The purified preparations seem to be free from flavins, since no spectrophotometric and fluorometric evidence thereof was obtained.

SUMMARY

1. 3-Hydroxyanthranilic acid oxidase has been purified from rat and ox liver. The most active preparations of the enzyme oxidize 2500 μm-moles of 3-hydroxyanthranilic acid/min./mg. of protein.

2. The dependence of the reaction rate on the concentration of both substrates (3-hydroxyanthranilic acid and oxygen) was measured. $K_m$ for 3-hydroxyanthranilic acid is low (7 μM under the conditions of the activity test). At the oxygen pressure of the air the reaction rate is still far from maximal.

3. The purified enzyme solutions present an absorption maximum at 400 mμ and a peculiar fluorescence. There is not yet sufficient evidence to ascertain whether these bands are due to the enzyme itself or to impurities.

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REFERENCES


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The Metabolism of C₂ Compounds in Micro-organisms

6. SYNTHESIS OF CELL CONSTITUENTS FROM GLYCOLLATE BY PSEUDOMONAS SP.*

BY H. L. KORNBERG AND A. M. GOTTO

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

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The growth of micro-organisms on C₂ compounds as the sole source of carbon necessitates the occurrence of reactions whereby the C₂ substrate provides both metabolic energy and the carbon skeletons of cellular constituents. When the C₂ substrate is acetate, both these functions are fulfilled by the tricarboxylic acid cycle, with the glyoxylate cycle operating in addition to maintain the concentrations of intermediates utilized for growth. But this combination of reactions cannot explain growth on C₂ compounds which are more highly oxidized than acetate, such as glycollate.

Moreover, though studies with a mutant of Escherichia coli confirmed that the tricarboxylic acid cycle plays an essential role in growth on glycollate, they showed that this C₂ substrate was oxidized via a dicarboxylic acid cycle in the absence of a functional tricarboxylic acid cycle (Kornberg & Sadler, 1960). A different sequence of reactions must therefore occur to replenish from glycollate the supply of intermediates of the dicarboxylic and tricarboxylic acid cycles drained away to provide cell materials for the growing bacteria.

The main purpose of this paper is to present evidence for the nature of such a biosynthetic
sequence in *Pseudomonas* sp. growing on glycollate as sole carbon source. The results obtained further support the view that, like *E. coli*, *Pseudomonas* sp. derive metabolic energy under these conditions from the oxidation of glycollate via a dicarboxylic acid cycle in which glyoxylate is completely oxidized by a series of reactions in which acetyl-coenzyme A, malate, oxaloacetate and pyruvate play a catalytic role.

An outline of similar work with *Pseudomonas ovalis* Chester has been previously published (Kornberg & Gotto, 1959).

**MATERIALS AND METHODS**

**Maintenance and growth of organisms.** The original culture of *Pseudomonas* (B_{2aba}) was a gift from Dr J. Lascelles. The organisms were maintained on slopes containing 25 mm-sodium glycollate, 25 mm-NH_4Cl, 25 mm-sodium-potassium phosphate, pH 7.2, and salts (2 mg. of CaCl_2, 6H_2O, 0.2 mg. of MnSO_4?H_2O, 4 mg. of MgSO_4?7H_2O and 0.2 mg. of FeSO_4?6H_2O/100 ml. of medium), solidified with 2% (w/v) agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Cultures of the organism were grown at 30° and stored at 2°; they were subcultured at intervals of 2–3 weeks.

For experimental use, cells were grown aerobically with shaking at 30°, in Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) containing 300 ml of growth medium similar to the above but with the carbon and nitrogen sources at a concentration of 50 mm and the agar agar omitted. The bacteria were harvested by centrifuging at 0°, 1500 g for 15 min. during the logarithmic phase of growth (at cell densities of 0.3–0.4 g. dry wt./ml.). For experiments on the incorporation of isotope from [14C]glycollate by growing bacteria, the packed cells were re-suspended in fresh growth medium containing 10 mm-sodium glycollate. For the preparation of extracts or studies on the oxidation of substrates, the packed cells were washed with 0.9% (w/v) KCl and suspended in 0.9% (w/v) KCl.

**Incorporation of isotope from [14C]glycollate.** The procedures used for studies on the incorporation of [14C]glycollate into constituents of the ethanol-soluble fractions of bacteria, growing on glycollate, were similar to those described for *Pseudomonas* (Kornberg, 1958) and *E. coli* (Kornberg, Phizackerley & Sadler, 1960) metabolizing [14C]acetate.

**Assay and identification of labelled compounds.** Labelled materials were located and identified on two-dimensional paper chromatograms by radioautography and co-chromatography with authentic unlabelled materials as described by Kornberg (1958). In addition, glycollic acid and glycergic acid were analysed by one-dimensional chromatography in ethanol-ammonia–water (80:4:16, Long, Quayle & Stedman, 1951), and amino acids by one-dimensional chromatography in water-saturated phenol in the presence of amoniac vapour (Consden, Gordon & Martin, 1944) and in the water–diethyamine–butanol–ethanol (5:2:10:10) system of Hardy, Holland & Nayler (1955). The phosphate groups of phosphorylated compounds were removed by incubation with Polidase-S (Schwarz Laboratories Inc., New York, U.S.A.) as described by Benson, Bassham & Calvin (1951); the resultant dephosphorylated compounds were analysed by two-dimensional chromatography (Kornberg, 1958).

**Preparation of cell extracts.** Freshly grown *Pseudomonas* (B_{2aba}) were disintegrated either by crushing in the Hughes press (Hughes, 1951), or by subjecting them to the output of a 600 w Mullard magnetostrictor oscillator, operating at 3.5A, for 2 min. The suspensions, in 0.9% KCl, of disintegrated bacteria obtained by either technique were centrifuged at 24 000 g for 15 min. at 0°; the supernatant solutions thus obtained were used for experimental purposes.

**Isotopic materials.** All isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [1-14C]glycollate was dissolved in the minimum quantity of water and purified by paper chromatography on Whatman no. 3 paper in ethanol–ammonia–water (80:4:16; Long et al., 1951). Sodium [2-14C]glycollate was prepared from the hydrolysis of [2-14C]bromoaetic acid by a procedure modified from that used by Jayasuriya (1956). To 1 ml. of aqueous [2-14C]bromoaetic acid (containing 12 mg. of material and 200 μc of 14C) was added 1 ml. of 1 M-KOH, and the mixture was kept at 80° for 3 hr. The solution was evaporated under O_2-free N_2 to less than 1 ml., cooled to room temperature, neutralized with 6N-HCl and streaked on to Whatman no. 3 paper. After chromatography in ethanol–ammonia–water (Long et al., 1951) the radioactive band on the air-dried paper was located by radioautography and eluted with water. The solution was adjusted to pH 9.0 with 0.2M-2-amino-2-hydroxymethylpropene-1,3-diol (tris) buffer and stored at −2°.

