Microbial Growth on C₁ Compounds

SYNTHESIS OF CELL CONSTITUENTS BY METHANE- AND METHANOL-GROWN *PSEUDOMONAS METHANICA*

By PATRICIA A. JOHNSON AND J. R. QUAYLE*

Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry,
University of Oxford

(Received 4 November 1964)

1. A study has been made of the incorporation of carbon from [¹⁴C]methane, [¹⁴C]methanol and [¹⁴C]bicarbonate by cultures of *Pseudomonas methanica* growing on methane, and [¹⁴C]methanol by cultures of the same organism growing on methanol. 2. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the cells, after incubation with labelled compound for periods up to 3 min., has been analysed by chromatography and radioautography. 3. Over 90% of the radioactivity fixed from [¹⁴C]methane or [¹⁴C]methanol at the earliest times of sampling appeared in phosphorylated compounds. Glucose phosphate and fructose phosphate together constituted the largest part of the radioactive phosphates (70–90%); phosphoglycerate was a relatively minor component (2–17%). Other compounds becoming labelled during the incubation included glycine, serine, glutamate, aspartate, malate, citrate and alanine. 4. The first stable products of [¹⁴C]bicarbonate fixation were malate and aspartate (containing between them over 90% of the total radioactivity fixed at the earliest times of sampling). 5. The percentage of the total radioactivity fixed that was contained in each of the radioactive compounds has been plotted against time. The slopes of the curves obtained show that hexose phosphates are primary stable products of [¹⁴C]methane and [¹⁴C]methanol incorporation and that aspartate and malate are primary stable products of [¹⁴C]bicarbonate incorporation. 6. No carboxydismutase activity has been found in cell-free extracts of the organism. This fact, together with the other findings, shows that an autotrophic metabolism involving the ribulose diphosphate cycle of carbon dioxide fixation cannot be operating.

Pseudomonas methanica is an aerobic organism that grows on either methane or methanol as sole carbon source in an otherwise mineral medium (Söhngen, 1906; Dworkin & Foster, 1956; Leadbetter & Foster, 1958). Little work has been done on the metabolism of the organism apart from that of Leadbetter & Foster (1958), who found that the specific radioactivity of cells of various strains of *P. methanica* grown under an atmosphere of methane + air + ¹⁴CO₂ was always much less than that of the exogenous ¹⁴CO₂, showing that the cells could not have been synthesized exclusively from exogenous carbon dioxide, or from respiratory carbon dioxide in equilibrium with the exogenous carbon dioxide. The authors concluded from this that the metabolism of the organism on methane as growth substrate was heterotrophic rather than

autotrophic. The nature of this heterotrophic metabolism has remained obscure. As part of an investigation of this problem, the present paper records a study of the path of carbon incorporation from [¹⁴C]methane, [¹⁴C]methanol and ¹⁴CO₂ into cell constituents when the organism is growing on either methane or methanol.

MATERIALS AND METHODS

Maintenance and growth of the organism. Cultures of *P. methanica* were a gift from Professor J. W. Foster (Department of Microbiology, University of Texas, Austin, Texas, U.S.A.). The growth medium employed was that described by Dworkin & Foster (1956). Agar slopes and 10 ml. starter cultures were incubated at 30° in a large desiccator gassed with methane + air (50:50) mixture. The gas mixture was sterilized by passage through a sterile cotton-wool filter attached to the desiccator inlet tap. After 2–3 days' growth the starter cultures were used to inoculate larger quantities of sterile medium (from 500 ml. to 2 l., as

* Present Address: Department of Biochemistry, University of Sheffield.

required), which were vigorously aerated with the same gas mixture in a closed-circuit apparatus at 30°. The gas was circulated by means of a peristaltic pump ('W.M.H.R.' Flow Inducer; Laboratory Glassblowers Co., High Wycombe, Bucks.). Bacterial growth was determined by measurement with a nephelometer (Evans Electro-selenium Ltd., Halstead, Essex) previously calibrated against dry wt. of cells/ml. of bacterial suspension. Growth of aging cultures was difficult to measure owing to clumping of the cells.

