GLYCOLATE METABOLISM IN *ESCHERICHIA COLI*[1]

ROBERT W. HANSEN[2] AND JAMES A. HAYASHI[3]

Department of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois

Received for publication November 14, 1961

ABSTRACT

HANSEN, ROBERT W. (University of Illinois College of Medicine, Chicago) AND JAMES A. HAYASHI. Glycolate metabolism in *Escherichia coli*. J. Bacteriol. **83**:679–687. 1962.—This study of glycolate-adapted *Escherichia coli* indicates that the most probable route for utilization of the substrate includes glyceric acid, 3-phosphoglyceric acid, and the tricarboxylic acid cycle. A glyceric acid dehydrogenase, which reduces tartronic semialdehyde to glycerate in the presence of reduced diphosphopyridine nucleotide, and a kinase, which catalyzes the formation of 3-phosphoglycerate from glyceric acid and adenosine triphosphate, were shown to be present. Carbon recoveries in growing cultures and manometric data obtained with resting cells showed the complete oxidation of glycolate to carbon dioxide. Measurements of the oxidation of tricarboxylic acid cycle intermediates indicated that these compounds are oxidized without lag and at a rate commensurate with the rate of glycolate oxidation. Assays of the enzymes characteristic of known pathways of terminal oxidation, such as isocitratase, malate synthetase, isocitric dehydrogenase, and condensing enzyme, provided further evidence for an operating tricarboxylic acid cycle. A postulated pathway for the utilization of glycolic acid is as follows: glycolate → glycerate → 3-phosphoglycerate → pyruvate → tricarboxylic acid cycle.

In *Escherichia coli*, the tricarboxylic acid cycle has been established as the major pathway for the oxidation of acetate and compounds giving rise to acetate (Swim and Krampitz, 1954; Wheat, Rust, and Ajl, 1956). The route of oxidation of compounds of a higher oxidation state, such as glycolic and glyoxylic acids, has not been as well established, although the tricarboxylic acid cycle has been implicated by the work of several investigators (Bachrach, 1957; Kalnitsky and Barron, 1947; Kornberg and Gotto, 1959). Recently, Kornberg and Gotto (1961) showed that, in a pseudomonad, glycolic acid is oxidized by a route involving the dicarboxylic acid cycle (Kornberg and Sadler, 1960).

Krakow and Barkulis (1956) reported that extracts of glycolate-adapted *E. coli* catalyzed the formation of a three-carbon intermediate and carbon dioxide from 2 moles of glyoxylic acid. The three-carbon intermediate was later identified as tartronic semialdehyde (Krakow, Hayashi, and Barkulis, 1959; Krakow, Barkulis, and Hayashi, 1961; Kornberg and Gotto, 1961). It was also demonstrated that there was present, in the same extracts, a diphosphopyridine nucleotide (DPN)-dependent dehydrogenase which reduced tartronic semialdehyde to a compound tentatively identified as glyceric acid. If the above two reactions observed in the extracts are components of the pathway leading to the tricarboxylic acid cycle, it is reasonable that a glyceric acid kinase could also be present in the glycolate-adapted *E. coli*. Glyceric acid kinase has been reported in mammalian cells (Ichihara and Greenberg, 1957) and in microorganisms (Black and Wright, 1956); a glyceric acid dehydrogenase specific for hydroxypyruvate has been reported to occur in extracts of parsley leaves (Stafford, Maqaldi, and Vennesland, 1954).

The following report indicates that extracts of glycolate-adapted *E. coli* contain a glyceric acid kinase and a glyceric acid dehydrogenase which reacts with tartronic semialdehyde but not with hydroxypyruvate. Also, evidence is presented for a functioning tricarboxylic acid cycle during the oxidation of glycolic acid. These data indicate

[1] This work is taken from a thesis submitted by the senior author to the University of Illinois Graduate College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[2] Present address: Hektoen Institute, Chicago, Ill.