Sodium [1-14C]glyoxylate was prepared from the calcium salt by treatment with the Na_2 form of Dowex-50 resin; it was assayed by direct counting of the 2-dinitrophenylhydrazone isolated on paper chromatograms (El Hawary & Thompson, 1953). [14C]Glyceric acid was prepared enzymically from sodium [1-14C]glyoxylate. A sonic extract of glycollate-grown *Pseudomonas* (B_{2aba}) (containing 1.0 mg. of protein) was incubated anaerobically at 30° for 15 min., in a Warburg manometer gassed with O_2-free N_2, with 2.5 μmoles of sodium [1-14C]glyoxylate, (giving 1.25 × 10^6 counts/min. under the conditions of radioassay used), 100 μmoles of potassium phosphate (pH 7.2), 0.5 μmole of thiamine pyrophosphate, 2.5 μmoles of reduced diphophorydride nucleotide (DPNH), 10 μmoles of MgCl_2 and water to 1 ml. Evolved CO_2 was trapped with 400 μmoles of KOH placed in the centre well. The cup was detached and the contents of the main compartment were mixed with 3 ml. of absolute ethanol. The precipitated material was removed by centrifuging and washed with 1 ml. of 20% (v/v) aqueous ethanol. The combined supernatant solutions were evaporated to a small volume at 60° under O_2-free N_2 and chromatographed two-dimensionally as described by Kornberg (1958). A small portion of the radioactive material was mixed with authentic D(-)-glyceric acid and chromatographed one-dimensionally (Long et al., 1951) and two-dimensionally (Kornberg, 1958): the radioactive spot located by radioautography occupied the same position and had the same shape as the carrier D(-)-glyceric acid, which was located by spraying the chromatogram with 0.04% (w/v) ethanolic bromocresol green adjusted to pH 8 with NaOH. The [14C]glyoxylate acid thus prepared and identified, which contained exactly half
the total radioactivity of the sodium [1-14C]glyoxylate used as starting material, was eluted from the chromatogram with water, adjusted to pH 7 with 20 mm-potassium phosphate, and used for experimental purposes.

Other materials used. Phenazine methosulphate, sodium glyoxylate, coenzyme A and pyridine nucleotides were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A.; 2:6-dichlorophenol-indophenol was from British Drug Houses Ltd., Poole. Generous gifts of lithium hydroxy-
pyruvate were received from Professor P. D. Dixon, F.R.S.; calcium \( \text{d}^\text{(-)} \text{glycerate} \) from Dr P. W. Kent; highly purified sodium glyoxylate from D. I. Zelitch (Connecticut Agricultural Experimental Station, New Haven, U.S.A.); sodium 3-phosphoglycerate from Dr J. R. Quayle; phosphotransacetylase from Mr N. Carr, and sodium pyruvate and oxaloacetic acid from Professor Sir Hans Krebs, F.R.S. All other reagents used were of the highest purity commercially available.

Enzyme assays

Glycolic oxidase. This enzyme (Tolbert, Claggett & Burris, 1949; Zelitch & Ochoa, 1953) was assayed by measuring the reduction of potassium ferricyanide or 2:6-dichlorophenol-indophenol (Zelitch & Ochoa, 1953) or the formation of glyoxylate semicarbazone (Olson, 1959) in a Cary recording spectrophotometer.

In manometric assays of this enzyme, the main compartments of Warburg manometer cups contained 150 \( \mu \)moles of potassium phosphate buffer, pH 7.2, 0.5 ml of sonic extract (containing 8–15 mg. of protein) and water to a final volume of 1.7 ml. Strips of Whatman no. 40 filter paper and 400\( \mu \)moles of KOH were placed in the centre wells. Sodium glycollate (20\( \mu \)moles) and 0.2 ml of 1% (w/v) phenazine methosulphate were added from the side arm after thermal equilibration at 30°. The gas phase was air. Cups without phenazine methosulphate, without glycollate and without glyoxylate and phenazin methosulphate were used as controls.

The stoichiometry of glycollate oxidation was determined by measuring \( O_2 \) uptake in the presence of semicarbazide. The procedure was the same as that just described, except that the main compartment of the Warburg flasks also contained 50\( \mu \)moles of semicarbazide adjusted to pH 5. At the end of the experiment 0.20 ml of 12\( N \) HCl was added to the contents of the flasks, and, after centrifuging, glyoxylate was estimated in the supernatant solution by the procedure of Friedemann & Haugen (1943). The 2:4-dinitrophenylhydrazone of glyoxylate was identified by chromatography in the solvent of El Hawary & Thompson (1953).

Glyoxylate carboligase. This enzyme (Krakow & Barkulis, 1956; Krakow, Hayashi & Barkulis, 1959) was assayed manometrically by measuring the rate of anaerobic evolution of \( CO_2 \) from glyoxylate. Double-armed Warburg flasks contained 40\( \mu \)moles of phosphate buffer, pH 6.4, 0.5\( \mu \)moles of thiamine pyrophosphate, 10\( \mu \)moles of MgCl\(_2\), 5\( \mu \)moles of sodium D(-)-glycerate, 0.4\( \mu \)moles of DPNH, 2000 units of crystalline lactic dehydrogenase (C. F. Boehringer und Söhne, Mannheim, Germany) and extracts, prepared in a Hughes press, of glycollate-grown Pseudomonas. A cuvette without DPNH was used as the blank. Reactions were followed in a Cary recording spectrophotometer.

Pyruvic oxidase. The pyruvic-oxidase activity of sonic extracts of Pseudomonas was measured by the dismutation assay described by Korkes (1955). The evolution of \( CO_2 \) was measured with Warburg manometers as pyruvate was dissimilated to lactate and acetyl phosphate in the presence of lactate dehydrogenase, phosphotransacetylase, catalytic amounts of diphosphopyridine nucleotide and coenzyme A, phosphate buffer and a sonic extract from glycollate-grown Pseudomonas sp. At the end of the reaction acetyl phosphate was estimated as the hydroxamic acid (Lipmann & Tuttle, 1945).

Malate synthetase (Wong & Ajl, 1956), citrate-condensing enzyme (Ochoa, Stern & Schneider, 1951) and isocitratase (Olson, 1954, 1959; Smith & Günsalus, 1954, 1955, 1957). These enzymes were estimated spectrophotometrically as described by Dixon & Kornberg (1959).

Malic dehydrogenase. This enzyme was estimated spectrophotometrically by measurements of the rate of decrease in extinction at 340 m\( \mu \) consequent upon the oxidation of DPNH by oxaloacetic acid. The assay system used was that described previously (Kornberg, 1958).