When methanol was used as carbon source the same mineral medium was employed with the addition of 0.5% (v/v) of methanol after sterilization.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. Solutions of [^{14}C]methanol and [^{14}C]bicarbonate were prepared as described by Large, Peel & Quayle (1961). [^{14}C]Methane was supplied in sealed ampoules and was transferred to the incubation mixture directly, as described below.

Special chemicals. Cylinders of methane were obtained from the National Coal Board, Point of Ayr Colliery, Flintshire. Typical analysis of the gas gave the following figures (by vol.): methane, 85.9; oxygen, 2.95; nitrogen, 9.46; carbon dioxide, 1.69; carbon monoxide, 0.0001.

Bacterial incorporation of [^{14}C]methanol and [^{14}C]bicarbonate. Cultures of methanol-grown bacteria in the exponential growth phase were centrifuged at room temperature and rapidly resuspended in solutions containing the growth substrate in the original concentration, phosphate buffer, pH 7.0 (10mm), and ammonium chloride (10mm), to give a concentration of about 5mg. dry wt. of bacteria/ml. (see below). Portions (5 or 10ml.) of these suspensions were placed at 30° in cylindrical glass vessels (10cm. $\times$ 2.5cm. diam.) with sintered-glass bottoms and vigorously aerated by blowing a stream of air through the sintered glass. After 10 min. aeration the labelled compound (100 or 200 μC of ^{14}C ; see below) was added and samples (approx. 1 ml.) were withdrawn at suitable intervals with a hypodermic syringe.

When experiments were performed with methane-grown bacteria, the air stream was replaced by a stream of methane+air (50:50) mixture circulated in closed circuit from a 10l. polythene reservoir. After the bacterial suspension had been gassed for 10–15 min. at 30° the outlet from the reaction vessel was disconnected from the closed circuit to allow the labelled compound to be introduced and samples of the bacterial suspension to be taken. The gas mixture in the 10l. reservoir was now passed directly through the suspension and led into the fume cupboard during the incubation (about 5 min.). Each sample taken was immediately squirted into 4 ml. of aq. 95% (v/v) ethanol at room temperature and the resulting suspensions of killed cells were prepared for chromatography as described by Large *et al.* (1961). In each experiment a control with 1 ml. of a boiled cell suspension and a proportional volume of tracer solution was included.

Bacterial incorporation of [^{14}C]methane. [^{14}C]Methane was transferred from sealed glass ampoules by means of the modified Toepler pump shown in Fig. 1. The [^{14}C]methane (4.2 ml. at S.T.P.; 500 μC) was contained in the ampoule *A* (internal vol. 14.6 ml.). The ampoule was sealed on to the apparatus with a soft iron core *B* enclosed. The apparatus was connected to a high-vacuum line via tube *C* and flask *D*

Fig. 1. Apparatus used for incubating *P. methanica* with [^{14}C]methane.

was evacuated with tap *E* shut and taps *F* and *G* open to the pump. Tap *G* was closed, and the glass seal in the ampoule broken by raising iron core *B* with a magnet and allowing it to drop on to the seal. After allowing 5 min. for the [^{14}C]methane to distribute itself throughout the apparatus, tap *F* was shut. Since the volume of flask *D* was 150 ml., approx. 90% of the [^{14}C]methane was enclosed between the closed taps *E* and *F*. Mercury was now run into flask *D* from reservoir *H* until the [^{14}C]methane was enclosed in the upper part of flask *D*. The gas was then finally led into a 10 ml. plastic hypodermic syringe via the tap *F* and a three-way stainless-steel tap *J*. Tap *J* was then closed off and the syringe removed from the rubber connexion *K*. In the syringe *I*, before it had been attached to the connexion *K*, was placed 5 ml. of air and 5 ml. of solution containing phosphate buffer, pH 7.0 (10mm), and ammonium chloride (10mm). The displacement of the syringe plunger during the entry of [^{14}C]methane into the syringe enabled a check to be made of the completeness of transfer of the gas from the ampoule to the syringe. The detached syringe was then shaken by hand for 5 min. to saturate the solution inside with [^{14}C]methane. Meanwhile a culture of methane-grown *P. methanica* was harvested in the exponential phase of growth and resuspended in 10mm-phosphate buffer, pH 7.0, containing ammonium chloride (10mm), to give a bacterial concentration of 8–10mg. dry wt./ml. This suspension was gassed with methane+air (50:50) for 15 min. and then 5 ml. of the suspension drawn into syringe *I* through a hypodermic needle *L* attached to one arm of tap *J*. The tap was closed and the syringe rapidly shaken by hand. Samples (approx. 1 ml.) were squirted through *L* at suitable time-intervals into separate 4 ml. portions of ethanol. The resulting suspensions of killed cells were then treated as described above.

