# The Utilization of Glycollate by *Micrococcus denitrificans*: the β-Hydroxyaspartate Pathway

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1. *Micrococcus denitrificans* utilized glycollate as sole carbon source for aerobic growth. Glyoxylate was utilized less well, and though glycine alone did not support growth it enhanced growth on glyoxylate. 2. During growth on glycollate, $^{14}C$ was incorporated from [2-$^{14}$C]glycollate into glycine and thence into aspartate, malate and glutamate. No phosphoglycerate was labelled at the earliest times. 3. Glyoxylate was the first product of glycollate utilization, and glycollate oxidase was inducibly formed on transfer of the organism to glycollate-containing media. 4. Extracts of glycollate-grown *M. denitrificans* contained negligible glyoxylate-carboligase activity and only low tartronate semialdehyde-reductase activity. 5. *erythro*-β-Hydroxyaspartate is a key intermediate in glyoxylate utilization by this organism. Enzymes catalysing (*a*) the synthesis of *erythro*-β-hydroxyaspartate from glyoxylate and glycine, and (*b*) the conversion of *erythro*-β-hydroxyaspartate into oxaloacetate, were inducibly formed during growth on glycollate and on other substrates yielding glyoxylate. Methods for the assay of these enzymes were developed. 6. It is concluded that in *M. denitrificans* the biosynthesis of cell materials from glycollate is accomplished by the 'β-hydroxyaspartate pathway', a novel metabolic route that may also perform a catabolic role in glyoxylate oxidation.

In many bacteria that use glycollate or glyoxylate as the sole source of carbon for growth, the replenishment of tricarboxylic acid-cycle intermediates as these are drained away during growth is achieved through the operation of the glycerate pathway (Kornberg & Gotto, 1961; Kornberg, 1961). This pathway is initiated by the action of glyoxylate carboligase (Krakow, Barkulis & Hayashi, 1961), which catalyses the decarboxylative condensation of 2mol. of glyoxylate to yield 1mol. of carbon dioxide and 1mol. of the $C_3$ compound, tartronate semialdehyde. After a series of transformations at the $C_3$ level, pyruvate (or phosphopyruvate) is formed, which must either be carboxylated to yield oxaloacetate, or, after oxidation to acetyl-CoA, condensed with a third mol. of glyoxylate to yield malate (Kornberg & Sadler, 1960); in either case, the formation of intermediates of the tricarboxylic acid cycle from glycollate is accomplished (Morris, 1963).

The present paper reports the absence of glyoxylate-carboligase activity from extracts of *Micrococcus denitrificans* grown on glycollate, and presents evidence for the existence therein of a novel means of glyoxylate utilization that effects the direct synthesis of a $C_4$ dicarboxylic acid by the condensation of two $C_2$ compounds. Portions of this work have

been reported in a preliminary form (Kornberg & Morris, 1962*a,b*, 1963).

## MATERIALS AND METHODS

*Maintenance and growth of organisms.* The strain of *M. denitrificans*, obtained from Dr June Lascelles, had been originally supplied by Dr. W. Verhoeven. It was maintained on slopes of glycollate growth medium solidified with 1% of Ionagar no. 2 (Oxoid). Cultures of the organism were grown aerobically at 30° and stored at 2°; they were subcultured weekly. For experimental use, organisms from the glycollate–agar slopes were used to inoculate 1l. of medium containing: 6g. of $K_2HPO_4$, 4g. of $KH_2PO_4$, 3·2g. of $NH_4Cl$, 0·2g. of $MgSO_4,7H_2O$, 0·04g. of $CaCl_2$, 0·008g. of $MnSO_4$, 0·005g. of $FeSO_4,7H_2O$, 0·015g. of $Na_2MoO_4,2H_2O$ and, as sole carbon source, 2·5g. of sodium glycollate. The inoculated medium was shaken in a 2l. conical flask at 30° in a Gyrotory incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., U.S.A.). The bacteria were harvested during the exponential phase of growth (at cell densities of 0·4–0·6mg. dry wt./ml.) by centrifuging at 1500*g.* for 10min. at 2°. For experiments on the incorporation of $^{14}C$ from [2-$^{14}$C]glycollate into growing organisms, they were resuspended to a cell density of about 8mg. dry wt./ml. in fresh growth medium containing glycollate (5mM). For studies on the oxidation of substrates, and for preparation of extracts, the organisms were washed with, and resuspended in, 10mM-sodium–potassium phosphate buffer, pH7·0.

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*Measurement of growth.* Growth was followed turbidimetrically at 680mμ with a Unicam SP.600 spectrophotometer. The relation between $E_{680}$ and dry wt. was determined and found to be linear up to a reading of approx. 0·8.

*Incorporation of $^{14}C$ from [2-$^{14}C$]glycollate.* The procedures used for study of the incorporation of $^{14}C$ from [2-$^{14}C$]-glycollate into constituents of the ethanol-soluble fraction of organisms growing on glycollate were similar to those described for *Pseudomonas* (Kornberg, 1958), and *Escherichia coli* (Kornberg, Phizackerley & Sadler, 1961) metabolizing acetate.

*Assay and identification of labelled compounds.* Isotopically 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, amino acids were analysed by high-voltage paper-strip electrophoresis in 0·2M-acetic acid–pyridine buffer, pH4·0 (60cm. strip length; 8kv; 60mA for 45min.).

*Preparation of cell extracts.* Suspensions of freshly grown organisms (10mg. dry wt./ml. in 0·01M-tris–HCl buffer, pH8) were subjected for 7–10min. at 0° to the output of an MSE ultrasonic disintegrator operating at 1·5A, or were passed through a chilled French pressure cell (American Instrument Co., Silver Spring, Md., U.S.A.) at 12000lb./in.$^2$. In experiments to test for glyoxylate-carboligase activity, extracts were also prepared at —20° in the Hughes (1951) press. All extracts were centrifuged at 20000g. for 10min. at 2°; the supernatant solutions thus obtained were used for experimental purposes.

*Determination of protein.* Soluble protein was generally measured by the method of Lowry, Rosebrough, Farr & Randall (1951); a standard curve was prepared with crystalline bovine plasma albumin (Armour Laboratories, Eastbourne, Sussex).

*Identification of keto acids.* Keto acids were analysed as their 2,4-dinitrophenylhydrazones by one-dimensional ascending paper chromatography in the solvent system of El Hawary & Thompson (1953).

