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Glycine Decarboxylase in *Rhodopseudomonas spheroides* and in Rat Liver Mitochondria

By G. H. TAIT
Department of Chemical Pathology, St Mary's Hospital Medical School, London W.2, U.K.

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1. Glycine decarboxylase and glycine–bicarbonate exchange activities were detected in extracts of *Rhodopseudomonas spheroides* and in rat liver mitochondria and their properties were studied. 2. The glycine decarboxylase activity from both sources is stimulated when glyoxylate is added to the assay system. 3. Several proteins participate in these reactions and a heat-stable low-molecular-weight protein was purified from both sources. 4. These enzyme activities increase markedly when *R. spheroides* is grown in the presence of glycine, glyoxylate, glycollate, oxalate or serine. 5. All the enzymes required to catalyse the conversion of glycine into acetyl-CoA via serine and pyruvate were detected in extracts of *R. spheroides*; of these glycine decarboxylase has the lowest activity. 6. The increase in the activity of glycine decarboxylase on illumination of *R. spheroides* in a medium containing glycine, and the greater increase when ATP is also present in the medium, probably accounts for the increased incorporation of the methylene carbon atom of glycine into fatty acids found previously under these conditions (Gajdos, Gajdos-Török, Gorchein, Neuberger & Tait, 1968). 7. The results are compared with those obtained by other workers on the glycine decarboxylase and glycine–bicarbonate exchange activities in other systems.

Gajdos, Gajdos-Török, Gorchein, Neuberger & Tait (1968) reported that, when *Rhodopseudomonas spheroides* was illuminated in a medium containing succinate, fumarate and glycine (mixture IS), the methylene carbon atom of glycine, but not the carboxyl carbon atom, was incorporated into the fatty acid moieties of the phospholipids. This incorporation was greatly stimulated when ATP or Fe^{2+} was also present in the medium. The methylene carbon atom of glycine was also incorporated to a significant extent into the methyl groups of choline (Gorchein, Neuberger & Tait, 1968). To account for these results it was suggested (Gorchein *et al.* 1968) that the following metabolic pathway operated: glycine + methylenetetrahydrofolate → serine → pyruvate → acetyl-CoA → fatty acids, and that some of the methylenetetrahydrofolate was derived from the methylene carbon atom of glycine. This last reaction is catalysed by glycine decarboxylase, an enzyme system that has been studied in extracts from mammalian, plant and bacterial cells (Sato, Kochi, Sato & Kikuchi, 1969; Cossins & Sinha, 1966; Klein & Sagers, 1966*a*,*b*, 1967*a*,*b*).

All the enzymes involved in the pathway described above have now been detected in extracts of *R. spheroides*, and other enzymes involved in the biosynthesis and degradation of serine and glycine have also been assayed. In addition, the present paper is concerned with the properties of glycine decarboxylase. The activity is relatively low in extracts from cells grown in a medium containing malate and glutamate as carbon and nitrogen sources, but it is about five- to tenfold higher if glycine, glyoxylate, glycollate, oxalate or serine is added to the medium. The increase in glycine decarboxylase activity that occurs on illumination of cells in mixture IS is markedly higher if ATP or Fe^{2+} is also present. Like the glycine decarboxylase system studied by Klein & Sagers (1966*a*,*b*, 1967*a*,*b*) in *Peptococcus glycinophilus*, several proteins participate in the oxidation of glycine to methylenetetrahydrofolate, CO_2 and ammonia. With two of these proteins, one of which is a small protein that is stable to boiling, glycine–bicarbonate exchange activity can be demonstrated. The heat-stable protein has been extensively purified from *R. spheroides*. A similar protein has been purified from rat liver mitochondria, whose glycine decarboxylase has also been studied. Motokawa & Kikuchi

(1969*a*) have published details of their purification of this heat-stable protein from rat liver mitochondria.

EXPERIMENTAL

Chemicals. [1-^{14}C]Glycine and [2-^{14}C]glyoxylic acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. NAD$^+$, NADH, NADP$^+$ and NADPH were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Folic acid was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K., Sephadex G-100 from Pharmacia, Uppsala, Sweden, and DEAE-cellulose from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. The lithium salt of hydroxypyruvic acid was prepared by Dr M. Matthew. Tetrahydrofolic acid was purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., and from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. It was also prepared by reducing folic acid with $NaBH_4$ in a manner similar to that described by Hillcoat & Blakley (1964) and Scrimgeour & Vitols (1966). To a suspension of 10mg of folic acid in 3ml of water was added 100mg of $NaBH_4$. After about 5min the pH was carefully adjusted to about 2.5 by the addition of conc. HCl. The solution became quite hot during the acidification, and H_2 was evolved. N_2 was then bubbled through the solution and the tube was stoppered and cooled in ice. A copious crystalline precipitate formed and on occasions the whole mixture set solid. It was then warmed to 5°C and centrifuged at 5°C for 10min at 20000*g*. The very pale yellow or colourless supernatant was removed and kept under N_2. The pellet, under N_2, was warmed slightly, and recentrifuged. By repeated warming and centrifuging three solutions were obtained. These solutions had spectra in acid, at neutral pH and in alkali identical with those reported for tetrahydrofolic acid (Rabinowitz, 1960). Any folic acid left or dihydrofolic acid formed was probably precipitated on acidification, as the final precipitate was markedly yellow. The solutions obtained, containing about 10μmol of tetrahydrofolic acid/ml, were neutralized, mercaptoethanol was added to a concentration of 200μmol/ml, N_2 was bubbled through the solutions, and they were stored in stoppered tubes at −20°C. The material prepared in this way gave results identical with those given by commercially obtained tetrahydrofolic acid when used as cofactor for serine hydroxymethyltransferase (EC 2.1.2.1) methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) or glycine decarboxylase. The presence of some borate in the preparation did not appear to affect the activity of these enzymes.

Organisms. Rhodopseudomonas spheroides (National Collection of Industrial Bacteria, N.C.I.B. 8253) was obtained from Dr J. Lascelles, and was maintained in stab culture as described by Lascelles (1956).

