Synthesis of Cell Constituents by Methane-Grown *Methylococcus capsulatus* and *Methanomonas methanooxidans*

By A. J. LAWRENCE, M. B. KEMP* AND J. R. QUAYLE

Department of Microbiology, University of Sheffield, Sheffield S10 2TN, U.K.

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1. A study was made of the incorporation of carbon from [^{14}C]methanol by cultures of *Methylococcus capsulatus* and *Methanomonas methanooxidans* growing on methane. 2. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the cells, after incubation with labelled substrate for periods of up to 3 min, was analysed by chromatography and radioautography. 3. Over 80% of the radioactivity fixed by *Methylococcus capsulatus* at 30°C at the earliest times of sampling appeared in phosphorylated compounds, of which glucose phosphate constituted 60%. 4. Most of the radioactivity fixed by *Methanomonas methanooxidans* at 30°C at the earliest times of sampling appeared in serine, malate, aspartate and an unknown compound(s) tentatively suggested to be folate derivative(s). At 16°C, [^{14}C]methanol was fixed predominantly into serine and the unknown compound(s). 5. Extracts of *Methylococcus capsulatus* contain an enzyme system that catalyses the condensation of formaldehyde and ribose 5-phosphate to give a mixture consisting mainly of fructose phosphate and allulose phosphate. No similar activity was detected in extracts of *Methanomonas methanooxidans*. A convenient method was developed for assay of this enzyme system. 6. The enzyme system catalysing the condensation of formaldehyde with ribose 5-phosphate is particle-bound in both *Methylococcus capsulatus* and *Pseudomonas methanica* and is unstable in the absence of Mg^{2+} . 7. Extracts of *Methanomonas methanooxidans* contain high activities of D-glycerate-NAD oxidoreductase, whereas extracts of *Methylococcus capsulatus* and *Pseudomonas methanica* contain negligible activities of this enzyme. 8. These results indicate that during growth of *Methylococcus capsulatus* on methane, as with *Pseudomonas methanica*, cell constituents are made by the ribose phosphate cycle of formaldehyde fixation. This contrasts with *Methanomonas methanooxidans*, whose assimilation pathway resembles in some features that of *Pseudomonas* AM1 growing on methanol.

Previous work has led to the suggestion that the net incorporation of C_1 units by *Pseudomonas methanica*, during growth on methane or methanol, proceeds by way of a modified pentose phosphate cycle (Johnson & Quayle, 1965; Kemp & Quayle, 1966, 1967). The present paper describes investigations carried out on the two other well-authenticated methane-utilizing organisms, *Methylococcus capsulatus* (Foster & Davis, 1966) and *Methanomonas methanooxidans* (Stocks & McCleskey, 1964) to see whether the same or different pathways operate for synthesis of cell constituents.

MATERIALS AND METHODS

Maintenance and growth of the organisms. *P. methanica* was grown as described previously (Johnson & Quayle, 1965; Kemp & Quayle, 1966).

Cultures of *M. capsulatus* were a gift from the late

Professor J. W. Foster (Department of Microbiology, University of Texas, Austin, Tex., U.S.A.). The organism was grown at 37°C in the medium described by Foster & Davis (1966), using the same techniques as described for *P. methanica*. Starter cultures (500 ml) were used to inoculate 10 l of sterile mineral-salts medium in a fermentor (model MF214; New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). In this case the culture was gassed with a methane-air (5:95) mixture at 1 l/min.

Cultures of *M. methanooxidans* were a gift from Dr P. Stocks (Department of Microbiology, University of Southern Mississippi, Hattiesburg, Miss., U.S.A.). The bacterium was grown at 30°C in the salts medium of Jayasuriya (1955) under a gas mixture of methane-air (50:50). Batch cultures were grown in a similar way to *M. capsulatus*.

Bacterial growth was determined by measurement of extinction at 610 nm with a spectrophotometer (model SP.600; Unicam Instruments Ltd., Cambridge, U.K.) previously calibrated against dry wt. of cells/ml of bacterial suspension. The cells were harvested by centrifugation and if not used immediately were freeze-dried and stored at -15°C .

* Present address: Microbiology Unit, Department of Biochemistry, University of Oxford, Oxford, U.K.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. [^{14}C]Methanol was purified by vacuum distillation. [^{14}C]Formaldehyde was either purchased in the form of an aqueous solution or obtained by heating [^{14}C]paraformaldehyde (1mCi) with 5ml of water in a stoppered tube at 100°C for 1h.

