The Metabolism of Glyoxylate by Cell-Free Extracts of *Pseudomonas* Sp.

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1. Extracts of *Pseudomonas* sp. grown on butane-2,3-diol oxidized glyoxylate to carbon dioxide, some of the glyoxylate being reduced to glycollate in the process. The oxidation of malate and isocitrate, but not the oxidation of pyruvate, can be coupled to the reduction of glyoxylate to glycollate by the extracts. 2. Extracts of cells grown on butane-2,3-diol decarboxylated oxaloacetate to pyruvate, which was then converted aerobically or anaerobically into lactate, acetyl-coenzyme A and carbon dioxide. The extracts could also convert pyruvate into alanine. However, pyruvate is not an intermediate in the metabolism of glyoxylate since no lactate or alanine could be detected in the reaction products and no labelled pyruvate could be obtained when extracts were incubated with [1-^{14}C]glyoxylate. 3. The ^{14}C was incorporated from [1-^{14}C]glyoxylate by cell-free extracts into carbon dioxide, glycollate, glycine, glutamate and, in trace amounts, into malate, isocitrate and α-oxoglutarate. The ^{14}C was initially incorporated into isocitrate at the same rate as into glycine. 4. The rate of glyoxylate utilization was increased by the addition of succinate, α-oxoglutarate or citrate, and in each case α-oxoglutarate became labelled. 5. The results are consistent with the suggestion that the carbon dioxide arises by the oxidation of glyoxylate via reactions catalysed respectively by isocitratase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase.

Hullin & Hassall (1962), using cell-free extracts of a *Pseudomonas* sp. grown on butane-2,3-diol as sole source of carbon, showed that a cyclic pathway operated in the organism whereby butane-2,3-diol is dissimilated to acetate. The organism is, in effect, growing on acetate as sole source of carbon. It was also demonstrated that synthesis of tricarboxylic acid-cycle intermediates took place via the glyoxylate cycle. Extracts exhibited high activities of isocitratase [*threo*-D$_s$-isocitrate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.1] and malate synthase [L-malate glyoxylate-lyase (CoA-acetylating), EC 4.1.3.2], the two key enzymes of the glyoxylate cycle.

Hassall & Hullin (1962) showed that the same extracts of *Pseudomonas* sp. metabolized glyoxylate to glycollate and carbon dioxide. The reduction of glyoxylate to glycollate was shown to be catalysed by a constitutive glyoxylate reductase (glycollate–NADP oxidoreductase), which was partially purified and found to be dependent on NADP for activity and to be distinct from any NAD-linked or NADP-linked lactate dehydrogenase system. The authors suggested that the oxidation of glyoxylate to carbon dioxide might occur via the mediation of the dicarboxylic acid cycle (Kornberg & Sadler, 1961), involving the successive formation of malate, pyruvate and acetyl-CoA. This suggestion, together with other possible routes of carbon dioxide formation from glyoxylate, has now been investigated. A preliminary account of part of this work has already been published (Hullin & Bailey, 1964).

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MATERIALS AND METHODS

Maintenance and growth of organism. The organism was originally isolated from soil by S. Dagley by enrichment culture on butane-2,3-diol as sole carbon source. Full details of the maintenance of cultures, growth procedures, harvesting of cells and preparation of cell-free extracts were as given by Hullin & Hassall (1962).

Determination of protein concentration. The method of Sols (1947) and latterly the method described by Warburg & Christian (1941) was adopted, with a solution of crystalline bovine serum albumin for the preparation of a standard curve.

Materials. Butane-2,3-diol, 2-hydroxybutan-3-one (both redistilled before use), glycolaldehyde, thiamine pyrophosphate, CoA and sodium pyruvate were obtained from Koch–Light and Co. Ltd., Colnbrook, Bucks. NAD$^+$, NADH, NADP$^+$ and NADPH were from the Sigma

Chemical Co., St Louis, Mo., U.S.A. Bovine serum albumin was from Armour Laboratories, Chicago, Ill., U.S.A.

[1,2-$^{14}C_2$]Glyoxylic acid was from Calbiochem, Los Angeles, Calif., U.S.A. Other isotopically labelled compounds were purchased from The Radiochemical Centre, Amersham, Bucks. Sodium glyoxylate monohydrate, synthesized as described by Metzler, Olivard & Snell (1954), was a gift from P. W. Trudgill. With the exception of ethyl acetate, organic solvents used were of reagent grade. Other chemicals used were of A.R. grade (British Drug Houses Ltd., Poole, Dorset). [^{14}C]Glyoxylate and [^{14}C]-glycollate were checked for radiochemical purity by paper chromatography of the free acid followed by radioautography. Some batches of sodium [1-^{14}C]glyoxylate, found to be 80% pure, were separated from impurities that were principally tartrate and glycollate by paper chromatography and radioautography. The area containing [^{14}C]-glyoxylic acid was eluted with water and the purity of the eluted material finally checked by two-dimensional chromatography. [^{14}C]Pyruvate was used as quickly as possible after opening the containing vessel; its purity was checked by paper chromatography of the 2,4-DNP-hydrazones followed by radioautography.

Sodium DL-isocitrate was prepared by hydrolysis of the lactone by the method of Olson (1959).

Buffers. 0·05M-Phosphate buffer, pH 7·2, was prepared from 50ml. of 0·2M-KH_2PO_4 plus 34·9ml. of 0·2N-NaOH, diluted to 200ml. with distilled water. 0·02M-Tris buffer was adjusted to the required pH with 2N-HCl.