Growth of organisms. Organisms were grown semi-anaerobically in the light at 32–34°C for 40–44h in medium S of Lascelles (1956) supplemented by $FeSO_4$ (10μM) and $MnCl_2$ (5μM) in 3-litre conical flasks filled almost to the top. The illumination was supplied and the temperature was maintained by 60W bulbs at appropriate distances from the flasks. Except where stated otherwise organisms grown in this way were used. When additional organic compounds were present in the medium, solutions of them were autoclaved separately and then added to the sterile medium. Organisms were also grown in the dark at 32°C by bubbling air or O_2 continuously through 1.5 litres of medium in 3-litre conical flasks.

Preparation of extracts. After growth, organisms were harvested by centrifuging at 4°C, washed with 0.05M-phosphate buffer (KH_2PO_4+NaOH), pH7.5, and resuspended in the same buffer to a concentration of approx. 80mg dry wt./ml. The suspension was either used immediately or kept frozen at −20°C until required. Suspensions of organisms were disrupted by passing them twice through a French pressure cell at a pressure of 15000lb/in^2. The extract was then diluted with 2vol. of cold 0.05M-phosphate buffer, pH7.5, and homogenized by hand in an all-glass homogenizer. Alternatively, suspensions of approx. 40mg dry wt./ml were disrupted by irradiation in an MSE 50 WUltrasonic Disintegrator operated at maximum output for 3min. During this time suspensions were maintained at close to 0°C by immersing the tube in crushed ice. Suspensions obtained by both methods were centrifuged at 25000*g* for 10min and the supernatants, termed 'crude extract', were removed. The crude extract was centrifuged at 100000*g* for 1h, and the resulting supernatant was removed and was dialysed against 0.05M-phosphate buffer, pH7.5, at 4°C overnight. This preparation is called 'enzyme extract' in the present work.

Illumination of cell suspensions. Illumination of cell suspensions was carried out in mixture IS (Gibson, Neuberger & Tait, 1962), which contains glycine, fumarate succinate and salts.

Preparation of mitochondria from rat liver. Mitochondria from rat liver were prepared essentially as described by Hogeboom (1955). After being washed in 0.25M-sucrose the mitochondrial pellet was suspended in 0.05M-phosphate buffer, pH7.5, to a concentration of 10–20mg of protein/ml and kept frozen at −20°C. Mitochondria were disrupted by freezing and thawing this suspension four or five times over a period of 8h. The suspension was then centrifuged at 25000*g* for 10min. The supernatant, called 'mitochondrial extract', was removed and the pellet was resuspended in buffer to the original volume. By this technique about 60% of the mitochondrial protein was present in the supernatant.

Determinations. The dry weight of organisms, their bacteriochlorophyll content and the coproporphyrin excreted on illumination in mixture IS were determined as described by Gibson *et al.* (1962). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Fractions eluted from columns were monitored for protein by measuring the extinction at 280nm.

Enzyme assays. (*a*) Glycine decarboxylase. This activity was measured in two ways. In the first methylenetetrahydrofolate formed by glycine decarboxylase was oxidized by methylenetetrahydrofolate dehydrogenase, an NADP$^+$-requiring enzyme present in extracts, to methylidynetetrahydrofolate which was measured spectrophotometrically after acidification of the reaction mixtures (Sagers & Gunsalus, 1961). In the second method, $^{14}CO_2$ formed in assays performed with [1-^{14}C]glycine was measured. Since the second method was used in most experiments the

spectrophotometric method is not described in detail. The results by both methods were in good agreement.

The radioactivity assay method was as follows. The assay mixture contained, in a total volume of 0.575ml, 25μmol of tris–HCl buffer, pH8.1, 6μmol of glycine, 0.1μCi of [1-^{14}C]glycine, 0.05μmol of pyridoxal phosphate, 0.2μmol of NAD^+, 0.2μmol of tetrahydrofolate, 5μmol of mercaptoethanol and enzyme extract; the final pH was 7.8. Solutions of all the reagents added, except enzyme, were stored under N_2. The contents of the assay were mixed by passing N_2 vigorously over the surface, and the tube was stoppered. After incubation for up to 3h at 37°C, the rubber stopper was replaced by one to which a Whatman glass-fibre disc (8mm diam.), soaked with 20μl of 2M-NaOH, was attached by a pin. This stopper was then taken off momentarily, 50μl of 3M-H_2SO_4 injected into the tube and the stopper replaced. The tube was incubated at 37°C for 30min with occasional shaking. The disc was then transferred to a vial containing 5ml of scintillator [a mixture of 2 parts of toluene containing 0.4% 2,5-diphenyloxazole and 0.1% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 1 part of Triton X-100]. It was left at 5°C in a Nuclear–Chicago scintillation counter for about 16h with occasional shaking and the radioactivity was then determined. The efficiency of counting was tested by acidifying solutions of $KH^{14}CO_3$, collecting the $^{14}CO_2$ on discs soaked with NaOH and counting for radioactivity as described above. Under these conditions 0.01μCi gave 12550c.p.m. In all experiments controls were done without tetrahydrofolate or without enzyme. In these a small amount of volatile radioactivity, probably due to slight decomposition of the [1-^{14}C]glycine on storage, was obtained. From the radioactivity, after correction for the control, the amount of CO_2 formed in assays was calculated. Activities are expressed as nmol of CO_2 formed/3h per mg of protein.

(*b*) Glycine–bicarbonate exchange activity. This activity was assayed essentially as described by Klein & Sagers (1966*a*). The assay mixture contained, in a total volume of 0.575ml, 20μmol of tris–HCl buffer, pH7.25, 6μmol of glycine containing 0.1μCi of [1-^{14}C]glycine, 0.05μmol of pyridoxal phosphate, 5μmol of mercaptoethanol, 20μmol of $NaHCO_3$ and enzyme extract; the final pH was 7.5. After incubation for up to 3h at 37°C in stoppered tubes, the radioactivity in the bicarbonate was determined as described above. Controls were done without enzyme or without $NaHCO_3$. Activity is expressed as nmol of bicarbonate exchanged/3h per mg of protein.

(*c*) Methylenetetrahydrofolate dehydrogenase. This enzyme was assayed as described by Hatefi, Osborn, Kay & Huennekens (1957). The assay mixture contained, in a total volume of 3ml, 300μmol of phosphate buffer, pH7.5, 5μmol of formaldehyde, 0.45μmol of tetrahydrofolate, 10μmol of mercaptoethanol, 0.5μmol of $NADP^+$ and enzyme extract containing 0.25–1.0mg of protein. Incubation was at 22°C in cells with a 1cm light-path. Enzyme was added last and the extinction at 340nm was followed for 2min. Activity is expressed as μmol of NADPH formed/h per mg of protein.