[3] Present address: Department of Biochemistry, Presbyterian-St. Luke's Hospital, Chicago, Ill.

that the glyoxylate shunt (Kornberg and Madsen, 1958) and the dicarboxylic acid cycle are less likely routes of terminal oxidation of glycolic acid in these organisms.

MATERIALS AND METHODS

Growth of organisms and preparation of extracts. *E. coli* (Crooks' strain), maintained on nutrient agar (Difco), was adapted to glycolate utilization by transferring a loopful of the stock culture to 10 to 500 ml of the following medium: NH_4Cl, 1.0 g; Na_2HPO_4, 6.0 g; KH_2PO_4, 2.0 g; NaCl, 0.5 g; $MgSO_4 \cdot H_2O$, 0.2 g; glycolic acid, 5.0 g; H_2O, 1 liter. These cultures were placed on a rotary shaker and incubated for 24 hr at 37 C. Larger amounts of cells for manometric experiments and for the preparation of cell-free extracts were obtained by inoculating 6 liters of the medium with 500 ml of an adapted culture and then incubating at 37 C for 24 hr. The culture was aerated by passing air through sterile cotton filters into medium-porosity sintered-glass spargers. Cells from these cultures were harvested in a Sharples refrigerated supercentrifuge and washed three times at 4 C with 0.0001 M phosphate buffer (pH 7.2). Extracts of glycolate-adapted cells were prepared by grinding with alumina and extracting with buffer, according to Wheat et al. (1956). Due to high endogenous oxidation of reduced DPN (DPNH) in the crude extracts, it was necessary to fractionate the extracts to obtain glyceric dehydrogenase in sufficient purity for assay. The fraction precipitating between 25 and 51% of ammonium sulfate saturation was found to contain all of the glyceric dehydrogenase activity and no endogenous DPNH oxidase activity.

Protein was determined by the biuret method, with crystalline bovine albumin as the standard.

Manometric assays. Manometric experiments were performed according to the general methods given by Umbreit, Burris, and Stauffer (1949). Experiments on the oxidation of glycolic acid were made using 10 μmoles of glycolic acid and 7.5 mg (dry wt) of washed glycolate-adapted cells in 2.35 ml of 0.1 M potassium phosphate buffer at pH 7.0 and 30 C. During oxygen-uptake experiments, 0.15 ml of 10% KOH was contained in the center well; this reagent was omitted when evolved CO_2 was determined. The quantity of CO_2 was calculated according to Umbreit et al. (1949). The oxidation of tricarboxylic acid cycle intermediates by resting cells was determined using 20 μmoles of substrate and 10 mg (dry wt) of cells in 2.35 ml of 0.1 M phosphate buffer (pH 7.2) in the main compartment and 0.15 ml of 10% KOH in the center well. The temperature was 30 C.

Enzyme assays. 1) Titrimetric:—Glyoxylic acid carboligase and glyceric kinase were assayed titrimetrically according to the general method described by Schwartz and Meyers (1958). Carboligase was determined as described by Krakow et al. (1961) in a reaction mixture containing 30 μmoles of sodium glyoxylate, 2 μmoles of $MgCl_2$, 0.3 mg thiamine pyrophosphate (TPP), 1.5 to 3.0 mg of enzyme protein, and water to 5.0 ml. The pH was adjusted to and maintained at 7.0 during the reaction by the addition of 0.1 N HCl.

Glyceric acid kinase in the crude extracts was determined titrimetrically, using the method of Schwartz and Meyers (1958). The reaction mixture contained 5.3 μmoles of DL-glyceric acid, 100 μmoles of potassium fluoride, 9.1 μmoles of disodium adenosine triphosphate (ATP), 1.56 mg of extract protein, and water to make 5.0 ml. The mixture, without ATP, was adjusted to pH 7.4, and the reaction initiated by the addition of ATP solution which had been adjusted previously to pH 7.4. The pH was maintained by addition of 0.05 N NaOH. The amount of produce was calculated from the amount of hydrogen ion which appeared during the phosphorylation.