To check that the methane used contained no non-volatile impurities and to verify the reproducibility of the results of the above experiment a second incubation was performed as follows. A second batch of methane-grown *P. methanica* was harvested and resuspended as described above to give a concentration of approx. 10mg. dry wt. of bacteria/ml. A sample of this suspension (1 ml.) was boiled for 10 min. Meanwhile, 1 ml. of the suspending medium was drawn into the syringe *I* and [^{14}C]methane (0.59 mg.,

0.1mc) was added as described above. The syringe was shaken by hand for 10 min., after which the cooled suspension of killed cells was added and vigorous shaking continued. Samples (approx. 0.5 ml.) were squirted into tubes containing 4.0 ml. of aq. 95% ethanol at 15, 165 and 240 sec. The experiment was then repeated with 1 ml. of live cells and the resulting ethanolic suspensions of killed cells from both experiments were evaporated and chromatographed.

Chromatographic analysis and radioautography. The techniques of chromatography, radioautography, identification of labelled compounds and radioassay have been described by Large *et al.* (1961). In the present experiments the enzyme preparation used to dephosphorylate phosphate compounds (Polidase S; Schwarz Laboratories Inc., Mount Vernon, N.Y., U.S.A.) was purified by precipitation with ammonium sulphate according to the method of Cohen (1953).

In one experiment (see below) the phosphate areas from the two-dimensional chromatograms were eluted and rechromatographed before dephosphorylation. For this rechromatography, sheets of Whatman no. 4 paper were washed with 1.0% (w/v) oxalic acid, repeatedly washed in water and air-dried. The aqueous eluates of the phosphate areas of the parent chromatograms (concentrated where necessary) were applied to the origin positions of the washed papers and rechromatographed two-dimensionally with the same solvent systems. The papers were developed for 48 hr. in each solvent.

This resulted in improved separation of the sugar phosphates and revealed the presence of a compound tentatively identified as uridine diphosphate glucose (UDP-glucose) on the following grounds: (1) Polidase S was without effect on the compound. (2) The compound co-chromatographed exactly with UDP-glucose (located by ultraviolet photography). Insufficient of the radioactive compound was present to allow further tests to be carried out.

Preparation of cell-free extracts. Methane- or methanol-grown bacteria were suspended in 10 mM-phosphate buffer, pH 7.5, in the proportion of 1 g. wet wt. of bacteria/2 ml. of buffer and disintegrated by two successive extrusions from

a precooled French pressure cell (American Instrument Co., Washington, D.C., U.S.A.) operating at 9000–11000 lb./in.². The resulting extracts were centrifuged for 15 min. at 25000g at 2°.

Enzymic activities in cell-free extracts. Carboxydismutase was assayed by Dr I. O. Walker by the procedure of Weissbach, Horecker & Hurwitz (1956), by using purified D-glyceraldehyde 3-phosphate-NAD oxidoreductase (phosphorylating) (EC 1.2.1.12) and ATP-D-3-phosphoglycerate 1-phosphotransferase (EC 2.7.2.3) obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase were assayed as described by Johnson & Quayle (1964).

Preparation of buffers. Phosphate buffers were prepared by mixing 0.2 M-Na₂HPO₄ and 0.2 M-KH₂PO₄ to give the required pH values.

