Microbial Growth on C₁ Compounds

2. SYNTHESIS OF CELL CONSTITUENTS BY METHANOL- AND FORMATE-GROWN *PSEUDOMONAS* AM 1, AND METHANOL-GROWN *HYPHOMICROBIUM VULGARE*

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(Received 28 April 1961)

The preceding paper (Peel & Quayle, 1961) described the isolation of an organism, *Pseudomonas* AM 1, which can grow on methanol or formate as sole sources of carbon and energy. The present paper describes an investigation of the path of carbon incorporation from [¹⁴C]methanol, [¹⁴C]formate, and [¹⁴C]bicarbonate into cell constituents during growth of this organism on methanol or formate. Similar experiments, involving [¹⁴C]methanol and [¹⁴C]bicarbonate, have been performed with methanol-grown *Hyphomicrobium vulgare*, and the data obtained with the two organisms are compared.

METHODS

Maintenance and growth of the organisms. The maintenance and growth of cultures of *Pseudomonas* AM 1 have been described (Peel & Quayle, 1961).

A culture of *H. vulgare* was a gift from Dr R. Näveke (Staatsinstitut für Allgemeine Botanik, Jungiusstrasse 6, Hamburg 36, Germany). This organism was maintained on agar slopes, containing 0.1 M-methylamine hydrochloride as carbon source, and grown in liquid culture on methanol (0.5%, v/v), in similar media to those used for *Pseudomonas* AM 1. With the liquid culture, the growth flasks were not shaken, and the organism was harvested after about 2 weeks. *H. vulgare* grew very slowly as a slime on the internal glass surface of the culture flask, and was harvested by scraping it off the glass and centrifuging the resulting suspensions.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. [¹⁴C]Methanol was supplied in a sealed tube under vacuum. One end of the tube was cooled in liquid air and the other end was then opened. Water was added to the methanol whilst the tube was still in liquid air. After slow thawing, the solution of [¹⁴C]methanol was transferred to a stoppered tube and stored at -10°.

Ba¹⁴CO₃ was decomposed with an excess of 2N-HClO₄ and the liberated ¹⁴CO₂ was trapped in the theoretical quantity of NaOH by using a transfer unit similar to that described by Aronoff (1956).

Sodium [¹⁴C]formate was chromatographed on Whatman no. 4 filter paper with a solvent system (Long, Quayle & Stedman, 1951) consisting of ethanol-aq. NH₃ soln. (sp.gr. 0.88)-water (80:4:16, v/v). After development, the papers were sprayed with 0.5 M-K₂HPO₄ and dried in air, and the formate area was located by radioautography. The radioactive area was eluted with water into one arm of a transfer unit (Aronoff, 1956) and the formic acid was liberated with

5N-H₂SO₄ and distilled *in vacuo* into the theoretical quantity of NaOH solution.

Measurement of specific activity of radioactive bacterial cells. The specific activity of cells after growth on radioactive substrates was measured by two different methods, outlined below:

(a) Direct plating of bacterial cells. Samples of the growing culture were withdrawn at intervals, their cell density was measured with a nephelometer (Evans Electro-selenium Ltd., Halstead, Essex), and the suspensions of cells were centrifuged at 0°. The supernatant solutions were poured off and each sample of the cells was resuspended in 0.5 ml. of water; samples (0.1-0.2 ml. containing < 0.6 mg. dry wt.) of the resuspended cells were plated on glass planchets (3 cm.² area) on a rotating turntable in a stream of warm air. Each planchet was washed with 0.1 ml. of formic acid (90% w/v; A.R.) and dried, and the radioactivity assayed, unless stated otherwise, with a gas-flow counter (Model D-47, Nuclear Instrument and Chemical Corp., Chicago, U.S.A.). A blank determination showed that a negligible amount of [¹⁴C]methanol remained on the planchets after this treatment.

(b) Combustion of bacterial cells. The cells were washed three times with aq. 0.9% KCl to remove exogenous radioactive compounds and were then totally oxidized to CO₂ in the combustion apparatus described by Sakami (1955a). The evolved CO₂, collected as Na₂¹⁴CO₃ in 2.5N-NaOH, was converted into Ba¹⁴CO₃ by precipitation with aq. 5% (w/v) BaCl₂. The Ba¹⁴CO₃ was plated on paper disks (2.69 cm.² area) as described by Sakami (1955b), assayed for radioactivity in the gas-flow counter, and corrected for self-absorption.

Bacterial incorporation of [¹⁴C]methanol, [¹⁴C]formate and [¹⁴C]bicarbonate. Cultures of bacteria in the logarithmic growth phase were centrifuged at room temperature and resuspended in solutions containing the growth substrate, buffer and, in some cases, other salts (see text) to give a concentration of about 3 mg. dry wt. of bacteria/ml. With *H. vulgare* it was not practicable to ascertain if the cells were in the logarithmic phase of growth when harvested, nor could the cell density be estimated nephelometrically. Instead, the harvested organism was pressed between filter paper to remove excess of water and then weighed out directly into the incubation mixtures to give a final concentration corresponding to about 3 mg. dry wt./ml.

