3. In stationary conditions, the quantities of the three keto acids can be calculated from their radioactivities. These quantities are small and correspond to the lactic acid or carbon dioxide generated during a few minutes only.

4. The average amount of each keto acid within a cell increases in parallel with the supply of glucose, but the ratio of the three keto acids does not change appreciably. Thus the enzymes of the citric acid cycle can, to a large extent, cope with an increase in the supply of pyruvic acid.

5. Pyruvic acid diffuses, from both mesenchyma and tumour tissue, into the medium where it is acted upon by lactic dehydrogenase. In the given conditions, the inverse Pasteur effect can partly be explained as being due to this reaction.

6. Oxaloacetic acid and α -oxoglutaric acid diffuse into the medium from tumour, but not from mesenchyma tissue. This loss is attributed to some malfunction of the mitochondria in tumour tissue. But it is shown that this malfunction need not express itself in any great increase of the ratio of pyruvic acid to oxaloacetic acid or α -oxoglutaric acid within the cells or any great decrease in the of rate respiration.

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Carbon Assimilation by *Pseudomonas oxalaticus* (OX 1)

1. FORMATE AND CARBON DIOXIDE UTILIZATION DURING GROWTH ON FORMATE

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Some non-photosynthetic micro-organisms can derive all their carbon and energy needs from oxalate or formate. It has been suggested that the organisms might obtain energy by oxidizing these compounds to carbon dioxide, and then synthesize cellular constituents from carbon dioxide (Bhat & Barker, 1948; Schatz, Isenberg & Trelawney, 1953; van Niel, 1954; Jayasuriya, 1955; Jakoby & Bhat, 1958). The isolation by Khambata & Bhat (1953) of *Pseudomonas oxalaticus* (OX 1), capable of growing on either formate or oxalate as the sole carbon and energy source, provided a suitable organism to investigate the reactions leading from these substrates to cell constituents. Results presented here and in the next paper are concerned with the carbon assimilation by *P. oxalaticus*

(OX 1) grown on formate. A preliminary account of part of this work has been published (Quayle & Keech, 1958).

METHODS

Maintenance and growth of organism. Cultures of *Pseudomonas oxalaticus* (OX 1) (Khambata & Bhat, 1953) were obtained from the National Collection of Industrial Bacteria (Culture no. 8642) and maintained on slopes consisting of: 5 mm-potassium oxalate, 3 mm-sodium phosphate-potassium phosphate buffer, pH 7.0, essential salts [50 mg. of $(\text{NH}_4)_2\text{SO}_4$, 10 mg. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 mg. of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg. of CaSO_4 /100 ml. of medium], 0.1% of yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.) and 1% (w/v) of agar-agar (Davis, New Zealand). Stock cultures were grown at 30°, stored at 2° and subcultured every 4 weeks.

The medium used for liquid growth was the same as that used by Jayasuriya (1955) except that 100 mM-sodium formate was used as the carbon source. A portion (200 ml.) of this medium was inoculated with the organism from an agar slope and shaken in a Fernbach flask at 30° for approximately 48 hr. Growth was determined by measurements with a nephelometer (Evans Electroselenium Ltd., Essex) previously calibrated against dry weight of cells/ml. of bacterial suspension. The bacteria were harvested by centrifuging at room temperature while in the logarithmic growth phase at a cell density of 0.07–0.1 mg. dry wt./ml. (mean generation time was 3.5–5.0 hr.).

Manometric methods. Consumption of O_2 and CO_2 production were measured in the conventional Warburg manometric apparatus. For manometric experiments the washed bacteria were suspended in 0.9% KCl to a final concentration of approximately 2 mg. dry wt./ml. Generally 1 ml. of cell suspension was added to 100 μ moles of sodium phosphate-potassium phosphate buffer, pH 7.0, contained in the main compartment of the Warburg vessel, and 40 μ moles of substrate was added from the side arm.

