Synthesis of Cell Constituents from Glycine by a *Pseudomonas*

By S. DAGLEY, P. W. TRUDGILL AND A. G. CALLELY*

Department of Biochemistry, University of Leeds

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Janke & Tayenthal (1937) identified glyoxylic acid as an intermediate in the degradation of glycine by *Pseudomonas fluorescens*. Campbell (1955) prepared cell-free extracts of a species of *Pseudomonas* which oxidized glycine to ammonia, carbon dioxide and water and showed that glyoxylic and formic acids were intermediates in the oxidation. Since glycine is converted into glyoxylate, it was suggested (Dagley, 1958; Callely & Dagley, 1959) that reaction sequences found in bacteria metabolizing glycollate (Kornberg & Gotto, 1959; Kornberg & Gotto, 1961a) and oxalate (Quayle & Keech, 1959; Quayle, Keech & Taylor, 1961) might also be used by bacteria when growing with glycine as sole carbon source. The present work supports the view that tartronic semialdehyde is a reaction intermediate in bacterial metabolism under these conditions.

MATERIALS AND METHODS

Maintenance and growth of organisms. The original culture of *Pseudomonas* (A) was a gift from Professor B. C. J. G. Knight and was one of four species isolated from poultry house deep-litter (Bachrach, 1957). Stock cultures were maintained on nutrient-agar slopes and when sealed with Parafilm (A. Gallenkamp and Co. Ltd., London, E.C. 2) could be kept for several months at 4° without desiccation.

For experimental use, cells were grown with forced aeration at 30° in a medium adjusted with NaOH to pH 7.2 and containing, per litre: KH_2PO_4 , 2 g.; $(\text{NH}_4)_2\text{SO}_4$, 0.5 g.; glycine, 1.4 g. (0.02 M); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g. After growth for 18 hr. cells were harvested in a Sharples centrifuge and washed once by resuspension in phosphate buffer (KH_2PO_4 ; 2 g./l.; pH 7.2) and centrifuged at 3° for 10 min. at 4300g. Growth of cultures was followed by measurement of their turbidities in a Spekker photoelectric absorptiometer (Dagley, Dawes & Morrison, 1950).

Preparation of cell extracts. Freshly grown cells of *Pseudomonas* (A) were disintegrated either in the Hughes (1951) press or by exposure for 5 min. at 0° to the output of a MSE-Mullard ultrasonic disintegrator at an average frequency of 20 kcy./sec. Each 10 g. of cells crushed in the press were ground with 1 g. of polishing alumina, grade 3/50 (Griffin and George Ltd., Manchester), and 25 ml. of buffer (KH_2PO_4 ; 2 g./l.; pH 7.2). This procedure facilitated both the dispersal of the crushed cells in the buffer and the subsequent clarification of the viscous extract by centri-

fuging at 3° and 10 500g for 45 min. Protein concentrations of extracts were determined by the method of Sols (1947).

Paper chromatography. Paper chromatography of carboxylic acids was carried out by the ascending method on Whatman no. 1 papers: one-dimensionally in phenol-formic acid-water (500:13:167, w/v/v; Kornberg, 1958), and two-dimensionally, first in this solvent and then in butanol-acetic acid-water (120:30:50, by vol.; Wood, 1958) or in ether-benzene-formic acid-water (70:30:14:10, by vol.) followed by propanol-formic acid-water (6:3:1, by vol.; Weimberg, 1959). For 2,4-dinitrophenylhydrazones of keto acids, ascending chromatograms were run on Whatman no. 4 papers: one-dimensionally in butanol-ethanol- $(\text{NH}_4)_2\text{CO}_3$ buffer (80:22:28, by vol.; Dagley, Fewster & Happold, 1952), and two-dimensionally, first in this solvent and then in butanol-benzene- $(\text{NH}_4)_2\text{CO}_3$ buffer (80:5:15, by vol.; Fewster & Hall, 1951). Radioactive compounds were located and identified on chromatograms by radioautography and co-chromatography with authentic unlabelled materials. Non-volatile carboxylic acids used as carriers were located by spraying with a 1% solution of aniline and xylose in 95% (v/v) methanol (Wood, 1958). Descending reverse-phase chromatograms of mono- and bis-2,4-dinitrophenylhydrazones were run on silicone-treated Whatman no. 1 paper in *N*-dimethylformamide-water-chlorobenzene-heptane (7:1:16:4, by vol.; Meigh, 1956).

Microassays of labelled compounds. Reactions with ^{14}C -labelled substrates were carried out in Pyrex glass tubes (8 mm. $\times$ 70 mm.) tightly plugged with non-absorbent cotton wool. Volumes of reactants, 0.02–0.10 ml., were accurately delivered into the tubes from Pasteur pipettes filled and discharged by manipulation of a screw clip around the rubber teat. The pipettes were calibrated from blood pipettes and checked by weighing the water delivered into capped polythene vessels (15 mm. $\times$ 30 mm.). The total volume of each complete reaction mixture was 0.2–0.3 ml. After incubation at 30°, reactions were stopped by placing the tubes in a boiling-water bath for 1 min. They were then cooled in ice, 0.02 ml. of 3 *N*-HCl was added, and the precipitate removed by centrifuging. Usually, 0.05 ml. of liquid was applied to the chromatography paper. Radioactive spots were located by radioautography and eluted with water into capped polythene vessels, and volumes determined by weighing. Samples (0.02 ml.) were plated out on the smooth sides of 19 mm. disks of Whatman no. 1 paper, dried for 10 min. at a distance of 12 cm. from a 100 w light bulb and counted in a Tracerlab S.C. 16G windowless flow counter.