Manometric studies of oxygen uptake and carbon dioxide evolution. The conventional Warburg respirometer was used, as described by Umbreit, Burris & Stauffer (1957), to follow O_2 uptake. Evolution of CO_2 was measured in an atmosphere of N_2, by using double-armed flasks and adding acid at the end of the experiment to release bound CO_2. $^{14}CO_2$ was counted as $Ba^{14}CO_3$ samples of infinite thickness in a Tracerlab SC16G windowless gas-flow counter.

Determination of glyoxylate, pyruvate and α-oxoglutarate. These were determined in the presence of each other by the method of Friedemann & Haugen (1943) as modified by Olson (1959).

Determination of glycollic acid. The method used was that of Dagley & Rodgers (1953).

Chromatography of 2,4-dinitrophenylhydrazones of keto acids. Usually keto acids were converted into their 2,4-DNP-hydrazones, which were then chromatographed as described by Cavallini, Frontali & Toschi (1949). One-dimensional ascending chromatograms were run in butan-1-ol–ethanol–ammonium carbonate buffer (40:11:14, by vol.) (Dagley, Fewster & Happold, 1952). Two-dimensional ascending chromatograms were run in the first direction in the above solvent and then in the second direction in the solvent system butan-1-ol–benzene–ammonium carbonate buffer (16:1:3, by vol.) (Fewster & Hall, 1951). The 2,4-DNP-hydrazones appeared as yellow spots in visible light and as dark spots in u.v. light. Further characterization was obtained by spraying the chromatograms with 2% (w/v) KOH in aq. 90% (v/v) ethanol.

Analysis of samples obtained from incubation experiments. Reaction mixtures were deproteinized by adding an equal volume of 2N-H_2SO_4 and centrifuging at 14000*g* for 20min. The supernatant solutions were continuously extracted with ether for 48hr. in Kutcher–Steudel liquid/liquid extractors, and samples of concentrated extract containing 0·5–1·0μmole of the acid were applied to sheets of Whatman no. 1 paper. Samples (5μl.) of 2% (w/v) solutions of known acids were chromatographed with the unknown acids in one-dimensional ascending chromatograms. Solvent systems employed were: phenol–90% (w/v) formic acid–water (500:13:167, w/v/v) (Kornberg, 1958); butan-1-ol–propionic acid–water (Calvin & Benson, 1949), as described by Kornberg (1958); butan-1-ol–acetic acid–water (12:3:5, by vol.) (Smith, 1958); ether–acetic acid–water (13:3:1, by vol.) (Denison & Phares, 1952); benzene–ether–90% (w/v) formic acid–water (15:35:7:5, by vol.) (Weimberg, 1959); butan-1-ol–pyridine–water (1:1:1, by vol.) (Morrison, 1953). The spots of organic acid were located by an aniline–xylose reagent (Wood, 1958).

Reactions involving [^{14}C]glyoxylate and [^{14}C]pyruvate. Reactions were carried out in a total volume of 0·2–0·3ml. and stopped by heating at 100° for 1min. followed by cooling to 0° and adding 0·05ml. of 2N-HCl. Precipitated protein was removed by centrifuging and 0·05ml. of each clear supernatant solution was analysed by single- and two-dimensional chromatography in the various solvent systems listed below. The accurate measurement of the reactants and the sample to be chromatographed was achieved with a calibrated finely-drawn Pasteur pipette. Chromatograms were developed by ascending chromatography until the solvent had moved 10in. The radioactive compounds were located by radioautography on Kodirex X-ray film for a number of days determined by the amount of radioactivity present on the chromatograms. Radioautographs with faint spots, occasionally obtained, were intensified as described by Moses & Edwards (1960). Each spot was cut from the chromatogram and eluted with water into a small weighed polythene container, and the volume of the eluent (less than 0·2ml.) was determined by re-weighing. Duplicate 0·02ml. samples of each eluted fraction were applied to the smooth sides of 2·4cm.-diam. Whatman no. 1 filter-paper disks mounted on Tracerlab E7B planchets, and counted in a Tracerlab SC16G windowless gas-flow counter for a minimum of 5000 counts. This method of plating gave S.D. ±4·1%.

The radioactivity of appropriate areas on chromatograms was frequently assayed by counting *in situ* with a mica end-window β-tube (General Electric Co. type EHM2/5) standing on a variety of masks so that the radioactivity of each area could be determined without overlapping.

Radioactive compounds were identified by two-dimensional co-chromatography with authentic unlabelled samples of the compound in two solvent pairs. The following pairs of solvent systems were used: phenol–90% (w/v) formic acid–water (500:13:167, w/v/v) (Kornberg, 1958) and equal parts (by vol.) of butan-1-ol–water (89:6, v/v) and propionic acid–water (63:79, v/v), mixed immediately before use (Calvin & Benson, 1949); butan-1-ol–pyridine–water (1:1:1, by vol.) (Morrison, 1953) and either benzene–ether–90% (w/v) formic acid–water (15:35:7:5, by vol.) (Weimberg, 1959) for carboxylic acids or butan-1-ol–acetone–water–diethylamine (5:5:5:1, by vol.) (Hardy, Holland & Nayler, 1955) for amino acids.