Preparation of tracer solutions. Radioactive chemicals were purchased from The Radiochemical Centre, Amersham, Bucks. By means of a transfer unit similar to that described by Aronoff (1956), barium [^{14}C]carbonate was decomposed with excess of 2N-HClO₄ and the liberated $^{14}CO_2$ trapped in the theoretical quantity of NaOH. The pH of the solution was adjusted finally to 7.5.

Sodium [^{14}C]formate was chromatographed on Whatman no. 4 filter paper with a solvent system of Long, Quayle & Stedman (1951) consisting of ethanol-aq. NH_3 soln. (sp.gr. 0.880)–water (80:4:16, by vol.). After development, the papers were sprayed with 0.5M- K_2HPO_4 , 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), the formic acid was liberated with 5N- H_2SO_4 and distilled *in vacuo* into the theoretical quantity of NaOH solution.

Bacterial incorporation of [^{14}C]formate and [^{14}C]bicarbonate. Bacteria, which had been grown in Fernbach culture flasks, were centrifuged at room temperature and resuspended in a small volume of their supernatant medium. A sample of this suspension containing from 14 to 21 mg. dry wt. of bacteria was added to fresh growth medium (9 ml.) containing 10 mM-sodium formate (or 100 mM-sodium formate in the [^{14}C]bicarbonate experiment). The suspension was shaken in a 50 ml. Erlenmeyer flask at 30° for 10 min. (or 35 min. in the [^{14}C]bicarbonate experiment) to ensure that growth had re-commenced. At the end of this time, 200 μ c of ^{14}C tracer solution was added to bring the final volume of the suspension to 10 ml. Samples (1 ml.) were withdrawn with a hypodermic syringe at suitable time intervals over 4 min., and transferred immediately to 3 ml. of boiling 95% (v/v) ethanol.

To study the incorporation under conditions of vigorous aeration, the above-described experiment was repeated with a cylindrical vessel (10 cm. $\times$ 2.5 cm. diam.) fitted with a sintered-glass bottom. Vigorous aeration in this suspension was maintained by blowing a stream of air through the sintered glass.

A comparison was made of [^{14}C]formate incorporation into bacteria growing in shaken cultures in air and in air + CO_2 (95:5, v/v). In this experiment, two cultures of the organism were harvested while in the logarithmic phase

of growth. The cells were resuspended in two Fernbach flasks, each containing 200 ml. of fresh growth medium, at a cell density of about 0.06 mg. dry wt./ml.; the substrate in each flask was 100 mM-sodium [^{14}C]formate, containing 200 μ c of ^{14}C . Samples (20 ml.) of the cultures were withdrawn at intervals, their growth was measured nephelometrically and then they were centrifuged at 0°. The supernatant medium was poured off and the cells were resuspended in 0.5 ml. of water; samples (0.2 ml.) were immediately plated on to 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.), dried and the radioactivity assayed with a mica end-window, lead-shielded G.M. tube (General Electric Co. Ltd.) A blank determination showed that a negligible amount of radioactive formate remained on the planchets after this treatment. A coincidence correction was applied to the counts obtained.

Treatment of aqueous ethanolic suspensions of killed cells, and radioassay. The hot ethanolic suspensions described previously were centrifuged and the resulting precipitates washed with 1 ml. of hot aqueous 20% (v/v) ethanol. The combined supernatant fractions were evaporated to dryness at 60° under N_2 and redissolved in aqueous 20% (v/v) ethanol ready for application to the origin positions of paper chromatograms. The ethanol-insoluble fractions were freed from nucleic acid contamination by a procedure similar to that of Roberts, Abelson, Cowie, Bolton & Britten (1955), by suspension in 2 ml. of 5% (w/v) trichloroacetic acid at 100° for 30 min. The resulting insoluble protein residues were washed with 1 ml. of acidified ethanol, re-centrifuged and hydrolysed in 0.5 ml. of 6N-HCl at 110° for 15 hr. in sealed tubes. The total radioactivity contained in the protein hydrolysates was measured by evaporation, at 60°, of 10–250 μ l. samples on silicone-treated glass coverslips (21 mm. diam.) supporting circles of lens tissue. The tissues were secured by means of 0.05 ml. of 10% (w/v) mannitol solution.