RESULTS

Analysis of the pathways of incorporation of labelled substrates into cell constituents

Incorporation of [¹⁴C]methane by methane-grown Pseudomonas methanica. The results of the experiment described in the Materials and Methods section are given in Table 1. They show that at the earliest time virtually the only non-volatile compounds labelled are phosphorylated compounds. The curves obtained by plotting against time the percentage of the total radioactivity fixed that was contributed by each constituent of the samples are shown in Fig. 2. A pronounced initially-negative slope is shown by the curve for phosphorylated compounds (other than triose phosphate, which is separated from the total phosphate area in the two-dimensional chromatographic system employed). The curve for triose phosphate itself shows a negative slope, although the amount of

Table 1. *Distribution of radioactivity among the non-volatile labelled components of the ethanol-soluble fractions of methane-grown Pseudomonas methanica incubated with [¹⁴C]methane*

[¹⁴C]Methane (3 mg., 500 µc) was added at zero time to a 10 ml. suspension of bacteria (5 mg. dry wt./ml.) in 10 mM-phosphate buffer, pH 7.0, containing ammonium chloride (10 mM), as described in the Materials and Methods section. Samples (approx. 1 ml.) were removed at known times into 4 ml. of aq. 95% (v/v) ethanol, centrifuged and the precipitates extracted with 0.4 ml. of aq. 20% (v/v) ethanol. The combined extracts were evaporated and resuspended in 0.25 ml. of 20% (v/v) ethanol, and 0.1 ml. of each sample was analysed by chromatography and radioautography.

Radioactivity (counts/min.)

Time (sec.)							Unknown compounds	Total
	Triose phosphate	Other phosphates	Glycine	Serine	Glutamate	Aspartate		
11	21	296	—	—	—	—	19	336
21	26	424	—	—	—	—	15	465
35	30	583	—	—	—	39	36	688
50	38	1038	—	20	12	104	—	1221
75	46	879	—	55	29	136	6	1156
94	38	1051	—	29	36	217	32	1403
113	59	1874	19	66	88	456	92	2651
152	57	1630	8	152	24	430	165	2465

Fig. 2. Variation with time of the percentage distribution of ^{14}C incorporated from $[^{14}\text{C}]\text{methane}$ into the constituents of the ethanol-soluble fraction of *P. methanica* growing on methane. ■, Phosphorylated compounds other than triose phosphate; ▼, triose phosphate; ○, glycine+serine+glutamate; ●, aspartate.

radioactivity incorporated in this compound was very small and does not permit an accurate evaluation of the slope of this curve. The curves for all the other compounds identified showed positive slopes; these were serine, glycine, glutamate and aspartate.

In Table 2 values are given of an analysis of the total phosphate areas of the parent chromatograms. In the first sample more than 70% of the total radioactivity incorporated appeared in glucose phosphate and fructose phosphate. This percentage decreased with time, rapidly for fructose phosphate and less rapidly for glucose phosphate. Phosphoglycerate acquired an increasing percentage of the total radioactivity incorporated. The only other identified compound present was ribose phosphate, which contained a constant low percentage of the total radioactivity incorporated.

The second incubation performed with live cells and $[^{14}\text{C}]\text{methane}$ yielded results identical with those just described, whereas the boiled cells incorporated no measurable radioactivity, showing that the radioactive methane contained no non-volatile impurities and also that no non-enzymic methane fixation occurred.

Table 2. Distribution of radioactivity among the labelled phosphates obtained from the ethanol-soluble fractions of suspensions of methane-grown *Pseudomonas methanica* incubated with $[^{14}\text{C}]\text{methane}$

The phosphorylated areas from the chromatograms described in Table 1 were eluted, concentrated to 0.2 ml. and incubated at 30° overnight with phosphatase from Polidase S. The resulting dephosphorylated non-volatile compounds were analysed by two-dimensional chromatography. Values in parentheses represent the percentages of total radioactivity on the chromatogram present in that particular compound.