Portions (9-10 ml.) of these bacterial suspensions were placed at 30° in cylindrical vessels (10 cm. × 2.5 cm. diam.) fitted with sintered-glass bottoms and vigorously aerated by blowing a stream of moist air through the sintered glass. To each suspension was added 200 μC of ¹⁴C, and 1 ml. samples were withdrawn with a hypodermic syringe at suitable time intervals over 5 min. All samples were

transferred immediately to 3 ml. of boiling aq. 95% (v/v) ethanol. The ethanolic suspensions of killed cells were centrifuged and the resulting precipitates extracted with 0.5 ml. of boiling aq. 20% (v/v) ethanol. The combined supernatants were evaporated to dryness at 60° under N_2 , taken up in 0.1 ml. of aq. 20% (v/v) ethanol and applied to the origin position of paper chromatograms.

Chromatographic analysis and radioautography. The papers (Whatman no. 4; 18½ in. × 22 in.) were developed two-dimensionally in a descending direction, by using first the solvent system of Kornberg (1958): phenol-90% (w/v, A.R.) formic acid-water (500 g.: 13 ml.: 167 ml.). The solvent system *n*-butan-1-ol-propionic acid-water (47:22:31, by vol.; Benson *et al.* 1950) was used to develop the chromatogram in the second dimension. Both solvents were allowed to run to the edge of the paper.

Radioautographs were made from the chromatograms by the procedure developed by Calvin and his co-workers (see Calvin, 1950-51), with Blue Brand X-ray film (Kodak Ltd.). The radioactive areas located on the paper were assayed for radioactivity directly with a mica end-window Geiger-Müller tube (General Electric Co.; type 2B2) and the count was corrected for background activity.

Under the conditions of chromatography used, glutamic acid, serine and glycine did not usually separate from each other. The radioactive area containing them was cut out, eluted with water, and the amino acids were separated by further chromatography or electrophoresis (see below). The proportion of each amino acid in the radioactive area on the parent chromatogram was assumed to be that found after separation, even though losses of total count occurred during the overall procedure.

Identification of labelled compounds. The radioactive compounds were eluted from the chromatograms with water and subjected to co-chromatography or co-electrophoresis with authentic samples of the suspected compound. Exact matching of the resulting radioactive area and the coloured area developed with an appropriate spray reagent was necessary for identification. Carboxylic acids were re-chromatographed either in the original solvent systems or ethanol-aq. NH_3 soln. (sp.gr. 0.88)-water (80:4:16, by vol.; Long *et al.* 1951), or subjected to high-voltage electrophoresis at 100 v/cm. in 0.5 M-acetic acid adjusted to pH 4.0 with pyridine (Gross, 1959). Amino acids were either re-chromatographed in butan-1-ol-acetone-water-diethylamine (40:40:20:8, by vol.; Hardy, Holland & Nayler, 1955) or subjected to high-voltage electrophoresis at 100 v/cm. in 0.75 M-formic acid (pH 2.0; Gross, 1959). Both these methods separated glutamic acid, glycine and serine from each other; the electrophoretic system also separated aspartic acid from glutamic acid. Dephosphorylation of phosphorylated compounds was carried out by incubation of the eluted compounds with Polidase-S (Schwarz Laboratories, Inc., New York, U.S.A.) according to the procedure of Benson, Bassham & Calvin (1951), and the resulting compounds were chromatographed two-dimensionally in water-saturated phenol and butanol-propionic acid-water (47:22:31, by vol.; Benson *et al.* 1950).

Trehalose was identified as follows: (a) by co-electrophoresis with authentic trehalose in 0.05 M-sodium borate at 100 v/cm. for 30 min. (Cabib & Leloir, 1958); (b) by its partial hydrolysis in *N*-HCl at 100° for 30 min., which yielded glucose as the sole product; (c) by admixture with authentic trehalose, after which the specific radioactivity

of the resulting material remained constant over two successive recrystallizations from aq. 80% (v/v) ethanol.

Sprays. Carboxylic acids were located on chromatograms by spraying with a solution (0.2%, w/v) of bromocresol green in ethanol and adjusting to pH 7 with aq. NH_3 solution; amino acids by spraying with 0.2% (w/v) ninhydrin in ethanol containing 0.1% (v/v) of collidine, followed by heating at 100°; aldose sugars by spraying with a solution of aniline (0.9 ml.) and phthalic acid (1.7 g.) in 100 ml. of water-saturated butanol, followed by heating at 105° (Partridge, 1949); fructose by spraying with 1% (w/v) naphtharesorcinol in ethanol-2*N*-HCl (10:90, v/v; Forsyth, 1948) followed by heating to 90° for 10 min.; sedoheptulose by spraying with a solution of orcinol (0.5 g.) and trichloroacetic acid (15 g.) in 100 ml. of water-saturated butanol, followed by heating at 100° (Klevstrand & Nordal, 1950); trehalose by spraying first with $AgNO_3$ in aq. acetone, followed by 0.5*N*-NaOH in aq. ethanol (Trevelyan, Procter & Harrison, 1950).

Preparation of cell-free extracts. Cell-free extracts of methanol-grown *Pseudomonas* AM1 were prepared either by crushing frozen bacteria in a Hughes (1951) press, or by ultrasonic disintegration. For formate-grown *Pseudomonas* AM1 and methanol-grown *H. vulgare*, the method of ultrasonic disintegration was not used. For crushing the cells, 1 g. wet wt. of bacteria was suspended in 2 ml. of 30 mM-tris buffer, pH 7.5, containing about 200 µg. each of crystalline ribonuclease and deoxyribonuclease (L. Light and Co. Ltd., Colnbrook, Bucks.). The suspension was crushed in a Hughes press which had been cooled to -25°. For preparing sonic extracts, 1 g. wet wt. of cells was suspended in 5 ml. of solution containing 100 m-moles of cysteine and 150 m-moles of tris buffer, pH 7.8. This suspension was cooled to 0° and subjected for 2 min. to the output at 25 kcyc./sec. of a 600 w Mullard magnetostriktor oscillator. The extracts resulting from both processes of cell breakage were either used as such, or after centrifuging at 25000g for 10 min. in a refrigerated centrifuge.