(*d*) Serine hydroxymethyltransferase. This enzyme was assayed as described above for methylenetetrahydrofolate dehydrogenase except that L-serine (10μmol) was used instead of formaldehyde, and enzyme extract containing 0.5–2mg of protein was used. This assay depends on the oxidation by methylenetetrahydrofolate dehydrogenase of methylenetetrahydrofolate formed by serine hydroxymethyltransferase. Activity with formaldehyde was about five times that with serine, showing that when serine hydroxymethyltransferase is assayed by this method methylenetetrahydrofolate dehydrogenase is not rate-limiting. Activity is expressed as μmol of NADPH formed/h per mg of protein.

(*e*) Glyoxylate–glutamate transaminase (EC 2.6.1.4). The assay mixture contained, in a total volume of 0.2ml, 10μmol of phosphate buffer, pH7.5, 2μmol of sodium [2-^{14}C]glyoxylate (0.01μCi/μmol), 2μmol of sodium glutamate and enzyme extract containing 0.05–0.15mg of protein. Pyridoxal phosphate did not stimulate activity when fresh enzyme extracts were used, so it was not normally added. Incubation was at 37°C for 45min. Then 0.2ml of a 50% (v/v) suspension of Zeo-Karb 225 (H^+ form) in water and 0.6ml of water were added. After being mixed, the suspension was centrifuged briefly and the supernatant was removed and discarded. The pellet was washed with 2ml of water. The amino acids were eluted from the resin by adding 1ml of M-NH_3, mixing and centrifuging. The supernatant was removed and a sample was pipetted on to a 6.25cm^2 aluminium planchet. It was dried, and radioactivity was then determined in a Nuclear–Chicago gas-flow counter with a Micromil end-window, operated at the centre of the plateau. Under these conditions 1μmol of [2-^{14}C]glyoxylate (0.01μCi/μmol) gave 7000c.p.m. Control assays were done without adding glutamate. Activity is expressed as μmol of glycine formed/h per mg of protein.

(*f*) Hydroxypyruvate reductase (glycerate dehydrogenase) (EC 1.1.1.29). The assay mixture contained, in a total volume of 3ml, 200μmol of tris–HCl buffer, pH7.6, 1.5μmol of lithium hydroxypyruvate, 0.5μmol of NADH or NADPH (both gave almost the same activities) and enzyme extract containing 0.5–1.0mg of protein. Incubation was at 22°C in cells with a 1cm light-path, and the extinction at 340nm was followed for 2min. Activity is expressed as μmol of NAD^+ ($NADP^+$) formed/h per mg of protein.

(*g*) Glycollate dehydrogenase (EC 1.1.1.26). The assay mixture contained, in a total volume of 3ml, 200μmol of tris–HCl buffer, pH7.6, 20μmol of sodium glyoxylate, 0.5μmol of NADPH and enzyme extract containing 1–2mg of protein. Incubation was at 22°C in cells with a 1cm light-path, and the extinction at 340nm was followed for 2min. Activity is expressed as μmol of $NADP^+$ formed/h per mg of protein.

(*h*) Serine dehydratase (EC 4.2.1.13). This enzyme was assayed at pH8.0 essentially as described by Greenberg (1962). Undialysed enzyme extract was used. Activity is expressed as μmol of pyruvate formed/h per mg of protein.

(*i*) Pyruvate dehydrogenase. This enzyme system was assayed at pH7.5 and at 22°C as described by Korkes (1955), the reduction of NAD^+ to NADH being followed in the presence of pyruvate, CoA, thiamin pyrophosphate and Mg^{2+}. Crude extract, prepared as described above, was used for the assay. Activity is expressed as μmol of NADH formed/h per mg of protein.

RESULTS

Glycine decarboxylase in extracts of R. spheroides. When extracts of *R. spheroides* were assayed for glycine decarboxylase activity by the methods described in the Experimental section the production of methylidynetetrahydrofolate and of CO_2 both increased linearly for up to 3h. The production of methylidynetetrahydrofolate in the spectrophotometric assay was dependent on the presence of glycine and of $NADP^+$, was increased by adding pyridoxal phosphate and increased slightly by adding NAD^+. In the radioactivity assay method the production of $^{14}CO_2$ from [1-^{14}C]glycine was dependent on tetrahydrofolate. Some $^{14}CO_2$ was formed in the absence of added pyridoxal phosphate and NAD^+, but the amount was markedly increased when both were present. In assays where both methylidynetetrahydrofolate and $^{14}CO_2$ were measured approximately the same amounts of each were formed. All the results to be described below were obtained with the radioactivity assay method, and incubations were for 3h.

When glycine decarboxylase activity was measured with different amounts of enzyme extract it was found that activity was only linearly proportional to protein concentration at relatively high protein concentrations; i.e. the specific activity (per mg of protein) was low with small amounts of protein (Fig. 1). Plots of activity versus glycine concentration (Fig. 2) were sigmoid in shape, rather than hyperbolic as predicted on the basis of Michaelis–Menten kinetics, suggesting co-operativity of substrate binding. Double-reciprocal (Lineweaver–Burk) plots were curves convex to the 1/[substrate] axis. Hill plots (Hill, 1910) gave slopes of close to 2 for both the curves in Fig. 2.

Enzyme extracts exhibited marked glycine decarboxylase activity over the pH range 7.5–9.0, with an optimum at about pH 8. Activities were about the same in both tris–HCl and phosphate buffers. Activity was sensitive to the concentration of buffer, the activity in 0.175M-tris–HCl being only 30% of the activity in 0.04M-tris–HCl.

The effect of a number of compounds related to glycine, structurally or metabolically, on glycine decarboxylase activity was tested. Assays were done with 6μmol of glycine and the same amount of glyoxylate, glycollate, pyruvate, malate, acetate or oxalate. Of these compounds only glyoxylate had any effect; it markedly stimulated activity. The effect of glyoxylate was therefore tested further. It was found that in its presence (6μmol/assay) plots of activity versus protein concentration were linear even at low protein concentration (Fig. 3) and that plots of activity versus glycine concentration were hyperbolic instead of sigmoid (Fig. 4). A double-reciprocal plot of the results of the experiment done in the presence of glyoxylate was linear. The extent of stimulation by glyoxylate increased as the amount per assay was increased to 6μmol, but with higher amounts the activity fell.