Time (sec.)	Radioactivity (counts/min.)					Total
	Glucose	Fructose	Ribose	Glycerate	Unknown compounds	
11	87 (37.8)	89 (38.6)	—	40 (17.4)	14 (6.1)	230
21	154 (34.0)	108 (23.9)	20 (4.4)	155 (34.3)	15 (3.3)	452
35	281 (34.0)	150 (18.0)	49 (6.0)	287 (35.0)	56 (6.8)	823*
50	318 (39.3)	171 (21.1)	16 (2.0)	304 (37.6)	—	809
75	236 (37.0)	138 (21.7)	21 (3.0)	239 (37.5)	—	634
94	266 (31.0)	151 (17.6)	24 (2.8)	412 (48.1)	4 (0.5)	857
113	483 (33.6)	251 (17.4)	42 (2.9)	663 (46.1)	—	1439
152	546 (36.6)	250 (16.7)	41 (2.8)	629 (42.1)	27 (1.8)	1493

* In this sample the phosphate area of the parent chromatogram was lost, so a 0.1-ml. sample of the original reaction mixture was treated with phosphatase from Polidase S and chromatographed as described above.

Table 3. Distribution of radioactivity among the non-volatile labelled components of the ethanol-soluble fractions of methane-grown *Pseudomonas methanica* incubated with [¹⁴C]methanol

Radioactivity (counts/min.)										
Time (sec.)	Triose phosphate					Glycine	Glutamate	Malate	Alanine	
	Phosphates	Aspartate	Citrate	Serine	Aspartate				Succinate + fumarate	Other compounds
9	11 279	434	—	48	9	6	48	71	—	89
24	25 790	453	—	224	15	15	370	503	132	1189
37	35 895	824	67	415	94	148	1023	1055	479	1448
75	49 483	1042	128	840	470	745	3539	3393	2318	6428
162	28 050	840	144	1040	983	3760	2733	5958	1995	8677
										Total
										12 170
										29 903
										44 064
										75 148
										60 250

[¹⁴C]Methanol (15 μmoles; 100 μC) was added at zero time to 5 ml. of a suspension of bacteria (5.4 mg. dry wt./ml.) in 10 mm-phosphate buffer, pH 7.0, containing ammonium chloride (10 mm), gassed with methane + air (50:50) as described in the Materials and Methods section. Samples (approx. 1 ml.) were removed at known times and treated as described in Table 1.

Fig. 3. Variation with time of the percentage distribution of ¹⁴C incorporated from [¹⁴C]methanol into the constituents of the ethanol-soluble fraction of *P. methanica* growing on methane. ■, Phosphorylated compounds other than triose phosphate; ○, glycine + serine + glutamate; ▼, triose phosphate; ●, aspartate.

Incorporation of [¹⁴C]methanol by methane-grown Pseudomonas methanica. The results of this experiment are recorded in Table 3. Over 90% of the radioactivity fixed at the earliest times was found in phosphorylated compounds, this percentage falling with increasing time (Fig. 3). A small percentage of the total radioactivity fixed was found in aspartate, serine, glycine, glutamate, malate, alanine, succinate and fumarate. The area containing phosphates was eluted from the chromatogram, dephosphorylated with the phosphatase from Polidase S and rechromatographed in the same solvent system. The total radioactivity recovered in non-volatile compounds was substantially less than that present in the original phosphate area of the parent chromatograms (Table 4). It is not known at present how much of these losses is due to the presence of phosphates of volatile compounds. Of the non-volatile compounds detected, fructose contained the greatest percentage of the radioactivity at the earliest times. This percentage decreased with time. Glycerate contained relatively small amounts of radioactivity.

Apart from the relatively lower labelling of phosphoglycerate observed in this experiment, the overall pattern obtained is very similar to that of the previous experiment involving [¹⁴C]methane incorporation.

Incorporation of [¹⁴C]methanol by methanol-grown Pseudomonas methanica. The results obtained with methanol-grown cells are shown in Tables 5

Table 4. *Distribution of radioactivity among the labelled phosphates obtained from the ethanol-soluble fractions of methane-grown Pseudomonas methanica incubated with [¹⁴C]methanol*

The phosphorylated compounds were eluted from the chromatograms described in Table 3, and the eluates concentrated to approx. 0.2 ml. and incubated overnight at 30° with phosphatase from Polidase S (see the Materials and Methods section). The resulting non-volatile dephosphorylated compounds were analysed by two-dimensional paper chromatography and located by radioautography. Values in parentheses represent the percentages, present in the particular compounds, of the total radioactivity on the chromatogram.