Protein determinations. Protein was estimated by the method of Folin & Ciocalteu (1927).

Chemicals. Ribulose 1:5-diphosphate was prepared by incubating ribose 5-phosphate and ATP with purified phosphoriboisomerase and phosphoribulokinase prepared from spinach according to the method of Horecker, Hurwitz & Weissbach (1956).

RESULTS

Effect of flushing Pseudomonas AM1 with carbon dioxide-enriched air while growing on ^{14}C -labelled substrates

In order to estimate the amount of cellular carbon derived from the growth substrate which had passed through a stage readily exchangeable with carbon dioxide during growth, two cultures of the organism were grown on each ^{14}C -labelled substrate. The $^{14}CO_2$ derived from oxidation of the substrate was removed from one culture of each pair by passing a stream of air-carbon dioxide (99:1, v/v) through the flask. The remaining flask of each pair was flushed with carbon dioxide-free air. The specific radioactivity of the cells in each culture was then measured and compared.

Attempts to perform this type of experiment with formate-grown *Pseudomonas* AM1 were not successful, owing to difficulties experienced in growing the cells in a stream of carbon dioxide-free air. The experiment was not attempted with *H. vulgare*, owing to its very slow rate of growth.

Growth on [^{14}C]methanol. Growth medium (400 ml.) containing [^{14}C]methanol (100 m-moles; 75 μC of ^{14}C) as carbon source was inoculated with *Pseudomonas* AM1. The suspension was divided equally between two conical culture flasks, one of which was flushed with carbon dioxide-free air and the other with carbon dioxide-air (1:99, v/v). The flasks were shaken at 30° and the bacteria were grown through about four generations, as determined nephelometrically. Samples were withdrawn at intervals, and the specific radioactivity of the cells was determined by direct plating on glass planchets followed by assay of radioactivity with a lead-shielded, end-window Geiger-Müller tube. The specific activity of the cell material rose to a constant value in each case, and remained constant during two generations of growth (Fig. 1). The cells grown in air reached an activity of 5.4 counts/min./ μg . dry wt. of cell material. Those

grown in air-carbon dioxide reached an activity of 2.7 counts/min./ μg . dry wt. The experiment was repeated, with [^{14}C]methanol of higher specific activity (50 m-moles; 500 μC of ^{14}C), and this time the specific activity of the cells was measured as $\text{Ba}^{14}\text{CO}_3$ derived from the cells by total combustion. The specific activity of the cells grown in air was found to be 6090 counts/min./mg. of $\text{Ba}^{14}\text{CO}_3$, a figure reduced to 3330 counts/min./mg. of $\text{Ba}^{14}\text{CO}_3$ in the cells grown in air-carbon dioxide.

The results from these experiments, which are in close agreement, indicate that at least 50 % of the carbon entering the cells from methanol passed through a stage exchangeable with exogenous carbon dioxide.

Growth on [^{14}C]succinate. The previous experiment was repeated with cultures of *Pseudomonas* AM1 growing on uniformly labelled [^{14}C]succinate. The specific radioactivity of the cells was measured by total combustion to $^{14}\text{CO}_2$, which was assayed as $\text{Ba}^{14}\text{CO}_3$. Flushing the cells with air-carbon dioxide (99:1), instead of air, reduced the specific activity of the cells from 375 to 349 counts/min./mg. of $\text{Ba}^{14}\text{CO}_3$, i.e. a reduction of 7 %.

Analysis of path of labelled substrates into cell constituents

Incorporation of [^{14}C]methanol and [^{14}C]bicarbonate by methanol-grown *Pseudomonas* AM1. A culture of bacteria in the logarithmic growth phase was centrifuged and resuspended in a solution of 20 mM-methanol in 10 mM-tris buffer (pH 7.8) to give a concentration of 3 mg. dry wt. of bacteria/ml. Two 10 ml. portions of this suspension were then used for incubation with [^{14}C]methanol and [^{14}C]bicarbonate respectively. Chromatographic analysis, followed by radioautography, showed that radioactivity from either tracer rapidly appeared in a large number of compounds. The distribution of radioactivity among the labelled compounds formed during the first 2 min. after the addition of isotope is shown in Tables 1 and 2. The curves obtained by plotting against time the percentage of the total radioactivity fixed which was contributed by each constituent of the samples are shown in Figs. 2 and 3. With [^{14}C]methanol the only curves clearly showing a negative slope immediately after the introduction of isotope are those of aspartate and serine, the latter containing the majority of the radioactivity in the first sample. The initial slope of the glycine curve may also be negative, although the very small amount of radioactivity appearing in this compound does not permit an accurate evaluation of this. Other compounds such as malate, phosphorylated compounds, citrate, glutamate and the disaccharide, trehalose, acquired initially increasing percentages of the total radioactivity fixed. At the end of 2 min., trehalose