Unlabelled carrier carboxylic acids were located by spraying with an aniline–xylose reagent (Wood, 1958), and amino acids by spraying with 0·2% (w/v) ninhydrin in acetone (Smith, 1953) to which pyridine was added to 2% (v/v) immediately before use.

Chromatography of ammonium salts of carboxylic acids.

A 0·05ml. sample of supernatant solution was added to a slight excess of aq. ammonia (sp.gr. 0·88) and 0·05ml. samples of this mixture were analysed by ascending chromatography in ethanol–aq. ammonia (sp.gr. 0·88)–water (20:1:4, by vol.) (Long, Quayle & Stedman, 1951).

Assay of oxaloacetate-decarboxylase activity. Reactions were carried out at 30° for 30min. in double side-armed Warburg respirometers filled with N_2. The reaction mixture contained 5mg. of bacterial extract, 5μmoles of $MgSO_4$ and 10μmoles of oxaloacetate in a total volume of 2·8ml. of phosphate buffer (KH_2PO_4, 2·0g./l.; adjusted to pH7·2). The reaction was terminated by adding 0·2ml. of 50% (w/v) trichloroacetic acid. In control respirometers, boiled extract was used. The enzyme activity of extracts was compared with that containing 2mg. of protein extract from cells of the *Pseudomonas* (G) grown on tartrate isolated by Dagley & Trudgill (1963). The latter extract was known to contain a high concentration of oxaloacetate decarboxylase.

RESULTS

The anaerobic metabolism of glyoxylate by cell-free extracts of *Pseudomonas* sp. was investigated manometrically. The results for glyoxylate utilization and carbon dioxide and glycollate production were similar to those obtained by Hassall & Hullin (1962). Glycollate and carbon dioxide production accounted for over 80% of the glyoxylate utilized.

The oxidation of glyoxylate to carbon dioxide and water via formate was demonstrated in *Pseudomonas* sp. by Campbell (1955). Hypophosphite, a specific inhibitor of formate dehydrogenase (Takamiya, 1953), was added to extracts metabolizing glyoxylate to test the possibility that formate was an intermediate. No effect on the rate of carbon dioxide evolution was produced by the inhibitor. When [1,2-$^{14}C_2$]glyoxylate was the substrate, no radioactive formate was detected as a product, thus confirming that formate is not an intermediate.

Hassall & Hullin (1962) had suggested that the carbon dioxide produced from glyoxylate might arise via the mediation of catalytic quantities of acetyl-CoA to form malate and the operation of the dicarboxylic acid cycle described by Kornberg & Sadler (1960). The reaction sequence would involve the successive formation from malate of oxaloacetate, pyruvate and acetyl-CoA, and further reaction of the latter with more glyoxylate. The presence in extracts of the organism of the enzymes of the dicarboxylic acid cycle, namely malate synthase, malate dehydrogenase (L-malate–NAD oxidoreductase, EC 1.1.1.37) and oxaloacetate decarboxylase (oxaloacetate carboxy-lyase, EC 4.1.1.3), would also be required. The presence of malate synthase was demonstrated by Hullin & Hassall (1962) and confirmed by us. Malate-dehydrogenase activity, expressed as μl. of oxygen uptake/mg. of protein/hr., was 10·2 for extracts of cells grown on butane-2,3-diol and 15·6 for extracts of cells grown on succinate.

Table 1. *Effect of semicarbazide on the oxidation of* L-*malate by cell-free extracts of* Pseudomonas *sp.*

Each reaction mixture initially contained, in a total volume of 2·8ml., 10μmoles of L-malate, 0·5ml. of cell extract containing 10mg. of protein, 1·2ml. of 0·05M-phosphate buffer, pH7·2, 5μmoles of $MgSO_4$ and, where stated, 100μmoles of semicarbazide. The reactions were carried out at 30° for 100min., when O_2 uptake had ceased.

Growth substrate	O_2 uptake (μg.atoms)	CO_2 evolved (μmoles)
Succinate	10·5	15·0
Butane-2,3-diol	10·5	14·5
Butane-2,3-diol (in the presence of semicarbazide)	10·5	10·0

If malate (10μmoles) is completely metabolized by extracts via oxaloacetate and pyruvate to acetyl-CoA, the expected oxygen uptake would be 20μg.atoms and the carbon dioxide evolution would be 20μmoles. However, 10·5μg.atoms of oxygen and 14·5μmoles of carbon dioxide were the values obtained (Table 1). No keto acids could be detected in the reaction mixture, demonstrating that the low values were not due to oxaloacetate or pyruvate accumulation. In the presence of semicarbazide, the oxygen uptake was again 10·5μg.atoms but the carbon dioxide evolution was decreased to 10μmoles owing to trapping by semicarbazide of oxaloacetate and pyruvate, which were shown to be present in the reaction mixture by the isolation and identification of their 2,4-DNP-hydrazones.

The values for oxygen uptake and carbon dioxide evolution are readily understood on the basis of the anaerobic metabolism of pyruvate by extracts to lactate, carbon dioxide and acetyl-CoA (see below). Ten μmoles of malate would be oxidized to 10μmoles of oxaloacetate with the uptake of 10μg.atoms of oxygen; decarboxylation of the oxaloacetate would yield 10μmoles of carbon dioxide, and the 10μmoles of pyruvate thus formed would then yield another 5μmoles of carbon dioxide without further oxygen uptake.