Time (sec.)	Radioactivity (counts/min.)						Total
	Glucose	Sedoheptulose	Fructose	Ribose	Glycerate	Unknown compounds	
9	2311 (31.2)	996 (13.4)	3129 (42.2)	219 (2.9)	292 (3.9)	459 (6.2)	7406
24	6580 (36.3)	2422 (13.3)	6050 (33.3)	675 (3.7)	1730 (9.5)	686 (3.8)	18143
37	6687 (30.6)	5507 (25.2)	6279 (28.7)	891 (4.1)	1360 (6.2)	1125 (5.1)	21849
75	14393 (42.4)	4746 (14.0)	9647 (28.4)	1372 (4.0)	1646 (4.8)	2149 (6.3)	33953
162	8942 (46.9)	2412 (12.6)	2600 (13.6)	1434 (7.5)	1361 (7.1)	2306 (12.1)	19055

Table 5. *Distribution of radioactivity among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown Pseudomonas methanica incubated with [¹⁴C]methanol*

[¹⁴C]Methanol (15 μ moles, 100 μ C) was added at zero time to 5 ml. of a bacterial suspension (5 mg. dry wt./ml.) in 0.5% (v/v) methanol in 10 mM-phosphate buffer, pH 7.0, containing ammonium chloride (10 mM). Samples (approx. 1 ml.) were removed by hypodermic syringe and treated as described in Table 1. The residues after evaporation were resuspended in 0.3 ml. of 20% (v/v) ethanol and 0.15 ml. of each sample was used for chromatographic analysis.

Time (sec.)	Radioactivity (counts/min.)									Total
	Triose	Other phosphorylated compounds	Glycine	Serine	Glutamate	Aspartate	Malate	Citrate	Alanine	
16	11	215	—	—	—	7	19	—	—	263
27	44	762	—	—	—	5	79	23	—	964
47	73	1212	—	—	33	82	136	36	—	1648
69	126	2346	—	40	170	215	254	100	85	3476
85	111	2717	—	47	174	240	316	124	145	4381

and 6 and Fig. 4, which show that the distribution of radioactivity is very similar to that observed in the experiments described above. One difference is that considerably less radioactive phosphoglycerate was produced: less than 2% of the total radioactivity incorporated was found in this compound, whereas when methane-grown cells were incubated with [¹⁴C]methane phosphoglycerate contained approx. 15% of the total radioactivity incorporated at the earliest time of sampling.

Incorporation of [¹⁴C]bicarbonate by methane- and methanol-grown Pseudomonas methanica. The analysis of the incorporation of ¹⁴C from NaH¹⁴CO₃ by methane-grown cells is given in Table 7 and Fig.

5. These show a pattern that is completely different from that obtained when incorporation of [¹⁴C]-methane or [¹⁴C]methanol is studied. Phosphorylated compounds did not become radioactive until a later stage of the experiment, and at earliest times most of the incorporated radioactivity was found in aspartate (80%), malate (15%) and citrate (5%). The curve for aspartate showed a negative slope. No radioactive serine was formed during the experiment, and only a little radioactive glycine appeared in the last two samples. Glutamate contained radioactivity at 24 sec. and its curve showed a positive slope thereafter.

When this experiment was performed with

Table 6. *Distribution of radioactivity among the labelled phosphates obtained from the ethanol-soluble fractions of suspensions of methanol-grown Pseudomonas methanica incubated with [14C]methanol*

The remaining 0.15 ml. samples of the ethanolic solutions obtained as described in Table 5 were chromatographed on oxalate-washed papers (see the Materials and Methods section). The separated phosphate areas were eluted, concentrated to approx. 0.2 ml. and incubated overnight with phosphatase from Polidase S. The resulting non-volatile dephosphorylated compounds were identified by co-chromatography. Values in parentheses represent the percentages, present in the particular compounds, of the total radioactivity on the chromatogram.