Special chemicals. Cylinders of methane were obtained from the Middlesex County Council, Main Drainage Dept., Mogden Works, Isleworth, Middlesex, U.K. D-Ribose 5-phosphate (sodium salt) was prepared from the barium salt of D-ribose 5-phosphate as described by Kemp & Quayle (1966).

Incorporation of [^{14}C]methanol by whole cells. Incorporation of [^{14}C]methanol by methane-grown cells utilizing methane was carried out as described by Johnson & Quayle (1965), with the exception that during the incubation with *Mtn. methanooxidans* the bacterial suspension was gassed with methane-air (5:95) instead of (50:50).

Preparation of cell-free extracts. Freeze-dried cells were suspended in 10 vol. of buffer mixture (see the text for buffer composition) and crushed in a Hughes (1951) press at -15°C, and the crude extract was centrifuged at 90 000g for 30 min. Unless specified differently in the text, the pellet was extracted thrice with 10 vol. portions of the same buffer mixture, with centrifuging each time. For routine use, the three supernatants obtained from the three extractions of the pellet were combined and the combined extract was used as such. The supernatant obtained from the first centrifugation of the crude extract was not generally used.

Chemical determinations. Protein was determined by the method of Folin and Ciocalteu as described by Layne (1957), with bovine serum albumin as standard.

Chromatography and radioautography. The methods of chromatography, radioautography, identification of labelled compounds and radioassay were as described by Large, Peel & Quayle (1961), unless specified otherwise.

Dephosphorylation of phosphorylated compounds was carried out after their elution from chromatograms with 0.2–0.5 ml of water. To this solution was added 20 μg of potato acid phosphatase [Boehringer Corp. (London) Ltd., London W.5, U.K.] dissolved in 20 mm-sodium acetate buffer, pH 4.8, containing 10 mm-MgCl₂. A drop of toluene was added and the mixture incubated at 37°C for 16 h. It was then chromatographed two-dimensionally as before, and the resulting radioactive sugars were identified by two-dimensional co-chromatography with authentic samples with the same solvent systems as used for the initial chromatography. The carrier sugars were located by the reagents described by Kemp & Quayle (1966). Identification of carboxylic acids was achieved by one-dimensional co-electrophoresis with authentic samples for 1 h at a potential gradient of 40 V/cm with as buffer 0.5 M-acetic acid solution adjusted with pyridine to pH 4.0 (Gross, 1959). After radioautography of the radioactive sample the carrier compound was detected by spraying the electrophoresis paper with 0.2% (w/v) Bromocresol Green in ethanol adjusted to pH 7.0 with ammonia. Amino acids were identified by one-dimensional co-electrophoresis with authentic samples at 40 V/cm with as buffer formic acid–water (3:37, v/v) (Hartley & Jevons, 1962). The carrier amino acid was

detected with 0.25% (w/v) ninhydrin in acetone followed by brief heating at 105°C.

Measurement of radioactivity. The radioactivity of paper chromatograms was measured with a mica-end-window Geiger–Müller tube (type 2B2) coupled to a scalar type D657 (Panax Equipment Ltd., Redhill, Surrey, U.K.). At least 1000 counts were recorded and corrections were made for background counts.

The radioactivity in the precipitated barium hexose phosphate from the hexose phosphate synthetase assays (see below) was measured as follows. Each glass-fibre disc containing the dried precipitate was placed in a scintillation vial with 0.5 ml of NE213 liquid scintillator (Nuclear Enterprises Ltd., Sighthill, Edinburgh, U.K.). The samples were assayed for radioactivity in a liquid-scintillation counter (model 6801; Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.) and quench corrections by the channels-ratio method were applied. The radioactivity of each sample was measured for 10 min and background counts were subtracted.

Incorporation of [^{14}C]formaldehyde by cell-free extracts. The incorporation of [^{14}C]formaldehyde by cell-free extracts of *Mtl. capsulatus* and *Mtn. methanooxidans* was performed in a similar way to that described by Kemp & Quayle (1966) except that the system was scaled up ten-fold and MgCl₂ was added. The complete system contained 100 μmol of sodium phosphate buffer, pH 7.0, 10 μmol of ribose 5-phosphate, 10 μmol of MgCl₂, 10 μmol of [^{14}C]formaldehyde containing 12 μCi of ^{14}C , and 0.5 ml of extract (containing 0.25 mg of protein) in a total volume of 1 ml. The reaction was started by adding extract and stopped after 15 min at 30°C by the addition of 4 ml of ethanol. Control incubations are described in the text. The mixtures were centrifuged, the supernatants evaporated to dryness *in vacuo* at 40°C and the residues each taken up in 0.2 ml of aq. 20% (v/v) ethanol. Samples (0.1 ml) of the ethanolic extracts were chromatographed two-dimensionally in the same way as described for whole-cell incubations. After radioautography the chromatograms were assayed for radioactivity with a mica-end-window Geiger–Müller tube.