The metabolism of malate, which normally requires the presence of oxygen, was investigated in extracts manometrically under anaerobic conditions. The evolution of carbon dioxide was insignificant when malate alone was the substrate but was about twice that from glyoxylate when a mixture of glyoxylate and malate was present. The coupling of the oxidation of malate catalysed by malate dehydrogenase to the reduction of some of the glyoxylate to glycollate was further demonstrated by the results obtained with [1-^{14}C]glyoxylate as substrate (Table 2). Table 2 shows that

Table 2. *Incorporation of radioactivity from* [1-^{14}C]*glyoxylate into glycollate by dialysed extracts of* Pseudomonas *sp. grown on butane-2,3-diol*

Each incubation mixture contained 0·05 ml. of sodium [1-^{14}C]glyoxylate (12·5 μc/ml.; 6·8 mM), 0·05 ml. of tris buffer, pH 7·2, 0·05 ml. of cell extract containing 1 mg. of protein, 0·05 ml. of each addition containing 1·5 μmoles of L-malate or isocitrate, and 0·5 μmole of NADPH or 0·25 μmole of NADP$^+$. The cell extract had previously been dialysed against 0·02 M-tris buffer, pH 7·2, for 18 hr. with three changes of buffer. The mixtures were incubated at 30° for 30 min. and the reactions terminated and assayed for radioactivity as described in the Materials and Methods section.

Additions	Distribution of radioactivity (% of initial radioactivity of glyoxylate)			
	Glyoxylate	Glycollate	Malate	Lost count
None	98	2		
NADPH	43	57		
Isocitrate	24	19		57
Isocitrate + NADP$^+$	21	24		55
Malate	27	25	3	45
Malate + NADP$^+$	19	43	2	36

the extracts could also couple the oxidation of isocitrate to the reduction of glyoxylate. In the presence of malate or isocitrate, part of the ^{14}C was lost either as carbon dioxide or as volatile or fugitive acids during chromatography.

Oxaloacetate-decarboxylase activity. Extracts of cells grown on butane-2,3-diol possessed an activity of 10·3 μmoles of carbon dioxide evolved/mg. of protein/hr. compared with 36·2 for extracts of tartrate-grown organism.

Metabolism of pyruvate by cell-free extracts. There was no significant oxygen uptake when pyruvate was metabolized by cell-free extracts. The extracts catalysed an anaerobic evolution of 5 μmoles of carbon dioxide during the complete metabolism of 10 μmoles of pyruvate. An organic acid with R_F values 0·79 and 0·75 in the phenol–water–formic acid and butan-1-ol–acetic acid–water solvents respectively was found in the reaction products (cf. R_F values 0·80 and 0·74 for lactate in the same two solvents).

The results suggest that the extracts catalyse the dismutation of 2 molecules of pyruvate to lactate, carbon dioxide and acetyl-CoA. This was confirmed by investigations of the metabolism of labelled pyruvate by extracts (Table 3). The formation of malate, presumably by the reaction of some of the acetyl-CoA, formed from pyruvate, with endogenous glyoxylate would explain why the radioactivity of the acetate formed is slightly lower than the radioactivity of the lactate. The formation of alanine probably occurs via transamination of some of the pyruvate by amino acids present in the extract.

Lactate was never detected as a product of the metabolism of glyoxylate by cell extracts. Hence if the extracts metabolize glyoxylate via the dicarboxylic acid cycle and via pyruvate, the oxidation of pyruvate to acetyl-CoA must be coupled to the reduction of glyoxylate to glycollate, not to the reduction of more pyruvate to lactate. If this is so, the presence of glyoxylate in extracts metabolizing pyruvate should greatly decrease the formation of lactate from pyruvate. The results of experiments with [1-^{14}C]pyruvate as substrate (Fig. 1) show that this is not so in that the presence of glyoxylate increased the incorporation of ^{14}C into lactate from 20 to 28%. In the absence of glyoxylate, alanine and lactate were the only labelled products obtained from [1-^{14}C]pyruvate. A similar experiment with [1-^{14}C]glyoxylate as substrate demonstrated the formation of radioactive malate, glycine, glutamate and glycollate. Paper chromatography in the butan-1-ol–acetic acid–water system separated all the reaction products except glycollate and malate, which

Table 3. *Metabolism of sodium* [3-^{14}C]*pyruvate by extracts of* Pseudomonas *sp. grown on butane-2,3-diol*

The reaction mixture contained 0·1 ml. (2 mg. of protein) of extract of cells grown on butane-2,3-diol, 0·05 ml. of phosphate buffer, pH 7·2, and 0·1 ml. of sodium [3-^{14}C]-pyruvate (10 μc/ml.; 5·2 mM). The mixture was incubated at 30° for 30 min. and the reaction products were determined by the techniques described in the Materials and Methods section. The initial pyruvate radioactivity was 3300 counts/min.