The remainder of the protein hydrolysate was analysed chromatographically for labelled amino acids. Each sample was evaporated at 80° under N_2 ; the residue was resuspended in 0.5 ml. of water and re-evaporated. The resulting residue was finally taken up in 0.25 ml. of water and chromatographed in a similar manner to the ethanol-soluble fraction.

Chromatographic analysis and radioautography. The solutions prepared from either the ethanol-soluble or ethanol-insoluble fractions of the reaction mixture were applied to chromatographic paper (Whatman no. 4, 18½ in. $\times$ 22 in.) and chromatographed two-dimensionally in a descending direction. The chromatogram was developed first across the machine direction of the paper with the solvent system of Kornberg (1958): 500 g. of phenol–13 ml. of 90% (w/v, A.R.) formic acid–167 ml. of water. The solvent system butanol–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 counted directly with a mica end-window G.M. tube (General Electric Co., type 2B2) and the count was corrected for

Table 1. *Oxidation of formate by washed suspensions of Pseudomonas oxalaticus*

Manometer cups contained, in the main compartment, washed bacteria (2.4 mg. dry wt.) in 42 mM-sodium phosphate, pH 7.0, and inhibitor where indicated; the side arm contained substrate; the centre well contained 400 μ moles of KOH. Total volume of contents, 3 ml. Incubation was at 30° for 1 hr. The results in parentheses refer to a second experiment performed on a different batch of cells.

Substrate	Cells alone		Cells + 1.1 mM-DNP		O_2 uptake by cells + 1 mM- NaH_2PO_4 (μ l.)
	O_2 uptake (μ l.)	CO_2 output (μ l.)	O_2 uptake (μ l.)	CO_2 output (μ l.)	
(a) None	31 (32)	41 (40)	32	33	—
(b) Formate (30 μ moles)	258 (230)	480 (480)	324	543	0
Theoretical values*	336	672	336	672	—
Q_{O_2}	-270 (-144)	—	-310	—	0
R.Q. observed	1.87 (2.10)		1.68		—
R.Q. theoretical	2.00		2.00		—

* Theoretical values for complete oxidation of formate to CO_2 .

background activity. The relationship between counting a sample of radioactive glucose mounted on a lens-tissue planchet (see above) and the same sample counted *in situ* after paper chromatography was found to be (planchet counts: paper counts) 1.5:1.

Identification of labelled compounds. The radioactive compounds were eluted from the chromatogram with water and co-chromatographed in appropriate solvent systems 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 in ethanol-aq. NH_3 soln. (sp.gr. 0.880)-water (80:4:16, by vol.; Long *et al.* 1951). Amino acids were re-chromatographed in ethanol-butanol-water (10:10:5, by vol.; Hardy, Holland & Nayler, 1955) or water-saturated phenol in the presence of NH_3 vapour (Consden, Gordon & Martin, 1944). The latter solvent was found particularly useful for the separation of aspartate, glutamate, serine and glycine. Phosphorylated compounds were re-chromatographed two-dimensionally in methanol-90% formic acid-water (80:15:5, by vol.) and methanol-aq. 35% (w/v) NH_3 soln.-water (60:10:30, by vol.; Bandurski & Axelrod, 1951). Dephosphorylation 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).