*Estimation of glyoxylate.* Glyoxylate was assayed as its 2,4-dinitrophenylhydrazone. To 1ml. of a solution of glyoxylate (10–100mμmoles) was added 0·33ml. of 0·1% 2,4-dinitrophenylhydrazine in 2N-HCl, and the mixture was incubated for 10min. at 30°. Then 1·67ml. of 10% (w/v) NaOH was added and the colour read against a reagent blank at 445mμ in a Unicam SP.500 spectrophotometer. In this assay $\epsilon_{445}$ for glyoxylate was $2·1 \times 10^4$.

*Identification of diastereomers of β-hydroxyaspartate.* The *erythro* and *threo* isomers of DL-β-hydroxyaspartate were identified by high-voltage paper-strip electrophoresis, either in 0·05M-oxalate buffer, pH3·4 (Jenkins, 1961), or in 0·2M-acetic acid–pyridine buffer, pH4·0 (60cm. strip length; 8kv; 60mA for 45min.). These compounds migrated towards the anode and were completely separated from each other (and from glycine). The amino acids were revealed by development with ninhydrin, the β-hydroxyaspartates giving a distinctive brown colour.

*Spectrophotometric procedures.* All spectrophotometric assays were performed at $20 \pm 1°$ in silica cuvettes (1·5ml. volume; 1cm. light-path), with an Optica model CF-4 recording spectrophotometer.

*Radioactive materials.* All radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [2-$^{14}C$]glycollate was dissolved in the mini-

mum quantity of water and purified before use by paper chromatography on solvent-washed Whatman no. 3 paper in ethanol–aq. ammonia (sp.gr. 0·88)–water (20:1:4, by vol.) (Long, Quayle & Stedman, 1951). *erythro*- and *threo*-DL-β-Hydroxy[1,4-$^{14}C_2$]aspartate were prepared from [1-$^{14}C$]-glycine and [1-$^{14}C$]glyoxylate by the method of Kornguth & Sallach (1960). The purity of all $^{14}C$-labelled compounds was checked by two-dimensional paper chromatography or high-voltage paper electrophoresis, followed by radioautography with Kodak Blue Brand X-ray film.

*Other materials used.* Samples of *erythro*- and *threo*-DL-β-hydroxyaspartate were at first synthesized by the method of Kornguth & Sallach (1960) but were later purchased from California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A. *erythro*- and *threo*-DL-β-Hydroxy-β-methylaspartate were generously given by Dr T. Jenkins, University of California, Berkeley. Oxaloacetate 4-carboxy-lyase (EC 4.1.1.3), purified from *Pseudomonas ovalis* Chester (Horton & Kornberg, 1964), was kindly provided by Dr A. Horton. Malate dehydrogenase (EC 1.1.1.37) was prepared eightfold purified as the 50–75% saturated ammonium sulphate fraction of an extract of succinate-grown *M. denitrificans*. Phenazine methosulphate and sodium glyoxylate were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. Cytochrome *c*, reduced nicotinamide nucleotides, oxaloacetic acid and sodium pyruvate were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other reagents were of the highest purity commercially available.

*Assay of glycollate oxidase (EC 1.3.1).* This enzyme was assayed by measuring the glycollate-dependent reduction of 2,6-dichlorophenol-indophenol at 600mμ or of cytochrome *c* at 550mμ, in the presence of cyanide and catalytic amounts of phenazine methosulphate (Zelitch & Ochoa, 1953; Gotto, 1961). The specific activity was calculated as μmoles of glycollate oxidized/mg. of protein/hr.

*Assay of glyoxylate carboligase.* This enzyme (Krakow & Barkulis, 1956; Krakow *et al.* 1961) was assayed manometrically by measuring the rate of anaerobic evolution of $CO_2$ from glyoxylate, as described by Kornberg & Gotto (1961).

*Assays of malate synthase (EC 4.1.3.2) and citrate synthase (EC 4.1.3.7).* These enzymes were estimated spectrophotometrically, as described by Dixon & Kornberg (1959).

*Assay of tartronate semialdehyde reductase (EC 1.1.1.g).* This enzyme was measured spectrophotometrically by NADH$_2$ oxidation by using a sample of tartronate semialdehyde prepared from glyoxylate with purified glyoxylate carboligase (Gotto & Kornberg, 1961).

*Assay of malate dehydrogenase (EC 1.1.1.37).* This enzyme proved to be some 40-fold more active with NADH$_2$ than with NADPH$_2$ and was present in such high activity in cell extracts of *M. denitrificans* that it could be assayed by the oxaloacetate-dependent oxidation of NADH$_2$, despite the NADH$_2$-oxidase activity of these extracts. Silica cuvettes contained (in 1ml. final vol.): 20μmoles of tris–HCl buffer, pH8·0, 1μmole of $MgCl_2$, cell extract (less than 5μg. of soluble protein) and about 0·1μmole of NADH$_2$. A cuvette containing the above system minus NADH$_2$ was used as the blank. After preliminary readings to ensure the absence of significant NADH$_2$-oxidase activity, the reaction was started by addition of 0·5μmole of oxaloacetate: enzyme activity was measured as the rate of decrease in extinction at 340mμ. Assuming $\epsilon_{340}$ of NADH$_2$ to be $6·2 \times 10^3$, the

specific activity of the enzyme was calculated as μmoles of NADH$_2$ oxidized/mg. of protein/hr.

*Assay of* erythro-β-*hydroxyaspartate aldolase. (a)* Spectrophotometric assay. In the presence of excess of *erythro-β-hydroxyaspartate dehydratase* and NADPH$_2$-linked malate dehydrogenase, this enzyme could be assayed by the glycine-dependent oxidation of NADPH$_2$ after the previous addition of glyoxylate. NADH$_2$ could not be used because of the considerable NADH$_2$-oxidase activity contained in the quantities of extract required for demonstration of *erythro-β-hydroxyaspartate-aldolase* and *erythro-β-hydroxyaspartate-dehydratase* activities (cf. malate dehydrogenase). Silica cuvettes contained (in 1 ml. final vol.): 20 μmoles of tris–HCl buffer, pH 8·0, 2 μmoles of MgCl$_2$, 0·01 μmole of pyridoxal phosphate, cell extract (containing about 150 μg. of soluble protein), approx. 0·1 μmole of NADPH$_2$ and 1·0 μmole of glyoxylate. The 'blank' cuvette contained the above minus NADPH$_2$, and the reaction was started by the addition of 3·0 μmoles of glycine. The rate of decrease in extinction at 340 mμ was measured spectrophotometrically. Assuming ε$_{340}$ of NADPH$_2$ to be 6·2 × 10$^3$, the specific activity was calculated as μmoles of NADPH$_2$ oxidized/mg. of protein/hr.