Enzyme assays

D-Glycerate–NAD oxidoreductase (hydroxypyruvate reductase, EC 1.1.1.29). This was assayed by the procedure of Large & Quayle (1963) by measuring the rate of disappearance of NADH spectrophotometrically in the presence of lithium hydroxypyruvate.

Enzyme system catalysing the condensation of formaldehyde and ribose 5-phosphate (hexose phosphate synthetase). This was measured in cell-free extracts by determining the incorporation of label from [^{14}C]formaldehyde into sugar phosphates with D-ribose 5-phosphate as substrate. Unless stated otherwise the assay mixture consisted of 20 μmol of sodium phosphate buffer, pH 7.0, 2 μmol of MgCl₂, 2 μmol of ribose 5-phosphate, 2 μmol of formaldehyde containing 0.02 μCi of ^{14}C , and bacterial extract and water to a total volume of 0.4 ml. The reaction was started by adding extract and incubation was for 5 min at 30°C. The reaction was stopped with 1.5 ml of ethanol, and 0.1 ml of 5% (w/v) barium acetate was added to precipitate sugar phosphates. The mixture was left for 10 min at 0°C and then filtered under reduced pressure

through a circle (2.5 cm²) of glass-fibre paper (Whatman GF/A). The precipitate was washed twice with 2 ml of ethanol-formaldehyde (19:1, v/v) and once with 2 ml of ethanol, sucked dry on the pump and then further dried under a radiant lamp for 5 min. Each disc was placed in a scintillation vial and assayed for radioactivity by liquid-scintillation counting (see above). One unit of activity is defined as that amount of enzyme catalysing the fixation of 1 μ mol of [¹⁴C]formaldehyde in 1 min under the conditions of assay.

RESULTS

Analysis of path of labelled substrate into cell constituents

Incorporation of [¹⁴C]methanol by methane-grown Mtl. capsulatus. At the earliest sampling time, virtually the only non-volatile compounds labelled were phosphates (Table 1). The curves obtained by plotting the percentage distribution of the fixed radioactivity against time (Fig. 1) showed a negative slope for phosphorylated compounds. The curves for all the other identified compounds had positive slopes; these are alanine, malate, aspartate and glycine+serine+glutamate. Elution, de-phosphorylation and rechromatography of the area of the parent chromatogram containing the phosphorylated compounds revealed the presence of free sugars. At the earliest time (16 s) the proportions of each sugar in the mixture were: glucose (60.1%), sedoheptulose (16.6%), fructose (21.2%), glyceric acid (1.3%) and ribose (0.8%). In subsequent samples the percentage of glucose remained constant, those of sedoheptulose and fructose decreased slightly and the glyceric acid and ribose percentages increased to 5.3% and 1.6% respectively after 107 s. These results are similar to those of Johnson & Quayle (1965) obtained with methane- and methanol-grown *P. methanica*.

Incorporation of [¹⁴C]methanol by methane-grown Mtn. methanooxidans. The first labelling experiment with whole cells of this organism was performed at 30°C under similar conditions to those used for *Mtl. capsulatus*. The results obtained are given in Table 2 and are very different from those relating to *P. methanica* and *Mtl. capsulatus*. In particular a considerable amount of radioactivity appeared at the earliest times of sampling in an unknown compound (or compounds) designated in Table 2 as 'compound X'. This chromatographed

Fig. 1. Variation with time of the percentage distribution of ¹⁴C incorporated from [¹⁴C]methanol into the constituents of the ethanol-soluble fraction of *Mtl. capsulatus* growing on methane at 30°C. ○, Phosphorylated compounds; △, malate; □, aspartate; ●, alanine; ▲, glycine+serine+glutamate.

Table 1. *Incorporation of [¹⁴C]methanol by Mtl. capsulatus at 30°C*

[¹⁴C]Methanol (22 μ mol; 200 μ Ci) was added at zero time to 10 ml of a suspension of bacteria (10.5 mg dry wt./ml) in 10 mM-sodium phosphate buffer, pH 7.0, containing NH₄Cl (10 mM). The suspension was gassed with methane-air (5:95) in a vessel (10 cm $\times$ 2.5 cm diam.) with a sintered-glass bottom. Samples (approx. 1 ml) were removed at intervals into 4 ml of aq. 95% (v/v) ethanol and centrifuged, and the phosphates extracted with 0.5 ml of aq. 20% (v/v) ethanol. The combined extracts were evaporated and redissolved in 0.25 ml of aq. 20% (v/v) ethanol, and 0.1 ml of each sample was analysed by chromatography and radioautography.