Pyruvate formation from oxaloacetate. This was estimated spectrophotometrically by measuring the difference in the rates of decrease in extinction at 340 m\( \mu \) when extracts of

strips. Samples of Ba\(_{14}CO_3\) were prepared in duplicate from the contents of the centre wells as described by Sakami (1955) and were counted with a lead-shielded Geiger–Müller mica end-window counter tube. The observed counts/min. were corrected for background radiation and for self-absorption to infinite thinness.

Conversion of glyoxylate into glycerate. The procedure used for measuring the formation of [1-14C]glycerate from sodium [1-14C]glyoxylate was as described above. When unlabelled glyoxylate was used as substrate, the glycerate formed was isolated by chromatography on Dowex-1 (Cl\(^-\) form, \( \times 10, 200–400 \) mesh) and was estimated by the colorimetric procedure of Bartlett (1959).

Conversion of glycerate into pyruvate. The formation of pyruvate from glycerate was measured by incorporating extracts of glycollate-grown Pseudomonas (B\(_{aba}\)) prepared in a Hughes press (Hughes, 1951) with 5\( \mu \)moles of sodium \( \text{d}^\text{(-)} \text{glycerate}, 5\mu \)moles of glutathione, 10\( \mu \)moles of MgCl\(_2\), 100\( \mu \)moles of phosphate buffer, pH 7.2, 0.20\( \mu \)mole of adenosine triphosphate (ATP) and water to 1 ml. The reaction mixture was incubated at 30° for 30 min. The reaction was terminated by the addition of 0.2 ml of 50% (w/v) trichloroacetic acid. After centrifuging, pyruvate was estimated as its 2:4-dinitrophenylhydrazone (Friedemann & Haugen, 1943) and identified by chromatography (El Hawary & Thompson, 1953).

The rate of formation of pyruvate from glycerate was determined spectrophotometrically by measuring the rate of decrease in extinction at 340 m\( \mu \) consequent upon conversion of the pyruvate into lactate in the presence of DPNH and lactic dehydrogenase. Quartz cuvettes (vol. 3 ml., 1 cm. light path) contained 100\( \mu \)moles of tris buffer, pH 7.4, 10\( \mu \)moles of MgCl\(_2\), 5\( \mu \)moles of reduced glutathione, 0.2\( \mu \)mole of ATP, 5\( \mu \)moles of sodium \( \text{d}^\text{(-)} \text{glycerate}, 0.4\( \mu \)mole of DPNH, 2000 units of crystalline lactic dehydrogenase (C. F. Boehringer und Söhne, Mannheim, Germany) and extracts, prepared in a Hughes press, of glycollate-grown Pseudomonas. A cuvette without DPNH was used as the blank. Reactions were followed in a Cary recording spectrophotometer.
Pseudomonas sp. were incubated with oxaloacetic acid and DPNH in the presence and absence of lactic dehydrogenase. All spectrophotometric assays were performed at 22±1°C.

RESULTS

Oxidation of substrates by whole cells

Washed suspensions of glycollate-grown Pseudomonas rapidly oxidized glycollate, glyoxylate, glycine, pyruvate and malate, but only slowly oxidized 2-oxoacids, α-oxoglutarate and succinate (Table 1). Citrate was not oxidized. The rate of oxygen uptake with glycollate was sufficient to account for the oxidation of 2-1 μmoles of this substrate/mg. dry wt./hr. The total quantities of oxygen absorbed, corrected for endogenous respiration, were 73% of the amounts required for complete oxidation of glycollate or glyoxylate.

Of the substrates tested, malate and succinate were rapidly, and α-oxoglutarate was slowly, oxidized by washed suspensions of the succinate-grown Pseudomonas (Table 1). Glycollate was slowly oxidized after a lag period of 30 min., during which time a glycollate 'permease' (for review, see Cohen & Monod, 1957) may have been formed; glycollic oxidase was found in only trace amounts in cell-free extracts of succinate-grown cells.

Incorporation of labelled glycollate

When [1-14C]glycollate or [2-14C]glycollate was added to suspensions of Pseudomonas growing on glycollate as sole carbon source, radioactivity was incorporated into both the ethanol-soluble and protein fractions. The incorporation of radioactivity into the ethanol-soluble fraction increased linearly with time for the duration of the experiment (2–3 min.).

Distribution of radioactivity from [2-14C]glycollate.

The distribution of isotope amongst the labelled components of the ethanol-soluble fractions was analysed by two-dimensional chromatography and radioautography. Label from [2-14C]glycollate rapidly appeared in malate, phosphoglycerate, glycine, citrate and glutamate (Fig. 1). In the samples taken at the earliest times, malate contained the greatest proportion of radioactivity (approx. 28% of the total 14C incorporated). Approx. 25% of the radioactivity was present in phosphoglycerate and 15% in glutamate. Citrate and glycine each contained 13–14% of the total radioactivity, and approx. 4% was found in aspartate. These proportions changed with time: the proportion of total isotope incorporated into glutamate increased and, after 2 min., represented more than 40% of the total radioactivity of the samples. At this time the phosphoglycerate area contained approx. 13% of the incorporated 14C, glycine approx. 6, malate approx. 17, citrate 10 and aspartate 8%. The three curves with the most distinct negative slopes were those of phosphoglycerate, glycine and malate. Initially these three compounds contained approx. 65% of the total.
radioactivity, but after 2 min. only 35% of the
total radioactivity was found in them. Hence it
must be concluded that glycollate enters the meta-
bolic pathways operating in Pseudomonas (Baba)at sites close to glyoxylate (or glycine), phospho-
glycerate and malate.

Oxidation of glycollate by cell extracts

Extracts, obtained by ultrasonic disintegration
of glycollate-grown Pseudomonas slowly formed
glyoxylate from glycollate. The formation of this
keto acid, measured spectrophotometrically as the
increase in extinction at 252 m\(\mu\) consequent upon
the formation of glyoxylate semicarbazone (Olson,
1959), was dependent upon the presence of cell
extract, glycollate and semicarbazide (Fig. 2). The
addition of electron acceptors, such as 2,6-dichloro-
phenol-indophenol, potassium ferricyanide or phen-
azide methosulphate, greatly stimulated glyoxy-
late formation and enabled the reaction to be
followed manometrically by the uptake of oxygen.
Fig. 3 shows results obtained with phenazine
methosulphate, which was the most effective of the
electron acceptors tested. Although the extract, in
the absence of added electron acceptors, catalysed
the uptake of 58 \(\mu\)l. of oxygen in 15 min., addition
of phenazine methosulphate increased this gas
absorption to 205 \(\mu\)l. When this experiment was
performed in the presence of semicarbazide, de-
composition of the semicarbazone formed with
acid 2,4-dinitrophenylhydrazine, and chromat-
ography of the resultant 2,4-dinitrophenylhydra-
zones (El Hawary & Thompson, 1953) showed
glyoxylate to be the sole keto compound present.
Comparison of the quantities of oxygen absorbed
and the quantities of keto acid produced (Friede-
mann & Haugen, 1943) established the stoicheio-
metry of the oxidation of glycollate (Table 2) to be:

\[
glycollate + \frac{1}{2}O_2 \rightarrow \text{glyoxylate} + H_2O.\]