The glycine decarboxylase system of *Peptococcus glycinophilus* (Klein & Sagers, 1967*a*) contains four different proteins: P_1, a pyridoxal phosphate-containing protein; P_2, a heat-stable protein of low

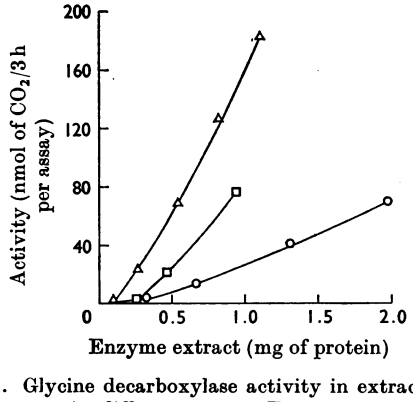

Fig. 1. Glycine decarboxylase activity in extracts from cells grown in different ways. Enzyme extracts were prepared and assayed for glycine decarboxylase activity as described in the Experimental section. Extracts were made from cells grown anaerobically in the light (○), anaerobically in the light in the presence of 10mM-glyoxylate (△) and in air in the dark (□).

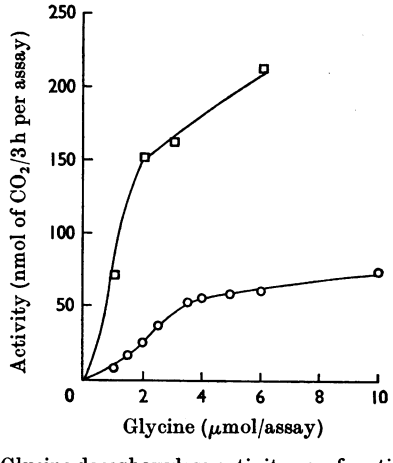

Fig. 2. Glycine decarboxylase activity as a function of the concentration of glycine in the assay. Enzyme extracts were prepared and assayed for glycine decarboxylase activity as described in the Experimental section. Present in assays were enzyme extracts from cells grown anaerobically in the light (○; 1.98mg of protein) or from cells grown anaerobically in the light in the presence of 10mM-glyoxylate (□; 1.4mg of protein).

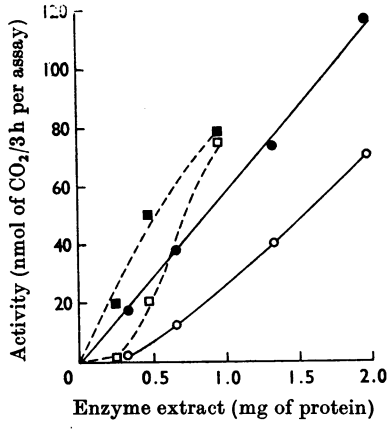

Fig. 3. Effect of glyoxylate on glycine decarboxylase activity. Assays for glycine decarboxylase were performed as described in the Experimental section in the absence (○ and □) and in the presence (● and ■) of glyoxylate (6μmol/assay). Enzyme extracts were from cells grown anaerobically in the light (——) and from cells grown under air in the dark (----).

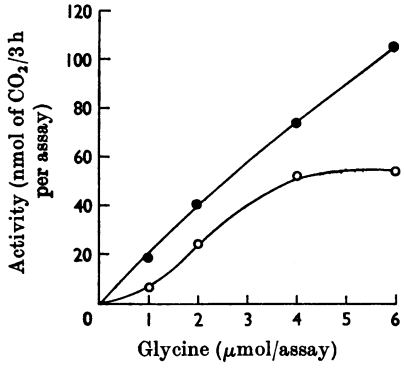

Fig. 4. Effect of glyoxylate on glycine decarboxylase activity in the presence of different amounts of glycine. Glycine decarboxylase activity was assayed as described in the Experimental section, with enzyme extracts from cells grown anaerobically in the light (1.98mg of protein) in the absence (○) and in the presence (●) of 6μmol of sodium glyoxylate.

molecular weight; P_3, a flavoprotein; P_4, a protein required for the transfer of the methylene carbon atom of glycine to tetrahydrofolate. To test whether the system in *R. spheroides* was similar, enzyme extracts were fractionated with ammonium sulphate. Fractions precipitated between 25 and 50% saturation with ammonium sulphate and between 50 and 75% saturation with ammonium sulphate were prepared. When each fraction was tested for activity separately, the activity was very low, whereas when equal volumes of the two fractions were recombined full activity was demonstrated (Table 1). The active component(s) in the 50–75%-saturated-ammonium sulphate fraction was not destroyed by boiling. Thus it appears that only a heat-stable factor, possibly equivalent to the protein P_2 described by Klein & Sagers (1967*a*), is present in this fraction. The activity given by the 25–50%-saturated-ammonium sulphate fraction increased as increasing amounts of the 50–75%-saturated-ammonium sulphate fraction were added. The low activity exhibited by each fraction separately, which suggested that complete separation had not been achieved, was stimulated by glyoxylate to a much greater extent with the 25–50% saturated-ammonium sulphate fraction than with the 50–75%-saturated-ammonium sulphate fraction. Glyoxylate also stimulated activity when equal volumes of both fractions were assayed together, but it was less effective in assays with excess of the 50–75%-saturated-ammonium sulphate fraction. The increased activity obtained by adding excess of the heat-stable factor suggested that in the original enzyme extract this factor might not be present in saturating amount. To test this, activity was measured with different amounts of enzyme extract plus a constant amount of boiled enzyme extract; it was found that the plot of activity versus protein concentration was linear even at low enzyme concentration (contrast with Fig. 1). Pindar (1968), when assaying glycine decarboxylase activity in extracts of *Arthrobacter globiformis*, found that plots of activity against protein were not linear at low protein concentration, but became linear on adding a boiled extract.

These results all suggest that the glycine decarboxylase system of *R. spheroides* is a complex one, that a number of proteins are required and that these factors are only loosely attached to one another, conditions favouring protein association increasing the specific activity, and factors favouring protein dissociation lowering the specific activity.