Time (s)	Radioactivity (c.p.m.)						Total
	Phosphates	Alanine	Malate	Aspartate	Glycine+serine+glutamate	Other compounds	
16	3572	38	20	90	80	298	4098
26	4651	104	58	308	124	418	5663
39	5480	154	105	439	217	312	6707
51	7955	236	149	620	215	459	9634
78	7966	386	268	887	344	356	10207
107	13899	871	580	1591	965	591	19497

Table 2. Incorporation of [^{14}C]methanol by *Mtn. methanooxidans* at 30°C

[^{14}C]Methanol (22 μmol ; 200 μCi) was added at zero time to 5 ml of a suspension of bacteria (6 mg dry wt./ml) in 10 mM-phosphate buffer, pH 7.0, containing NH_4Cl (10 mM), gassed with methane-air (5:95) as described in Table 1. Samples (approx. 1 ml) were removed at intervals and treated as described in Table 1.

Time (s)	Radioactivity (c.p.m.)									
	Phosphates	Alanine	Malate	Aspartate	Glycine	Serine	Succinate+ fumarate	Compound X	Other compounds	Total
9	280	39	442	899	38	648	201	642	430	3619
18	1241	233	749	1715	694	1851	542	337	1217	8579
27	1467	275	1392	1212			734	687		8263
35	3215	543	1721	1459	412	728	781	754	1317	10930
43	1783	501	1209	1418	474	598	839	425	877	8223
52	1688	595	1568	1330	447	582	717	441	1494	8862

as a long streak in the phenol-formic acid solvent, the leading edge of the streak ran with the solvent front and the trailing edge terminated at R_f approx. 0.5. When the chromatogram was developed in the second dimension with butanol-propionic acid, the streak migrated with R_F 0.1–0.2. The identity of the compound(s) comprising this streak is not known. It does not co-chromatograph with either *N*-methylglutamate or sarcosine, compounds that have been implicated in methylamine metabolism during growth of species of *Pseudomonas* on methylamine as sole carbon and energy source (Shaw, Tsai & Stadtman, 1966; Kung & Wagner, 1968). On the other hand, tetrahydrofolic acid when chromatographed in this two-dimensional solvent system gives a long streak in a closely similar position to that of the unknown compound. Since serine is also an early labelled intermediate, it might be thought that a folate derivative could be functioning as a carrier of radioactive C_1 units for hydroxymethylation of glycine. Hence we suggest tentatively that compound X might be a folate derivative or mixture of folate derivatives. Reduced folate compounds are very unstable and break down to a complex mixture under the conditions used in the analysis of the labelled compounds, hence further identification was not attempted in these experiments.

A further difference between the labelling pattern in *Mtn. methanooxidans* and those in *P. methanica* and *Mtl. capsulatus* is that in the first organism serine is rapidly labelled whereas phosphorylated compounds are labelled relatively slowly. This is clearly brought out when the results are plotted graphically, as shown in Fig. 2. The slopes for the two compounds, aspartate and compound X, are negative; the initial slope for malate appears negative but this cannot be regarded as certain on the basis of only the first and second points, and, further, the net fixation of [^{14}C]methanol into components of the ethanol-soluble fraction virtually ceased after 18 s.

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]methanol into the constituents of the ethanol-soluble fraction of *Mtn. methanooxidans* growing on methane at 30°C. O, Phosphates; Δ , malate; $\square$, aspartate; $\bullet$, glycine; $\blacktriangle$, serine; $\blacksquare$, compound X.

In view of the complexity of the labelling pattern, the incorporation of [^{14}C]methanol into methane-grown *Mtn. methanooxidans* was repeated at 16°C instead of 30°C. The organism grows at 16°C at 10–20% (depending on the conditions of shaking) of the rate at 30°C. Incubation at this temperature greatly lowered the rate of incorporation of label (Table 3) and simplified the graph of percentage incorporation (Fig. 3). Fixation of [^{14}C]methanol continued throughout the incubation although the rate of fixation decreased with time. Under these conditions compound X was clearly represented as a primary port of entry for the methanol carbon. Serine was labelled at the earliest times, but in the absence of a definitely positive or negative slope it cannot be deduced whether serine is labelled simultaneously with compound X or is derived from it. Radioactive phosphates are included with

Table 3. Incorporation of [^{14}C]methanol by *Mtn.* methanooxidans at 16°C

The experimental procedure was identical with that described in Table 2, except that the temperature of incubation was 16°C instead of 30°C.