Time (sec.)	Radioactivity (counts/min.)							
	Glucose	Fructose	Ribose	Glycerate	Mannose	Uridine nucleotide*	Sedoheptulose	Unknown compounds Total
16	91 (42.2)	99 (46.0)	—	5 (2.3)	—	8 (3.7)	—	12 (3.7) 215
27	267 (39.6)	324 (48.2)	16 (2.4)	14 (2.1)	—	30 (4.5)	—	20 (3.0) 671
47	262 (27.6)	354 (37.2)	49 (5.2)	59 (6.2)	39 (4.1)	86 (9.0)	—	101 (10.6) 950
69	385 (31.3)	436 (35.6)	44 (3.6)	29 (2.4)	165 (13.4)	45 (3.7)	62 (5.0)	57 (4.6) 1223
85	491 (26.6)	681 (37.0)	36 (2.0)	35 (2.0)	164 (8.9)	97 (5.3)	174 (9.5)	159 (8.6) 1837

* Probably UDP-glucose (see the Materials and Methods section).

Fig. 4. Variation with time of the percentage distribution of ¹⁴C incorporated from [¹⁴C]methanol into the constituents of the ethanol-soluble fraction of *P. methanica* growing on methanol. ■, Phosphorylated compounds other than triose phosphate; ▼, triose phosphate; ○, glycine + serine + glutamate; ●, aspartate; □, malate; ▽, alanine.

methanol-grown cells the total incorporation of radioactivity obtained was small and was found to be mainly in aspartate with a little in malate and citrate. Insufficient incorporation occurred in the early samples to allow the results to be plotted graphically, but it was clear that the pathway of

incorporation corresponded to that observed in methanol-grown cells.

Absence of carboxydismutase in cell-free extracts of the organisms. Assays of cell-free extracts of methane-grown *P. methanica* prepared in the French pressure cell failed to demonstrate the presence of carboxydismutase under any conditions.

DISCUSSION

The findings described in the Results section confirm the tentative conclusion of Leadbetter & Foster (1958) that the growth of *P. methanica* on methane is heterotrophic rather than autotrophic. The same conclusion can now also be drawn for the growth of this organism on methanol as carbon source. There is only one feature of the findings that might initially suggest that an autotrophic metabolism was operating, namely the very rapid incorporation of radioactivity from either [¹⁴C]methane or [¹⁴C]methanol into phosphorylated compounds. However, that this must only be a superficial resemblance to the incorporation patterns obtained in similar experiments with autotrophic organisms is consequent on the following facts: (a) The composition of the labelled phosphates is not typical of autotrophic metabolism. In all three experiments phosphoglycerate contained less than 17% of the radioactivity fixed in phosphates at early times. This may be contrasted with values of over 70% for photosynthetic autotrophs (Calvin, 1951) or 80% for *Pseudomonas oxalaticus* growing

Table 7. Distribution of radioactivity among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methane-grown *Pseudomonas methanica* incubated with [^{14}C]-bicarbonate

The $\text{NaH}^{14}\text{CO}_3$ (100 μmoles ; 200 μC) was added at zero time to 10 ml. of a bacterial suspension (7.2 mg. dry wt./ml.) in 10 mM-phosphate buffer, pH 7.0, containing ammonium chloride (10 mM), vigorously aerated with methane+air (50:50) mixture. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as described in Table 1.

