on trimethylamine. The growth-substrate specificity of hyphomicrobia indicates that

Table 1. *Specific activities of C₁-oxidizing enzymes in crude sonic extracts of restricted facultative methylotrophs*

All values are expressed as munits/mg of protein; N.T., not tested.

Growth substrate	Isolate ...	Type M				Type L			
		W6A		W3A1		S2A1		PM6	
		Trimethyl-amine	Glucose	Trimethyl-amine	Glucose	Trimethyl-amine	Glucose	Trimethyl-amine	Citrate
Trimethylamine dehydrogenase		78	0	126	2	0	N.T.	0	N.T.
Trimethylamine mono-oxygenase		0	N.T.	0	N.T.	134	0	37	0
Trimethylamine N-oxide demethylase		0	N.T.	0	N.T.	870	0	3600	0
Dimethylamine mono-oxygenase		26	0	193	0	27	0	25	28
Primary amine dehydrogenase		75	0	165	0	0	N.T.	0	N.T.
Methanol dehydrogenase		125	116	216	240	0	N.T.	0	N.T.
Formaldehyde dehydrogenase		0	0	24	14	0	N.T.	0	N.T.
(2,6-dichlorophenol-indophenol)									
Formaldehyde dehydrogenase (NAD ⁺)		0	0	3	0	0	N.T.	0	N.T.
Formate dehydrogenase		0	N.T.	3	N.T.	0	N.T.	0	N.T.

Table 2. *Specific activities of hydroxypyruvate reductase and of hexulose phosphate cycle enzymes in crude sonic extracts of trimethylamine-grown methylotrophs*

All values are expressed as munits/mg of protein. Hexose diphosphatase was measured in 40mM-glycine-NaOH buffer, pH 9.5, containing 3.5mM-MnCl₂ and in organisms S2A1 and PM6 it was also measured without MnCl₂ but with or without 10mM-MgCl₂ instead (values in parentheses). Sedoheptulose diphosphatase was measured in 40mM-glycine-NaOH buffer, pH 9.5, containing 10mM-MgCl₂.

Isolate ...	Obligate		Type M		Type L	
	46B	C2A1	W3A1	W6A	S2A1	PM6
Hydroxypyruvate reductase	0*	0	0 (0†)	0 (0†)	0 (7†)	0 (6†)
Hexulose phosphate synthase-hexulose phosphate isomerase	975*	935	220 (152†)	87 (73†)	95 (71†)	195 (109†)
6-Phosphofructokinase	0	0	0	0	87	61
Fructose diphosphate aldolase	1	1	1	0	32	54
Transketolase	159	216	560	294	252	263
Transaldolase	61	111	100	86	3	2
Ribose phosphate isomerase	1420	1670	1920	1640	285	432
Ribulose phosphate 3-epimerase	640	640	386	225	1390	741
Hexose diphosphatase	0	0	0	0	31 (0)	12 (0)
Sedoheptulose diphosphatase	0	0	0	0	33	30

* Data of Colby & Zatman (1972).

† Glucose-grown.

‡ Citrate-grown.

they can be included with the restricted facultatives (see Colby & Zatman, 1975) and trimethylamine dehydrogenase has thus not been observed in any typical facultative methylotroph.

The specific activities of other C₁-oxidizing enzymes in crude extracts of these organisms after growth on trimethylamine or on non-C₁ substrates are given in Table 1. These results show that routes exist in all four restricted facultatives for the conversion of trimethylamine into methylamine and formaldehyde, and, at least in the type M organisms,

for the further conversion of methylamine into formaldehyde and NH₃. Only low or zero amounts of formaldehyde and formate dehydrogenases were detected in extracts of the type M organisms; such extracts do, however, contain high activities of methanol dehydrogenase and it is possible that the latter enzyme might be responsible for formaldehyde oxidation in these organisms (Ladner & Zatman, 1969; Heptinstall & Quayle, 1970; Patel & Hoare, 1971). A different situation is found in the type L organisms PM6 and S2A1 where 2mol of NADH

are required for the oxidation of trimethylamine to methylamine and formaldehyde. The oxidation of formaldehyde must provide all the energy and reducing power required for the growth of these organisms on trimethylamine despite the absence of dehydrogenases for formaldehyde, formate and methanol. This problem of energy generation during the methylotrophic growth of these two organisms is discussed below.

Specific activities of hydroxypyruvate reductase and hexulose phosphate synthase in extracts of methylotrophs

The absence of hydroxypyruvate reductase (Table 2) in extracts of the restricted facultatives and of the obligates 4B6 and C2A1 indicate that the serine pathway of formaldehyde assimilation does not occur in these organisms. This is confirmed by the finding (R. B. Cox & L. J. Zatman, unpublished observations) that extracts of trimethylamine-grown C2A1, W6A, W3A1, PM6 and S2A1 organisms also lack malyl-CoA lyase (EC 4.1.3.24), another key enzyme of the

serine pathway (Hersh & Bellion, 1972; Bellion & Hersh, 1972; Salem *et al.*, 1973; Harder *et al.*, 1973; Cox & Zatman, 1973).