Reaction product	Radioactivity (counts/min.)
Alanine	1135
Lactate	1055
Acetate	925
Malate	75
Lost count	110
Pyruvate	0

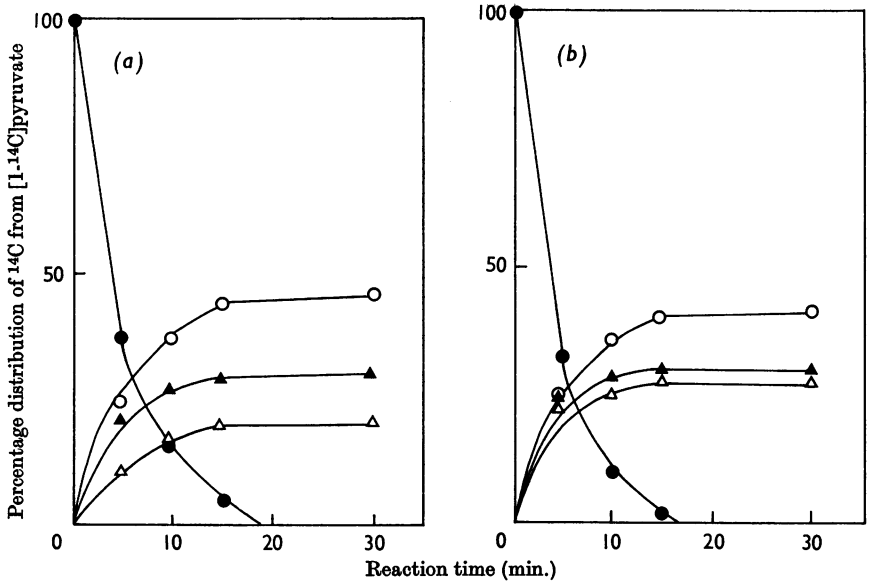

Fig. 1. Effect of glyoxylate on the metabolism of [1-^{14}C]pyruvate by *Pseudomonas* sp. extract. Each reaction mixture initially contained 0·1 ml. of sodium [1-^{14}C]pyruvate (10 μc/ml.; 5 mM) and 0·1 ml. of cell extract (2 mg. of protein) in a total volume of 0·25 ml. The reaction, carried out at 30°, was ended by heating at 100° for 1 min. followed by the addition of 0·05 ml. of 2 N-HCl. (*a*) No additions; (*b*) 1 μmole of glyoxylate added. ○, Lost count; ●, pyruvate; ▲, alanine; △, lactate.

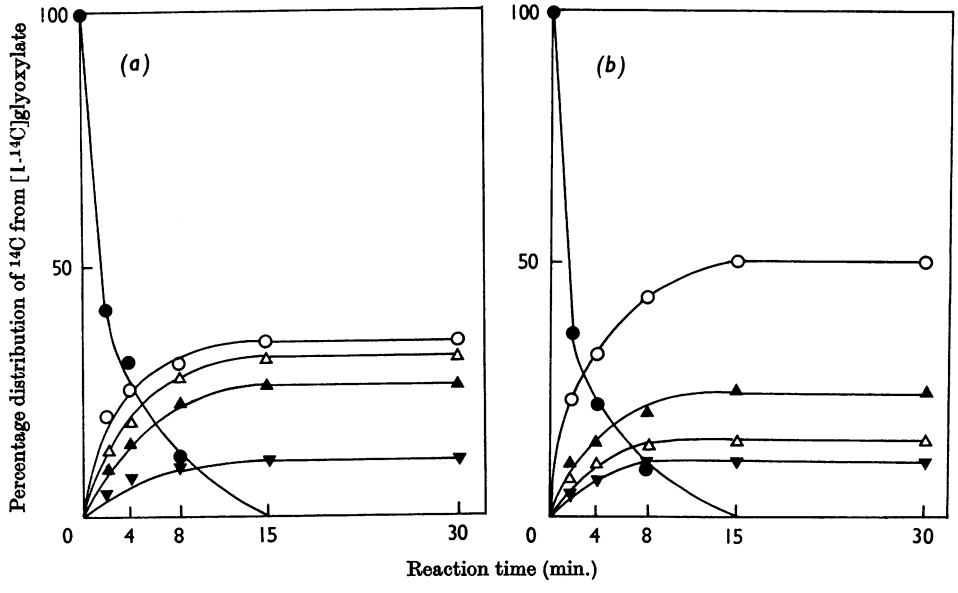

Fig. 2. Effect of pyruvate on the metabolism of [1-^{14}C]glyoxylate by *Pseudomonas* sp. extract. Each reaction mixture initially contained 0·1 ml. of cell extract (1 mg. of protein) and 0·1 ml. of sodium [1-^{14}C]glyoxylate (1 μc/ml.; 8 mM) in a total volume of 0·25 ml. The reaction, carried out at 30°, was stopped by heating at 100° for 1 min. followed by the addition of 0·05 ml. of 2 N-HCl. Radioactive assay of the reaction products, after paper chromatography in butan-1-ol–acetic acid–water solvent, was carried out as described in the Materials and Methods section. (*a*) No additions; (*b*) 1 μmole of pyruvate added. ○, Lost count; ●, glyoxylate; ▲, glycollate + malate; △, glycine; ▼, glutamate.