Isotopic materials. All isotopic materials were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [^{14}C]glyoxylate was diluted with unlabelled glyoxylate for use and unless otherwise stated contained 8 $\mu\text{moles/ml}$. with an activity of 1 $\mu\text{C/ml}$. Labelled glyceric acid was prepared in solution when required by the action

* Present address: Department of Botany, University College, Cardiff.

of sonic extracts of *Pseudomonas* (A) on sodium glyoxylate and DPNH; the yield of [^{14}C]glycerate from sodium [^{14}C]glyoxylate was 95% of the theoretical value.

Other materials used. Sodium glyoxylate was prepared from tartaric acid by periodate oxidation (Radin, Metzler, Bloom & Westerfield, 1955) and purified by repeated crystallization from aqueous acetone. Reduced pyridine nucleotides were from Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP, ADP, coenzyme A, glycolaldehyde and glyceric acid were from L. Light and Co. Ltd., Colnbrook, Bucks. Barium 2-phosphoglycerate, barium 3-phosphoglycerate and barium silver phosphoenolpyruvate were from C. F. Boehringer und Soehne, Mannheim, Germany. We thank Professor Frank Dickens, F.R.S., for a gift of lithium hydroxypyruvate and Mr P. J. Chapman for disodium 2,4-dioxovalerate and methyl 2,4-dioxovalerate. All other reagents used were of the highest purity commercially available.

Determination of materials. Glycolaldehyde forms the same bis-2,4-dinitrophenylhydrazone as glyoxal, and the present method is a modification of the determination of diacetyl as its bis-2,4-dinitrophenylhydrazone (Neuberg & Straus, 1945). To 3 ml. of a solution containing 0.0–2 μmole of glycolaldehyde was added 5 ml. of 0.2% 2,4-dinitrophenylhydrazine in 2N-HCl, and after incubation for 1 hr. at 100° the precipitated bis-2,4-dinitrophenylhydrazone was centrifuged with 0.2 g. of kaolin, washed once with 3 ml. of 2N-HCl, twice with water and drained. The precipitate was extracted twice with 5 ml. of 0.3% sodium ethylate solution at room temperature, the extracts were made up to 25 ml. with sodium ethylate solution, and the extinctions were read at 580 μm in a spectrophotometer and referred to a curve prepared from standard glycolaldehyde solutions similarly treated.

Glyoxylate and pyruvate were determined by the method of Friedemann & Haugen (1943).

Determination of enzyme activities. Glyoxylate carboxylase (Krakow & Barkulis, 1956; Krakow, Barkulis & Hayashi, 1961) was assayed by measurement either of the rate of disappearance of [^{14}C]glyoxylate or the rate of anaerobic evolution of CO_2 from glyoxylate. For the latter, double-armed Warburg flasks which contained in the main compartments 15 μmoles of phosphate buffer, pH 7.2, and enzyme solution were gassed with O_2 -free N_2 and thermally equilibrated at 30°. The reaction was started by adding sodium glyoxylate from side arm 1 and stopped by tipping 0.2 ml. of 50% (w/v) trichloroacetic acid from side arm 2, when all CO_2 was expelled from solution. When DPNH was added it was placed in the side arm with glyoxylate. Volumes were made up to 3 ml. with distilled water.

Tartronic semialdehyde reductase (Kornberg & Gotto, 1961b) was assayed by measuring spectrophotometrically at 340 μm the rate of oxidation of DPNH in the presence of an excess of the C_3 compound produced enzymically from sodium glyoxylate before addition of DPNH. There is strong evidence that this C_3 compound is tartronic semialdehyde.

RESULTS

Accumulation of pyruvate in cultures

Cells grown in glycine and mineral salts medium were harvested after 20 hr. in the stationary phase and resuspended in an equal volume of aerated

growth medium at 30°. During a lag in growth of 4 hr. there accumulated in the culture a keto acid, the 2,4-dinitrophenylhydrazone of which appeared to be identical with that of pyruvate from its absorption spectrum in alkali and its behaviour on chromatograms in the solvents described by Dagley *et al.* (1952) and Fewster & Hall (1951). When growth began, pyruvate disappeared (Fig. 1). Cell suspensions taken at the end of the exponential phase of growth did not lag in a fresh medium and there was no accumulation of pyruvate.

Pyruvate might be formed from glycine by conversion into serine; however, serine-dehydrase activity (Binkley, 1943; Wood & Gunsalus, 1949) could not be detected when suspensions of washed cells grown with glycine were incubated anaerobically with 50 μmoles of L-serine/3 ml.