Fig. 1. Effect of flushing *Pseudomonas* AM1 with CO_2 -enriched air during growth on [^{14}C]methanol. The specific activity of the cellular material was determined by direct plating on glass planchets. The experimental conditions are described in the text. Δ , $\blacktriangle$, Growth and specific radioactivity respectively of cells grown under CO_2 -free air; $\circ$, $\bullet$, growth and specific radioactivity respectively of cells grown under air- CO_2 (99:1).

contained the highest percentage of radioactivity. Chromatographic analysis of the compounds resulting from dephosphorylation of the phosphate area of the chromatogram showed a mixture of phosphates of glyceric acid, glucose, fructose, ribose and sedoheptulose to be present. For incubation of [^{14}C]bicarbonate with the organism, most of the count at early times was contained in malate. From 10 sec. onwards the malate curve showed a steep negative slope; whether the apparent rise in the curve between 4 and 10 sec. is of significance or is due to the errors attendant on the low fixation obtained in the earliest sample is not known at present. Both glycine and serine showed initially negative slopes, although the total count in either compound was relatively small. As with [^{14}C]methanol, radioactivity rapidly accumulated in trehalose at later times.

Incorporation of [^{14}C]formate and [^{14}C]bicarbonate by formate-grown Pseudomonas AM1. Similar experiments to those described above were performed on [^{14}C]formate and [^{14}C]bicarbonate incorporation by formate-grown *Pseudomonas* AM1. The distribution of radioactivity among the labelled compounds formed is shown in Tables 3 and 4, and the curves of percentage of total radioactivity fixed which was contributed by each constituent of the sample, plotted against time, are shown in Figs. 4 and 5. With [^{14}C]formate as tracer, the curves belonging to serine and malate show pronounced negative slopes initially, whereas the curves relating to phosphorylated compounds and, later, trehalose, show positive slopes (Fig. 4). The percentage of radioactivity contained in glycine remained approximately constant. When [^{14}C]bicarbonate was used (Fig. 5), only the curve relating to

Table 1. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown Pseudomonas AM1 incubated with [^{14}C]methanol*

[^{14}C]Methanol (38 μmoles ; 200 μC of ^{14}C) was added at zero time to a 10 ml. suspension of bacteria (30 mg. dry wt.) in 20 mM-methanol, 10 mM-tris buffer (pH 7.8), under vigorous aeration. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and transferred at known times into 3 ml. of hot ethanol. The precipitates were extracted with 1 ml. of aq. 20% (v/v) ethanol and the combined extracts analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window Geiger-Müller tube.

Time (sec.)	Radioactivity (counts/min.)									Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds	Other compounds	
4	9	0	18	0	6	0	0	5	9	47
9	22	0	23	0	6	0	0	9	6	66
14	52	0	37	0	10	0	0	29	11	139
20	100	11	38	0	11	7	0	56	35	258
24	101	10	41	12	12	8	12	81	44	321
30	133	9	71	0	16	14	84	135	72	534
46	90	17	48	11	18	31	75	188	91	569
60	57	17	47	30	22	56	137	191	154	711
122	167	50	60	200	19	74	614	372	599	2155

Table 2. *Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown Pseudomonas AM1 incubated with sodium [^{14}C]bicarbonate*

Sodium [^{14}C]bicarbonate (100 μmoles ; 200 μC of ^{14}C) was added at zero time to a 10 ml. suspension of bacteria (30 mg. dry wt.) under conditions identical with those described in Table 1.

Time (sec.)	Radioactivity (counts/min.)									Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds	Other compounds	
4	62	23	10	0	17	20	0	2	44	178
10	134	28	19	9	30	28	0	6	61	315
15	283	43	40	18	51	67	0	45	274	821
21	273	55	28	19	42	118	0	49	320	904
26	184	41	24	18	37	81	16	54	290	745
32	189	70	31	47	42	142	33	121	435	1110
55	127	46	35	132	62	302	198	185	471	1558
64	122	49	18	137	63	343	254	154	351	1491
121	358	109	59	494	63	617	1066	271	884	3921

malate showed an initially negative slope. Radioactive glycine was present at the earliest times, containing approximately 7% of the total radioactivity fixed.

Incorporation of [^{14}C]methanol and [^{14}C]bicarbonate by methanol-grown *Hyphomicrobium vulgare*. Similar experiments to those described above were performed on [^{14}C]methanol and [^{14}C]bicarbonate incorporation by methanol-grown *H. vulgare*. The distribution of radioactivity amongst the labelled compounds formed is shown in Tables 5 and 6, and the curves of percentage of total radioactivity fixed which is contributed by each constituent of the sample, plotted against time, are shown in Figs. 6 and 7.

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 *Pseudomonas* AM1, growing on methanol. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

With the [^{14}C]methanol incorporation, radioactive serine and glycollate contained most of the initially incorporated tracer, and the curves relating to both these compounds showed clear negative slopes throughout the experiment (Fig. 6). All other compounds showed initially positive slopes. The percentage of radioactivity contained in glycine fluctuated between 3 and 6% of the total. No trehalose was detected. When [^{14}C]bicarbonate was the isotope used (Fig. 7), the curves relating to malate and aspartate showed initially negative slopes; curves of other compounds (including glycine) showed positive slopes. No radioactive glycollate was detected.