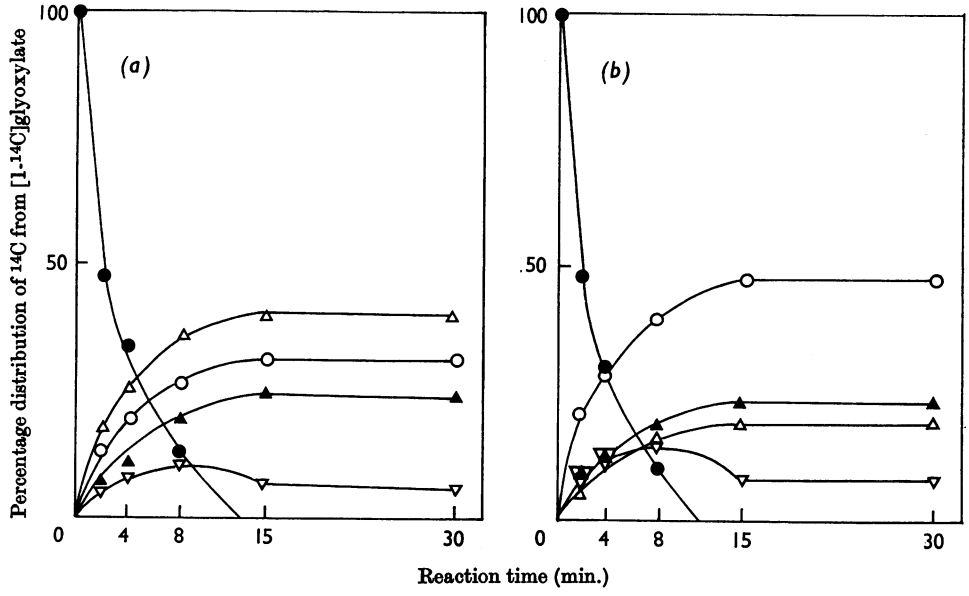

Fig. 3. Effect of pyruvate on the metabolism of [1-^{14}C]glyoxylate by *Pseudomonas* sp. extract. The details are as described for Fig. 2, except that paper chromatography of the reaction products was carried out in phenol–water–formic acid solvent. (*a*) No additions; (*b*) 1 μmole of pyruvate added. ○, Lost count; ●, glyoxylate; △, glycine+glutamate; ▽, malate; ▲, glycollate.

moved together on the chromatogram (Fig. 2). These last two products are resolved by chromatography in the phenol–water–formic acid system, which fails to separate glycine and glutamate (Fig. 3). The addition of pyruvate (1 μmole), one of the intermediates of the cycle, did not increase the incorporation of ^{14}C into glycollate, as would be expected if glyoxylate is metabolized via the dicarboxylic acid cycle.

Glycollate can be formed by the reduction of glyoxylate catalysed by the enzyme glyoxylate reductase. Malate can be formed from glyoxylate and endogenous acetyl-CoA. The formation of glycine presumably occurs by transamination of glyoxylate with endogenous amino acids. The pathway of glutamate formation is discussed below.

Further evidence that glyoxylate is not metabolized via pyruvate by extracts was provided by the demonstration that 'carrier' pyruvate, isolated as the 2,4-DNP-hydrazones, contained no significant amount of radioactivity after incubation of cell extract with [1-^{14}C]glyoxylate.

Short-time incubation of [1-^{14}C]*glyoxylate with extracts.* [1-^{14}C]Glyoxylate was incubated with extract of *Pseudomonas* sp., grown on butane-2,3-diol, for time-intervals up to 6min. to determine whether products, undetected in the previous longer incubations, appear in the early stages of the reaction. Radioactive isocitrate was formed at a rate similar to glycine in the first minute, but after 6min. very little radioactivity was found in this compound (Fig. 4), thus explaining why isocitrate had not been detected in previous experiments. Isocitrate could be formed from the reaction of glyoxylate with endogenous succinate catalysed by the enzyme isocitratase, which is present at a high level of activity in cells grown on butane-2,3-diol (Hullin & Hassall, 1962). The results of experiments in which [1-^{14}C]glyoxylate was incubated with cell extracts in the presence of unlabelled carrier compounds (Table 4) show that glyoxylate, in the presence of succinate, can be readily converted into labelled α-oxoglutarate, which would appear to be an intermediate in the metabolism of glyoxylate by extracts. The formation of glutamate from glyoxylate could be explained by the transamination of the α-oxoglutarate with endogenous amino acids.

The results indicate a reaction sequence in which glyoxylate reacts with endogenous succinate to form isocitrate; the isocitrate is converted into α-oxoglutarate and oxidative decarboxylation of the latter compound to succinate would complete a cyclic system by means of which glyoxylate is completely oxidized. The enzymes isocitratase, isocitrate dehydrogenase and oxalosuccinate decarboxylase respectively would catalyse these successive reactions.

DISCUSSION

The oxidation of glyoxylate to carbon dioxide and water has been studied in a number of systems. Campbell (1955), using extracts of glycine-grown *Pseudomonas* sp., claimed that glyoxylate was oxidized via formate to carbon dioxide. This report was based on the formation of labelled formate and carbon dioxide when the extracts were shaken for 6hr. with 2mM-hypophosphite, which inhibits the oxidation of formate (Campbell, 1954). The mechanism of the reaction may be the same as that suggested by Wood (1964) for the metabolism of glyoxylate by extracts of a *Pseudomonas* sp. grown on phenylacetate, namely the condensation of glyoxylate with catalytic amounts of acetyl-CoA to form malate, which then is converted into oxaloacetate and pyruvate before the final metabolism of pyruvate to formate and acetyl-CoA by the 'phosphoroclastic fission' reaction. This last reaction, however, is fermentative and has not so far been found to occur in obligate aerobes such as *Pseudomonas*. The oxidation of glyoxylate to formate and carbon dioxide has also been reported as an intermediate step in the oxidation of glyceric acid by a soil diphtheroid (Taylor & Juni, 1959).

In contrast with these observations, however, and similar to the results of the present work, Bachrach (1957), Kornberg & Gotto (1959, 1961) and Kornberg & Sadler (1961) failed to detect formate as a product of glyoxylate oxidation by extracts of several species of *Pseudomonas* and *Escherichia coli*; neither did hypophosphite have any effect on the metabolism of glyoxylate by these extracts.