The marked stimulation of oxygen uptake by
phenazine methosulphate, and the yellow colour of

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**Fig. 2.** Formation of glyoxylate from glycollate by sonic
eextracts of glycollate-grown Pseudomonas (Baba). The complete
system (\(\bullet\)) contained in 3 ml., 100 \(\mu\)moles of
potassium phosphate, pH 6.0, 10 \(\mu\)moles of MgCl\(_2\), 60 \(\mu\)moles
of freshly neutralized semicarbazide, 0-06 ml. of a
sonic extract of glycollate-grown Pseudomonas (Baba)
(containing 1-0 mg. of soluble protein) and 10 \(\mu\)moles
of sodium glycollate. Glyoxylate formation was measured in
a Cary recording spectrophotometer as the rate of change of
extinction at 252 m\(\mu\) consequent upon the formation of
glyoxylate semicarbazone (Olson, 1959). From control
cuvettes, the extract (O) or glycollate (A) or semi-
carbazide (\(\triangle\)) was omitted.

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**Fig. 3.** Oxidation of glycollate by sonic extracts of
Pseudomonas (Baba). The main compartments of Warburg
manometer flasks contained in 1-9 ml., 100 \(\mu\)moles of
sodium potassium phosphate, pH 7.2, 10 \(\mu\)moles of MgCl\(_2\),
1-0 ml. of sonic extract of Pseudomonas (Baba) grown on
glycollate or succinate (containing 15 mg. of soluble pro-
tein). The centre wells contained 0-2 ml. of 2\(\times\)-KOH. After
thermal equilibration at 30° for 10 min., 20 \(\mu\)moles of
sodium glycollate and 0-2 ml. of 1% \((w/v)\) phenazine
methosulphate were added from the side arms. The quanti-
ties (\(\mu\)l.) of \(O_2\) absorbed after tipping the side arms were
recorded over 40 min. 1, \(\bullet\), Complete system, glycollate-
grown cell extract; 2, \(\times\), as 1 but phenazine methosul-
phate omitted; 3, \(\Delta\), as 1 but glycollate omitted; 4, \(\bigcirc\), as 1
but glycollate and phenazine methosulphate omitted; 5, \(\blacktriangle\),
as 1 but containing extract of cells grown on succinate
instead of glycollate.
Table 2. Oxidation of glycollate to glyoxylate by extracts of glycollate-grown Pseudomonas (B2aba)

The main compartments of Warburg manometer flasks contained, in 2 ml, 200 μmoles of potassium phosphate, pH 6-0, 10 μmoles of MgCl₂, 1-0 ml of sonic extract of glycollate-grown Pseudomonas (B2aba) (containing 10 mg of protein) and 60 μmoles of semicarbazide. The centre wells contained 400 μmoles of KOH. After thermal equilibration at 30°, 20 μmoles of sodium glycollate and 0-2 ml of 1% (w/v) phenazine methosulphate were added from the side arm. At the end of the incubation period the cups were detached, and the contents were acidified with 0-5 ml of 6N-HCl and centrifuged. The glyoxylate content of the supernatant solution was estimated by the method of Friedemann & Haugen (1943); chromatography of the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953) showed glyoxylate to be the sole keto compound formed.

<table>
<thead>
<tr>
<th>Contents of side arms</th>
<th>Quantity of O₂ absorbed (μmoles)</th>
<th>Net (corr. for endogenous)</th>
<th>Quantity of glyoxylate formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system</td>
<td>6-2</td>
<td>5-6</td>
<td>11-2</td>
</tr>
<tr>
<td>Phenazine methosulphate omitted</td>
<td>3-2</td>
<td>2-6</td>
<td>5-3</td>
</tr>
<tr>
<td>Glycollate omitted</td>
<td>0-6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Complete system, but cell extract boiled before incubation</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3. Stoicheiometry of the anaerobic decarboxylation of glyoxylate

The main compartment of Warburg manometer cups contained, in 2 ml, 80 μmoles of potassium phosphate, pH 6-4, 0-5 μmole of thiamine pyrophosphate, 10 μmoles of MgCl₂ and 1-0 ml of sonic extract of glycollate-grown Pseudomonas (B2aba) (containing 9 mg of protein). The manometers were gassed with O₂-free N₂. After thermal equilibration at 30°, sodium glyoxylate contained in the side arms was tipped into the main compartments. After the evolution of CO₂ had ceased, analysis of the contents of the main compartments showed that all the glyoxylate added had been utilized but that a material giving an intense purple colour in the assay of Friedemann & Haugen (1943) had been formed.

<table>
<thead>
<tr>
<th>Quantity of glyoxylate added (μmoles)</th>
<th>Quantity of CO₂ evolved (μmoles)</th>
<th>Glyoxylate utilized CO₂ evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>5-5</td>
<td>2-0</td>
</tr>
<tr>
<td>22</td>
<td>11-1</td>
<td>1-98</td>
</tr>
<tr>
<td>33</td>
<td>16-6</td>
<td>1-98</td>
</tr>
<tr>
<td>11*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Cell extract boiled before incubation.

Table 4. Anaerobic decarboxylation of [1-14C]glyoxylate

The complete system contained, in the main compartments of double-armed Warburg manometer cups, 40 μmoles of potassium phosphate, pH 6-4, 0-5 μmole of thiamine pyrophosphate, 10 μmoles of MgCl₂, 0-10 ml of sonic extract of glycollate-grown Pseudomonas (B2aba) (containing 1-0 mg of protein) and water to 2-0 ml. The centre wells contained 200 μmoles of KOH. The first side arm contained 4 μmoles of sodium [1,14C]glyoxylate (giving 1-54 x 10⁶ counts/min. when assayed as BaCO₃ at infinite thinness). The second side arm contained 0-2 ml of 3N-HCl. The manometers were gassed with O₂-free N₂ and equilibrated at 30°. The reaction was started by tipping the contents of the first side arm into the main compartment; after 30 min, it was terminated by adding the contents of the second. The cups were detached and the contents of the centre wells were mixed with 400 μmoles of Na₂CO₃. The carbon contained in measured portions of this was precipitated as BaCO₃ and assayed for radioactivity, the observed counts/min. being corrected for background radiation and self-absorption to infinite thinness. The contents of the main compartments were centrifuged, mixed with 2 μmoles of sodium glyoxylate and chromatographed as the 2:4-dinitrophenylhydrazones (El Hawary & Thompson, 1953).