*(b)* Colorimetric assay. In this test-tube assay system, the rate of pyruvate formation from *erythro*-DL-β-hydroxy-β-methylaspartate was measured. The complete system contained (in 1 ml. final vol.): 30 μmoles of tris–HCl buffer, pH 8·0, 3 μmoles of MgCl$_2$, 0·02 μmole of pyridoxal phosphate and cell extract (less than 1 mg. of soluble protein). The same system, but with cell extract heated at 100° for 15 min. before use, was used as the reaction 'blank'. The tubes were incubated at 30° and the reaction was started by the addition of 6 μmoles of *erythro*-DL-β-hydroxy-β-methyl-aspartate (neutralized). At 1 min. intervals over a period of 5 min., 0·1 ml. samples were withdrawn into 0·9 ml. of water plus 0·33 ml. of 0·1 % 2,4-dinitrophenylhydrazine in 2 N-HCl. After incubation at 30° for 10 min., 1·67 ml. of 10 % NaOH was added and the colour developed read against a reagent 'blank' at 445 mμ in a Unicam SP.500 spectrophotometer. Assuming ε$_{445}$ for pyruvate 2,4-dinitrophenylhydrazone in this assay to be 1·8 × 10$^4$, the specific activity of the enzyme was calculated as μmoles of pyruvate produced/mg. of protein/hr.

*Assay of erythro-β-hydroxyaspartate dehydratase.* This enzyme was assayed spectrophotometrically by linking it with excess of NADPH$_2$-utilizing malate dehydrogenase (above) and following NADPH$_2$ oxidation at 340 mμ on the addition of *erythro*-DL-β-hydroxyaspartate. Silica cuvettes contained (in 1 ml. final vol.): 20 μmoles of tris–HCl buffer, pH 8·0, 2 μmoles of MgCl$_2$, 0·01 μmole of pyridoxal phosphate, cell extract (100–200 μg. of soluble protein) and about 0·1 μmole of NADPH$_2$ (plus, if necessary, purified malate dehydrogenase from succinate-grown *M. denitrificans*). A 'blank' cuvette contained the above minus NADPH$_2$. The reaction was started by the addition of 0·5 μmole of *erythro*-DL-β-hydroxyaspartate (neutralized), and the rate of decrease in extinction at 340 mμ was measured spectrophotometrically. Specific activity of the enzyme was calculated as μmoles of NADPH$_2$ oxidized/mg. of extract protein/hr.

## RESULTS

*Growth of the organism on glycollate and related* C$_2$ *compounds. M. denitrificans* grew well on a defined

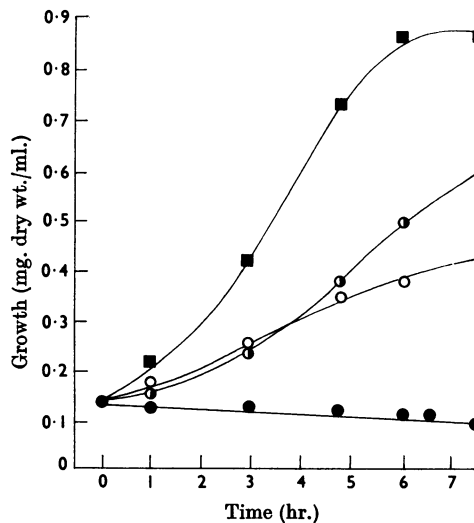

Fig. 1. Growth of *M. denitrificans* on glycollate and related C$_2$ compounds as sole carbon source. Organisms harvested after aerobic growth on glycollate were transferred to fresh media containing the substrates indicated: ■, glycollate (25 mM); ○, glyoxylate (25 mM); ●, glycine (5 or 25 mM); ◐, glyoxylate (25 mM) plus glycine (5 mM). Growth was followed turbidimetrically during continued aerobic incubation at 30°.

basal medium containing glycollate as the sole source of carbon when either nitrate or oxygen was the terminal hydrogen acceptor; aerobic growth was used as a routine. Less rapid growth was obtained with ethane-1,2-diol or glyoxylate in place of glycollate. Although no growth on glycine alone was observed in 8 hr., a mixture of glycine and glyoxylate was utilized somewhat more effectively than glyoxylate by itself (Fig. 1).

*Oxidation of substrates by whole organisms.* Washed suspensions of glycollate-grown *M. denitrificans* rapidly oxidized glycollate, glyoxylate and glycerate; malate, succinate and oxaloacetate were oxidized rapidly after a short lag; but glycine was oxidized only slowly (Table 1). The rate of oxygen uptake with glycollate was sufficient to account for the total oxidation of 2 μmoles of this compound/ mg. dry wt./hr. and the total quantity of oxygen absorbed, corrected for the endogenous respiration, was 55 % of the amount required for complete oxidation of the glycollate. Arsenite (0·02 M) partially inhibited the oxidation of glycollate and provoked release of a keto acid identified as glyoxylate by chromatography of its 2,4-dinitrophenylhydrazone (Fig. 2).

*Incorporation of* $^{14}$C *from* [$^{14}$C]*glycollate during growth.* When [1-$^{14}$C]- or [2-$^{14}$C]-glycollate was added to cultures of *M. denitrificans* growing on

Table 1. *Oxidation of substrates by washed suspensions of* M. denitrificans *grown on glycollate*

The main compartments of Warburg manometer flasks contained (in 3 ml.): 300 $\mu$moles of potassium phosphate buffer, pH 7·0, and washed organisms (10 mg. dry wt.). The centre wells contained 400 $\mu$moles of KOH. Substrates as indicated (10 $\mu$moles) were added from the side bulbs after thermal equilibration at 30°.

| Substrate added | $Q_{O_2}$ ($\mu$l. absorbed/hr./mg. dry wt.) |
|---|---|
| None | 5 |
| Glycollate | 77 |
| Glyoxylate | 59 |
| Glycine | 21 |
| erythro-$\beta$-Hydroxyaspartate | 7 |
| Oxaloacetate | 39* |
| Malate | 73* |
| Succinate | 73* |
| Glycerate | 54 |