Sprays. Carboxylic acids were located on chromatograms by spraying with ethanolic 50% (v/v) Universal Indicator (British Drug Houses Ltd., Poole, Dorset) adjusted to pH 10; amino acids by spraying with 0.2% (w/v) ninhydrin in butanol-2N-acetic acid (95:5, v/v); phosphorylated compounds by first spraying with 0.1% (w/v) $FeCl_3 \cdot 6H_2O$ in 80% (v/v) ethanol followed by 1% (w/v) salicylsulphonic acid in 80% (v/v) ethanol (Wade & Morgan, 1953). Aldose sugars were detected with aniline-phthalic acid (0.9:1.7, w/v) in 100 ml. of water-saturated butanol, followed by heating to 105° for 5 min. (Partridge, 1949); fructose was detected by spraying with ethanolic 1% (w/v) naphtharesorcinol-2N-HCl (10:90, v/v; Forsyth, 1948) followed by heating to 90° for 10 min.; sedoheptulose

by spraying with a solution of orcinol-trichloroacetic acid (0.5:15, w/v) in 100 ml. of water-saturated butanol, followed by heating at 100° (Klevstrand & Nordal, 1950).

RESULTS

Oxidation of formate. Washed suspensions of *P. oxalaticus* (OX 1) oxidized formate with Q_{O_2} values of -140 to -270 (μ l. of O_2 consumed/mg. dry wt. of bacteria/hr.; Table 1). The oxidation was completely inhibited in the presence of mM-sodium hypophosphite, a compound which is an inhibitor of formic dehydrogenase in some organisms (Takamiya, 1953; Campbell, 1954, 1955). Although different batches of cells gave respiratory quotients for this substrate ranging from 1.87 to 2.10 (theoretical value, 2.0), the volumes of O_2 consumed and CO_2 evolved were always below those required for complete oxidation. Values of 68-80% of the theoretical were generally obtained, which suggested an oxidative assimilation of a portion of the substrate.

In the presence of mM-2,4-dinitrophenol (DNP), the uptake of O_2 was raised to 96% of the theoretical value and the respiratory quotient dropped to 1.7 (Table 1). The increased uptake of O_2 observed in the presence of DNP, which is known to be an inhibitor of oxidative assimilation (Clifton, 1937), supports the suggestion that the incomplete oxidation of formate by washed organisms was due to assimilation of the substrate. In these calculations, gas exchanges observed in the absence of substrate were not subtracted from those observed in its presence.

Incorporation of [^{14}C]formate and [^{14}C]bicarbonate. Addition of either [^{14}C]formate or [^{14}C]bicarbonate to growing cultures resulted in incorporation of isotope into both the ethanol-soluble and protein fractions of the cells (Fig. 1).

The incorporation of [^{14}C]formate was studied under conditions of vigorous aeration in the

bubbler flask and the [^{14}C]bicarbonate experiment was conducted in a shaken Erlenmeyer flask to avoid any possible sweeping out of the isotope. Separate experiments had shown that the growth rates of the organism under these two conditions were not significantly different. The observed average incorporation rates of [^{14}C]formate and [^{14}C]bicarbonate carbon into the ethanol-soluble fractions of the cells were 1620 and 354 counts/min./mg. dry wt. of bacteria respectively. The specific activity of the [^{14}C]formate tracer used was four times that of the [^{14}C]bicarbonate, hence the observed incorporation rates in terms of $\mu\text{g. atoms}$ of carbon from either formate or bicarbonate are similar. However, since small quantities of both unlabelled formate and bicarbonate would be present at the start of the experiments, a very accurate comparison of the two incorporation rates cannot be made, owing to the resulting uncertainty of the initial specific activity of the tracers in the bacterial suspensions.

Fig. 1. Incorporation of ^{14}C from [^{14}C]formate and [^{14}C]bicarbonate into the protein and ethanol-soluble fractions of *Pseudomonas oxalaticus*. $\circ$, Ethanol-soluble fractions; $\bullet$, protein fractions; conditions were as described in Table 2 ([^{14}C]formate incorporation). $\blacktriangle$, Ethanol-soluble fractions; $\blacktriangledown$, protein fractions; conditions were as described in Table 3 ([^{14}C]bicarbonate incorporation). All the counts in the protein fractions have been reduced by a factor of 0.665 to correct for the different method of radioassay used from that used for the ethanol-soluble fraction.