Time (s)	Radioactivity (c.p.m.)						Total
	Malate	Aspartate + glutamate	Glycine	Serine	Compound X	Other compounds	
11	28	<10	0	384	508	195	1115
18	61	20	0	456	362	359	1258
24	108	62	0	531	426	486	1613
48	197	190	27	482	202	658	1756

Fig. 3. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]methanol into the constituents of the ethanol-soluble fraction of *Mtn. methanooxidans* growing on methane at 16°C. Δ , Malate; $\square$, aspartate + glutamate; $\blacktriangle$, serine; $\blacksquare$, compound X.

'Other compounds' in Table 3 and represented only a minor component of the total radioactivity fixed.

Incorporation of [^{14}C]formaldehyde by cell-free extracts

The results of the above experiments indicated that in *Mtl. capsulatus* a ribose phosphate cycle of formaldehyde fixation was operating, similar to that in *P. methanica*, whereas no evidence for the operation of this pathway could be found in *Mtn. methanooxidans*. It is thought that one of the key reactions of this modified pentose phosphate cycle is the condensation of formaldehyde with ribose 5-phosphate to give allulose 6-phosphate (Kemp & Quayle, 1966). Hence cell-free extracts of *Mtl. capsulatus* and *Mtn. methanooxidans* were tested for the presence of this enzyme system by incubation with [^{14}C]formaldehyde and ribose 5-phosphate, as described in the Materials and Methods section. The radioactive products were analysed by chromatography and radioautography. The complete

system from *Mtl. capsulatus* fixed much more radioactivity into the area expected for sugar phosphates than did the control incubations (Table 4); this area included the chromatogram origin. These areas were cut out from three of the chromatograms, as indicated in Table 4, and eluted with water. The eluates were incubated with acid phosphatase and rechromatographed. Much of the radioactivity now appeared as three spots that co-chromatographed with fructose, allulose and glucose respectively. Considerable radioactivity remained near the origin, but when these origin areas were eluted, treated with acid phosphatase and chromatographed again more radioactivity appeared in the same three sugars. It was concluded that a large proportion of the radioactivity remaining in the origin area arose from incompleteness of dephosphorylation by the phosphatase preparation. Similar behaviour was encountered by Kemp & Quayle (1966) using an acid phosphatase preparation from Polidase S (Schwartz Laboratories Inc., Mount Vernon, N.Y., U.S.A.). Phosphatase treatment of the origin areas of the chromatograms resulting from the 'zero time' and 'extract-omitted' control incubations did not result in liberation of radioactive sugars.

These results indicate that *Mtl. capsulatus* possesses an enzyme system similar to that of *P. methanica* catalysing the condensation of formaldehyde and ribose 5-phosphate to give hexose phosphate.

By contrast, when a similar experiment to that described above was performed with an extract of *Mtn. methanooxidans*, little radioactivity was found in the areas of the chromatograms corresponding to either free sugars or phosphorylated compounds. Elution of the origin area of the chromatogram resulting from the complete test system, followed by treatment with phosphatase and rechromatography, did not reveal the presence of any radioactive sugars. It is concluded that *Mtn. methanooxidans* does not possess an enzyme system catalysing the condensation of formaldehyde with ribose 5-phosphate.

Table 4. Incorporation of [^{14}C]formaldehyde by cell-free extracts of *Mtl. capsulatus*

The extract (0.25 mg of protein/ml) was prepared by crushing a suspension of freeze-dried cells in 10 vol. of a buffer mixture (20 mM-sodium phosphate buffer, pH 7.0, containing 2 mM-ribose 5-phosphate and 5 mM- MgCl_2) as described in the Materials and Methods section. The conditions of incubation, chromatography and radioautography were as described in the Materials and Methods section.

Reaction system	Radioactivity in parent chromatogram (c.p.m.)			Radioactivity in dephosphorylated compounds (c.p.m.)				Origin area of chromatogram
	Allulose	Fructose	Phosphates	Fructose	Allulose	Glucose		
Complete	355	325	37 672	6194	2959	461		17 588
Complete, but extract boiled	0	0	1363		Not dephosphorylated			
Complete, but reaction stopped at zero time	0	0	2783	0	0	0		918
Extract omitted	0	0	1609	0	0	0		128
Tracer only	0	0	493		Not dephosphorylated			

Table 5. Ribose 5-phosphate-dependent fixation of [^{14}C]formaldehyde by extracts of *Mtl. capsulatus*

The complete assay system was that described for hexose phosphate synthetase assay in the Materials and Methods section. The cell-free extract was prepared from methane-grown *Mtl. capsulatus* by crushing a suspension of freeze-dried cells in 10 vol. of a buffer mixture (20 mM-sodium phosphate buffer, pH 7.0, containing 5 mM- MgCl_2) as described in the Materials and Methods section; 0.03 ml was used in each incubation mixture. The ^{14}C fixed is expressed as the total amount of ^{14}C fixed in each assay tube during the 5 min incubation. The results are corrected for a background count of 63 d.p.m.