* After approx. 20 min. lag period (presumably due to induction of a dicarboxylic acid permease).
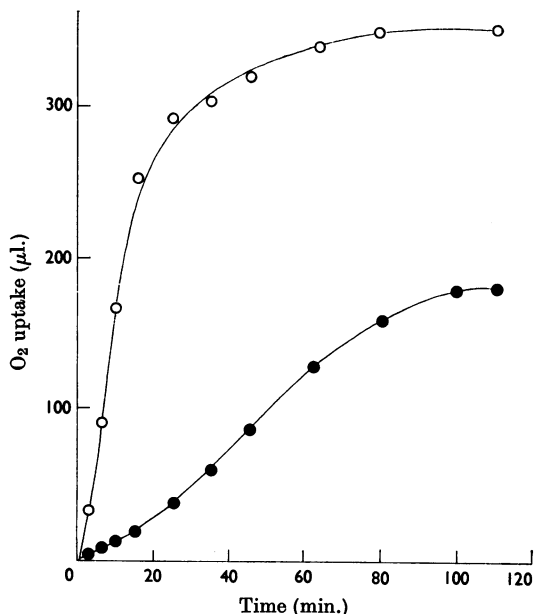


Fig. 2. Arsenite inhibition of glycollate oxidation by washed suspensions of *M. denitrificans*. The organisms were harvested during exponential aerobic growth on glycollate medium and their endogenous reserves depleted by 4 hr. aerobic shaking at 30° of a suspension (1 mg./ml.) in basal medium minus the added source of carbon. The 'depleted' organisms were reharvested and washed before use. The main compartments of Warburg manometers contained (in 2·8 ml.): 100 $\mu$moles of sodium–potassium phosphate buffer, pH 7·0, and 1 ml. of the suspension of organisms (13 mg. dry wt.). When required, 60 $\mu$moles of potassium arsenite were added. The centre wells contained 0·2 ml. of 2 N-KOH. After thermal equilibration at 30° for 10 min., 20 $\mu$moles of sodium glycollate, or in the endogenous controls 0·2 ml. of water, were tipped in from the side bulbs. The volumes ($\mu$l.) of $O_2$ absorbed after tipping the contents of the side bulbs were measured over 3 hr. The values recorded have been corrected for the endogenous $O_2$ uptake. The glyoxylate content of the final suspension was assayed at the conclusion of the experiment: $\bigcirc$, minus arsenite, 0·06 $\mu$mole of glyoxylate formed; $\bullet$, plus arsenite, 2·91 $\mu$moles of glyoxylate formed.

glycollate as sole carbon source, ^{14}C was rapidly incorporated into both the ethanol-soluble and protein fractions. This incorporation of ^{14}C into the ethanol-soluble fraction increased linearly with time for the duration of the experiment (60–90 sec.).

*Distribution of* [14]C *from* [2-^{14}C]glycollate. The distribution of ^{14}C among the labelled components of the ethanol-soluble fractions was analysed by two-dimensional chromatography and radioautography. Label from [2-^{14}C]glycollate rapidly appeared in glycine, malate, aspartate and glutamate (Fig. 3). In the sample taken at the earliest time (6 sec.) glycine contained by far the greatest proportion of radioactivity (approx. 50 % of the total ^{14}C incorporated). Approx. 8 % of the radioactivity was present as malate, 5 % as aspartate and 5 % as glutamate; no other single compound contained more than 5 % of label. Though the bulk of the radioactivity remained in glycine the proportions changed during the course of the experiment, that in malate increasing rapidly and that in glutamate somewhat less quickly, so that at 65 sec. the distribution of label was as follows: glycine, 32 %; malate, 22 %; glutamate, 12 %; aspartate, 5 %. This distribution of radioactivity derived from [2-^{14}C]glycollate differed markedly from that obtained with a *Pseudomonas* sp. that metabolized glycollate via the glycerate pathway (Kornberg & Gotto, 1961). In particular, phosphoglycerate, which with glycine was among the more important early recipients of radioactivity in the *Pseudomonas* sp., was not represented in the *Micrococcus* extracts, and the incorporation of label into malate, which in *Pseudomonas* showed a marked negative slope with time, in *Micrococcus* showed a positive slope. The results in

Fig. 3 were consistent with the hypothesis that glycollate in *M. denitrificans* entered a novel metabolic route stemming from glycine.

*Oxidation of glycollate by cell extracts.* Extracts obtained by ultrasonic disintegration of glycollate-grown *M. denitrificans* slowly oxidized glycollate ($Q_{O_2}$ 9 at 30°), the rate of oxidation being much enhanced ($Q_{O_2}$ 32 at 30°) by the addition of phenazine methosulphate (0·06 %). When the oxidation was allowed to proceed in the presence of semicarbazide, decomposition of the resultant semicarbazone with acid 2,4-dinitrophenylhydrazine and
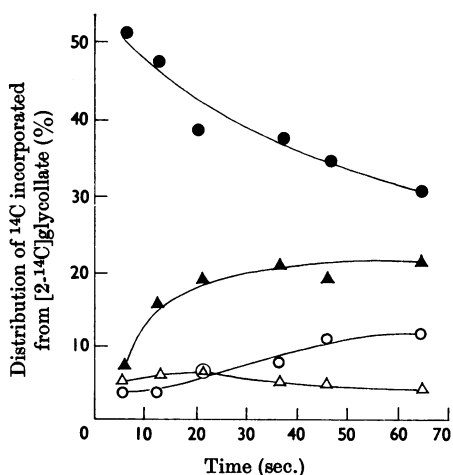
Fig. 3. Variation with time of the percentage distribution of $^{14}C$ from [2-$^{14}$C]glycollate incorporated by *M. denitrificans* growing on glycollate as sole carbon source, into glycine (●), malate, (▲) glutamate (○) and aspartate (△). The conditions are given in the Materials and Methods section.
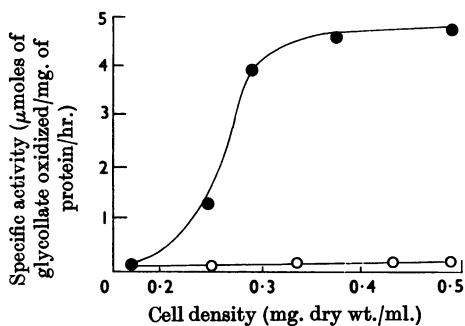


Fig. 4. Induced synthesis of glycollate oxidase. *M. denitrificans* was grown in a medium containing lactate as sole source of carbon. Harvested organisms were transferred to fresh medium containing lactate (5mM) and glycollate (25mM) (●), or lactate (25mM) (○), to give an initial cell density of approx. 0·2mg. dry wt./ml. Growth was followed turbidimetrically during aerobic incubation at 30° and samples were withdrawn at intervals for assay of the organisms' content of glycollate oxidase (see the Materials and Methods section).

chromatography of the product demonstrated that this was glyoxylate. Spectrophotometric assays of the glycollate oxidase content of cell-free extracts, obtained from *M. denitrificans* grown on various carbon sources, showed that the enzyme was virtually absent after growth on acetate or lactate or C₄ dicarboxylic acids such as succinate or aspartate, but was inducibly synthesized on transference of the organism to a medium in which growth was dependent on utilization of glycollate (Fig. 4).