<table>
<thead>
<tr>
<th>Contents of cup</th>
<th>Glyoxylate Radioactivity (counts/min.) in</th>
<th>Glyoxylate Radioactivity (counts/min.) in</th>
<th>10⁻⁴ × Radioactivity (counts/min.) in</th>
<th>[14C]Glyoxylate utilized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyoxylate (initial)</td>
<td>Glyoxylate (final)</td>
<td>CO₂</td>
<td>14CO₂ evolved</td>
</tr>
<tr>
<td>Complete system</td>
<td>15-4</td>
<td>0-0</td>
<td>7-9</td>
<td>1-95</td>
</tr>
<tr>
<td>Complete system, but cell extract boiled before incubation</td>
<td>15-4</td>
<td>15-4</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
the purified glycollate-oxidizing enzyme, suggest that it is a flavoprotein similar to the glycolic oxidase of spinach (Zelitch & Ochoa, 1953; Frigerio & Harbury, 1958).

Extracts of succinate-grown *Pseudomonas* catalysed the oxidation of glycollate at less than 4% of the rate observed with similar extracts prepared from glycollate-grown cells.

**Conversion of glyoxylate into a C₃ compound**

When extracts of glycollate-grown cells oxidized glycollate in the absence of semicarbazide as trapping agent, a keto compound accumulated which differed from glyoxylate and formed an orange, insoluble (probably bis) derivative with 2:4-dinitrophenylhydrazine. Glyoxylate was not detected under these conditions, which indicates that the keto compound formed was a product of the further metabolism of glyoxylate.

One such reaction of glyoxylate is its condensative decarboxylation. This reaction was discovered by Krakow & Barkulis (1956) in extracts of glycollate-grown E. coli, and the enzyme which catalysed it was named glyoxylate carboligase (Krakow et al. 1959). Extracts of glycollate-grown *Pseudomonas* (B₂aba) contained high activities of glyoxylate carboligase. Anaerobically, one molecule of carbon dioxide was evolved for each two molecules of glyoxylate present (Tables 3 and 4). After dialysis of the extracts against pyrophosphate buffer, pH 8.5, the rapid decarboxylation of glyoxylate required the addition of magnesium ions and thiamine pyrophosphate (Fig. 4). These findings are similar to those reported for *E. coli*, where the anaerobic condensation of glyoxylate yielded one molecule of carbon dioxide and one mole of a C₃ compound, which was assumed to be tartronic semialdehyde or its isomer, hydroxy- pyruvate [see (ii)].

Although both isomers of the C₃ product were readily interconverted by extracts of *E. coli* (Krakow & Barkulis, 1956; Krakow et al. 1959), extracts of glycollate-grown *Pseudomonas* under these conditions did not form hydroxypyruvate. The conclusion that the C₃ product formed by *Pseudomonas* (B₂aba) extracts differed from hydroxypyruvate rests on the following evidence:

(a) When dilute solutions of hydroxy(pyruvate and the enzymically formed material reacted with acid 2:4-dinitrophenylhydrazine, the reaction product of the latter compound but not of the former rapidly separated out from solution as orange needles, which indicated that a bis derivative had been formed.

(b) Alkaline solutions of the 2:4-dinitrophenylhydrazone of hydroxy(pyruvate are red in colour, and show an absorption maximum at 445 mμ. Similar solutions of the derivative obtained from the enzymically formed C₃ product are deep purple in colour, do not show an absorption maximum at 445 mμ but absorb strongly at 560-570 mμ. The observed spectrum also differs from that noted with solutions of the 2:4-dinitrophenylhydrazones of pyruvate, glyoxylate or glycolaldehyde, but is similar to that obtained with glyoxal or mesoxalic semialdehyde.

(c) The 2:4-dinitrophenylhydrazone of hydroxy(pyruvate formed a compact double spot, close to the lower of the two spots of glyoxylate 2:4-dinitrophenylhydrazone, in the chromatographic solvent system of El Hawary & Thompson (1953). It could be readily separated from the derivative obtained from the enzymic C₃ product, which tended to streak, but the bulk of which ran faster than hydroxy(pyruvate.

![Fig. 4. Evolution of CO₂ from glyoxylate catalysed by extracts of glycollate-grown *Pseudomonas* (B₂aba). The main compartments of Warburg-manometer flasks contained in 1-9 ml, 40 μmoles of potassium phosphate, pH 6.4, 10 μmoles of MgCl₂, 0.5 μmole of thiamine pyrophosphate and 0.5 ml. of a sonic extract of glycollate-grown *Pseudomonas* (B₂aba), which had been dialysed against pyrophosphate buffer, pH 8.5, and which contained 2.0 mg. of protein (●). The manometers were gassed with N₂ and equilibrated for 10 min. at 30°. Sodium glyoxylate (30 μmoles) was added from the side arms and the quantities of CO₂ evolved (μl.) were measured. Flasks from which MgCl₂ (x), thiamine pyrophosphate (△) or both MgCl₂ and thiamine pyrophosphate (▲) had been omitted were used as controls.](image-url)
Table 5. Conversion of $[1-^{14}C]$glyoxylate into $[^{14}C]$glycerate

The complete reaction mixture contained 100 $\mu$moles of tris buffer, pH 7.4, 0.5 $\mu$mole of thiamine pyrophosphate, 10 $\mu$mole of MgCl$_2$, 0.05 ml. of sonic extract of glycollate-grown *Pseudomonas* (B$_2$aba) (containing 1-0 mg. of protein), 0.45 $\mu$mole of DPNH, 0.45 $\mu$mole of sodium $[1-^{14}C]$glyoxylate (giving 2.5 $\times$ 10$^4$ counts/min. under the conditions of radioassay used), and water to 3-1 ml. The rate of the reaction was measured as the decrease in extinction at 340 m$\mu$m in a Cary recording spectrophotometer. At the end of the incubation, one half of the contents of the cuvettes was pipetted into 3 ml. of ethanol, centrifuged, and the supernatant solution was analysed by chromatography and radioautography (Kornberg, 1958). A portion of the other half was pipetted into tubes containing 0.2 ml. of saturated 2,4-dinitrophenylhydrazine in 2n-HCl. The derivatives formed were extracted into ethyl acetate and analysed by chromatography (El Hawary & Thompson, 1953) and direct radioassay of the labelled spots.

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Glyoxylate Radioactivity (counts/min.)</th>
<th>Glyoxylate Radioactivity (counts/min.)</th>
<th>Glycerate Radioactivity (counts/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glyoxylate (initial)</td>
<td>Glyoxylate (final)</td>
<td>Glycerate</td>
</tr>
<tr>
<td>Complete system</td>
<td>25-0</td>
<td>0-10</td>
<td>12-0</td>
</tr>
<tr>
<td>DPNH omitted</td>
<td>25-0</td>
<td>0-20</td>
<td>0-80</td>
</tr>
<tr>
<td>Complete system but extract boiled before incubation</td>
<td>25-0</td>
<td>25-0</td>
<td>0-00</td>
</tr>
<tr>
<td>Complete system; but hydroxyxypyruvate (1$\mu$mole) added*</td>
<td>25-0</td>
<td>0-15</td>
<td>12-7</td>
</tr>
<tr>
<td>DPNH omitted but hydroxyxypyruvate (1$\mu$mole) added*</td>
<td>25-0</td>
<td>0-20</td>
<td>0-40</td>
</tr>
</tbody>
</table>

* The 2,4-dinitrophenylhydrazone of the hydroxyxypyruvate, isolated after the incubation, was virtually devoid of radioactivity.