Reaction system	[^{14}C]HCHO fixed (net d.p.m.)
Complete	4289
Ribose 5-phosphate omitted	92
Extract omitted	7
Complete, but boiled extract used	59

Quantitative assay of enzyme system catalysing the condensation of formaldehyde and ribose 5-phosphate

Experiments had now shown the presence of the above enzyme system in cell-free extracts of *P. methanica* and *Mtl. capsulatus*, but a quantitative assay procedure was obviously needed for any further work. Since crude bacterial extracts possess many enzymes capable of metabolizing sugar phosphates, it was considered that the most useful assay for such extracts would be one based on ribose 5-phosphate-dependent fixation of formaldehyde rather than appearance of a particular hexose phosphate. Accordingly, an assay was developed in which [^{14}C]formaldehyde and ribose 5-phosphate were incubated with bacterial extract and the reaction was terminated by addition of 3.75 vol. of

Fig. 4. Progress curves for the reaction between [^{14}C]formaldehyde and ribose 5-phosphate catalysed by extracts of *P. methanica* and *Mtl. capsulatus*. The assay system was the hexose phosphate synthetase assay described in the Materials and Methods section. O, 0.1 ml of *P. methanica* extract; ●, 0.3 ml of *Mtl. capsulatus* extract; Δ, control lacking extract (incorporation of ^{14}C is measured in the same units as those for the *Mtl. capsulatus* extract).

ethanol and 0.25 vol. of aq. 5% (w/v) barium acetate. The radioactivity fixed was measured directly by liquid-scintillation counting of the precipitated sugar phosphates (see the Materials and Methods section). Checks were made, with [^{14}C]glucose 6-phosphate in place of [^{14}C]formaldehyde, that hexose phosphate at the lowest concentrations to be expected under the assay conditions was quantitatively precipitated. It was found that 1–100 nmol quantities of [^{14}C]glucose 6-phosphate dissolved in the assay mixture were precipitated to

the extent of over 90% by this procedure. It was not expected that the solubility of barium salts of other hexose phosphates would differ appreciably under these conditions from that of the barium salt of glucose 6-phosphate. There was no evidence from the channels ratio of any significant quenching occurring in the samples prepared from the artificial

assay mixtures, the efficiency of counting being 74%. When the method was used to assay enzyme activity in cell-free extracts, the channels ratio indicated a fall in efficiency of counting to 65%. This was allowed for in the conversion of the counting rate from c.p.m. into d.p.m. Under these assay conditions the fixation of [^{14}C]formaldehyde was dependent on the presence of ribose 5-phosphate and extract (Table 5) and showed a reasonably linear rate over 10 min incubation with extracts prepared from *P. methanica* or *Mtl. capsulatus* (Fig. 4). When a 5 min incubation period was used there was a linear relation between fixation of [^{14}C]formaldehyde and the amount of extract added (prepared from either *P. methanica* or *Mtl. capsulatus*) up to rates of 0.02 μmol of formaldehyde fixed/min. It was concluded that this assay method gives a reliable measure of the activity of the enzyme system (hexose phosphate synthetase) catalysing the condensation of formaldehyde and ribose 5-phosphate, and it was used as such in the subsequent experiments. Although evidence has been presented (Kemp & Quayle, 1966) suggesting that the hexose phosphate initially formed in extracts is allulose 6-phosphate, until this is proved with a purified enzyme the crude enzyme system will be referred to as 'hexose phosphate synthetase'.

Localization of hexose phosphate synthetase in Mtl. capsulatus. When extracts were prepared from freeze-dried *Mtl. capsulatus* by disruption in a Hughes press it was found that 93% of the total activity of hexose phosphate synthetase present in the suspension was sedimented at 90 000 g (Table 6). Synthetase could be extracted from the particulate fraction by repeated extraction with buffer, but even after three successive extractions, each followed by centrifugation, the resulting pellet still contained 30% of the starting activity of the original

Table 6. *Localization of hexose phosphate synthetase in extracts of Mtl. capsulatus*

A suspension of 0.1 g of freeze-dried cells in 1 ml of 20 mM-sodium phosphate buffer, pH 7.0, containing 2 mM-ribose 5-phosphate and 5 mM-MgCl₂ was disrupted in a Hughes press at -25°C. The crude extract was centrifuged at 90 000 g for 30 min. The pellet was successively extracted three times with portions (1 ml) of the same buffer; the resulting suspensions were centrifuged each time and the supernatants drawn off. The final pellet was resuspended in 1 ml of the same buffer mixture. The various fractions were assayed for hexose phosphate synthetase as described in the Materials and Methods section.