Fig. 3. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]bicarbonate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on methanol. ●, Malate; ▲, serine; □, glycine; ▼, phosphorylated compounds; ×, trehalose.

Table 3. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of formate-grown Pseudomonas AM1 incubated with [^{14}C]formate*

Sodium [^{14}C]formate (32.7 μmoles ; 200 μC of ^{14}C) was added at zero time to 8 ml. of a suspension of bacteria (31 mg. dry wt.) in 20 mM-sodium formate, 50 mM-tris buffer, pH 7.65, 1 mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

Time (sec.)	Radioactivity (counts/min.)							Phosphorylated compounds	Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose		
7	19	5	39	11	0	0	0	0	74
14	28	16	84	38	0	18	0	52	236
21	8	11	67	8	30	26	0	31	181
38	3	16	45	36	26	40	44	139	349
63	9	12	80	95	73	77	68	392	806
84	11	16	104	142	51	103	103	576	1106
122	11	20	74	144	186	97	281	549	1362

Table 4. Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of formate-grown *Pseudomonas* AM1 incubated with [^{14}C]bicarbonate

Sodium [^{14}C]bicarbonate (5.9 μmoles ; 140 μC of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (28.3 mg. dry wt.) in 20 mM-sodium formate, 50 mM-tris buffer, pH 7.65, 1 mM-sodium phosphate and essential salts. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

Time (sec.)	Radioactivity (counts/min.)								Other compounds	Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Trehalose	Phosphorylated compounds		
4	47	8	0	3	43	0	0	0	20	121
14	82	20	0	7	80	15	0	0	65	269
21	76	29	0	8	145	31	0	0	57	346
27	121	46	0	16	266	62	0	58	50	619
50	54	21	0	40	96	78	0	72	34	395
75	56	15	0	86	186	79	36	114	30	602
107	38	13	0	105	170	78	82	165	47	698

Fig. 4. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]formate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on formate. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

Fig. 5. Variation with time of the percentage distribution of ^{14}C incorporated from [^{14}C]bicarbonate into the constituents of the ethanol-soluble fraction of *Pseudomonas* AM1 growing on formate. ●, Malate; △, aspartate; □, glycine; ■, glutamate; ▼, phosphorylated compounds; ×, trehalose.

Absence of carboxydismutase in cell-free extracts of the organisms. Cell-free extracts of methanol-grown *Pseudomonas* AM1 and *H. vulgare*, and formate-grown *Pseudomonas* AM1, prepared by crushing the frozen bacteria, were assayed for the presence of carboxydismutase by incubating the extracts with [^{14}C]bicarbonate in the presence or absence of ribulose 1:5-diphosphate (Quayle & Keech, 1960). In no case did the presence of the

ribulose diphosphate cause any appreciable stimulation of [^{14}C]bicarbonate fixation. Cell-free extracts of methanol-grown *Pseudomonas* AM1 were also prepared by ultrasonic disintegration and showed no activity in the same assay. The possibility that an inhibitor of carboxydismutase was present in the cell-free extracts was eliminated by the finding that the activity of this enzyme in extracts of formate-grown *Pseudomonas oxalaticus* (Quayle & Keech, 1959b) was unaltered in the presence of extracts of either *Pseudomonas* AM1 or *H. vulgare*.

Table 5. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown Hyphomicrobium vulgare, incubated with [^{14}C] methanol*

[^{14}C]Methanol (37 μmoles ; 200 μC of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (120 mg. wet wt.) in 10 mm-methanol, 10 mm-ammonium chloride and 10 mm-sodium phosphate, pH 7.0. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

Time (sec.)	Radioactivity (counts/min.)								Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Phosphorylated compounds	Glycolate	Other compounds
6	3	0	44	0	9	0	0	27	15
12	34	9	94	13	58	10	0	42	25
18	44	23	101	35	85	5	0	43	23
24	74	25	103	48	126	3	0	47	26
30	92	25	133	65	178	5	0	72	28
47	142	62	155	103	239	7	69	65	56
62	149	72	133	211	267	9	205	52	76
100	209	95	154	163	292	8	244	58	267

Table 6. *Distribution of radioactive carbon among the non-volatile labelled components of the ethanol-soluble fractions of suspensions of methanol-grown Hyphomicrobium vulgare, incubated with sodium [^{14}C]bicarbonate*

Sodium [^{14}C]bicarbonate (8.1 μmoles ; 200 μC of ^{14}C) was added at zero time to 9 ml. of a suspension of bacteria (120 mg. dry wt.) in 10 mm-methanol, 10 mm-ammonium chloride and 10 mm-sodium phosphate, pH 7.0. Samples (approx. 1 ml.) were withdrawn by hypodermic syringe and treated as in Table 1.