Glycine–bicarbonate exchange activity in extracts of R. spheroides. Klein & Sagers (1966*a*) showed that extracts of *P. glycinophilus* catalysed exchange between the carboxyl carbon atom of glycine and bicarbonate in the absence of tetrahydrofolate and NAD^+. They further showed that only two of the proteins, P_1 and P_2, were required; they partially purified these proteins. Enzyme extracts of *R. spheroides* also catalysed this reaction when [1-^{14}C]-glycine, bicarbonate, pyridoxal phosphate and mercaptoethanol were present. The incorporation of radioactivity into CO_2 was completely dependent on bicarbonate and on a reducing agent. GSH, but not ascorbic acid, could replace mercaptoethanol. As found for glycine decarboxylase activity the

Table 1. *Partial separation of components required for glycine decarboxylase activity*

Fractions precipitated between 25 and 50% saturation with $(NH_4)_2SO_4$ (fraction 1) and 50–75% saturation with $(NH_4)_2SO_4$ (fraction 2) were prepared by adding solid $(NH_4)_2SO_4$ to a dialysed enzyme extract. The fractions were dissolved in equal volumes of buffer and dialysed. 'Boiled fractions' were placed in a boiling-water bath for 3min before use. Assays were done as described in the Experimental section. Glyoxylate (6μmol/assay) was added when stated. Activity is expressed either as nmol of CO_2/3h per mg of protein or as nmol of CO_2/3h per assay.

Expt. no.	Enzyme fractions	Addition	Sp. activity (per mg of protein)
1	Dialysed enzyme extract		26.0
	Fraction 1		7.4
	Fraction 2		5.4
	Fraction 1 + fraction 2 (equal volumes)		24.3
			Activity (per assay)
2	Fraction 1 (0.1ml) + fraction 2 (0.1ml)		74.2
	Fraction 1 (0.1ml) + boiled fraction 2 (0.1ml)		66.0
			Activity (per assay)
3	Fraction 1 (75μl)		6.7
	Fraction 1 (75μl)	Glyoxylate	49.5
	Fraction 2 (75μl)		8.0
	Fraction 2 (75μl)	Glyoxylate	29.0
	Fraction 1 (75μl) + fraction 2 (75μl)		51.2
	Fraction 1 (75μl) + fraction 2 (75μl)	Glyoxylate	91.0
	Fraction 1 (75μl) + fraction 2 (150μl)		77.2
	Fraction 1 (75μl) + fraction 2 (150μl)	Glyoxylate	93.0

specific activity (per mg of protein) in the exchange assay increased with the amount of extract added (cf. Fig. 1 and Pindar, 1968). Plots of exchange activity versus glycine concentration were also sigmoid in shape (cf. Fig. 2). On the other hand a plot of activity versus bicarbonate concentration was hyperbolic and a double-reciprocal plot was linear. From these an apparent K_m of 80mM for bicarbonate was calculated. The optimum activity was observed at pH7.75; at pH8.6 the activity was only about 15% of that at pH7.75. Glyoxylate (6μmol/assay) had no effect on exchange activity in the presence of 1 or 6μmol of glycine/assay.

When the ammonium sulphate fractions prepared above were assayed for glycine–bicarbonate exchange activity it was found that when the 25–50%-saturated-ammonium sulphate and 50–75%-saturated-ammonium sulphate fractions were assayed separately only low activity was exhibited, but when they were assayed together high activity was obtained. The factor in the 50–75%-saturated-ammonium sulphate fraction was stable to boiling, but that in the 25–50%-saturated-ammonium sulphate fraction was not.

The glycine–bicarbonate exchange activity of unfractionated enzyme extracts was almost completely inhibited by 10μM-Zn^{2+} and 10μM-Cu^{2+}. Ni^{2+} and Co^{2+} at that concentration had little effect, although at higher concentrations they did inhibit. This inhibitory effect is rather surprising, since the assay system contains a high concentration of mercaptoethanol, which might be expected to bind these cations and thereby prevent the inhibition.

Glycine decarboxylase and glycine–bicarbonate exchange activities in extracts from rat liver mitochondria. Richert, Amberg & Wilson (1962) detected glycine decarboxylase activity in avian liver and, while the work described in the present paper was in progress, Sato *et al.* (1969) detected both glycine decarboxylase and glycine–bicarbonate exchange activities in an extract of acetone-dried powder of rat liver mitochondria and studied the reactions in some detail. Some of the work described here confirms the findings of Sato *et al.* (1969), but additional findings, not made by them, are also reported.

A soluble fraction, obtained by centrifuging a suspension of broken mitochondria (see the Experimental section), catalysed glycine decarboxylase and glycine–bicarbonate exchange activities. As with extracts of *R. spheroides* the specific activities (per mg of protein) increased when small amounts of protein were used and only became constant when there was more than 1mg of protein in the assay mixture. In contrast with the results with extracts of *R. spheroides* a plot of activity versus glycine concentration was hyperbolic in shape (Fig. 5) and a double-reciprocal plot was linear. From these data on glycine decarboxy-

lase activity, an apparent K_m for glycine of 50mM was calculated. In the presence of glyoxylate (6μmol/assay), decarboxylase activity was increased about twofold in the presence of 1–6μmol of glycine (Fig. 5). The apparent K_m for glycine in the presence of glyoxylate, calculated from the linear double-reciprocal plot, was 25mM. Glyoxylate also appeared to increase the relative $V_{max.}$ by about twofold. Glyoxylate had no stimulatory effect on glycine–bicarbonate exchange activity; indeed, it was slightly inhibitory. The optimum pH for the decarboxylase activity was 6.7 in phosphate buffer and 7.0 in tris buffer. For glycine–bicarbonate exchange activity the highest activity was observed at pH6.9, the lowest pH used; at pH7.8 the activity was only 15% of that at pH6.9. Both activities were markedly inhibited by increasing the concentration of phosphate or tris buffer in the assay; at 0.14M-phosphate buffer the exchange activity was only 10% of that at 0.035M-phosphate buffer.

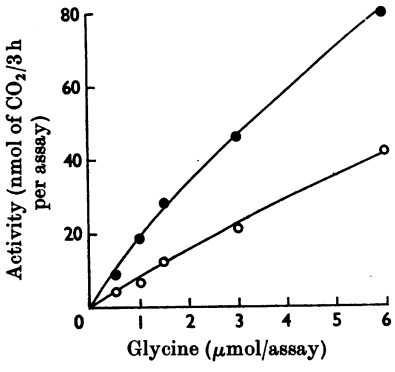

Fig. 5. Effect of glyoxylate on glycine decarboxylase activity of mitochondrial extract. Mitochondrial extract (1.38mg of protein), prepared as described in the Experimental section, was assayed for glycine decarboxylase activity in the absence (○) and in the presence (●) of 6μmol of sodium glyoxylate.