Oxidation of substrates by whole cells

Pyruvate, malate, fumarate, succinate, glycine, glyoxylate and formate were rapidly oxidized by

Fig. 1. Accumulation of pyruvate by *Pseudomonas* (A) in a glycine growth medium. The cells were harvested from 100 ml. of culture after 20 hr. in the stationary phase and resuspended in 75 ml. of fresh aerated growth medium at 30°. At time intervals, turbidities (upper curve) and pyruvate concentrations in centrifuged samples (lower curve) were measured.

cell suspensions taken at the onset of the stationary phase in glycine media. Acetate was oxidized rapidly after a lag of 10 min. in the respirometer and citrate slowly throughout; DL-alanine was oxidized rapidly and DL-serine slowly. Cells grown in succinate and mineral salts medium readily oxidized various compounds of the tricarboxylic acid cycle, except citrate, but glycine and glyoxylate were oxidized slowly.

Campbell (1955) showed that the oxidation of formate by extracts of another species of *Pseudomonas* was completely inhibited by addition of 2 mM-potassium hypophosphite, and glycine and glyoxylate were oxidized quantitatively to formate when hypophosphite was present. Addition of 2 mM-potassium hypophosphite inhibited oxidation of formate by whole cells of *Pseudomonas* (A) but not oxidation of glycine or glyoxylate, and no formate accumulated. Bachrach (1957) obtained similar results with whole cells of *Pseudomonas aeruginosa*. In contrast, oxidation of glycine and glyoxylate was decreased by the presence of inhibitors of the tricarboxylic acid cycle. Sodium fluoroacetate (0.1 mM) inhibited the oxidation of fumarate by washed cells completely and that of glycine and glyoxylate by 90%; in cultures, fluoroacetate (10 μ M) inhibited growth on glycine by 93%. The oxidation of glycine by cell suspensions was reduced by 95 and 35% in the presence of 10 mM- and 1 mM-sodium arsenite respectively. In the second case there accumulated 0.39 μ mole of pyruvate and 0.06 μ mole of α -oxoglutarate/3 ml., the keto acids being identified by chromatography and spectrophotometry of their 2,4-dinitrophenylhydrazones. Cell extracts of *Pseudomonas* (A) oxidized neither formate nor glycine, but succinate and citrate were oxidized rapidly.

Enzymic decarboxylation of glyoxylate

Krakov & Barkulis (1956), Krakow *et al.* (1961) and Kornberg & Gotto (1959, 1961a) studied the decarboxylation of glyoxylate by extracts prepared respectively from *Escherichia coli* and from species of *Pseudomonas* grown with glycollate as carbon source; they found that 2 moles of glyoxylate gave rise to 1 mole of carbon dioxide by an enzyme glyoxylate carboligase. Calley & Dagley (1959) obtained yields of carbon dioxide greater than this but somewhat lower than required by the equation: 2 glyoxylate $\rightarrow$ glycolaldehyde + 2CO₂. They suggested the existence of an enzyme in their cell extracts that slowly decarboxylated the C₃ intermediate as it accumulated from the more rapid action of the carboligase. This has been reinvestigated as follows. The evolution of carbon dioxide from glyoxylate, catalysed by a sonic extract of *Pseudomonas* (A) grown with glycine, was measured in a series of double-armed Warburg flasks, and the

reactions were stopped at intervals of time by tipping 50% (w/v) trichloroacetic acid from a side arm of the appropriate flask (Fig. 2). There was a rapid evolution of 1 mole of carbon dioxide/mole of glyoxylate within 5 min. and thereafter carbon dioxide continued to be liberated more slowly; this slower reaction was abolished when DPNH was present to reduce the C₃ intermediate to glycerate.

In the following experiments it was proved that the second slow reaction was not enzymic, contrary to the suggestion of Calley & Dagley (1959). Three series of tubes (8 mm. $\times$ 70 mm.) were set up to test three concentrations of cell extract. Each tube contained, in a volume of 0.2 ml., 0.75 μ mole of potassium phosphate buffer, pH 7.2, and 0.76 μ mole of sodium [1-¹⁴C]glyoxylate (540 counts/min./0.02 ml. when plated on filter paper). Reactions were started by addition of 0.05 ml. of sonic extract of *Pseudomonas* (A) containing 1.2, 0.6 and 0.15 mg. of protein in each series respectively, and

Fig. 2. Evolution of CO₂ from glyoxylate catalysed by crude extracts of glycine-grown *Pseudomonas* (A). The main compartments of double-armed Warburg flasks contained, in a total of 3 ml., 15 μ moles of potassium phosphate (pH 7.2) and 0.5 ml. of sonic extract (12 mg. of protein). Manometers were gassed with N₂ and equilibrated at 30°. Sodium glyoxylate (8 μ moles) with or without DPNH (5 μ moles) was added from side arm 1. Reactions were stopped by the addition of 0.2 ml. of 50% (w/v) trichloroacetic acid from side arm 2; the glyoxylate remaining and CO₂ evolved (μ moles) were then measured. DPNH added: Δ , CO₂ evolved; $\circ$, glyoxylate remaining. No DPNH added: $\blacktriangle$, CO₂ evolved; $\bullet$, glyoxylate remaining. Controls with no glyoxylate added: ∇ , DPNH added; $\blacktriangledown$, no DPNH added.