Time (sec.)	Radioactivity (counts/min.)								Total
	Malate	Glycine	Serine	Glutamate	Aspartate	Citrate	Phosphorylated compounds	Other compounds	
7	138	31	0	0	82	8	0	59	318
15	237	90	23	38	176	34	0	85	683
22	358	151	50	69	242	38	23	140	1071
29	371	216	82	125	380	47	58	200	1479
38	348	283	71	186	413	62	116	225	1704
52	338	333	84	376	510	76	162	305	2184
94	110	369	58	443	227	46	414	339	2006

DISCUSSION

The only known series of reactions which leads to the synthesis of polycarbon compounds from C_1 units is the ribulose diphosphate cycle of carbon dioxide fixation leading to triose phosphate synthesis, discovered by Calvin and his colleagues in photosynthetic tissue (Bascham *et al.* 1954). The cycle has since been found in many species of autotrophic bacteria (see Vishniac, Horecker & Ochoa, 1957; Quayle, 1961). The possibility that microbial growth on organic C_1 compounds might involve an autotrophic type of metabolism, in which the cellular carbon is synthesized from carbon dioxide, had already been foreseen (Bhat & Barker, 1948; van Niel, 1954). That such a mechanism actually operated, and involved the ribulose diphosphate cycle, was demonstrated by Quayle & Keech (1959*a, b*) with formate-grown *P. oxalaticus*. Hence, in any investigation of the method of microbial synthesis of cell constituents from C_1 compounds,

evidence for the operation of the ribulose diphosphate cycle should first be examined. The experiments reported in this paper eliminate the possibility of its operation in methanol- and formate-grown *Pseudomonas* AM1 and methanol-grown *H. vulgare*. This conclusion is based on the following two criteria: (a) the absence of a key enzyme, carboxydismutase, in cell-free extracts of the organisms; (b) the course of ^{14}C incorporation into cell constituents follows a totally different pattern from that observed in organisms in which the ribulose diphosphate cycle has been shown to operate. Such organisms show early labelling from $^{14}\text{CO}_2$ to occur predominantly in 3-phosphoglyceric acid, non-phosphorylated compounds becoming radioactive at a later time. In both *Pseudomonas* AM1 and *H. vulgare*, radioactivity from $^{14}\text{CO}_2$ is initially incorporated into C_4 acids and glycine, whereas phosphorylated compounds appear later. The slope of the curve obtained by plotting against time the percentage of the fixed radioactivity that was con-

Fig. 6. Variation with time of the percentage distribution of ^{14}C incorporated from $[^{14}\text{C}]$ methanol into the constituents of the ethanol-soluble fraction of *Hyphomicrobium vulgare* growing on methanol. ○, Glycollate; ●, malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds.

Fig. 7. Variation with time of the percentage distribution of ^{14}C incorporated from $[^{14}\text{C}]$ bicarbonate into the constituents of the ethanol-soluble fraction of *Hyphomicrobium vulgare* growing on methanol. ●, Malate; △, aspartate; ▲, serine; □, glycine; ■, glutamate; ▼, phosphorylated compounds.

tained in phosphorylated compounds is, in all cases, positive. This shows that these compounds are unlikely to be primary products of $^{14}\text{CO}_2$ fixation.

The conclusion that growth of *H. vulgare* on methanol does not involve an autotrophic metabolism is at variance with the conclusion reached by Nèveke & Engel (1955). These authors have summarized most of the work published on this organism and, while stating that it is not possible to decide whether the bulk of its carbon is fixed in the form of carbon dioxide or not, they list properties which they consider as constituting indirect evidence for the organism's being regarded as an autotroph: (a) slow growth (Mevius, 1953); (b) no growth on glucose, sucrose, mannose, or raffinose (Mevius, 1953); (c) the presence of $^{14}\text{CO}_2$ during growth leads to radioactivity appearing in amino acids isolated from the organism (Mevius, 1953; Nèveke, 1957); (d) the organism oxidizes formate and oxalate to carbon dioxide and water and releases 85–95% of the energy as heat (Korte & Engel, 1955). Since the nitrifying bacteria oxidize inorganic nitrogen compounds with the release of 93–95% of the available energy as heat, the authors state that the similarity in formate or oxalate oxidation by *H. vulgare* leads to the probability

that these substrates serve only as energy sources and not as carbon sources.

The first three criteria are very indirect, whereas the difficulties that attend both the measurement of the efficiency of energy utilization in autotrophic and heterotrophic bacteria and the interpretation of the experimental results are such (see Larsen, 1960) as to make an assignment of mode of metabolism based on such values uncertain. In our opinion, the data reported in the present paper argue more directly against the operation of an autotrophic type of metabolism in *H. vulgare* than the above data argue in its favour.

A comparison of the time courses of incorporation of labelled growth substrate or labelled bicarbonate by methanol- or formate-grown *Pseudomonas* AM 1, and methanol-grown *H. vulgare*, shows striking similarities. First, the incorporation of ^{14}C from the labelled substrate as compared with that from $[^{14}\text{C}]$ bicarbonate is roughly of the same order with each organism, indicating a substantial incorporation of carbon both in organic form and as carbon dioxide. This conclusion is also borne out in *Pseudomonas* AM 1 growing on $[^{14}\text{C}]$ methanol by the finding that the specific radioactivity of its cellular material is reduced by 50% when the culture

is flushed with air-carbon dioxide. If all the carbon fixed by the cell were passing through the stage of carbon dioxide, as an autotrophic metabolism would demand, this reduction might be expected to be of the order of 90–100%, as was found with formate-grown *P. oxalicus* (Quayle & Keech, 1959a); if most of the cell carbon were not passing through the stage of carbon dioxide, the reduction might be expected to be below about 20%, as was found with the succinate-grown *Pseudomonas* AM1 (7%). A similar type of experiment performed with acetate-grown *Pseudomonas* KB1 (Kornberg & Quayle, 1958) gave a reduction of 17% in the specific radioactivity of cellular protein. Secondly, from either [¹⁴C]methanol or [¹⁴C]formate the compound that contains most of the radioactivity at early times is serine. The percentage contained in serine of the total carbon fixed, when plotted against time, shows a pronounced negative slope, indicating that serine is a primary stable product of methanol or formate incorporation. With *H. vulgare*, glycollate is also indicated as being a primary product in methanol incorporation, although it was not detected in *Pseudomonas* AM1. Glycine was present to a fairly constant low percentage of the total count fixed throughout the experiments with each tracer. The patterns of incorporation observed with [¹⁴C]bicarbonate as tracer all show predominant early labelling in C₄ acids such as malate and aspartate and, in contrast with the experiments with [¹⁴C]methanol or [¹⁴C]formate, glycine contained more radioactivity than did serine. No glycollate was observed in any of the experiments.