Extracts of all the isolates contained hexulose phosphate synthase-hexulose phosphate isomerase, key enzymes of the hexulose phosphate cycle of formaldehyde assimilation [hitherto known as the ribose phosphate (Kemp & Quayle, 1967) or ribulose monophosphate (Anthony, 1975) cycle of formaldehyde assimilation]. This activity was apparently constitutive in the four restricted facultatives (Table 2).

Specific activities of other enzymes of the hexulose phosphate cycle of formaldehyde assimilation in obligate methylotrophs and restricted facultative methylotrophs

The presence of hexulose phosphate synthase-hexulose phosphate isomerase activity in extracts of the restricted facultatives W6A, W3A1, PM6 and S2A1 and of the obligates 4B6 and C2A1 (Table 2) suggested that these organisms use the hexulose phosphate cycle of formaldehyde assimilation (Kemp

Scheme 1. Hexulose phosphate cycle of formaldehyde assimilation and the KDPG variant

—, Modified from Kemp & Quayle (1967); ----, alternative KDPG route from F6P to C₃ compounds. R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; Xu5P, xylulose 5-phosphate; Hu6P, D-arabino-3-hexulose phosphate; S7P, sedoheptulose 7-phosphate; F6P, fructose 6-phosphate; F16P₂, fructose 1,6-diphosphate; E4P, erythrose 4-phosphate; DHAP, dihydroxyacetone phosphate; G3P, glyceraldehyde 3-phosphate; 6PG, 6-phosphogluconate; G6P, glucose 6-phosphate; KDPG, phospho-2-keto-3-deoxygluconate; HCHO, formaldehyde; HPS, hexulose phosphate synthase; HPI, hexulose phosphate isomerase; RPI, ribose phosphate isomerase; RuPE, ribulose phosphate epimerase; TA, transaldolase; TK, transketolase; A, fructose diphosphate aldolase; HDP, hexose diphosphatase; PFK, 6-phosphofructokinase; GPI, glucose phosphate isomerase; GPD, glucose 6-phosphate dehydrogenase; PGDH, phosphogluconate dehydratase; KDPGA, KDPG aldolase. The net reactions are: (a) Hexulose phosphate cycle of Kemp & Quayle (1967): 3HCHO + ATP → DHAP + ADP; and (b) KDPG variant of the hexulose phosphate cycle: 3HCHO → CH₃-CO-CO₂H + 2H₂.

& Quayle, 1967) as presented in Scheme 1. Confirmation of this was sought by assaying extracts of these organisms for the other enzymes of the pathway and the results are given in Table 2. These data show that the obligates and type M organisms lack 6-phosphofructokinase and fructose diphosphate aldolase, whereas extracts of the type L organisms lack transaldolase. It must be concluded that none of our obligate or restricted facultative methylotrophs use the assimilatory hexulose phosphate cycle in the form originally proposed by Kemp & Quayle (1967).

Specific activities of sedoheptulose diphosphatase and hexose diphosphatase in extracts of methylotrophs

The absence of transaldolase activity from extracts of the type L organisms suggested that a modified assimilatory hexulose phosphate cycle, involving sedoheptulose 1,7-diphosphate and sedoheptulose diphosphatase (or hexose diphosphatase) in place of transaldolase, might occur in these organisms; such a modified pathway would be analogous to one version of the ribulose diphosphate cycle of CO_2 fixation found amongst the autotrophs. Extracts of trimethylamine-grown isolates PM6 and S2A1 were therefore assayed for sedoheptulose diphosphatase and hexose diphosphatase and both enzymes were detected (Table 2). Hexose diphosphatase activity was dependent on the presence of MnCl_2 (3.5 mM); no activity was found when MgCl_2 (10 mM) replaced MnCl_2 . Sedoheptulose diphosphatase activity in the presence of MgCl_2 (10 mM) was double that found with MnCl_2 (3.5 mM). Sedoheptulose diphosphatase activities in extracts of citrate-grown PM6 or alanine-

grown S2A1 isolates were only 10% of those found in trimethylamine-grown organisms. It was thus concluded that a modified hexulose phosphate cycle (SDP variant) occurs in the two type L organisms. In this variant the transaldolase route shown in Scheme 1 for the conversion of fructose 6-phosphate and erythrose 4-phosphate into pentose phosphates is replaced by the SDP route shown in Scheme 2.