Fractions prepared from the mitochondrial extract with ammonium sulphate were inactive, and recombination of the fractions did not restore activity. When the mitochondrial extract was boiled for 3min a bulky precipitate formed. The resulting supernatant was found to have only 7% of the original protein. This supernatant markedly stimulated glycine decarboxylase and glycine-exchange activities when assayed together with the 25–50%-saturated-ammonium sulphate fraction from *R. spheroides*; that is, it can replace the heat-stable factor from *R. spheroides*. Thus it is probable that the mitochondrial glycine decarboxylase system consists of a number of proteins, at least one of which is similar to one from *R. spheroides*.

Table 2. *Effect on glycine decarboxylase and glycine–bicarbonate exchange activities of growing* R. spheroides *in different ways*

R. spheroides was grown from a small inoculum in medium S plus Fe^{2+} and Mn^{2+} semi-anaerobically in the light, unless stated otherwise, with the additions (10mM) as shown. At the end of growth cells were harvested and washed, and dialysed enzyme extract was prepared as described in the Experimental section. Specific activities are expressed as nmol of product/3h per mg of protein.

Expt. no.	Additions to growth medium	Glycine decarboxylase	Glycine–bicarbonate exchange
1	None	37	22
	Formate	21	15
2	None	46	27
	Glycollate	113	157
	Glucose	58	49
	Pyruvate	41	31
	Serine	91	122
3	None	51	27
	Glycine (5mM)	214	244
4	None	49	40
	Oxalate	100	150
5	None	50	25
	Glyoxylate	159	176
	Conditions of growth		
6	Grown semi-anaerobically in light	50	26
	Grown in air in dark	70	25
	Grown in oxygen in dark	30	14

Activities of enzymes in extracts of R. spheroides *grown in different ways and in different media.* The specific activities of glycine decarboxylase and glycine–bicarbonate exchange in extracts of *R. spheroides* grown semi-anaerobically in the light in a medium containing malate and glutamate are very low when compared with those in extracts of *P. glycinophilus* (cf. Sagers & Gunsalus, 1961). Organisms grown in the presence of glycine, glycollate, glyoxylate, oxalate or serine had much higher activities than did organisms grown in the normal medium (Table 2). Growing cells in the presence of pyruvate, glucose or formate had little effect on activity. It is also noteworthy that the specific activity of the exchange system increased more markedly than that of the decarboxylase system. The glycine decarboxylase but not the exchange activity of organisms grown in air was slightly higher than that of cells grown semi-anaerobically in the light. The rate of increase in these enzyme activities on adding glycine to growing cells is shown in Fig. 6. From these results it cannot be stated which of the compounds functions as inducer for synthesis of the proteins in the glycine decarboxylase system, but it is possible that there are enzyme systems in *R. spheroides* for the interconversion of serine, glycine, glyoxylate, glycollate and oxalate. The activities of three enzymes involved in the metabolism of these compounds were measured (Table 3) in enzyme extracts from cells grown in different ways. The activities of methylene tetrahydrofolate dehydrogenase and serine hydroxymethyltransferase were similar under all conditions of growth. The glyoxylate–glutamate transaminase activity varied to some extent but not as markedly as the glycine decarboxylase and glycine–bicarbonate exchange activities. The specific activities of these three enzymes are very much higher than those of the glycine decarboxylase and exchange. In addition, glycerate dehydrogenase and glycollate dehydrogenase were assayed in extracts of cells grown in the normal medium and with some of the additions shown in Tables 2 and 3 (Table 4). They had almost the same activities in all extracts tested. Serine dehydratase and pyruvate dehydrogenase were assayed only in extracts from normal cells (Table 4).

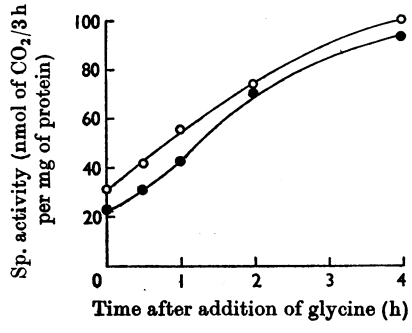

Fig. 6. Effect on glycine decarboxylase and glycine–bicarbonate exchange activities on adding glycine to a growing culture of *R. spheroides*. At 0 h glycine, to a final concentration of 5 mM, was added to a culture of *R. spheroides* growing semi-anaerobically in the light (0.225 mg dry wt./ml). Samples were taken from the growing culture at the times shown and enzyme extract was prepared from each. Glycine decarboxylase (○) and glycine–bicarbonate exchange (●) were assayed with the enzyme extracts (approx. 1 mg of protein/assay). At 4 h the culture had 0.325 mg dry wt./ml.

Illumination in mixture IS. Illumination of *R. spheroides* in mixture IS gives rise to excretion of coproporphyrin. This excretion is decreased or abolished by ATP or Fe^{2+}, and increased by ethionine or threonine (Gajdos *et al.* 1968). ATP and Fe^{2+} stimulate the incorporation of the methylene

Table 3. *Activities of some enzymes in extracts of cells grown in different media*

R. spheroides was grown from a small inoculum semi-anaerobically in the light medium S plus Fe^{2+} and Mn^{2+} with the additions (10 mM) shown. Enzyme extracts were prepared and assayed as described in the Experimental section. Specific activities are expressed as μmol of product formed/h per mg of protein.

Expt. no.	Addition to medium	Methylene-tetrahydrofolate dehydrogenase	Serine hydroxymethyl-transferase	Glyoxylate–glutamate transaminase
1	None	4.98	0.89	2.98
	Glycollate	4.80	1.14	1.40
	Glucose	4.32	0.73	6.32
	Pyruvate	5.59	0.76	2.66
	Serine	5.51	1.19	0.94
	Oxalate	3.24	0.29	1.42
2	None	3.48	0.82	0.76
	Glycine	4.20	0.84	0.61
	Glyoxylate	4.56	0.29	0.89

carbon atom of glycine into fatty acids whereas ethionine decreases it (Gajdos *et al.* 1968). Table 5 shows that on illumination of cells in mixture IS both glycine decarboxylase and glycine–bicarbonate exchange activities increased, that in the presence of ATP or Fe^{2+} the increase was greater and that in the presence of ethionine there was no increase in the activities. It was found that when organisms that had been grown on glycine or glyoxylate, and that had very high glycine decarboxylase activity (cf. Table 2), were illuminated in mixture IS they excreted coproporphyrin at the same rate as cells previously grown without these additions.