The similarities between the results obtained with methanol- and formate-grown *Pseudomonas* AM1 and methanol-grown *H. vulgare* indicate that an essentially similar pathway of carbon incorporation operates in all three instances. This proposed pathway probably involves substantial incorporation of both reduced carbon and carbon dioxide, although the relative contributions of exchange reactions and net carbon dioxide fixation to the observed carbon dioxide incorporation cannot yet be assessed. For the latter incorporation to be due largely to exchange reactions, these would, however, have to be quite unusually large.

It seems probable that the serine formed from either methanol or formate is made by hydroxymethylation of glycine by the well-known serine hydroxymethylase (Rabinowitz, 1960):

If this reaction represents the main step for synthesis of C₃ compounds from C₂ compounds, then a crucial problem lies in the prior synthesis of the C₂ compound, glycine, or its precursors, from C₁ units. Radioactive glycine is present at the earliest times

when either [¹⁴C]methanol, [¹⁴C]formate or [¹⁴C]bicarbonate is used and it is tempting to think that it (or possibly glycollate) may be arising from a condensation of two C₁ units derived respectively from organic substrate and carbon dioxide. Such a condensation might be a direct one or the result of a cyclic series of reactions. The rather low level of ¹⁴C fixation into glycine, relative to serine, does not support the idea of a direct condensation between two C₁ units. A second alternative, involving a cyclic series of reactions, might result in delayed appearance of radioactivity in glycollate or glycine, but would necessitate rapid labelling in members of such a synthetic cycle. The only other compounds rapidly labelled at the earliest times are malate and aspartate, and hence such C₄ compounds or their derivatives might thus be implicated. A third alternative is that the method of analysis, which deals only with stable, non-volatile compounds that are soluble in aqueous ethanol, is not detecting key intermediates in the metabolism.

At present no other details of the biosynthetic pathway are known, and further information is being sought from studies of the labelling patterns of some of the intermediates which become radioactive after short incubation times, and of the levels of enzymes involved in serine synthesis which are present in the organism.

A similar pathway of synthesis of cell constituents probably operates during the growth of *Pseudomonas* PRL-W4 on methanol. Kaneda & Roxburgh (1959a) showed with this organism that the first labelled compound to be formed from either [¹⁴C]methanol or [¹⁴C]bicarbonate was serine. No glycine or glycollate was detected. The concentration of tracer used was much less, and far fewer labelled compounds were detected, than in the present series of experiments, which might possibly account for the non-detection of glycine, for instance. Kaneda & Roxburgh (1959b) also carried out a series of isotopic experiments, mainly with resting cell suspensions of *Pseudomonas* PRL-W4, to test the effect of the presence of various combinations of methanol, formaldehyde, formate and carbonate on fixation of either [¹⁴C]methanol or ¹⁴CO₂ by the cells. On the basis of these experiments the authors suggested that two simultaneous pathways for the fixation of C₁ compounds exist: carbonate and formate are involved in one pathway, and methanol and formaldehyde in the other. It is presumed that both the formate and formaldehyde are incorporated as serine, possibly by a serine hydroxymethylase system.

It remains to be seen whether microbial growth on methane involves a metabolism broadly similar to that involved in growth on methanol. The isotopic experiments necessary to decide this have not been reported, although some work has been

carried out on *Pseudomonas methanica* by Leadbetter & Foster (1958) which indicates that during growth on methane a considerable proportion of the carbon incorporated by the cell does not pass through a stage readily exchangeable with carbon dioxide. It is thus unlikely that an autotrophic type of metabolism is involved.

SUMMARY

1. A study has been made of the incorporation of carbon from [^{14}C]methanol and [^{14}C]bicarbonate by cultures of *Pseudomonas* AM1 and *Hyphomicrobium vulgare* grown on methanol, and from [^{14}C]formate and [^{14}C]bicarbonate by cultures of *Pseudomonas* AM1 grown on formate.

2. The distribution of radioactivity within the non-volatile constituents of the ethanol-soluble fractions of the cells, after incubation for periods up to 2 min., has been analysed by chromatography and radioautography.

3. The first stable products of [^{14}C]methanol or [^{14}C]formate fixation were serine (containing approx. 50 % of the total count fixed) and C_4 dicarboxylic acids such as malate and aspartate. Radioactive glycolate was also observed in *Hyphomicrobium vulgare*. Radioactive phosphorylated compounds were present at the earliest time in methanol-grown *Pseudomonas* AM1, but only appeared at later times in the other two incubation experiments. Radioactive glycine appeared at early times of incubation, but its proportion of the total count fixed did not exceed 15 % and was less at all times than that of serine. Other compounds which appeared labelled during the incubations included glutamate, citrate and, in *Pseudomonas* AM1, trehalose.