the disappearance of glyoxylate with time was followed by chromatography, radioautography, elution and counting (Fig. 3a). These results were then used to study the effect of cell extract on the stability of the compound formed from glyoxylate. At intervals of time, each interval being measured from the point at which 90% of the initial glyoxylate had disappeared, 0.6 μ mole of DPNH was added to each one of a series of reaction mixtures. In this way the C_3 intermediate was assayed by rapid conversion into [^{14}C]glycerate, which was identified by chromatography and counted. From Fig. 3b it appears that the rate of disappearance of intermediate, about 50% in 59 min. at 30°, was independent of the amount of extract present, and accordingly its decomposition was not enzymically catalysed. At 100° all the intermediate decomposed to glycolaldehyde in 1 min.; the stoichiometry of the overall reaction agreed with the equation: 2 glyoxylate $\rightarrow$ glycolaldehyde + 2 CO_2 (Table 1).

Thiamine pyrophosphate and Mg^{2+} ions were cofactors for the enzymic decarboxylation of glyoxylate (Table 2) as shown also by Krakow *et al.* (1961) and Kornberg & Gotto (1961a). When DPNH

was present in the reaction, 1 mole was oxidized and 1 mole of glycerate was formed with the disappearance of 2 moles of glyoxylate (Table 3a and b).

Reactions and properties of the C_3 compound

Krakow & Barkulis (1956) found that hydroxypyruvate was formed by decomposition of glyoxylate, but they showed later (Krakow *et al.* 1961) that it was not the initial product of the reaction; this, they suggested, was its isomer tartronic semialdehyde, which was converted into hydroxypyruvate by the acid used in deproteinizing reaction mixtures. Although we incubated such mixtures with acid solutions of 2,4-dinitrophenylhydrazine on many occasions we found no trace of the 2,4-

Table 1. *Stoichiometry of the conversion of glyoxylate into glycolaldehyde*

Reaction mixtures contained, in 2.5 ml., 15 μ moles of potassium phosphate (pH 7.2), 2.5 μ moles of thiamine pyrophosphate, 2.5 μ moles of $MgCl_2$ and amounts of sodium glyoxylate and DPNH as stated. The reaction was started by addition of 0.5 ml. of dialysed cell extract (10 mg. of protein) and stopped after 15 min. by heating at 100° for 1 min.; this also converted the enzymic reaction product into glycolaldehyde. Residual glyoxylate was determined by the method of Friedemann & Haugen (1943) and glycolaldehyde as in the Materials and Methods section.

Glyoxylate added (μ moles)	Glyoxylate used (μ moles)	DPNH added (μ moles)	Glycolaldehyde formed (μ moles)
7.9*	0	0	0.1
7.9	6.7	0	3.3
3.9	3.5	0	1.7
3.9	3.8	4	0.2

* Cell extract boiled before incubation.

Fig. 3. Rate of decomposition of the C_3 compound formed from [^{14}C]glyoxylate, and its lack of dependence on cell-extract concentration. Reaction tubes (8 mm. $\times$ 70 mm.) contained, in 0.2 ml., 0.75 μ mole of potassium phosphate (pH 7.2) and 0.76 μ mole of sodium [^{14}C]glyoxylate (540 counts/min./0.02 ml. of sample counted). After equilibration at 30° amounts of cell extract were added containing: $\circ$, 1.2 mg. of protein; $\triangle$, 0.6 mg. of protein; ∇ , 0.15 mg. of protein. (a) Disappearance of glyoxylate at each extract concentration, determined by chromatography, radioautography, elution and counting of [^{14}C]glyoxylate. (b) Disappearance of C_3 compound, determined by conversion into [^{14}C]glycerate by addition of DPNH.

Table 2. *Requirement for thiamine pyrophosphate and magnesium ions of glyoxylate carboligase present in cell extracts of glycine-grown Pseudomonas (A)*

Reaction mixtures contained, in 9.5 ml., 17.2 μ moles of sodium glyoxylate, 7.5 μ moles of potassium phosphate (pH 7.2), 5 μ moles of thiamine pyrophosphate (TPP) and 5 μ moles of $MgSO_4$. Reactions at 30° were started by adding 0.5 ml. of a Hughes press extract of cells dialysed for 24 hr. against phosphate buffer. After 15 min. protein was precipitated by the addition of 50% (w/v) trichloroacetic acid, and residual glyoxylate determined.