Purification of heat-stable factors from R. spheroides *and from mitochondria.* The heat-labile protein involved in glycine–bicarbonate exchange activity in extracts of *R. spheroides*, i.e. the one precipitated between 25 and 50% saturation with ammonium sulphate, was unstable to purification, and a tenfold increase in specific activity was all that was obtained. However, the heat-stable factors from both *R. spheroides* and rat liver mitochondria were purified extensively and were obtained in almost pure form as judged by polyacrylamide-gel electrophoresis. Details of the purification procedures, which so far have only been done on a relatively small scale, are given below.

(*a*) From *R. spheroides*. Enzyme extract was prepared from cells grown in the presence of glycine, as described in the Experimental section. The extract (9.5mg of protein/ml) was fractionated by addition of solid ammonium sulphate and fractions precipitated at 30–50% saturation and 50–80% saturation were collected. The precipitates were dissolved in 0.05M-phosphate buffer, pH7.5, and dialysed against the same buffer. The dialysed solutions had about one-quarter the volume of the original enzyme extract; the 30–50%-saturated-ammonium sulphate fraction had about 23mg of protein/ml and the 50–80%-saturated-ammonium sulphate fraction had about 12mg of protein/ml. The 30–50%-saturated-ammonium sulphate fraction was used as a source of heat-labile factor, and glycine–bicarbonate exchange assays for heat-stable factor of both *R. spheroides* and mitochondria at various stages of purification were done in its presence. A 55ml portion of the 50–80%-saturated-ammonium sulphate fraction was applied to a column of DEAE-cellulose (5g) equilibrated with 0.05M-phosphate buffer, pH7.5. Elution was performed with a linear gradient made from 150ml each of 0.05M-phosphate buffer, pH7.5, and 0.5M-sodium chloride–0.05M-phosphate buffer, pH7.5; 3ml fractions were collected. Activity was eluted as a single peak at about 0.3M-sodium chloride. Active fractions having about 16 times the specific activity of the ammonium sulphate fraction were pooled (30ml) and the volume was reduced to about 2ml by putting the solution in a dialysis bag

Table 4. *Activities of enzymes in extracts*

R. spheroides was grown semi-anaerobically in the light in medium S plus Fe^{2+} and Mn^{2+}. Crude extract and enzyme extract were prepared and enzyme assays were done as described in the Experimental section. Specific activities are expressed as μmol of product formed/h per mg of protein.

Enzyme	Sp. activity
Glycerate dehydrogenase	4.8
Glycollate dehydrogenase	1.0
Pyruvate dehydrogenase	1.2
Serine dehydrogenase	1.0

Table 5. *Effect of illumination of cells in mixture IS with various additions on glycine decarboxylase and glycine–bicarbonate exchange activities*

R. spheroides, grown semi-anaerobically in the light, was suspended to a concentration of 1.5mg dry wt./ml in mixture IS with the additions stated. The suspensions were illuminated at 32–34°C for the times stated. The cells were then harvested and washed, and dialysed enzyme extracts were prepared. Enzymes were assayed as described in the Experimental section. Specific activities are expressed as nmol of product/3h per mg of protein.

Expt. no.	Addition	Time of illumination (h)	Glycine decarboxylase	Glycine–bicarbonate exchange
1	None	0	50	20
	None	5	92	42
	ATP (3mM)	5	130	98
2	None	0	22	7
	None	5	60	33
	Fe^{2+} (10μM)	5	100	96
3	None	0	38	19
	None	4.5	80	43
	DL-Ethionine (1mM)	4.5	44	15

and placing it in a beaker containing Carbowax. This concentrated solution was then applied to a column of Sephadex G-100 in 0.05M-phosphate buffer, pH 7.5. Inactive protein was eluted first and the heat-stable factor later. The elution volume was identical with that of chymotrypsinogen (mol.wt. 24000). By this step a further three- to four-fold purification was obtained. Active fractions were pooled and concentrated as above. The final specific activity was about 200 times that of the original enzyme extract, and the yield was about 2–3mg of protein. On electrophoresis of the purified material at pH 8.4 on polyacrylamide gel (Ornstein, 1964), there was found one major and one minor band of protein, both migrating towards the anode almost as fast as the tracker dye.

(*b*) From rat liver mitochondria. An extract of mitochondria was prepared by repeated freezing and thawing as described in the Experimental section. This extract was boiled for 3min and cooled, and the precipitate was removed by centrifuging. The supernatant obtained contained all the heat-stable factor, but only about 7% of the protein. The supernatant (80ml), containing 0.5mg of protein/ml, was concentrated with Carbowax to about 5ml. This solution was applied to a 1g column of DEAE-cellulose equilibrated with 0.05M-phosphate buffer, pH 7.5, and elution was performed as described above but with 100ml of each buffer. Activity was eluted in a single peak with approx. 0.2M-sodium chloride. This step produced a 20–30-fold purification. Active fractions were pooled, concentrated and chromatographed on Sephadex G-100. The elution volume of the active factor was identical with that of the one from *R. spheroides*, and a further three-fold purification was obtained. The final specific activity was about 300 times that of whole mitochondria, and about two to three times that of the purified fraction from *R. spheroides*. The final yield of material was about 200μg of protein. The purified material had an E_{280}/E_{260} ratio of 1.89, indicating that the material is a protein in nature and lacks nucleic acid (cf. Klein & Sagers, 1966*a*). The preparation showed a single band of protein on polyacrylamide-gel electrophoresis, with the band running towards the anode almost as fast as the tracker dye.

These procedures are fairly simple and there would not appear to be any difficulty in doing them on a larger scale to obtain sufficient material for further studies.