*Absence of the glycerate pathway.* *Escherichia coli* (Krakow *et al.* 1961) and *Pseudomonas* sp. (Kornberg & Gotto, 1959, 1961) utilize glyoxylate via the glycerate pathway, a key component of which is the enzyme glyoxylate carboligase (Krakow & Barkulis, 1956). Extracts of glycollate-grown *M. denitrificans* produced by a variety of means (see the Materials and Methods section) possessed no detectable glyoxylate-carboligase activity and only low levels of tartronate semialdehyde-reductase activity (Table 2). From these findings and the results of the [2-$^{14}$C]glycollate incorporation studies (Fig. 3) it was concluded that glyoxylate was utilized by *M. denitrificans* by some route other than the glycerate pathway.

*Utilization of glyoxylate by extracts.* Extracts of glycollate-grown *M. denitrificans* possessed considerable NADH₂-oxidase activity but did not oxidize added NADPH₂. On the addition of glyoxylate a slow but continuing oxidation of NADPH₂ was initiated. With the aid of [2-$^{14}$C]glyoxylate, and two-dimensional paper chromatography and radioautography of the product after protein precipitation, it was established that the material formed from glyoxylate was glycollate. The observed reaction was thus the result of a slight glyoxylate-reductase (EC 1.1.1.26) activity. However, when glycine was added subsequently, this slow oxidation of NADPH₂ was markedly stimulated (Fig. 5). When these C₂ compounds were added in the reverse order, it was found that, though glycine by itself did not cause the oxidation of NADPH₂, the subsequent addition of glyoxylate again produced the fast rate of NADPH₂ oxidation (Fig. 5). With a mixture either of [2-$^{14}$C]glycine plus glyoxylate or of glycine

Table 2. *Activities of key enzymes in* M. denitrificans *growing on glycollate compared with those in other bacteria in which the glycerate pathway is operative*

All organisms were grown aerobically on glycollate, at 30°, and harvested during the exponential phase of growth. Specific activities are expressed as μmoles of substrate utilized/mg. of protein/hr.

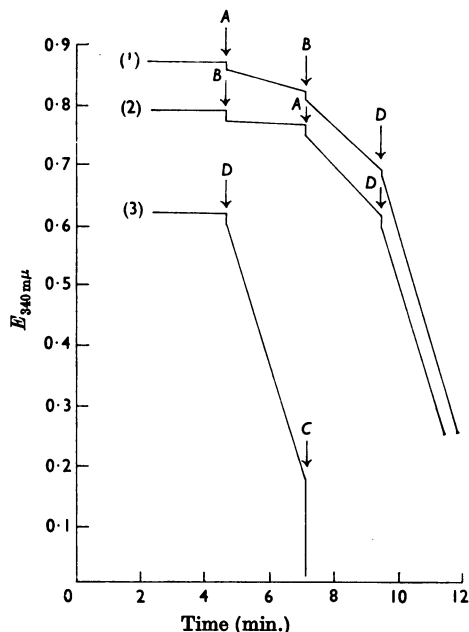| | Glycollate oxidase | Glyoxylate carboligase | Tartronate semialdehyde reductase | Malate synthase | Citrate synthase |
|---|---|---|---|---|---|
| *M. denitrificans* | 5·6 | 0 | 4·2 | 1·6 | 5·4 |
| *Pseudomonas* B₂aba (Kornberg & Gotto, 1961) | 3·5 | 41·0 | 47·0 | 104·0 | 24·8 |
| *E. coli* W (Sadler, 1961) | 9·2 | 28·0 | 250·0 | 35·0 | 12·0 |

Fig. 5. Oxidation of NADPH$_2$ by extracts of glycollate grown *M. denitrificans*. The complete system contained (in 1·0 ml.): 20 $\mu$moles of tris–HCl buffer, pH 8·0, 2 $\mu$moles of MgCl$_2$, cell extract (containing 150 $\mu$g. of soluble protein), approx. 0·1 $\mu$mole of NADPH$_2$ and 0·5 $\mu$mole of substrate as shown, i.e. glyoxylate (*A*), glycine (*B*) and oxaloacetate (*C*), but 1·0 $\mu$mole of *erythro*-DL-$\beta$-hydroxyaspartate (*D*) since only one enantiomorph is utilized (Gibbs & Morris, 1964b). Three experiments (1–3) are illustrated. The graphs were taken from a tracing made with an Optica recording spectrophotometer.

plus [2-$^{14}$C]glyoxylate. when the reaction was allowed to proceed to completion in the presence of excess of NADPH$_2$ the major labelled product proved to be malate (Table 3). Since the cell extract used was rich in malate dehydrogenase (EC 1.1.1.37), which in the crude state could utilize NADPH$_2$ (albeit less effectively than NADH$_2$), it was likely that the labelled malate arose from oxaloacetate, formed in turn from some product of interaction of glycine and glyoxylate. The most probable condensation product of these two C$_2$ compounds would be $\beta$-hydroxyaspartate (Metzler, Longenecker & Snell, 1954). When the diastereomers of this hydroxyamino acid were tested, it was found that the *threo* isomer was without activity, whereas *erythro*-DL-$\beta$-hydroxyaspartate was even more effective than an equimolar mixture of glycine and glyoxylate in promoting the oxidation of NADPH$_2$ in extracts of glycollate-grown *M. denitrificans* (Fig. 5).