2) Spectrophotometric:—Enzyme activities were determined spectrophotometrically, using 1.0-cm silica cuvettes and a Beckman DU spectrophotometer. The final volumes were 3.0 ml in all the assays. Isocitric dehydrogenase was determined by the method of Wheat et al. (1956); the mixture consisted of 100 μmoles of potassium phosphate buffer (pH 7.4), 0.2 mg of triphosphopyridine nucleotide (TPN), 1.0 μmoles of $MgCl_2$, and 5.0 μmoles of isocitric acid. The rate was determined by observing the increase in absorbance at 340 mμ. The TPN reduced (μmoles) was calculated from the extinction coefficient of reduced TPN (TPNH): 6.22×10^6 cm^4/mole (Horecker and Kornberg, 1948). Isocitratase was determined by the method of Dixon and Kornberg (1959). The reaction mixture contained potassium phosphate buffer (pH 7.0), 200 μmoles; $MgCl_2$, 20 μmoles; 2,4-dinitrophenyl

hydrazine hydrochloride, 10 μmoles; cysteine hydrochloride, 0.33 μmoles; isocitric acid, 10 μmoles; and extract protein, 1.56 mg. Observation of the increase in absorbancy at 234 mμ, at which wave length glyoxylic phenylhydrazone absorbs, gave the rate at which glyoxylate formed, and the amount was calculated from the extinction coefficient: 1.7×10^4 cm²/mole. Evaluation of the activities of malate synthetase and condensing enzyme was based on the decrease in absorbancy at 232 mμ during the hydrolysis of acetyl coenzyme A (Dixon and Kornberg, 1959). Reaction mixtures contained 50 μmoles acetyl coenzyme A, 3.0 μmoles glyoxylate or oxaloacetate, and 0.312 mg of extract protein. The reaction was initiated by the addition of glyoxylate or oxaloacetate and followed by observation of the absorbance at 232 mμ. The quantity of acetyl coenzyme A used was calculated from the extinction coefficient: 4.5×10^6 cm²/mole. For the determination of the malic enzyme, advantage was taken of the fact that carboxylation of pyruvate is accompanied by the oxidation of TPNH (Ochoa, Mehler, and Kornberg, 1948). To reaction mixtures containing 75 μmoles of glycylglycine buffer (pH 7.4), 0.4 μmoles of TPNH, 1.0 μmoles of $MnCl_2$, 10 μmoles of pyruvate, and 0.31 mg of extract protein were added 30 μmoles of $NaHCO_3$. The decrease in absorbance at 340mμ was noted, and the amount of TPNH oxidized was calculated from the extinction coefficient of TPNH. Glyceric dehydrogenase was assayed in the following mixture: potassium phosphate buffer (pH 7.0), 200 μmoles; DPNH, 0.36 μmoles; glyoxylate, 0.6 μmoles; extract protein, 0.572 mg; and water to make to 3.0 ml. All the components except TPP and $MgCl_2$ were mixed, and the change in absorbance at 340 mμ determined. After 3 to 5 min, TPP and $MgCl_2$ were added and the change in absorbance with time was again observed. The amount of product formed was determined by calculation from the extinction coefficient of DPNH: 6.22×10^4 cm²/mole (Horecker and Kornberg, 1948).

Product identification. Products formed during the enzyme reactions were identified by paper chromatographic techniques. In all cases the reaction mixtures were deproteinized by adding 1.0 ml of 50% trichloroacetic acid, and cations were removed by treating in a batch process with 10.0 g wet wt of Dowex-50 ion-exchange resin in the hydrogen form. After removal of the precipitated protein and the resin by centrifugation, the supernatant fluids were reduced in volume under diminished pressure.