DISCUSSION

Although glycine decarboxylase activity has been found in many cells (see below), detailed work on the properties of the system and on the constituents involved has only been published for *P. glycinophilus* (Klein & Sagers, 1966*a*,*b*, 1967*a*,*b*; Baginsky & Huennekens, 1967), *A. globiformis* (Kochi & Kikuchi, 1969; Pindar, 1968) and more recently for rat liver mitochondria (Sato *et al.* 1969). The results reported here for extracts of *R. spheroides* and of rat liver mitochondria show that some of the features are similar to those of the above systems, but there appear to be differences. In *P. glycinophilus* four proteins are required to catalyse decarboxylation of glycine and with only two of them is glycine–bicarbonate exchange exhibited. Although all four proteins have not been separated from extracts of *R. spheroides* or from mitochondria at least two proteins are involved in both reactions. With extracts of *R. spheroides* and of mitochondria, as with those of *A. globiformis* (Pindar, 1968), the specific activities of both glycine decarboxylase and glycine-bicarbonate exchange are higher at high than at low protein concentration. Glycine exerts a homotropic activation in both glycine decarboxylase and glycine–bicarbonate exchange assays with extracts of *R. spheroides*, but not of mitochondria. No homotropic activation by glycine has been found with extracts of other cells. Glyoxylate, whose effect on glycine decarboxylase does not appear to have been tested previously, stimulates activity; by contrast it has no effect on glycine–bicarbonate exchange activity. When an extract of *R. spheroides* is assayed in its presence a plot of activity versus glycine concentration is hyperbolic and a plot of activity versus protein concentration is linear even at low protein concentration. With mitochondrial extracts glyoxylate halves the apparent K_m for glycine and doubles the relative $V_{max.}$ for the reaction.

The heat-stable factor that is involved in both decarboxylase and exchange activities has been extensively purified from both *R. spheroides* and from mitochondria; it is not certain whether either has been purified completely. The factors from both sources seem to be acidic proteins with molecular weights of about 24000; they appear to be about equally effective in allowing glycine–bicarbonate exchange activity when assayed together with the 30–50%-saturated-ammonium sulphate fraction from *R. spheroides*. Motokawa & Kikuchi (1969*a*) have reported the purification of the heat-stable protein from rat liver mitochondria, by using techniques very similar to the ones reported here. They find a molecular weight of 17000 and show that the protein contains 1 disulphide group/molecule, whose thiol groups participate in electron transport during glycine metabolism. The factor from *P. glycinophilus* is also an acidic protein, but it appears to have a molecular weight of 10000 (Klein & Sagers, 1966*a*).

Activity of glycine decarboxylase in R. spheroides *grown in different ways.* All the enzymes required

for the conversion of glycine into acetyl-CoA via serine and pyruvate, and for the formation of methylenetetrahydrofolate from glycine, have been found in extracts of *R. spheroides*. Of these enzymes glycine decarboxylase has the lowest activity. The changes in the activity of glycine decarboxylase that occur on illuminating cells in the presence of different compounds (Table 5) probably account for the changes in the extent of incorporation of the methylene carbon atom of glycine into fatty acids found previously under these conditions (Gajdos *et al.* 1968). However, it does not appear that the ability of cells to convert glycine into fatty acids is related to their ability to excrete coproporphyrin as was suggested by Gajdos *et al.* (1968). It has been found (Tait, 1969) that the enzymic conversion of coproporphyrinogen into protoporphyrinogen under anaerobic conditions requires ATP and methionine and that Fe^{2+} is also involved. It seems likely that ATP and Fe^{2+} stimulate and ethionine inhibits formation of protoporphyrin and of haem, which is a feedback inhibitor of δ-aminolaevulate synthetase (Lascelles, 1968), and that in this way they affect the excretion of coproporphyrin.

In *R. spheroides* serine can probably be formed in two ways: from glycine by the action of glycine decarboxylase plus serine hydroxymethyltransferase or from glycerate (3-phosphate) via hydroxypyruvate (3-phosphate), a pathway known to occur in many cells (Meister, 1965). Although not all the enzymes of the latter pathway have been investigated the organism does have a very active glycerate dehydrogenase. The low activity of glycine decarboxylase suggests that under normal growth conditions in a medium containing malate and glutamate this enzyme is not of great importance in supplying methylenetetrahydrofolate for the formation of serine and other compounds. That it may be important under certain conditions is strongly suggested by the finding that glycine decarboxylase activity is markedly higher than normal in cells grown in the presence of glycine or a number of metabolically related compounds such as glyoxylate, glycollate and serine. The metabolism of oxalate has not been investigated, but since it also induces glycine decarboxylase it seems likely that it can be enzymically reduced to glyoxylate by the organisms.

Role of glycine decarboxylase in other cells. In *P. glycinophilus*, which grows on glycine as sole source of carbon and nitrogen, glycine decarboxylase is a key enzyme and the specific activity of a crude extract is much higher than that of an extract of *R. spheroides* (Sagers & Gunsalus, 1961).

In *Arthrobacter* species (Jones & Bridgeland, 1966; McGilvray & Morris, 1969) glycine decarboxylase and serine dehydratase activities are low in cells grown on pyruvate plus NH_4^+, but they are markedly higher in cells grown on glycine, or on threonine, which is rapidly converted into glycine. In *Escherichia coli* glycine decarboxylase has been found in glycine–serine auxotrophs that respond to either glycine or serine; it appears to be an adaptive enzyme, whose activity is perhaps controlled by the concentrations of C_1 derivatives of tetrahydrofolate (Crosbie, 1966; Newman & Magasanik, 1963). In liver mitochondria although the activity of glycine decarboxylase is low (present paper; Sato *et al.* 1969) the enzyme seems to have an important role in the catabolism of glycine. Yoshida, Kikuchi, Tada, Narisawa & Arakawa (1969) found that the primary lesion in non-ketotic hyperglycinaemia in a human infant was a deficiency of this enzyme. In plants also the enzyme system may be important (Cossins & Sinha, 1966). On the basis of labelling experiments with illuminated etiolated maize seedlings Shah, Rodgers & Goodwin (1968) suggested that the carotenes of chloroplasts were formed from CO_2 via glyoxylate, glycine, serine, pyruvate and acetyl-CoA.

The glycine decarboxylase system may also be involved in the synthesis of glycine in some organisms since extracts of *A. globiformis* (Kochi & Kikuchi, 1969) and of liver mitochondria (Motokawa & Kikuchi, 1969*b*) catalyse the conversion of methylenetetrahydrofolate, ammonia and CO_2 into glycine.

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