Distribution of radioactivity amongst labelled compounds in the ethanol-soluble fractions. Chromatographic analysis, followed by radioautography, showed that radioactivity either from [^{14}C]formate or [^{14}C]bicarbonate very rapidly appeared in a large number of compounds; over 30 labelled compounds in the ethanol-soluble fraction were visible after incubation for 90 sec. with [^{14}C]formate under the conditions of high aeration. The distribution of radioactivity amongst the labelled compounds formed during the first 90 sec. incubation with [^{14}C]formate or [^{14}C]bicarbonate is shown in Tables 2 and 3. Under the conditions of chromatography used, phosphorylated compounds ran together in one large spot near the origin of the chromatograms. The earliest time samples showed radioactivity only in this phosphate area and, to a minor extent, in malic acid. In the later samples radioactivity progressively appeared also in glutamic acid, aspartic acid and citric acid. The curves obtained by plotting time against the percentage of the total radioactivity contributed by each constituent of the sample are shown in Figs. 2 and 3. The most striking feature of both diagrams is the very high percentage of total radioactivity appearing in the earliest times in phosphorylated compounds. Furthermore, the negative slopes of these curves show that a primary fixation product was present in these compounds. Malate was labelled in both cases at the earliest times, to the extent of about 5 % of the total radioactivity. This figure rose slightly during the course of the incubation. These results show that isotope from either [^{14}C]formate or [^{14}C]bicarbonate was incorporated by the cells via phosphorylated compounds.

The composition of the phosphate areas on the chromatograms is shown in Table 4. Losses of count from the original phosphate area were always observed (compare Table 2); part of this may have been due to irreversible absorption of the phosphates on the paper and part to the loss of volatile compounds after dephosphorylation (e.g. pyruvic acid). In the earliest samples, 81 % of the recovered radioactivity in the dephosphorylated compounds was contained in glyceric acid, the remainder in glucose, fructose and sedoheptulose. Ribose became labelled in the later samples, although the amount of radioactivity incorporated into this compound was surprisingly low.

The composition of the phosphate areas in the [^{14}C]bicarbonate experiment was investigated by rechromatographing two-dimensionally in the solvent system of Bandurski & Axelrod (1951) (Table 5). Considerable losses in total count recovery were again encountered, but the only labelled compound that appeared in the phosphate chromatogram from the earliest sample was 3-phosphoglyceric acid (PGA).

Table 2. Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of *Pseudomonas oxalaticus* incubated with sodium [¹⁴C]formate

Sodium [¹⁴C]formate (47 μ moles; 200 μ C of ¹⁴C) was added at zero time to 10 ml. of a suspension of bacteria (21 mg. dry wt.) growing with vigorous aeration on 10 mM-sodium formate, 25 mM-potassium phosphate, pH 7.0, and essential salts. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and transferred into 3 ml. of hot ethanol. The precipitates were each washed with 1 ml. of hot aqueous 20% (v/v) ethanol and analysed by two-dimensional chromatography. The radioactive areas were located by radioautography and directly counted with a mica end-window G.M. tube.

Time (sec.)	Radioactivity (counts/min.)						Total
	Phos- phorylated compounds	Malate	Glutamate	Aspartate	Citrate	Other compounds	
12	623	35	0	0	0	130	788
25	1358	104	0	0	0	268	1730
53	2520	200	153	64	0	412	3349
63	2410	180	264	88	37	660	3639
91	3130	323	367	163	182	700	4865

Table 3. Distribution of radioactive carbon amongst the non-volatile labelled components of the ethanol-soluble fractions of growing suspensions of *Pseudomonas oxalaticus* incubated with sodium [¹⁴C]bicarbonate

Sodium [¹⁴C]bicarbonate (190 μ moles; 200 μ C of ¹⁴C) was added at zero time to 10 ml. of a suspension of bacteria (14.7 mg. dry wt.) growing in a 50 ml. Erlenmeyer flask on 100 mM-sodium formate, 25 mM-potassium phosphate, pH 7.0, and essential salts. Samples (1 ml.) were withdrawn by hypodermic syringe at known times and treated as described in Table 2.