Tube no.	Contents	Glyoxylate (initial) (μ moles)	Glyoxylate (final) (μ moles)
1	Complete system	17.2	2.7
2	Enzyme omitted	17.2	17.1
3	TPP and $MgSO_4$ omitted	17.2	17.1
4	TPP omitted	17.2	12.8
5	$MgSO_4$ omitted	17.2	10.6

dinitrophenylhydrazones of hydroxypyruvate; therefore detectable non-enzymic conversion of the C_3 compound into hydroxypyruvate in the presence of acid did not occur in the conditions we used. DPNH and TPNH were rapidly oxidized in the presence of active glyoxylate carboligase (Fig. 4), the cofactors for which are thiamine pyrophosphate and Mg^{2+} ions (Table 2). Oxidation was faster, even at one-tenth of the enzyme concentration, when glyoxylate was first incubated with active extract to form the C_3 compound; hydroxypyruvate was reduced much more slowly than the intermediate. We confirmed the observation of Krakow *et al.* (1961) that some of the C_3 compound is formed after prolonged incubation of hydroxypyruvate with cell extract since the initial rate of oxidation of DPNH was thereby increased.

Kornberg & Gotto (1961a) have summarized evidence that the C_3 compound is tartronic semialdehyde. However, since no authentic sample of tartronic semialdehyde has yet been described we have recorded some additional properties of

aqueous solutions obtained by enzymic decarboxylation of glyoxylate. On addition of 0.1% 2,4-dinitrophenylhydrazine in 2N-hydrochloric acid to a dilute solution of C_3 compound, orange-red crystals separated after 15 min. at 30° and from its purple colour with alkali this appeared to be a bis-2,4-dinitrophenylhydrazone. At first, examination of its properties was hindered by the non-enzymic decomposition of C_3 compound to glyceraldehyde, which also forms a bis derivative with the reagent; however, these crystals separate after 1 hr. at 30°; glyoxal gives an immediate precipitate of the same bis-2,4-dinitrophenylhydrazone as glyceraldehyde.

Since the bis derivatives streaked across the papers, chromatography with various solvents was in general of no value except for the reverse-phase

Table 3. Conversion of glyoxylate into glycerate by extracts of glycine-grown *Pseudomonas* (A)

(a) Oxidation of reduced diphosphopyridine nucleotide by cell extracts and glyoxylate

Silica cuvettes contained, in 3 ml. of reaction mixture at 20°, 15 μ moles of potassium phosphate (pH 7.2), 0.39 μ mole of DPNH, 1 μ mole of thiamine pyrophosphate, 1 μ mole of $MgCl_2$, 1 ml. of sonic extract (1 mg. of protein) and sodium glyoxylate. In control cuvettes DPNH was omitted. The amount of DPNH that reacted was calculated from the change in extinction at 340 m μ . The product of reaction was identified as glyceric acid.

Glyoxylate used (μ mole)	DPNH oxidized (μ mole)	DPNH oxidized Glyoxylate used
0.20	0.097	0.48
0.40	0.21	0.51
0.56	0.28	0.50

(b) Stoichiometry of the formation of [^{14}C]glycerate from [^{14}C]glyoxylate

Reaction tubes (8 mm. $\times$ 70 mm.) contained, in 0.2 ml., 0.75 μ mole of potassium phosphate (pH 7.2), 0.76 μ mole of sodium [^{14}C]glyoxylate and 0.6 μ mole of DPNH. Reactions at 30° were started by the addition of 0.05 ml. of cell extract (1.2 mg. of protein); after 5 min. protein was removed and 0.05 ml. of liquid from each tube was applied to chromatograms. Glyoxylate and glycerate were assayed by radioautography, elution and counting.

Glyoxylate added (μ mole)	Glyoxylate used (μ mole)	DPNH added (μ mole)	Glycerate formed (μ mole)
0.76*	0	0.60	0
0.76	0.76	0.0	0.06
0.76	0.76	0.60	0.37

* Cell extract boiled.

Fig. 4. Oxidation of reduced phosphopyridine nucleotides catalysed by sonic extracts of *Pseudomonas* (A) in the presence of glyoxylate or C_3 compound. (a), DPNH; (b), TPNH. In one experiment reaction mixtures contained, in 3 ml., 1 ml. of dialysed extract (1 mg. of protein) with 15 μ moles of potassium phosphate (pH 7.2), 0.5 μ mole of thiamine pyrophosphate, 0.5 μ mole of $MgCl_2$ and either 0.37 μ mole of DPNH or 0.27 μ mole of TPNH. Reactions were started by addition of: ∇ , sodium glyoxylate (1.6 μ moles); $\circ$, lithium hydroxypyruvate (0.8 μ mole); or $\bullet$, glycolaldehyde (0.8 μ mole). The decrease in extinction at 340 m μ was then measured. In control experiments extract (Δ) or glyoxylate (Δ) was omitted. In another experiment the cell extract was diluted and 1 ml. (0.1 mg. of protein) preincubated for 40 min. at 20° with sodium glyoxylate (1.6 μ moles), thiamine pyrophosphate (0.5 μ mole) and $MgCl_2$ (0.5 μ mole). Reactions (∇) were started by addition of either 0.37 μ mole of DPNH or 0.27 μ mole of TPNH.