*Metabolism of* erythro-DL-$\beta$-hydroxyaspartate *in extracts*. When *erythro*-$\beta$-hydroxyaspartate was

### Table 3. *Formation of malate from glyoxylate in extracts of glycollate-grown* M. denitrificans

The reaction mixtures contained (in 1 ml.): 20 $\mu$moles of potassium phosphate buffer, pH 7, 2 $\mu$moles of MgCl$_2$, 0·5 $\mu$mole of [1-$^{14}$C]glyoxylate (0·4 $\mu$c/$\mu$mole) and extract (containing 1 mg. of protein); other additions as indicated were 0·5 $\mu$mole of glycine and 1 $\mu$mole of NADPH$_2$. After incubation at 30° for 30 min. the reaction was stopped and the protein precipitated by the addition of 3 ml. of ethanol. One-tenth (0·4 ml.) of the ethanol extract was chromatographed two-dimensionally and radioautographed. Radioactivity was incorporated into two spots identified (after counting on the paper, elution and co-chromatography with authentic markers) as glycine and malate.

| | Radioactivity (counts/100 sec.) | |
| --- | --- | --- |
| Additions | In glycine | In malate |
| (1) [1-$^{14}$C]Glyoxylate only | 1018 | 361 |
| (2) [1-$^{14}$C]Glyoxylate+NADPH$_2$ | 1180 | 1240 |
| (3) [1-$^{14}$C]Glyoxylate+glycine | 1340 | 247 |
| (4) [1-$^{14}$C]Glyoxylate+glycine +NADPH$_2$ | 3480 | 3930 |
| (5) As (4), but extract omitted | 228 | 0 |

added to freshly prepared extracts of glycollate-grown *M. denitrificans* in tris–hydrochloric acid buffer, pH 8, it was rapidly metabolized to yield chiefly oxaloacetate with some pyruvate and less glyoxylate. The formation of oxaloacetate could be followed spectrophotometrically at 262 m$\mu$, or at 252 m$\mu$ in the presence of semicarbazide at pH 6·5 (Kornberg & Morris, 1962b). In the presence of semicarbazide, oxaloacetate was the sole labelled product of *erythro*-$\beta$-hydroxy[1,4-$^{14}$C$_2$]aspartate utilization (identified by chromatography of the 2,4-dinitrophenylhydrazone produced after acid hydrolysis of the semicarbazone).

*Evidence for the existence of* erythro-$\beta$-hydroxy-aspartate dehydratase. The conversion of *erythro*-$\beta$-hydroxyaspartate into oxaloacetate could occur by any one of at least three routes (Scheme 1). If *erythro*-$\beta$-hydroxyaspartate were acted on by a transaminase or oxidoreductase (deaminating), the immediate product would be oxaloglycollate (reaction 1): this might then give rise to oxaloacetate by some undisclosed pathway (possibly via tartrate). The product of the action of a deaminase akin to aspartase (reaction 2) would be the enol form of oxaloacetate, which would tautomerize to yield oxaloacetate whose keto group was derived from the $\beta$-carbon atom of *erythro*-$\beta$-hydroxyaspartate. Were *erythro*-$\beta$-hydroxyaspartate the substrate for a hydroxyamino acid dehydratase (reaction 3), then the oxaloacetate formed would possess a keto group originating in the $\alpha$-carbon atom of the *erythro*-$\beta$-hydroxyaspartate.
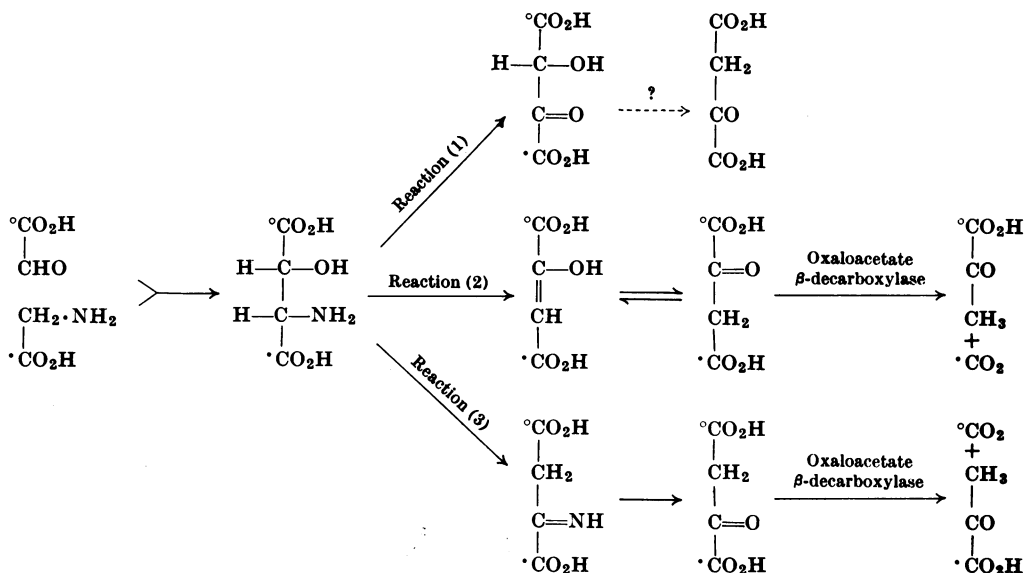
Scheme 1. Possible routes for the production of oxaloacetate from erythro-$\beta$-hydroxyaspartate.

Neither oxaloglycollate nor its decarboxylated derivative, hydroxypyruvate, was detected amongst the 2,4-dinitrophenylhydrazones prepared from the products of erythro-$\beta$-hydroxyaspartate utilization by extracts of glycollate-grown *M. denitrificans*. Further, no evidence was obtained of the conversion into oxaloacetate in these extracts of oxaloglycollate itself or of any isomer of tartrate. Reaction (1) was therefore discounted.