Time (sec.)	Radioactivity (counts/min.)						Total
	Phos- phorylated compounds	Malate	Glutamate	Aspartate	Citrate	Other compounds	
13	162	8	0	0	0	0	170
45	280	34	0	0	0	38	352
62	484	53	53	9	0	21	620
80	514	69	98	15	8	82	786

Fig. 2. Variation with time of the percentage distribution of ¹⁴C from [¹⁴C]formate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on formate. Conditions were as described in Table 2. O, Phosphorylated compounds; ●, malate; △, glutamate; ▲, citrate; ▼, aspartate.

Fig. 3. Variation with time of the percentage distribution of ¹⁴C from [¹⁴C]bicarbonate incorporated into the constituents of the ethanol-soluble fraction of *Pseudomonas oxalaticus* growing on formate. Conditions were as described in Table 3. O, Phosphorylated compounds; ●, malate; △, glutamate; ▲, aspartate.

Table 4. *Distribution of radioactive carbon amongst the labelled phosphates obtained from the ethanol-soluble fractions of growing suspensions of Pseudomonas oxalaticus incubated with sodium [^{14}C]formate*

The phosphorylated compounds were eluted from the chromatograms described in Table 2, and the eluates concentrated to 0.5 ml. and incubated overnight at 30° with 0.2 mg. of Polidase S. The resulting non-volatile dephosphorylated compounds were analysed by two-dimensional paper chromatography and located by radioautography.

Time (sec.)	Radioactivity (counts/min.)					Total
	Glyceric acid	Glucose and sedoheptulose	Fructose	Ribose	Other compounds	
12	373	73	13	0	0	459
25	263	372	63	<10	110 (Chromatogram origin)	818
53	358	600	130	<10	30	1128
63	394	593	150	<10	400 (Chromatogram origin)	1547
91	476	600	25	<10	35	1146

Fig. 4. Incorporation of ^{14}C from [^{14}C]formate into *Pseudomonas oxalaticus* growing in air and in air + CO_2 (95:5) at 30°. ○, Growth in air; ●, growth in air + CO_2 ; △, radioactivity incorporated by air-grown culture; ▲, radioactivity incorporated by culture grown in air + CO_2 .

Distribution of radioactivity amongst labelled compounds in protein hydrolysates. The first amino acid to become labelled in the protein fraction of cells growing on [^{14}C]formate was aspartic acid (Table 6). Glutamate and alanine followed, with radioactivity also appearing in three spots running closely together in the leucine area of the chromatogram. These compounds were not identified.

Incorporation of [^{14}C]formate in the presence and absence of added carbon dioxide. The growth rate of *P. oxalaticus* was higher (mean generation time 2 hr.) during growth in air + CO_2 (95:5) than it was (mean generation time 3.5 hr.) in air alone (Fig. 4). This could be due either to the higher concentration of CO_2 or to the establishment of an efficient buffer system. The total incorporation of ^{14}C into the cells under these two conditions was

Table 5. *Distribution of radioactive carbon amongst the labelled phosphates obtained from the ethanol-soluble fractions of growing suspensions of Pseudomonas oxalaticus incubated with sodium [^{14}C]bicarbonate*

The phosphorylated compounds were eluted from the chromatograms described in Table 3 and the eluates treated with Dowex-50 (H^+ form), analysed by two-dimensional paper chromatography and the phosphates located by radioautography. PGA was identified by co-chromatography with an authentic sample, and the sugar phosphates were eluted, dephosphorylated and identified chromatographically as described in Table 4.