Time (sec.)	Radioactivity (counts/min.)						Total phosphate	Unknown compounds	Total
	Glycine	Serine	Glutamate	Aspartate	Malate	Citrate			
7	—	—	—	213	38	13	—	—	264
15	—	—	—	372	59	25	—	6	462
23	—	—	87	372	58	30	—	—	547
29	—	—	140	476	75	39	—	—	730
39	—	—	209	469	71	34	58	6	852
51	—	—	264	648	101	31	82	70	1196
74	44	—	402	600	123	20	99	134	1422
108	63	—	405	429	105	15	76	70	1166
152	136	—	578	356	93	—	125	94	1382

Fig. 5. Variation with time of the percentage distribution of ^{14}C incorporated from $\text{NaH}^{14}\text{CO}_3$ into the constituents of the ethanol-soluble fraction of *P. methanica* growing on methane. ■, Total phosphorylated compounds; ○, glutamate; ▲, glycine; ●, aspartate; □, malate; △, citrate.

autotrophically on formate (Quayle & Keech, 1959). This percentage rises with time in the *P. methanica* experiments, and falls in experiments with autotrophic organisms. (b) [^{14}C]Bicarbonate is mainly incorporated into aspartate and malate. An autotrophic metabolism would result in rapid labelling of phosphates. (c) The presence of the

key enzyme of autotrophic metabolism, carboxy-dismutase, could not be demonstrated in cell-free extracts of *P. methanica*.

Previous work on intact cells of *P. methanica* (Dworkin & Foster, 1956; Brown & Strawinski, 1958) has indicated that methane is oxidized via methanol, formaldehyde and formate, and enzymes capable of catalysing these reactions have been demonstrated in cell-free extracts of *P. methanica* (Harrington & Kallio, 1960; Johnson & Quayle, 1964). Since the same pattern of isotopic incorporation results from the administration of [^{14}C]methane or [^{14}C]methanol to the organism while it is growing on methane, and a different pattern results from exposure to [^{14}C]bicarbonate, it follows that C_1 units are assimilated at reduction level(s) between methanol and formate by one pathway, and as carbon dioxide by another. It may be, as with other heterotrophic organisms, that incorporation of carbon dioxide is concerned mainly with the synthesis of C_4 compounds from C_3 compounds by established enzymic reactions, whereas the incorporation of reduced C_1 units into sugar phosphates is part of an unknown pathway for the necessary net synthesis of C_3 compounds from the C_1 substrate. It has not proved practicable to conduct tracer experiments with [^{14}C]formaldehyde owing to its chemical reactivity and toxicity.

Further information is needed before speculating on the nature of the biosynthetic pathway that apparently results in incorporation of reduced C_1 units into sugar phosphates. Nevertheless, the pattern of isotopic incorporation is so distinct from that encountered with methanol- and formate-grown *Pseudomonas* AM1 and methanol-grown *Hyphomicrobium vulgare*, where serine is initially

the most rapidly labelled compound formed from radioactive substrate (Large *et al.* 1961), that the possibility of there being some diversity in mechanisms of synthesis of cell constituents from C₁ units becomes rather likely, even among organisms not adopting an autotrophic metabolism, e.g. *P. oxalaticus* when growing on formate (Quayle & Keech, 1959).

We thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement, and Miss A. West and Mrs J. Poole for their technical assistance. This work was supported in part by the Rockefeller Foundation and the U.S. Public Health Service and also by the U.S. Air Force under Grant no. AF-EOAR 62-45 through the European Office, Office of Aerospace Research.

REFERENCES

- Brown, L. R. & Strawinski, R. J. (1958). *Bact. Proc.* p. 122.
Calvin, M. (1950-1951). *Harvey Lect.* **46**, 218.
Cohen, S. S. (1953). *J. biol. Chem.* **201**, 71.
Dworkin, M. & Foster, J. W. (1956). *J. Bact.* **72**, 646.
Harrington, A. A. & Kallio, R. E. (1960). *Canad. J. Microbiol.* **6**, 1.
Johnson, P. A. & Quayle, J. R. (1964). *Biochem. J.* **93**, 281.
Large, P. J., Peel, D. & Quayle, J. R. (1961). *Biochem. J.* **81**, 470.
Leadbetter, E. & Foster, J. W. (1958). *Arch. Mikrobiol.* **30**, 91.
Quayle, J. R. & Keech, D. B. (1959). *Biochem. J.* **72**, 623.
Söhngen, N. L. (1906). *Zbl. Bakt. (Abt. II)*, **15**, 513.
Weissbach, A., Horecker, B. L. & Hurwitz, J. (1956). *J. biol. Chem.* **218**, 795.