system of Meigh (1956) in which the derivatives ran as yellow spots, those of the C_3 compound and 2,4-dioxovaleric acid near the solvent front ($R_f > 0.9$) and those of glycolaldehyde and the methyl ester of 2,4-dioxovaleric acid more slowly (R_f 0.4–0.5). Pure samples were obtained by elution with the upper layer of the solvent system of Meigh (1956) and their spectra in alkali plotted; maxima were at 550 $m\mu$ for the C_3 intermediate and 565 $m\mu$ for glycolaldehyde (Fig. 5). The solutions were respectively red-purple and deep purple. The behaviour of the bis derivative of the C_3 compound on chromatograms suggested the presence of a carboxyl group. In agreement, a derivative formed after treating [^{14}C]glyoxylate with cell extract retained radioactivity which ran with $R_f > 0.9$, but when the enzyme product was first decarboxylated by heating to 100° for 1 min. the derivative was not radioactive, ran with R_f 0.5 and had a spectrum in alkali identical with that of the bis compound of glycolaldehyde. The bis-2,4-dinitrophenylhydrazone was much more stable than the C_3 compound from which it was formed and did not lose radioactivity in 2*N*-hydrochloric acid, 2% (w/v) potassium hydroxide in ethanol or when maintained at 100° for several hours. The bis derivative of glycolaldehyde was readily extracted by ethyl acetate from its solution in 1.5*N*-sodium hydroxide, but in accordance with its possession of a carboxyl group the derivative of the C_3 compound was extracted only after more prolonged shaking.

Fig. 5. Absorption spectra of the bis-2,4-dinitrophenylhydrazones of the C_3 compound and glycolaldehyde. The derivatives were purified by reverse-phase chromatography and eluted with chromatographic solvent. A clear area of the chromatogram was similarly treated to serve as a blank. Eluents were dried under vacuum and dissolved in 0.3% sodium ethylate, and their spectra recorded. ---, Glycolaldehyde derivative; —, C_3 compound derivative.

Sodium carbonate (10%, w/v) would not extract the C_3 derivative from its ethyl acetate solution, and by use of these solvents it was therefore possible to determine glyoxylate as its 2,4-dinitrophenylhydrazone in the presence of its decarboxylation products.

Enzymic conversion of glycerate into pyruvate

Extracts of *Pseudomonas* (A) grown with glycine catalysed a conversion of [^{14}C]glycerate into [^{14}C]pyruvate dependent on the presence of ATP (Table 4). During the conversion two reaction intermediates were formed (Fig. 6) which gave colour reactions on chromatograms with a reagent specific for organic phosphates (Burrows, Grylls & Harrison, 1952). Since they were also converted almost completely into glyceric acid by calf-intestinal-mucosa phosphatase (L. Light and Co. Ltd.) it may be assumed that the compounds were phosphoglyceric acids (Table 5). When incubated with ADP, sonic extracts of *Pseudomonas* (A) converted phosphoenolpyruvate, 2-phosphoglyceric acid and 3-phosphoglyceric acid into pyruvate (Table 6). Similar results were obtained when ADP was replaced by ATP. The overall conversion of [^{14}C]glyoxylate into [^{14}C]pyruvate in the presence of DPNH and ATP was also shown (Fig. 6).

Malate synthetase

This enzyme (Wong & Ajl, 1956) could not be studied by using undialysed extracts since glyoxylate was rapidly removed from reaction mixtures by glyoxylate carboligase. After dialysis against 0.02*M*-phosphate buffer, pH 7.2, for 36 hr. at 4° a cell extract catalysed the conversion of sodium [^{14}C]glyoxylate into [^{14}C]malate when sodium acetate, ATP, coenzyme A and Mg^{2+} ions were present (Table 7). The yield of malate was unaffected by addition of reduced glutathione.

Table 4. Conversion of glycerate into pyruvate by a cell extract of *Pseudomonas* (A)

Reaction mixtures at 30° contained, in 0.2 ml., 0.75 μ mole of [^{14}C]glycerate and 0.05 ml. of undialysed sonic extract (1.2 mg. of protein). The reaction was started by addition of 0.5 μ mole of ATP and stopped after 30 min. by heating to 100°. The reaction product was converted into a 2,4-dinitrophenylhydrazone, and identified as the derivative of pyruvate by co-chromatography and radioautography. Spots were eluted, plated out and assayed for radioactivity in a gas-flow counter.

ATP added (μ mole)	Radioactivity (counts/min.)		
	Glycerate (initial)	Glycerate (final)	Pyruvate formed
0.5*	226	228	(2)
0	230	228	0
0.5	228	30	132

* Cell extract boiled before incubation.

Fig. 6. Alteration with time of the percentage distribution of ^{14}C in the products formed by the action of a sonic extract of glycine-grown *Pseudomonas* (A) on sodium $[1-^{14}\text{C}]$ glyoxylate. Reaction tubes contained, in 0.2 ml., 0.75 μmole of potassium phosphate (pH 7.2), 0.76 μmole of sodium $[1-^{14}\text{C}]$ glyoxylate, 0.6 μmole of DPNH and 0.5 μmole of ATP. After equilibration at 30° reactions were started by the addition of 0.05 ml. of extract (1.2 mg. of protein). Glyoxylic acid (Δ), glyceric acid ($\bullet$), and phosphoglyceric acid ($\circ$, $\blacktriangle$) were determined by direct counting of radioactive spots after chromatography as free acids. Pyruvate ($\blacktriangledown$) was determined after chromatography of its 2,4-dinitrophenylhydrazone.