Time (sec.)	Radioactivity (counts/min.)			Total
	Glucose phosphate, fructose phosphate and sedoheptulose phosphate	Other compounds		
13	67	0	0	67
45	70	<10	8	88
62	49	84	46	179
80	90	112	86	288

Table 6. *Distribution of radioactive carbon in the protein hydrolysate of Pseudomonas oxalaticus incubated with sodium [^{14}C]formate*

The ethanol-insoluble fractions from Table 2 were heated in 2 ml. of 5% (w/v) trichloroacetic acid at 100° for 30 min. Insoluble residues were washed with 1 ml. of acidified ethanol and hydrolysed in 0.5 ml. of 6N-HCl at 110° for 15 hr. Samples were analysed by chromatography and radioautography as described in Table 2.

Time (sec.)	Radioactivity (counts/min.)			
	Aspartate	Glutamate	Alanine	Other compounds
91	66	0	0	0
180	263	122	0	237
304	300	288	244	1200

measured in each case over one complete generation, the range of cell densities being the same in both cases (Fig. 4).

Growth of the air-grown cells from a cell density of 0.063 mg. dry wt./ml. to 0.126 mg. dry wt./ml. was accompanied by an incorporation of radioactivity of 2990 counts/min./ml. of culture; that of the culture gassed with CO₂ was accompanied by an incorporation of 180 counts/min./ml. of culture.

This shows that at least 94 % $\left(100 - \frac{180 \times 100}{2990}\right)$ of the carbon entering the cell from formate passes through the stage of CO₂ or a compound which is in rapid equilibrium with extracellular CO₂.

DISCUSSION

The ability of some non-photosynthetic micro-organisms to exist on C₁ compounds as combined energy and growth substrates has been known for a long time. Amongst the well-characterized species may be mentioned those living by oxidation of methane (Hutton & ZoBell, 1949), formate (Kingma Boltjes, 1936; Mevius, 1953; Khambata & Bhat, 1953, 1955) and the methane fermentation of formate (Stadtman & Barker, 1951). The assimilation of their cellular carbon by carbon dioxide fixation, as previously suggested, would make such organisms analogous to the autotrophic bacteria, which live at the expense of the oxidation of inorganic substances. The difference between the two processes would thus be one of energy source only, being an organic and inorganic oxidation respectively. Some evidence for this type of metabolism of an organic substrate has been obtained by Schatz *et al.* (1953) with *Hydrogenomonas facilis*. Schatz *et al.* found that, when *H. facilis* oxidized lactate in the presence of [¹⁴C]carbon dioxide: 'the CO₂...assimilated was far greater than could be accounted for by the ordinary heterotrophic fixation'.

Studies of carbon dioxide fixation by cell-free extracts of the autotrophic bacteria *Thiobacillus thio-parus* (Santer & Vishniac, 1955), *T. denitrificans* (Trudinger, 1955, 1956), *T. thio-oxidans* (Suzuki & Werkman, 1958) and *Hydrogenomonas ruhlandii* (Santer & Vishniac, 1957) have shown the ability of the extracts to carry out enzymic carboxylation of a phosphorylated pentose to give PGA. Furthermore, a kinetic analysis of the distribution of radioactivity among the intermediates formed from [¹⁴C]carbon dioxide by *T. denitrificans* (Aubert, Milhaud & Millet, 1956, 1957; Milhaud, Aubert & Millet, 1956), *T. thio-oxidans* (Suzuki & Werkman, 1957) and *H. facilis* (Bergmann, Towne & Burris, 1958) indicates that the growth of these organisms depends on the operation

of a carbon dioxide-fixation cycle similar to that found in photosynthetic organisms (Bassham *et al.* 1954).