Franke & de Boer (1959) reported that extracts of *Aspergillus niger* anaerobically formed oxalate and glycollate from glyoxylate by a dismutation reaction; aerobically, carbon dioxide was obtained with little uptake of oxygen. In extracts of oxalate-grown *Pseudomonas oxalaticus*, Quayle & Taylor (1961) demonstrated the presence of an NADP-linked glyoxylate dehydrogenase that catalysed the

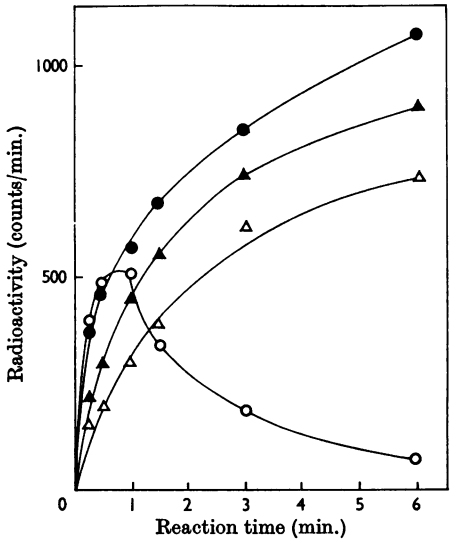

Fig. 4. Metabolism of [1-^{14}C]glyoxylate by extract of *Pseudomonas* sp. grown on butane-2,3-diol. Each reaction mixture initially contained 0·05ml. of cell extract (1mg. of protein), 0·05ml. of phosphate buffer, pH7·2, and 0·05ml. of sodium [1-^{14}C]glyoxylate (10μc/ml.; 5·46mM). The reactions were carried out at 30° and stopped by heating at 100° for 1min. followed by the addition of 0·05ml. of 2N-HCl. Identification and radioactive assay of the products were carried out as described in the Materials and Methods section. ●, Glycine; ▲, glycollate; △, glutamate; ○, isocitrate.

Table 4. *Effect on the radioactivity of glyoxylate and α-oxoglutarate of the addition of unlabelled carrier compounds to* Pseudomonas *sp. extracts metabolizing* [1-^{14}C]*glyoxylate*

Each incubation mixture contained 0·05ml. of sodium [1-^{14}C]glyoxylate (10μc/ml.; 5·46mM), 0·05ml. (1mg. of protein) of extract of cells grown on butane-2,3-diol, 0·05ml. of phosphate buffer, pH7·2, and 0·05ml. of unlabelled carrier compound (20mM). Two sets of incubations were carried out at 30° for 5 and 30min. respectively. The reactions were terminated and assayed for radioactivity as described in the Materials and Methods section. The initial glyoxylate radioactivity was 5560 counts/min. —, Not detected.

	Radioactivity (counts/min.)			
	Glyoxylate		α-Oxoglutarate	
Carrier	5min.	30min.	5min.	30min.
Succinate	210	—	520	86
Fumarate	2480	—	120	50
None	2430	—	80	60
Citrate	1475	—	125	80
α-Oxoglutarate	724	—	760	625
Malate	3010	—	125	60

oxidation of glyoxylyl-CoA to oxalyl-CoA, but the equilibrium of the reaction was strongly in favour of glyoxylyl-CoA. The oxidation of glyoxylate to oxalate has also been demonstrated in plants (Richardson & Tolbert, 1961; Millerd, Morton & Wells, 1963*a*,*b*) and animals (Nakada & Weinhouse, 1953; Ratner, Nocito & Green, 1944). Participation of oxalate in glyoxylate metabolism by extracts used in the present work was excluded by using [^{14}C]glyoxylate as substrate; no labelled oxalate was discernible among the reaction products at any stage of the reaction.

Nakada & Sund (1958) found that the decarboxylation of glyoxylate by washed liver homogenates was stimulated by glutamate. They suggested the occurrence of a sequence of reactions whereby glyoxylate first condensed with glutamate to yield *N*-glyoxyl-L-glutamic acid, which, under the catalytic influence of a glyoxylate decarboxylase, was converted into carbon dioxide and *N*-formyl-L-glutamic acid. This last compound was then hydrolysed to glutamate and formate, and the formate was finally converted into carbon dioxide and water. In contrast with the results of Campbell (1955) and in agreement with the scheme suggested above, Nakada & Sund (1958) showed that the aldehyde group of glyoxylate was transformed into formate with the concomitant oxidation of the carboxyl group to carbon dioxide. In a later investigation of the metabolism of glyoxylate by liver mitochondria, Crawhall & Watts (1962) confirmed that glutamate was needed for the decarboxylation of glyoxylate but could obtain no evidence for the occurrence of *N*-formyl-L-glutamic acid as an intermediate. In the extracts studied in the present work, the presence of glutamate was shown to inhibit the evolution of $^{14}CO_2$ from [1-^{14}C]glyoxylate.