Table 6. Oxidation of reduced diphosphopyridine nucleotide in the presence of glyoxylate

The reactions were followed in a Cary recording spectrophotometer at 340 m$\mu$. The 'test' cuvette contained 100 $\mu$moles of potassium phosphate, pH 7.4, 10 $\mu$mole of MgCl$_2$, 0.3 $\mu$mole of thiamine pyrophosphate, 0.05 ml. of a sonic extract of glycollate-grown *Pseudomonas* (B$_2$aba), 0.40 $\mu$mole of DPNH and sodium glyoxylate in a total volume of 3-0 ml. In the 'blank' cuvette DPNH was omitted. The product of the reaction was identified as glyceric acid.

<table>
<thead>
<tr>
<th>Glyoxylate used (umole)</th>
<th>DPNH oxidized (umole)</th>
<th>Glyoxylate used (umole)</th>
<th>DPNH oxidized (umole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>0-05</td>
<td>2-0</td>
<td></td>
</tr>
<tr>
<td>0-30</td>
<td>0-14</td>
<td>2-1</td>
<td></td>
</tr>
<tr>
<td>0-50</td>
<td>0-24</td>
<td>2-1</td>
<td></td>
</tr>
</tbody>
</table>

(d) The p-nitrophenylhydrazone of the enzymic C$_3$ product was readily separated from the similar derivatives of glyoxal, glycolaldehyde, hydroxyxypyruvate and glyoxylate in the chromatographic solvents of El Hawary & Thompson (1953).

(e) The p-nitrophenylhydrazone of the C$_3$ product formed from $[1-^{14}C]$glyoxylate was highly radioactive whereas hydroxyxypyruvate, present throughout the incubation, formed a derivative containing negligible $^{14}$C.

(f) The enzymic C$_3$ product reacted approx. 40 times as fast with DPNH and extracts of glycollate grown *Pseudomonas* as did a similar concentration of hydroxyxypyruvate.

(g) The quantities of labelled glycerate formed from labelled glyoxylate in the presence of DPNH and glycollate-grown cell extract (see below) were unaffected by the addition of hydroxyxypyruvate.
Table 7. Stoicheiometry of net formation of glycerate from glyoxylate

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Contents</th>
<th>Glyoxylate content (µmoles)</th>
<th>Glyoxylate utilized (µmoles)</th>
<th>Glyceric acid content (µmoles)</th>
<th>Glyceric acid formed (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Complete system</td>
<td>Initial: 5.0</td>
<td>Final: 1.6</td>
<td>Initial: 3.4</td>
<td>Final: 1.8</td>
</tr>
<tr>
<td>2</td>
<td>DPNH omitted</td>
<td>Initial: 5.0</td>
<td>Final: 0.0</td>
<td>Initial: 0.0</td>
<td>Final: 0.0</td>
</tr>
<tr>
<td>3</td>
<td>Glyoxylate omitted</td>
<td>Initial: 0.0</td>
<td>Final: 0.0</td>
<td>Initial: 0.0</td>
<td>Final: 0.0</td>
</tr>
<tr>
<td>4</td>
<td>Complete system, but enzyme boiled</td>
<td>Initial: 5.0</td>
<td>Final: 5.0</td>
<td>Initial: 0.0</td>
<td>Final: 0.0</td>
</tr>
</tbody>
</table>

and the hydroxypropyruvate added did not acquire radioactivity during this reaction (Table 5).

These results support the tentative identification of the C₃ product as tartronic semialdehyde.

Conversion of the C₃ compound into glycerate

Cell-free extracts of glycollate-grown Pseudomonas oxidized DPNH in the presence of glyoxylate. The reaction started after a short lag period, and required the addition of magnesium ions and thiamine pyrophosphate for maximal activity (Fig. 5). When the extract was preincubated for 5 min. with glyoxylate, the reaction rate was at least 10 times as great as that obtained without preincubation. In the presence of limiting amounts of added glyoxylate, one molecule of DPNH was oxidized per two molecules of glyoxylate added as substrate (Table 6). No accumulation of the C₃ compound was detected when the total amount of glyoxylate added was less than twice the amount of DPNH present. The demonstration of lag period, of the requirements for magnesium ions and thiamine pyrophosphate, of the increased rate after preincubation with glyoxylate and of the stoichiometry of DPNH oxidation indicated that the process being studied was not a single-step reduction of glyoxylate (possibly to glycollate) but the conversion of glyoxylate into the C₃ compound [reaction (ii)], followed by its reduction to glycerate in the presence of DPNH:

\[
\text{[CHO·CH(OH)·CO₂H] + DPNH + H⁺ → C₃ compound}
\]

Evidence for the overall conversion of glyoxylate into glycerate [reactions (ii)–(iii)] was obtained by incubating cell-free extracts of glycollate-grown Pseudomonas with [1-¹⁴C]glyoxylate and DPNH.

Table 8. Conversion of glycerate into pyruvate by extracts of glycollate-grown Pseudomonas

In a total volume of 2-0 ml the complete reaction system contained 100-µmoles of tris buffer, pH 7-4, 10-µmoles of MgCl₂, 5-µmoles of glutathione, 10-µmoles of sodium D-glycerate, 0-5 ml of extract from glycollate-grown Pseudomonas (Bₐaba) (containing 3-0 mg of protein) and varied amounts of ATP. The reaction mixtures were incubated at 30° for 30 min. The reactions were stopped by the addition of 0.2 ml of conc. HCl. The precipitates were removed by centrifuging. In the supernatant solutions pyruvate was estimated by the method of Friedemann & Haugen (1943), and the identity of the 2:4-dinitrophenylhydrzone was established by chromatography (El Hawary & Thompson, 1953).