A decision as to which of the remaining reactions (2 or 3) was responsible for oxaloacetate formation was made by supplementing the extracts with a specific oxaloacetate $\beta$-decarboxylase. Assuming utilization of [1-$^{14}$C]glycine plus unlabelled glyoxylate to yield predominantly erythro-$\beta$-hydroxy-[1-$^{14}$C]aspartate, the $\beta$-decarboxylation of the $^{14}$C-labelled oxaloacetate product would only yield [$^{14}$C]pyruvate if reaction (3) were operative. Were [1-$^{14}$C]glyoxylate and unlabelled glycine used as precursors of endogenously synthesized erythro-$\beta$-hydroxyaspartate, production of labelled pyruvate would indicate the occurrence of reaction (2), and of non-radioactive pyruvate, reaction (3). Accordingly a cell-free extract of glycollate-grown *M. denitrificans* (5mg. of protein) was supplemented with excess of oxaloacetate $\beta$-decarboxylase and incubated in 3ml. (final vol.), at 30° for 60min. under nitrogen, with tris–hydrochloric acid buffer, pH 8 (150 $\mu$moles), magnesium chloride (10 $\mu$moles), pyridoxal phosphate (30 m$\mu$moles) and either [1-$^{14}$C]-glyoxylate (20 $\mu$moles; 0·1 $\mu$c/$\mu$mole) plus unlabelled

glycine (20 $\mu$moles) or unlabelled glyoxylate (20 $\mu$-moles) plus [1-$^{14}$C]glycine (20 $\mu$moles; 0·1 $\mu$c/$\mu$-mole). The pyruvate formed was identified by paper chromatography of its 2,4-dinitrophenylhydrazone (see the Materials and Methods section), followed by radioautography. Unlabelled pyruvate was obtained when [1-$^{14}$C]glyoxylate was the labelled substrate, but when [1-$^{14}$C]glycine was used the label was retained in the pyruvate formed, which was highly radioactive. These results indicated that formation of oxaloacetate from erythro-$\beta$-hydroxyaspartate in these extracts proceeded via reaction (3) and that the responsible enzyme was an erythro-3-hydroxy-aspartate hydro-lyase (deaminating) akin to other hydroxyamino acid dehydratases (EC 4.2.1.13–16), and thus trivially named erythro-$\beta$-hydroxyaspartate dehydratase (Kornberg & Morris, 1962b). Purification of this enzyme (Gibbs & Morris, 1964b) has shown that it is a stable pyridoxal phosphate-activated enzyme, highly specific in its action on erythro-$\beta$-hydroxyaspartate, being completely without activity on serine, threonine, threo-$\beta$-hydroxy-aspartate and both erythro and threo diastereo-isomers of DL-$\beta$-hydroxy-$\beta$-methylaspartate.

*Enzymic formation of* erythro-$\beta$-hydroxyaspartate. No radioactive zone corresponding to erythro-$\beta$-hydroxyaspartate was detected by radioautography of the ethanol-soluble compounds formed at earliest times during the utilization of [2-$^{14}$C]glycollate by a growing culture of *M. denitrificans* (Fig. 3). This might well have been due to the existence of only a

very small pool of the free *erythro*-β-hydroxy-aspartate which would be expected if the dehydratase ensured rapid conversion of this intermediate into oxaloacetate and thence into the products such as malate, aspartate and glutamate, whose pool sizes allowed their detection. However, the production of *erythro*-β-hydroxyaspartate from glyoxylate and glycine could be demonstrated in extracts of glycollate-grown organisms. [2-$^{14}$C]Glycine (2 μ-moles; 0·1 μc/μmole) plus glyoxylate (2 μmoles) were incubated at 30° in 1 ml. (final vol.) with tris–hydrochloric acid buffer, pH 8 (20 μmoles), magnesium chloride (1 μmole), pyridoxal phosphate (10 mμmoles) and cell-free extract (2 mg. of protein). A duplicate tube contained the above but with cell extract that had been heated at 100° for 15 min. After 30 min. incubation, *erythro*-β-hydroxyaspartate (5 μmoles) was added and the protein precipitated with ethanol (3 ml.). High-voltage paper electrophoresis of the ethanol extract (see the Materials and Methods section), followed by radioautography, demonstrated the presence of labelled *erythro*-β-hydroxyaspartate only in the mixture that had contained unboiled extract. Although non-enzymic interaction of glycine and glyoxylate is known to occur, the product is a mixture of the *erythro* and *threo* forms of β-hydroxyaspartate (Metzler *et al.* 1954; Kornguth & Sallach, 1960). Only *erythro*-β-hydroxyaspartate was produced by the extract-catalysed condensation reaction. The enzyme responsible for this would be expected to be an aldolase resembling others of this class that act on hydroxyamino acids (EC 4.1.2.5–6). It is characteristic of such reactions that the equilibrium is far to the side of cleavage of the hydroxyamino acid, another reason to suppose that *erythro*-β-hydroxy-aspartate should not accumulate in *M. denitrificans* metabolizing glycollate. Indeed, when *erythro*-β-hydroxyaspartate was added to freshly prepared extracts of glycollate-grown organisms, among the keto acids isolated there appeared not only the oxaloacetate and pyruvate derived from the action of *erythro*-β-hydroxyaspartate dehydratase but also glyoxylate resulting from the action of the aldolase. The assay and further study of this enzyme (*erythro*-β-hydroxyaspartate glycine-lyase; Gibbs & Morris, 1964a) has been made possible by the finding that it will cleave *erythro*-DL-β-hydroxy-β-methylaspartate to form glycine and pyruvate (Gibbs & Morris, 1964a; see also the Materials and Methods section).

*Induced formation of enzymes metabolizing* erythro-β-hydroxyaspartate. High activities of *erythro*-β-hydroxyaspartate aldolase and *erythro*-β-hydroxyaspartate dehydratase were only found in extracts prepared from *M. denitrificans* grown on glycollate and on glyoxylate or on carbon sources known to be catabolized via glyoxylate (such as ethane-1,2-diol or allantoin). Extracts from cells grown on acetate or on various C$_3$ and C$_4$ compounds were virtually devoid of these enzymes (Table 4). When aspartate-grown organisms were introduced into a medium containing aspartate (5 mM) plus glycollate (25 mM), growth resumed with no marked diauxic lag, though utilization of glycollate required the induced synthesis of several enzymes, including glycollate oxidase, *erythro*-β-hydroxyaspartate aldolase and *erythro*-β-hydroxyaspartate dehydratase (Fig. 6). Transference of organisms from growth media containing other C$_4$ or C$_3$ compounds to a medium containing glycollate as main source of carbon for growth invariably resulted in the induction of the enzymes of the β-hydroxyaspartate pathway.