Okuyama, Tsuiki & Kikuchi (1965) have shown that cell-free extracts of *Rhodopseudomonas spheroides* will catalyse an α-oxoglutarate-dependent oxidation of glyoxylate to carbon dioxide. The extracts contained enzymes that would catalyse (*a*) the condensation of glyoxylate with α-oxoglutarate to give β-hydroxy-α-oxoadipate, (*b*) the decarboxylation of the latter to yield α-hydroxyglutarate and (*c*) the dehydrogenation of α-hydroxyglutarate to regenerate α-oxoglutarate. The authors suggested that these reactions could cause the cyclic oxidation of glyoxylate to carbon dioxide and water, catalytic amounts of α-oxoglutarate being required to start the cycle. Glutamate is formed in our system and it might be argued that it could arise by transamination of α-oxoglutarate derived from glyoxylate by the pathway of Okuyama *et al.* (1965), which could also account for the oxidation of glyoxylate. However, unlabelled α-oxoglutarate was obtained from [1-^{14}C]glyoxylate by Okuyama *et al.* (1965) in their system, whereas labelled glutamate and α-oxoglutarate were obtained by us.

Kornberg & Sadler (1960, 1961) obtained evidence for a cyclic pathway of oxidation of glyoxylate by a mutant of *E. coli*. In this dicarboxylic acid cycle, glyoxylate reacts with catalytic quantities of acetyl-CoA, catalysed by malate synthase, to form malate, which is then reconverted into acetyl-CoA via oxaloacetate and pyruvate. Some evidence was obtained initially in support of the possibility that carbon dioxide production from glyoxylate in the extracts studied by us occurred via the dicarboxylic acid cycle. The extracts are rich in malate synthase (Hullin & Hassall, 1962) and could metabolize malate via oxaloacetate to pyruvate. Glyoxylate can act as a hydrogen acceptor, being reduced to glycollate in the process. In addition, it was demonstrated that, although malate alone was not metabolized anaerobically by extracts, it was removed in the presence of glyoxylate. Further, the oxidation of malate by extracts could bring about the reduction of [1-^{14}C]glyoxylate to [1-^{14}C]-glycollate, and labelled malate could always be detected as a reaction product when [1-^{14}C]glyoxylate was incubated with extract.

However, in contrast with these observations, the experiments on the metabolism of pyruvate by the extracts indicated that glyoxylate was not metabolized via the dicarboxylic acid cycle. Extracts metabolized pyruvate, aerobically or anaerobically, by a dismutation reaction to acetyl-CoA and lactate. Since lactate was never detected as a product of glyoxylate metabolism by the extracts, if the metabolism of glyoxylate proceeds via pyruvate, then the oxidation of pyruvate to acetyl-CoA must be coupled to the reduction of glyoxylate to glycollate, not to the reduction of pyruvate to lactate. Yet in the presence of glyoxylate the amount of lactate produced from pyruvate is increased. In addition, the presence of pyruvate had little effect on the rate of utilization of glyoxylate by extracts or on the amount of glycollate formed. Finally, when [1-^{14}C]glyoxylate was incubated with carrier quantities of unlabelled pyruvate, no ^{14}C appeared in the pyruvate isolated as the 2,4-DNP-hydrazones at the end of the experiment. These observations, together with the evidence that alanine was produced from pyruvate by extracts but never from glyoxylate, indicated that pyruvate is not an intermediate in the metabolism of glyoxylate by the extracts of *Pseudomonas* used.

When [1-^{14}C]glyoxylate was incubated with extract for 10min. or more the ^{14}C appeared principally in carbon dioxide, glycine, glutamate and glycollate and, to a small extent, in malate, α-oxoglutarate and isocitrate. In experiments up to 6min., the ^{14}C was incorporated into isocitrate as fast as it was into glycine. The rate of glyoxylate

utilization can be increased by the addition of succinate, α-oxoglutarate or citrate, and in each case α-oxoglutarate becomes labelled. These results demonstrate that carbon dioxide can arise more readily by the oxidation of glyoxylate via the series of reactions catalysed by isocitratase, isocitrate dehydrogenase and α-oxoglutarate dehydrogenase respectively than via the dicarboxylic acid cycle.

Bachrach (1957), using a glycine-grown *Pseudomonas* sp., had suggested that glyoxylate may be oxidized by a similar cycle. Endogenous succinate is always present in the extracts used by us; the extracts are also rich in isocitratase (Hullin & Hassall, 1962). Labelled isocitrate, α-oxoglutarate, glutamate (presumably from transamination of α-oxoglutarate with endogenous amino acids) and glycollate can always be detected as reaction products when [1-^{14}C]glyoxylate is incubated with extracts. The glycollate would be obtained through some of the glyoxylate acting as a hydrogen acceptor through the mediation of nicotinamide–adenine dinucleotides. It has been demonstrated that the oxidation of isocitrate can bring about the reduction of [1-^{14}C]glyoxylate to [1-^{14}C]glycollate.

It seems likely that the oxidation of glyoxylate via this cyclic pathway with the concomitant reduction of some of the substrate to glycollate occurs in extracts, but that it has no significance in the whole cell. Hullin & Hassall (1962) showed that no ^{14}C was incorporated into glycollate from [1-^{14}C]acetate by cells utilizing butane-2,3-diol. The glyoxylate-reductase reaction was not readily reversible and no evidence could be obtained for the metabolism of glycollate by any other pathway. Glycollate was not metabolized by crude cell extracts even when fortified with various cofactors. The possibility that glycollate is produced by whole cells and then excreted into the medium was investigated under various conditions of aeration, but none was found. Cells growing exponentially on butane-2,3-diol took up [1-^{14}C]glycollate from the medium but no metabolism of the labelled glycollate was detected.

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