<table>
<thead>
<tr>
<th>Contents</th>
<th>Pyruvate formed (µmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete system (0-2 µ mole of ATP)</td>
<td>1-2</td>
</tr>
<tr>
<td>Complete system (1-0 µ mole of ATP)</td>
<td>1-3</td>
</tr>
<tr>
<td>Complete system (10 µ moles of ATP)</td>
<td>1-2</td>
</tr>
<tr>
<td>Complete system but ATP omitted</td>
<td>0-10</td>
</tr>
<tr>
<td>Glycerate omitted</td>
<td>0</td>
</tr>
<tr>
<td>Complete system, enzyme boiled</td>
<td>0</td>
</tr>
</tbody>
</table>

For each two molecules of [1-¹⁴C]glyoxylate utilized, one molecule of radioactive glycerate accumulated (Table 5); no labelled glycollate was detected. The formation of radioactive glycerate was dependent upon the addition of DPNH. Similarly, the net formation of one molecule of glycerate from two molecules of glyoxylate (Table 7) was shown to be

\[
\text{CH₄(OH)·CH(OH)·CO₂H + DPN⁺ → C₃ compound}
\]

dependent on the presence of glyoxylate, DPNH and cell extract. These results further show that, as expected for reactions (ii) and (iii), the carbon dioxide evolved for the condensative decarboxyl-
ation of glyoxylate arose from the carboxyl groups of one of the glyoxylate molecules involved, and that the glycerate formed contained only half the radioactivity of the total glyoxylate entering this sequence but the same quantity as the C₃ compound found as its first step.

Conversion of glycerate into pyruvate

Cell-free extracts of glycollate-grown *Pseudomonas* catalysed the conversion of glycerate into pyruvate (Table 8). The conversion was dependent upon the addition of a catalytic amount of ATP, indicating that the glycerate was probably phosphorylated to form phosphoglycerate and that the ATP was regenerated in the subsequent conversion of phosphoenolpyruvate into pyruvate. The overall reaction is represented by:

\[ \text{CH}_3\text{(OH)}\cdot\text{CH(OH)}\cdot\text{CO}_2\text{H} \rightarrow \text{CH}_3\text{CO}\cdot\text{CO}_2\text{H} + \text{H}_2\text{O} \]

The rate of the overall process was measured by converting the pyruvate into lactate in the presence of DPNH and crystalline lactic dehydrogenase. Under these conditions 7·2 μmoles of lactate were formed from glycerate/hr./mg. of protein in the extract. No lactate was formed if the extract, glycerate or ATP, was omitted (Fig. 6).

When radioactive glycerate, enzymically prepared by incubating extracts of glycollate-grown *Pseudomonas* with [1-¹⁴C]glyoxylate and DPNH [reactions (ii)–(iii)], was used as the substrate for pyruvate formation, radioactive pyruvate was isolated at the end of the reaction (Table 9). This demonstrates the overall conversion of [1-¹⁴C]glyoxylate into radioactive pyruvate, being the sum of reactions (ii)–(iv).

Rates of enzymic reactions in extracts of *Pseudomonas* (B₂aba)

Sonic extracts of *Pseudomonas* (B₂aba) grown on succinate differed markedly from those grown on glycollate in their ability to catalyse relevant reactions (Table 10). Glycollate was oxidized [reaction (i)] 50 times as fast by extracts of glycollate-grown cells as by those grown on succinate; similarly the rates of the glyoxylate carboligase reaction [reaction (ii)] and the formation of pyruvate from glycerate [reaction (iv)] occurred respectively at at least 50 and 700 times the rates in the former than in the latter extracts. This is evidence for the view that these reactions play a role in the metabolism of glycollate, and are not of importance when glycollate (or glyoxylate) are not major substrates.

Both extracts grown on glycollate and on succinate contained malate synthetase and con-

---

Table 9. Conversion of [¹⁴C]glycerate into pyruvate

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Radioactivity of pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Complete system</td>
<td>510</td>
</tr>
<tr>
<td>(2) As (1) but ATP omitted</td>
<td>24</td>
</tr>
<tr>
<td>(3) As (1) but extract boiled before incubation</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 6. Conversion of glycerate into lactate catalysed by extracts of glycollate-grown *Pseudomonas* (B₂aba). The complete system (●) contained, in 3 ml., 100 μmoles of tris buffer, pH 7·6, 10 μmoles of MgCl₂, 5 μmoles of reduced glutathione, 0·2 μmole of ATP, 2000 units of crystalline lactic dehydrogenase, 0·40 μmole of DNP and 0·05 ml. of sonic extract of glycollate-grown *Pseudomonas* (B₂aba) (containing 0·35 mg. of soluble protein). The reaction was started by addition of 5 μmoles of sodium D(−)-glycerate and was measured as the change in extinction with time at 340 μm concomitant with the oxidation of DNP. Control cuvettes contained the above system but omitting cell extract (△) or ATP (×) or glycerate (○).
Table 10. Enzymic reactions in extracts of Pseudomonas (B2aba)

The rates of the reactions in sonic extracts are expressed as specific activities (μmoles of substrate transformed/mg. of soluble protein/hr.). For conditions of assay, see Methods section.

<table>
<thead>
<tr>
<th>Reaction no.</th>
<th>Enzymic activity</th>
<th>Glycollate Specific activity</th>
<th>Succinate Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>Glycollic oxidase</td>
<td>3.5</td>
<td>0.07</td>
</tr>
<tr>
<td>(ii)</td>
<td>Glyoxylate carboligase</td>
<td>41.0</td>
<td>0.80</td>
</tr>
<tr>
<td>(iii)</td>
<td>Formation of glycerate from C4 compound</td>
<td>47.0</td>
<td>—</td>
</tr>
<tr>
<td>(iv)</td>
<td>Formation of pyruvate from glycerate</td>
<td>7.2</td>
<td>0.01</td>
</tr>
<tr>
<td>(v)</td>
<td>Malate synthetase</td>
<td>104</td>
<td>6.1</td>
</tr>
<tr>
<td>(vi)</td>
<td>Malic dehydrogenase</td>
<td>143</td>
<td>157</td>
</tr>
<tr>
<td>(vii)</td>
<td>Formation of pyruvate from oxaloacetate</td>
<td>102</td>
<td>130</td>
</tr>
<tr>
<td>(viii)</td>
<td>Pyruvate oxidase</td>
<td>1.2</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>Condensing enzyme</td>
<td>24.8</td>
<td>42.6</td>
</tr>
<tr>
<td>—</td>
<td>isocitratase</td>
<td>0.21</td>
<td>—</td>
</tr>
</tbody>
</table>

The condensing enzyme (cf. Kornberg & Lund, 1959), but differed in the absolute levels of these enzymes and in their relative amounts. Glycollate-grown cells contained 17 times as much malate synthetase but less than two-thirds as much condensing enzyme as carbon to the cell. The oxidation of glycollate in E. coli, strain w, has been shown to be effected, at least in part, via a dicarboxylic acid cycle (Kornberg & Sadler, 1960), in which the component reactions are

\[
\text{glycollate} + \frac{1}{2} \text{O}_2 \rightarrow \text{glyoxylate} + \text{H}_2\text{O} \quad \text{(i)}
\]

\[
\text{glyoxylate} + \text{acetyl-coenzyme A} + \text{H}_2\text{O} \rightarrow \text{malate} + \text{coenzyme A} \quad \text{(v)}
\]

\[
\text{malate} + \frac{1}{2} \text{O}_2 \rightarrow \text{oxaloacetate} + \text{H}_2\text{O} \quad \text{(vi)}
\]

\[
\text{oxaloacetate} \xrightarrow{\text{possibly via phosphopyruvate}} \text{pyruvate} + \text{CO}_2 \quad \text{(vii)}
\]

\[
\text{pyruvate} + \frac{1}{2} \text{O}_2 + \text{coenzyme A} \rightarrow \text{acetyl-coenzyme A} + \text{CO}_3 + \text{H}_2\text{O} \quad \text{(viii)}
\]

\[
\text{Sum: glycollate} + 1\frac{1}{2}\text{O}_2 \rightarrow 2\text{CO}_2 + 2\text{H}_2\text{O}. \quad \text{(ix)}
\]