Table 4. *Activities of enzymes responsible for formation and utilization of* erythro-β-hydroxyaspartate *in* M. denitrificans *grown on various carbon sources*

Ultrasonic extracts prepared from harvested washed organisms and clarified by centrifuging at 20000g for 10 min. were assayed for *erythro*-β-hydroxyaspartate-aldolase and *erythro*-β-hydroxyaspartate-dehydratase activities as described in the Materials and Methods section. Units of the aldolase are expressed as μmoles of pyruvate produced/hr. from *erythro*-DL-β-hydroxy-β-methylaspartate; units of the dehydratase are expressed as μmoles of NADPH$_2$ oxidized/hr. in the presence of excess of malate dehydrogenase. —, Not assayed.

| | Specific activity (units/mg. of protein) | |
| --- | --- | --- |
| Carbon source for growth | *erythro*-β-Hydroxyaspartate aldolase | *erythro*-β-Hydroxyaspartate dehydratase |
| Glycollate | 185 | 11·4 |
| Glyoxylate | 194 | 15·0 |
| Ethane-1,2-diol | 170 | 10·5 |
| Allantoin | — | 11·2 |
| Acetate ⎫ Lactate ⎪ Succinate ⎬ Malate ⎪ Fumarate ⎪ Aspartate ⎭ | < 0·2 | < 0·02 |

*Utilization of* erythro-β-hydroxyaspartate *by whole organisms.* No growth of *M. denitrificans* was obtained on a medium containing *erythro-β*-hydroxyaspartate (20mM) as the sole source of carbon, nor was *erythro-β*-hydroxyaspartate appreciably oxidized by washed suspensions of glycollate-grown organisms (Table 1). Yet *erythro-β*-hydroxyaspar-

tate was inhibitory to growth of the organism on other carbon sources, e.g. on malate (20mM) it was wholly inhibitory to growth at 10mM, and on glycollate (20mM) it was inhibitory to growth at 30mM.

## DISCUSSION

The utilization of glyoxylate in *M. denitrificans* proceeds by a sequence of enzymic reactions unique in being the only route known to effect the direct condensation of two $C_2$ compounds, without prior activation to CoA derivatives, to yield a $C_4$ dicarboxylic acid precursor of a tricarboxylic acid-cycle intermediate. This β-hydroxyaspartate pathway is summarized in Scheme 2.

The preliminary loss of carbon dioxide with subsequent recarboxylation, which is a feature both of the glycerate pathway (Kornberg & Gotto, 1961) and of the utilization of glycine via serine (Sagers & Gunsalus, 1961; Morris, 1963), is avoided in the β-hydroxyaspartate pathway, which would thus appear to be a more economic metabolic route. The relatively low activity of malate synthase in glycollate-grown *M. denitrificans* (Table 3) further suggests that the β-hydroxyaspartate pathway also plays a catabolic role in this organism, in contrast with the situation in which the glycerate pathway performs only an anaplerotic function, the energy-yielding catabolic function being assumed by the dicarboxylic acid cycle (Kornberg & Sadler, 1960).

At present, the β-hydroxyaspartate pathway has only been demonstrated to operate during growth of *M. denitrificans* on glyoxylate. Even *Azotobacter* sp. that may form β-hydroxaspartate during growth (Virtanen & Saris, 1957) do not form the enzymes of the β-hydroxyaspartate pathway during growth on ethane-1,2-diol, but use instead the more usual glycerate pathway (Morris, 1964). This exceptional behaviour of *M. denitrificans* has yet to be explained.
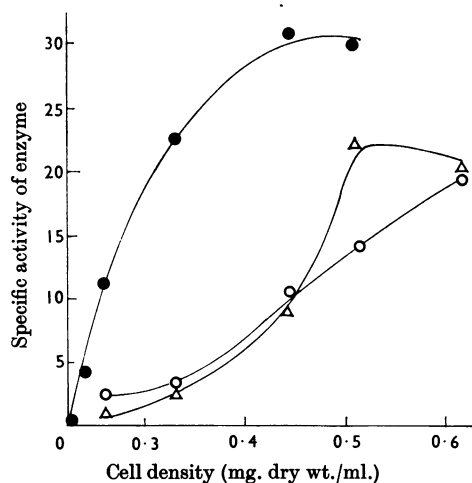
The properties of the novel enzymes of this path-



Fig. 6. Induction of enzymes of the β-hydroxyaspartate pathway. *M.denitrificans* was grown in a medium containing aspartate (25mM) as the sole source of carbon. Harvested organisms were transferred to fresh medium containing aspartate (5mM) and glycollate (25mM) to give an initial cell density of approx. 0·2mg. dry wt./ml. Growth was measured turbidimetrically during aerobic incubation at 30° and samples were withdrawn at intervals for assay of enzymic content (see the Materials and Methods section). For convenient graphic representation, specific activities of the enzymes (μmoles of substrate utilized/mg. of protein/hr.) were multiplied as follows: ●, glycollate oxidase, by 4; ○, *erythro*-β-hydroxyaspartate dehydratase, by 2; △, *erythro*-β-hydroxyaspartate aldolase, by 0·1.
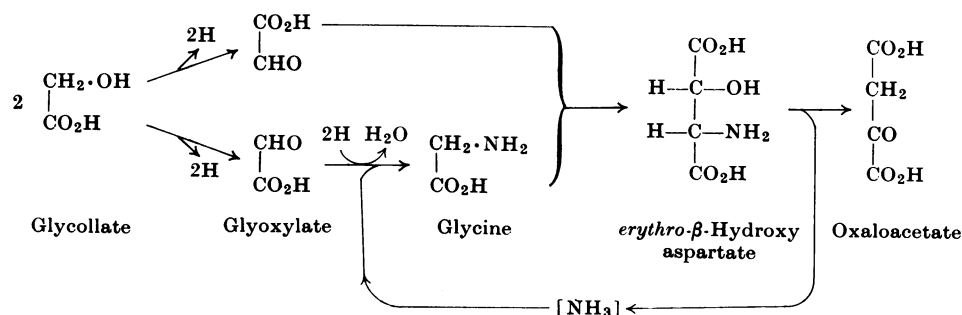


Scheme 2. The β-hydroxyaspartate pathway.

way are now being studied (Gibbs & Morris, 1964a,b). Also being examined is the interrelationship between glyoxylate and glycine in this organism. It is notable that whereas in extracts of glycollate-grown *M. denitrificans* glyoxylate very readily gives rise to glycine, the reverse, i.e. deamination of glycine, occurs only very slowly and the organism grows only very poorly on glycine. Glyoxylate can be converted into glycine in these extracts by transamination with aspartic acid (H. L. Kornberg & J. G. Morris, unpublished work), but it is possible that some more direct method of reductive amination might also operate.

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