The alternative to this autotrophic mechanism in organisms growing on C₁ organic compounds would be the assimilation of a C₁ unit through an unknown series of condensation reactions. Such reactions would have to accomplish an overall chemical reduction when the carbon source is at a higher oxidation level than that of cell carbon.

The finding that PGA and, to a minor extent, malate, were the first labelled compounds to be formed from either [¹⁴C]formate or [¹⁴C]bicarbonate by *P. oxalaticus* grown on formate suggests that the main pathway of carbon assimilation into cellular constituents is via carboxylation of ribulose 1:5-diphosphate, together with a minor incorporation into malate (or possibly oxaloacetate as well). The early labelling of phosphate esters of glucose, fructose, sedoheptulose and ribose is consistent with the operation of a ribulose 1:5-diphosphate-regenerating cycle. The appearance of aspartate as the first labelled amino acid residue in the protein fractions would follow from carboxylation of pyruvate or of phosphopyruvate or of both, the two compounds being derived from radioactive PGA.

The assimilation of formate carbon into the cell by these means necessitates carbon dioxide being an intermediary metabolite, unless in these organisms ribulose 1:5-diphosphate can react directly with formate. No such reaction could be demonstrated with cell-free extracts (Quayle & Keech, 1959). Furthermore, the fact that at least 94 % of the carbon assimilated by the cell was exchangeable with carbon dioxide indicates that assimilation of formate is preceded by oxidation to carbon dioxide.

These findings contrast with those obtained with *Pseudomonas* KB1 grown on [¹⁴C]acetate (Kornberg & Quayle, 1958), where it was found that cells growing in a stream of oxygen+carbon dioxide (95:5) synthesized protein of specific radioactivity 15-17 % less than that synthesized in a stream of oxygen. The deduction that a maximum of 15-17 % of the carbon incorporated into the cell passes through the stage of carbon dioxide has been criticized on the grounds of insufficiently rapid equilibration between intra- and extra-cellular carbon dioxide. The findings with *P. oxalaticus* tend to invalidate this criticism.

The results reported in this paper therefore show that a major pathway of carbon incorporation from formate into cellular constituents occurs by a carbon dioxide-fixation cycle, similar to that found in photosynthetic organisms and other autotrophic organisms. Studies carried out with cell-free extracts of *P. oxalaticus* confirm this conclusion (Quayle & Keech, 1959).

SUMMARY

1. The incorporation of carbon from either [^{14}C]formate or [^{14}C]bicarbonate by cultures of *Pseudomonas ovalaticus* (OX1) grown on formate has been studied.

2. Isotope appeared in both the aqueous ethanol-soluble and protein fractions of the cells during 5 min. incubation periods.

3. The distribution of radioactivity within the non-volatile constituents of these fractions, after incubation for from 12 sec. to 5 min., has been analysed by chromatography and radioautography. Over 80 % of the radioactivity incorporated at the earliest time (12 sec.) into the ethanol-soluble fractions was present in phosphorylated compounds, and 5 % in malate, with either [^{14}C]formate or [^{14}C]bicarbonate. After incubation for 1 min., radioactivity also appeared in citrate, aspartate and glutamate.

4. In 12 sec., over 80 % of the radioactivity initially incorporated into phosphorylated compounds was present in 3-phosphoglyceric acid; phosphates of glucose, fructose, sedoheptulose and ribose (to a minor extent) became labelled in the later samples.

5. The specific radioactivity of cellular material obtained from cells grown on [^{14}C]formate in air was reduced 17-fold by bubbling air+carbon dioxide (95:5, v/v) through the growing culture. Thus at least 94 % of the carbon incorporated must have passed through the stage of carbon dioxide or a compound in ready equilibrium with it.

6. These results show that the organism synthesizes cell constituents from carbon dioxide by a cycle similar to that found in photosynthetic organisms and other autotrophic organisms. The necessary energy is derived from oxidation of the formate to carbon dioxide.

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