Fraction	Hexose phosphate synthetase activity	
	Specific activity (units/mg of protein)	Total amount in fraction (units)
Crude extract before centrifugation	0.94	14.7
Supernatant after centrifugation	0.63	1.3
Supernatant from first extraction of pellet	0.69	0.86
Supernatant from second extraction of pellet	1.15	0.62
Supernatant from third extraction of pellet	3.75	0.94
Resuspended pellet	0.37	4.4

Table 7. *Effect of Mg²⁺ on hexose phosphate synthetase*

Cell-free extracts were prepared from freeze-dried *P. methanica* and *Mtl. capsulatus* as described in the Materials and Methods section, with 20 mM-sodium phosphate buffer, pH 7.0. Then 0.01 ml of each extract was assayed for hexose phosphate synthetase in the presence or absence of 5 mM-MgCl₂ in the assay system described in the Materials and Methods section. Samples of the extracts were then diluted tenfold with 20 mM-sodium phosphate buffer, pH 7.0, with or without the addition of 1 mM-MgCl₂. Then 0.1 ml of each diluted preparation was assayed in the presence or absence of 5 mM-MgCl₂ in the assay system.

Enzyme extract	Diluent	Concn. of MgCl ₂ in assay mixture (mM)	[^{14}C]HCHO fixed (net d.p.m.)	
			<i>P. methanica</i> extract	<i>Mtl. capsulatus</i> extract
Boiled	—	5	53	16
Undiluted (0.01 ml)	—	—	2300	154
Undiluted (0.01 ml)	—	5	7841	383
Diluted (0.1 ml)	20 mM-Phosphate	—	1200	153
Diluted (0.1 ml)	20 mM-Phosphate	5	5000	222
Diluted (0.1 ml)	20 mM-Phosphate-1 mM-MgCl ₂	5	7464	311

suspension. Similar behaviour has been encountered with *P. methanica*. Since the specific activity of the enzyme extracted from the pellet was higher than that in the initial supernatant, for routine purposes the latter was not usually combined with the extracts of the pellet (see the Materials and Methods section).

Effect of Mg^{2+} on hexose phosphate synthetase. It was found that the activity of hexose phosphate synthetase in *P. methanica* or *Mtl. capsulatus* was stimulated two- to three-fold by the presence of 5mM-magnesium chloride in the assay mixture (Table 7). Further, with *P. methanica*, dilution of the enzyme extract with Mg^{2+} -free phosphate buffer before assay caused a loss of 48% of the activity if the assay was conducted in the absence of Mg^{2+} , or 36% if the assay was conducted in the presence of 5mM-magnesium chloride. If 1mM-magnesium chloride was included in the phosphate buffer used for dilution, 80–90% of the activity present in the undiluted extracts of either organism could be recovered. This indicates that the enzyme system is not only stimulated by Mg^{2+} but is unstable in its absence, and hence 5mM-magnesium chloride was added to all buffers used in the preparation of cell-free extracts or in their dilution. Sometimes a similar effect was observed for ribose 5-phosphate, and hence 2mM-ribose 5-phosphate was also added as a routine to the buffer mixture.

When extracts of the three methane-grown organisms were prepared in the presence of 5mM-magnesium chloride and 2mM-ribose 5-phosphate and assayed for hexose phosphate synthetase, specific activities of 1–2 μ mol of formaldehyde incorporated per min/mg of protein were observed for *P. methanica* and *Mtl. capsulatus*, whereas no activity could be detected for *Mtn. methanooxidans*.

Specific activity of hydroxypyruvate reductase in *P. methanica*, *Mtl. capsulatus* and *Mtn. methanooxidans*. Extracts of these methane-grown organisms, prepared in 20mM-sodium phosphate buffer, pH 7.0, when assayed for hydroxypyruvate reductase (see the Materials and Methods section) gave specific activities of 0.01, 0 and 1.2 μ mol of NADH oxidized/min per mg of protein for *P. methanica*, *Mtl. capsulatus* and *Mtn. methanooxidans* respectively. In contrast with the hexose phosphate synthetase, hydroxypyruvate reductase was not particle-bound, and hence the supernatant from the first centrifugation of the crude extract (see the Materials and Methods section) was used for assay of the latter enzyme.