Neither sedoheptulose diphosphatase nor hexose diphosphatase was detected in extracts of trimethylamine-grown 4B6, C2A1, W3A1 or W6A thus precluding the occurrence of the SDP variant of the hexulose phosphate cycle in these obligate and type M organisms. These four organisms therefore lack hexose diphosphatase and fructose diphosphate aldolase which are important for glyconeogenesis. If the absence of these two enzymes from extracts of the trimethylamine-grown organisms reflects a constitutive inability to synthesize these enzymes (fructose diphosphate aldolase is also absent from glucose-grown W3A1 and W6A isolates although such organisms have not been examined for hexose diphosphatase activity), this must prevent glyconeogenesis in these organisms from a wide range of potential growth substrates; on this criterion alone, growth would be restricted to sugars and/or compounds that can give rise to sugars directly, e.g. C_1 compounds. These lesions, together with the incomplete tricarboxylic acid cycle found in these organisms (Colby & Zatman, 1975) may well explain their restricted range of growth substrates. The ability of organisms PM6 and S2A1 to synthesize hexose diphosphatase and fructose diphosphate

Scheme 2. SDP route to pentose phosphates occurring in the two type L restricted facultative methylotrophs PM6 and S2A1

The symbols used are given in the legend to Scheme 1. SDP, Sedoheptulose diphosphatase; S17P₂, sedoheptulose 1,7-diphosphate. The net reaction of the SDP variant of the hexulose phosphate cycle is: $3\text{HCHO} + 2\text{ATP} \rightarrow \text{DHAP} + \text{P}_i + 2\text{ADP}$.

Table 3. Specific activities of enzymes of the Entner–Doudoroff pathway in crude sonic extracts of trimethylamine-grown methylotrophs

All values are expressed as munits/mg of protein.

	Isolate	...	Obligate		Type M		Type L	
			4B6	C2A1	W3A1	W6A	S2A1	PM6
Glucokinase			7	4	28	28	45	55
Glucose phosphate isomerase			695	670	1400	1020	710	915
Glucose 6-phosphate dehydrogenase (NADP ⁺)			975	790	1310	954	308	287
Glucose 6-phosphate dehydrogenase (NAD ⁺)			134	138	176	206	0	0
Phosphogluconate dehydrogenase (NADP ⁺)			575	50	122	61	594	710
Phosphogluconate dehydrogenase (NAD ⁺)			39	134	189	189	79	99
Phosphogluconate dehydratase+phospho-2-keto-3-deoxygluconate aldolase			48	30	32	22	0	0

Scheme 3. Hexulose phosphate synthase-mediated cyclic pathway for formaldehyde oxidation

The symbols used are those given in the legend to Scheme 1. *PGD*, Phosphogluconate dehydrogenase. Net reaction: $\text{HCHO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 4\text{H}$.

aldolase is consistent with their ability to grow on citrate, glutamate or alanine.

Enzymes of the Enter–Doudoroff pathway in extracts of the methylotrophs

Extracts of trimethylamine-grown 4B6, C2A1, W6A and W3A1 isolates contain phosphogluconate dehydratase, phospho-2-keto-3-deoxygluconate aldolase and glucose phosphate isomerase (Table 3). These results suggested that a KDPG variant of the hexulose phosphate cycle occurs in these obligate and type M organisms, in which enzymes of the Entner–Doudoroff pathway effect the conversion of fructose 6-phosphate into C_3 compounds as shown in

Scheme 1. In the preceding paper Colby & Zatman (1975) concluded from studies of the growth-substrate specificity and activities of tricarboxylic acid-cycle enzymes, that the type M organisms W6A and W3A1 were very similar physiologically to the obligates C2A1 and 4B6. This close relationship is confirmed in the present studies in that both groups (i) use the same KDPG variant of the hexulose phosphate cycle, and (ii) use the trimethylamine dehydrogenase route rather than the trimethylamine mono-oxygenase route for trimethylamine oxidation.

Phosphogluconate dehydratase and phospho-2-keto-3-deoxygluconate aldolase were not detected in extracts of the type L organisms (Table 3). Glucose phosphate isomerase was present at high

specific activities although it is not involved in the SDP variant of the hexulose phosphate cycle that apparently occurs in these type L organisms; it is, however, required for the cyclic pathway of formaldehyde oxidation postulated below.

A hexulose phosphate synthase-mediated cyclic pathway for the complete oxidation of formaldehyde to CO₂

Extracts of the obligates and of all the restricted facultatives contain high specific activities of glucose phosphate dehydrogenase and phosphogluconate dehydrogenase (Table 3). These two enzymes, together with hexulose phosphate synthase, hexulose phosphate isomerase and glucose phosphate isomerase, constitute a cyclic mechanism for the complete oxidation of formaldehyde to CO₂ as shown in Scheme 3. We refer to this pathway as the dissimilatory hexulose phosphate cycle and suggest that it be distinguished from the formaldehyde assimilation pathway by designation of the latter as the assimilatory hexulose phosphate cycle. The dissimilatory cycle is probably responsible for the generation of energy during the methylotrophic growth of the type L organisms which apparently have no other mechanism for formaldehyde oxidation (see Table 1). The high activities of the enzymes of the dissimilatory hexulose phosphate cycle in extracts of the obligates and the type M organisms suggest that the cycle plays an important role in the methylotrophic growth of these isolates as well. In the type L organisms the glucose phosphate dehydrogenase is NADP⁺-specific whereas the phosphogluconate dehydrogenase activity is much higher with NADP⁺ than with NAD⁺; in these circumstances operation of the dissimilatory hexulose phosphate cycle would yield NADPH rather than NADH. This could explain the very high NADPH oxidase activities found in trimethylamine-grown isolates PM6 and S2A1 (Colby & Zatman, 1975).

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