The observation that isotope from [2-14C]-glycollate was incorporated initially by glycollate-grown Pseudomonas (B2aba) into glycine (presumably by amination of glyoxylate, which is not detected by the chromatographic procedures used) and into malate indicates that this dicarboxylic acid cycle serves also in Pseudomonas (B2aba) to provide metabolic energy from the oxidation of glycollate. This is supported by the finding that the levels of malate synthetase (v) in extracts of Pseudomonas (B2aba) grown on glycollate or on other substrates catabolized chiefly to glyoxylate (such as allantoin) are 4–5 times as high as those of the citrate-forming condensing enzyme, whereas in extracts of the same organisms grown on succinate, or other substrates catabolized chiefly to intermediates of the tricarboxylic acid cycle, the malate-synthetase activity is approx. one-seventh of that of the condensing enzyme; moreover, the absolute levels of condensing enzyme are lower in glycollate-grown cells than in cells grown on substrates catabolized to intermediates of the tricarboxylic acid cycle. This suggests that during the growth of Pseudomonas (B2aba) on glycollate, malate synthetase is involved both in respiration and in the biosynthesis of cellular materials, whereas
condensing enzyme fulfils chiefly the latter function.

Whereas the operation of this dicarboxylic acid cycle serves to supply metabolic energy to the cell from the catabolism of glycollate, it cannot explain growth on glycollate. Cellular constituents, such as carbohydrate and protein, are synthesized by a series of reactions in which intermediates of the dicarboxylic and tricarboxylic acid cycles serve as starting materials (for review, see Roberts, Abelson, Cowie, Bolton & Britten, 1955; Davis, 1955; Ehrensvärd, 1955; Kornberg, 1959). Growth therefore necessarily involves the removal of such intermediates from these cycles, and cannot take place unless reactions occur to effect the net formation of these compounds from glycollate.

The patterns of distribution of isotope incorporated from [2-14C]glycollate by Pseudomonas (B2aba) growing on it indicated that, besides glyoxylate and malate, phosphoglyceric acid was rapidly formed from the C6 substrate. Glutamate synthesized under similar conditions but in which [1-14C]-glycollate was the substrate, was almost exclusively labelled in the α-carboxyl group (H. L. Kornberg & A. M. Gotto, unpublished work). This latter observation confirms the earlier work of Jayasuriya (1955) who found that approx. 7 times as much isotope was incorporated into citrate from [2-14C]glycollate as from [1-14C]glycollate, and that with the former isotopic substrate, all six carbon atoms of citrate were labelled whereas, with the latter, isotope was incorporated only into two of the three carboxyl groups. Both the isotope distribution observed in brief incubations and the pattern of labelling of citrate and glutamate support the operation of the biosynthetic sequence out-

Scheme 1. Postulated route for the formation of cell constituents from glycollate by Pseudomonas (B2aba). The overall reaction leading from glycollate to malate by this sequence is

\[ 3 \text{glycollate} \rightarrow \text{malate} + 2\text{CO}_2 + 6\text{H}. \]

Roman numbers in parentheses refer to reactions discussed in the text.
Studies of (cf. glyoxylate biosynthetic reactions of which coenzyme A would result at a C3 compound biosynthetic growth in Keesch and of the initial isotope would still be half yield then would be unlabelled. Condensation of tartronic semialdehyde (Chow et al., 1959), which is phosphorylated to phospho-

The quantitative net formation of glycerate from glyoxylate under these conditions was demonstrated.

Summary

1. Washed suspensions of *Pseudomonas* (B2aba), grown on glycollate, readily oxidized glycollate, glyoxylate, glycerate, pyruvate and malate. Of these substrates, only malate was readily oxidized by the organisms grown on succinate.

2. *Pseudomonas* (B2aba) growing on glycollate incorporated 14C from [2-14C]glycollate most rapidly into glycine, phosphoglycerate and malate.

3. Extracts of such cells catalysed the oxidation of glycollate to glyoxylate, which was stimulated by phenazine methosulphate.

4. Such extracts further catalysed the anaerobic condensative decarboxylation of glyoxylate to carbon dioxide and a C3 compound, which differed from hydroxypropionate and was probably tartronic semialdehyde. This reaction required magnesium ions and thiamine pyrophosphate for maximal activity. One molecule of carbon dioxide was evolved for each two molecules of glyoxylate added; experiments with [1-14C]glyoxylate showed that the evolved carbon dioxide arose from the carboxyl group of one of the glyoxylate molecules reacting.

5. The tartronic semialdehyde formed was enzymically reduced to glycric acid with the concomitant stoichiometric oxidation of reduced diphasphopyridine nucleotide.

6. In the presence of magnesium ions, thiamine pyrophosphate and reduced diphasphopyridine nucleotide, such cell extracts catalysed the incorporation of isotope from [1-14C]glyoxylate into carbon dioxide and glycric acid, this overall reaction being:

\[2 \text{glyoxylate} + \text{DPNH} + \text{H}^+ \rightarrow \text{glycerate} + \text{CO}_2 + \text{DPN}^+.\]

Further evidence for the operation of the postulated biosynthetic sequence, all the component reactions of which have been demonstrated to occur in extracts of glycollate-grown *Pseudomonas* at rates of the order necessary to account for the growth of the organisms on glycollate (mean generation time, 3 hr.), is provided by the observation that both the enzyme conversion of glycerate into pyruvate (iv) and glyoxylate carboligase (ii) occur in high activity only in cells grown on substrates the major catabolic product of which is glyoxylate (cf. Gray, Gerhart & Brooke, 1959). The studies of Callely & Dagley (1959) and of Quayle & Keech (1959) indicate that this or a closely similar biosynthetic sequence operates also in *Pseudo-

monas* growing on glycinc or oxalate as sole carbon source.
(B₄aba). The former extracts also contained 17 times as much malate synthetase, but less than two-thirds as much condensing enzyme, as the latter.

10. On the basis of these results, a scheme is proposed to account for the biosynthesis of cell constituents from glycocollic acid. It is further concluded that the oxidation of glycocollic by *Pseudomonas* (B₄aba) proceeds at least in part via a dicarboxylic acid cycle.

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REFERENCES