DISCUSSION

The present results indicate that during growth of *Mtl. capsulatus* on methane a cycle of reactions operates that is similar to that proposed for growth

of *P. methanica* on methane or methanol (Kemp & Quayle, 1966, 1967). The main evidence for this conclusion is: (a) both organisms incorporate labelled substrate most rapidly into phosphorylated compounds, consisting predominantly of hexose phosphates; (b) cell-free extracts of both organisms contain an enzyme system, at comparable specific activity, that catalyses the condensation of formaldehyde with pentose phosphate to give a mixture of hexose phosphates, consisting predominantly of fructose phosphate and allulose phosphate. By these criteria, a totally different pathway must operate in *Mtn. methanooxidans* during growth on methane. Phosphorylated compounds are not the earliest compounds to be labelled from radioactive substrate, nor can any hexose phosphate synthetase activity be detected in cell-free extracts of the organism. The exact nature of the carbon-assimilation pathway that operates in *Mtn. methanooxidans* is not known, but there are several features that suggest a similarity to the 'serine pathway' of methanol-grown *Pseudomonas* AM1 (Large & Quayle, 1963). The labelling experiments performed with whole cells of *Mtn. methanooxidans* show that serine and C_4 dicarboxylic acids, such as malate and aspartate, are among the most rapidly labelled compounds. This is in accord with the pattern of uptake of labelled substrate found during growth of *Pseudomonas* AM1 on methanol or formate (Large *et al.* 1961). Further, one of the key enzymes of the serine pathway, hydroxypyruvate reductase, which is present in methanol-grown *Pseudomonas* AM1 at high specific activity (94 μ mol of NADH oxidized per hr/mg of protein) (Large & Quayle, 1963), is also present in extracts of *Mtn. methanooxidans* at comparable specific activity (72 μ mol of NADH oxidized per hr/mg of protein). This is in contrast with *P. methanica* and *Mtl. capsulatus*, extracts of which contain no detectable activity of this enzyme.

The known methane-utilizing bacteria possess in common the striking nutritional similarity of growing only on methane or methanol as carbon sources. Foster & Davis (1966) suggested that, if the allulose phosphate pathway was found to be a general mechanism of methane assimilation for bacteria that can grow only on methane and methanol, these bacteria should be classified together using the prefix '*Methylo*-' in their generic name, e.g. *Methylomonas* in place of *Methanomonas*. The present results already show that operation of the ribose phosphate cycle of formaldehyde fixation is not peculiar to all obligate methylotrophs and call for further comparative studies of the many new methane-utilizing organisms isolated by Whittenbury and Wilkinson and their colleagues (Whittenbury, Davies, McLee, Phillips & Wilkinson, 1968; Whittenbury, 1969). It will also be of interest to

see whether the particulate nature of the enzyme system catalysing the condensation of formaldehyde with pentose phosphate in *P. methanica* and *Mtl. capsulatus*, as found in the present study, can be correlated with the elaborate internal membrane system found in methane-utilizing bacteria (Whittenbury, 1969; Proctor, Norris & Ribbons, 1969).

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REFERENCES

- Foster, J. W. & Davis, R. H. (1966). *J. Bact.* **91**, 1924.
Gross, D. (1959). *Nature, Lond.*, **184**, 1298.
Hartley, F. K. & Jevons, F. R. (1962). *Biochem. J.* **84**, 134.
Hughes, D. E. (1951). *Br. J. exp. Path.* **32**, 97.
Jayasuriya, G. C. N. (1955). *J. gen. Microbiol.* **12**, 419.
Johnson, P. A. & Quayle, J. R. (1965). *Biochem. J.* **95**, 859.
Kemp, M. B. & Quayle, J. R. (1966). *Biochem. J.* **99**, 41.
Kemp, M. B. & Quayle, J. R. (1967). *Biochem. J.* **102**, 94.
Kung, H. F. & Wagner, C. (1968). *Bact. Proc.* p. 115.
Large, P. J., Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 470.
Large, P. J. & Quayle, J. R. (1963). *Biochem. J.* **87**, 386.
Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 448. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Proctor, H. M., Norris, J. R. & Ribbons, D. W. (1969). *J. appl. Bact.* **32**, 118.
Shaw, W. V., Tsai, L. & Stadtman, E. R. (1966). *J. biol. Chem.* **241**, 935.
Stocks, P. K. & McCleskey, C. S. (1964). *J. Bact.* **88**, 1071.
Whittenbury, R. (1969). *Process Biochem.* **4** (no. 1), 51.
Whittenbury, R., Davies, S. L., McLee, A. G., Phillips, K. C. & Wilkinson, J. F. (1968). *J. gen. Microbiol.* **55**, xviii.