4. The first stable products of [^{14}C]bicarbonate fixation were the C_4 acids, malate and aspartate (containing between them 40–70 % of the total count fixed) and glycine. Other compounds becoming labelled included serine, phosphorylated compounds, glutamate, and, in *Pseudomonas* AM1, trehalose.

5. The percentage of the total count fixed which was contained in each of the radioactive compounds was plotted against time. The slopes of the curves obtained show that serine is a primary product of [^{14}C]methanol or [^{14}C]formate incorporation (and glycolate in *Hyphomicrobium vulgare*), and that malate or aspartate is a primary product of [^{14}C]bicarbonate incorporation.

6. The specific radioactivity of cellular material obtained from *Pseudomonas* AM1 grown on [^{14}C]methanol in air was reduced by one-half on bubbling air-carbon dioxide (99:1, v/v) through the growing culture. This shows that at least 50 % of the carbon incorporated must have passed through the

stage of carbon dioxide or a compound in ready equilibrium with it.

7. No carboxydismutase activity has been found in cell-free extracts of the organisms. This fact, together with the other data, shows that an autotrophic metabolism involving the ribulose diphosphate cycle of carbon dioxide fixation cannot be operating.

We wish to thank Professor Sir Hans Krebs, F.R.S., for his interest and encouragement, and Miss A. West for technical assistance. This work was supported by the Rockefeller Foundation, United States Public Health Service, and the Office of Scientific Research of the Air Research and Development Command of the United States Air Force, through its European Office, under Contract no. AF 61 (052)–180, and was done during the tenure of Medical Research Council Training Scholarships (P. J. L. and D. P.).

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Composition of Phospholipids of Rabbit, Pigeon and Trout Muscle and Various Pig Tissues

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(Received 17 April 1961)

Studies on the distribution and structure of the phospholipids in certain mammalian tissues and subcellular components have been reported previously (Gray & Macfarlane, 1958; Gray, 1960*a, b*; Macfarlane, Gray & Wheeldon, 1960; Macfarlane, 1961*a*). The object of the present work was to obtain a general picture of the pattern of fatty components in phospholipids in other normal tissues, and to examine further the distribution of choline plasmalogen. This compound is less than 1% of the total phospholipid in brain (Klenk, Debuch & Daun, 1953; Webster, 1960) and a minor component in liver and spleen; but it is a major component (36%) in ram semen and in ox and pig heart (Lovern, Olley, Hartree & Mann, 1957; Gray, 1960*a*; Klenk & Debuch, 1955; Marinetti & Erbland, 1957; Gray & Macfarlane, 1958). It appeared possible that choline plasmalogen is associated directly with a contractile process, though the small amount found in cod muscle (Garcia, Lovern & Olley, 1956) indicated a considerable species difference; Hartree & Mann (1959), on the other hand, suggested that fatty acids derived from plasmalogen participate in the aerobic endogenous metabolism of sperm.

METHODS

The estimation of P, total N, amino N, choline, aldehyde, inositol, fatty acid ester groups, alkali-labile P and phosphomonoester were carried out as in Gray & Macfarlane (1958). The method for the estimation of ethanolamine and serine (Axelrod, Reichental & Brodie, 1953) was modified

slightly by substituting an acid hydrolysis and chloroform-methanol-extraction procedure used by Dr C. Long (personal communication) for the usual alkaline hydrolysis. Chromatography of phospholipids on silicic acid-impregnated paper was done in diisobutyl ketone-acetic acid-water (40:20:3, by vol.) at 2° and of glycerides in light petroleum (b.p. 60–80°)–diisobutyl ketone (96:6, v/v) at room temperature (Marinetti, Erbland & Kochen, 1957). Chromatography on Whatman no. 1 acid-washed paper in butan-1-ol–water–conc. NH₃ (100:15:2, by vol.; Coulon-Morelec, Faure & Maréchal, 1960) was also used (*R_F* cardiolipin 0.74; phosphatidic acid 0.48). Chromatography of water-soluble esters obtained by mild hydrolysis was done according to Dawson (1954).

Myofibrils were prepared from rabbit muscle by the method of Perry & Grey (1956); the lipid was compared with that extracted from a portion of the whole muscle from the same animal. Muscle from rainbow trout and pigeons was extracted within 1 hr. after death, and pig tissue obtained from a slaughterhouse within 2 hr.

The extraction of lipid from the tissues was based on the method of Folch, Lees & Sloane-Stanley (1957) and Gray (1960*b*). In general the tissue was blended mechanically with 5 vol. of cold chloroform-methanol (1:1, v/v) and re-extracted twice with 1 vol. of chloroform-methanol (2:1, v/v). The combined extracts were washed two or three times with 0.2 vol. of salt solution (0.04 M-MgCl₂ or 0.05 N-NaCl), and the chloroform solution was dried and evaporated. The residue was dissolved in light petroleum and dialysed according to van Beers, de Iongh & Boldingh (1958) to separate most of the non-P lipid (neutral lipid fraction) from the phospholipid.

The phospholipid was fractionated on silicic acid as in Gray (1960*b*) and neutral lipid by the system of Hirsch & Ahrens (1958). The isolation of the fatty acids from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine and of the fatty acids and aldehydes from the ethanolamine, serine and choline plasmalogen was carried out as described by Gray (1960*b*).

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