Table 5. Action of alkaline phosphatase on reaction intermediates in the conversion of glycerate into pyruvate

Reaction mixtures for the preparation of these intermediates contained, in 0.25 ml., 0.75 μmole of potassium phosphate (pH 7.2), 0.6 μmole of DPNH, 0.5 μmole of ATP and 0.8 μmole of sodium $[1-^{14}\text{C}]$ glyoxylate (1.25 μC). Reactions at 30° were started by the addition of 0.05 ml. of sonic extract (1.2 mg. of protein) and stopped after 8 min. by heating at 100° for 1 min. To 0.05 ml. of the mixture was added 0.05 ml. of a solution of 1 mg. of alkaline phosphatase (calf intestinal mucosa) in glycine-NaOH buffer, pH 9.5, containing 5 mM-magnesium acetate. After 30 min. at 30° protein was removed and the products were assayed by chromatography, radioautography and counting.

Alkaline phosphatase added	Reaction intermediates (% of total count)	Glycerate (% of total count)
Boiled	90	10
Active	8	92

Table 6. Conversion of phosphoenolpyruvic acid, 2-phosphoglyceric acid and 3-phosphoglyceric acid into pyruvic acid by cell extracts

Reaction mixtures at 30° contained, in 0.16 ml., 0.75 μmole of potassium phosphate (pH 7.2), 0.5 μmole of ADP and 0.4 μmole of substrate. The reactions were started by addition of 0.05 ml. of sonic extract of glycine-grown cells (1.2 mg. of protein) and stopped after 20 min. Pyruvate was identified as its 2,4-dinitrophenylhydrazone and estimated by the method of Friedemann & Haugen (1943).

Substrate added	ADP added (μmole)	Pyruvate formed (μmole)
Phosphoenolpyruvate	0.5*	0.04
	0	0.03
	0.5	0.30
2-Phosphoglycerate	0.5*	0.02
	0	0.03
	0.5	0.14
3-Phosphoglycerate	0.5*	0.04
	0	0.02
	0.5	0.20

* Cell extract boiled before incubation.

Table 7. Malate synthetase in extracts of glycine-grown *Pseudomonas* (A)

Reaction mixtures contained, in 0.35 ml., 0.75 μmole of potassium phosphate (pH 7.2), 0.76 μmole of sodium $[1-^{14}\text{C}]$ glyoxylate, 1 μmole of MgCl_2 , 1.6 μmoles of ATP, 1.6 μmoles of sodium acetate and 0.1 μmole of coenzyme A. Reactions at 30° were started by addition of 0.05 ml. of sonic extract (1.1 mg. of protein) that had been dialysed for 24 hr. against phosphate buffer (pH 7.2) and were stopped after 30 min. when protein was removed and solutions were chromatographed. Malate was located and counted directly with a Geiger-Müller end-window tube; its identity was confirmed by co-chromatography and radioautography.

Tube no.	Contents	Malate (% of total counts)
1	Complete system	55
2	Complete system*	6
3	Sodium acetate omitted	0
4	ATP omitted	0
5	Coenzyme A omitted	0
6	MgCl_2 omitted	11

* Cell extract boiled before incubation.

DISCUSSION

Campbell (1955) proved that glycine was oxidized by a species of *Pseudomonas* to carbon dioxide, ammonia and water with glyoxylic acid and formic acid as reaction intermediates. Cells grew in a medium containing, in addition to glycine, 1 g. of yeast extract/l. This concentration would support abundant growth of *Pseudomonas* (A) which, in our experiments, was supplied with

no source of carbon except glycine. It is possible that the enzyme system studied by Campbell (1955) operates for the removal of glycine, rather than for its utilization, by cells assimilating other nutrients. Our extracts differed chiefly from his in their failure to oxidize glyoxylate, which instead was vigorously decarboxylated. The significance of this reaction in the economy of the cell has already been discussed for bacteria utilizing as sole sources of carbon glycollate (Kornberg & Gotto, 1959), glycine (Callely & Dagley, 1959) and oxalate (Quayle & Keech, 1959). If intermediates of the tricarboxylic acid cycle are used for biosynthesis of cell constituents, ancillary reactions must operate for their replenishment when bacteria grow with C_2 compounds as sole source of carbon. When the C_2 compound is acetate or fatty acids that give rise entirely to acetyl-coenzyme A (Callely, Dagley & Hodgson, 1958), reactions occur by which glyoxylate is formed to enable the cycle to be replenished with malate, synthesized from acetyl-coenzyme A and glyoxylate (Kornberg & Krebs, 1957). When the C_2 acid contains more oxygen than acetate and gives rise to glyoxylate, enzymes convert some glyoxylate into the acetyl-coenzyme A needed for malate synthesis (Kornberg & Gotto, 1961a). Our experiments with whole cells, cell extracts and inhibitors suggest that the tricarboxylic acid cycle operates in *Pseudomonas* (A) grown with glycine as carbon source. The following enzymes which lie on the proposed pathway of conversion of glycine into acetyl-coenzyme A were present in extracts of these cells: glyoxylate carboligase, a reductase for conversion of the product of this reaction into glycerate ('tartronic semialdehyde reductase'), and enzymes for converting glycerate into pyruvate; and their cofactors were those found by other workers. Malate synthetase was also present. Cell suspensions converted glycine into pyruvate during a lag period before cell division began (Fig. 1), whereas serine dehydrase could not be detected in such cells. The proposed conversion of glycine into pyruvate, which would then give acetyl-coenzyme A, is as follows:

Sum:

The conversion of glycerate into pyruvate (reaction iv) is assumed to include well-established reactions

of the Embden-Meyerhof scheme since we have shown (Fig. 6 and Tables 4-6) that phosphate esters of glyceric acid are involved.

The C_3 product of glyoxylate-carboligase action is assumed to be tartronic semialdehyde for reasons summarized by Kornberg & Gotto (1961a). Solutions at pH 7.2 when incubated for less than 1 hr. at 30° may contain appreciable amounts of its decomposition product, glycolaldehyde; this second decarboxylation is non-enzymic (Fig. 3b). The bis-2,4-dinitrophenylhydrazone is more stable than the tartronic semialdehyde from which it is formed, but samples may be contaminated with the derivative of glycolaldehyde which it resembles closely. The two bis derivatives can be well separated by reverse-phase chromatography, and when eluted give spectra in alkali differing by 15 mμ in the wavelengths of their absorption maxima. The behaviour of the bis derivative of glycolaldehyde formed from [1- ^{14}C]glyoxylate is consistent with the presence of a carboxyl group in the bis-2,4-dinitrophenylhydrazone of tartronic semialdehyde; this is radioactive when produced from [1- ^{14}C]glyoxylate.

SUMMARY

1. When *Pseudomonas* (A) grown with glycine as sole carbon source was harvested after 20 hr. in the stationary phase and suspended in fresh medium, cell division was delayed for 4 hr. and pyruvate accumulated. Fluoroacetate and arsenite inhibited oxidation of glycine by these cells. They contained no serine dehydrase.

2. Extracts of cells grown with glycine catalysed the evolution of one molecule of carbon dioxide from two molecules of glyoxylate. This enzyme (glyoxylate carboligase) required thiamine pyrophosphate and magnesium ions for maximal activity. The product, presumed to be tartronic semialdehyde, decomposed spontaneously at pH 7.2 at the rate of 50 % in 50 min. to give glycolaldehyde.

3. The bis-2,4-dinitrophenylhydrazone of tartronic semialdehyde was separated from that of glycolaldehyde (glyoxal) by reverse-phase chromatography, and its absorption spectrum in alkali obtained.

4. Cell extracts contained an enzyme (tartronic semialdehyde reductase) that catalysed reduction of tartronic semialdehyde to glycerate. When coupled with glyoxylate carboligase, one molecule of glycerate was formed from two molecules of glyoxylate, and one molecule of reduced diphosphopyridine nucleotide was oxidized.

5. Cell extracts converted glycerate into pyruvate, phosphate esters of glyceric acid being formed as reaction intermediates. Malate synthetase was present in extracts.

6. From these results it appears that compounds of the tricarboxylic acid cycle are synthesized from glycine by a scheme similar to that suggested for another species of *Pseudomonas* grown with glycollate as carbon source (Kornberg & Gotto, 1961a).

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The Total Intracellular Concentration of Solutes in Yeast and Other Plant Cells and the Distensibility of the Plant-Cell Wall

By E. J. CONWAY AND W. McD. ARMSTRONG

Biochemical Department, Merville House, University College, Dublin,

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It is primarily intended to determine in yeast and other plant cells the total intracellular molar concentration of free solutes, including ion species, which may be conveniently stated as total intracellular molarity, the molarity of any single solute being the moles/l. of intracellular water (or kg. of such water without appreciable relative error).

In determining total intracellular molarities a variant of the cryoscopic method after breakage of the cell walls by freezing in liquid oxygen and thawing has been used in the experiments described, attention being given to the volume of the extracellular fluid as well as to the possibility of autolytic changes increasing the total molarity in

the frozen and thawed mixture. Such changes occur rapidly even at 0° in certain animal tissues (Conway, Geoghegan & McCormack, 1955).

The conversion of freezing-point depression so determined into total molecular concentrations involves the use of a conversion factor which is here taken as 1.86° per unit molarity. This, applied in general, is no doubt approximate but sufficiently accurate for present purposes.

It is also necessary to know the volume of the extracellular water. For centrifuged yeast this has been dealt with in some detail by Conway & Downey (1950), and similar considerations have been applied here to plant tissues in general as described under Methods.