Glyceric acid and glycolic acid were both identified in two different solvent systems: *n*-butanol–acetic acid–water (100:22:50), as described by Stepka (1952), developed by the ascending technique for 15 hr and dried at room temperature; and *n*-butanol–formic acid–water, according to Barnabas and Joshi (1955), developed by the descending technique for 15 hr and dried at room temperature. Acid spots were visualized by spraying the dried chromatograms with 0.04% bromcresol green in 95% ethanol, adjusted to pH 5.5. Acids appeared as yellow spots on a light green background. Phosphate esters of hydroxy acids were identified by descending chromatography in methyl Cellosolve–methylethyl ketone–3 N NH_4OH (7:2:3; Mortimer, 1952) and by ascending chromatography in methanol–NH_4OH–water (6:1:30; Bandurski and Axelrod, 1951). Chromatograms were developed for 15 to 18 hr and dried at room temperature. Phosphates were visualized by spraying the chromatograms with Hanes-Isherwood spray (Bandurski and Axelrod, 1951), drying in air, and exposing to ultraviolet light for 15 min in a blueprinting apparatus. Phosphates appeared as dark blue spots on a light blue background.

Chemical analyses. Glycolic acid was determined by the method of Calkins (1953). Glyceric acid was assayed by the chromotropic acid assay of Bartlett (1959) and the periodate oxidation method of Frisell, Meech, and MacKenzie (1954). No compound present in the reaction mixtures, other than glycerate, reacted in these methods. Acid-labile phosphate was determined according to Lowry and Lopez (1946) after hydrolysis for 7 min in 1.0 N HCl in a boiling-water bath.

TABLE 1. *Glycolate utilization by Escherichia coli*

Glycolate	Growth experiment		Resting cell oxidation		
	Carbon	CO_2	Carbon	CO_2	O_2
	μM	μM	μM	μM	μM
Added	3300		20		
Remaining	1132				
Used	2168				
Calculated		2168		20	15
Found		2060*		19.4	14.3

* Recovery = 95.0%.

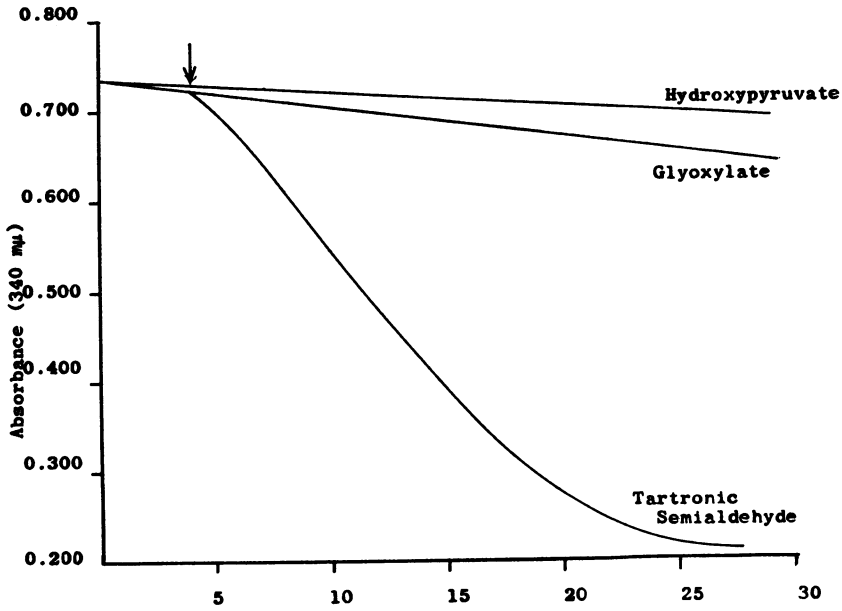

FIG. 1. *Specificity of glyceric dehydrogenase. Reaction mixture: KH_2PO_4 buffer (pH 7.0), 200 μM; DPNH, 0.36 μM: sodium glyoxylate, 0.6 μM; enzyme extract, 0.572 mg of protein. Final volume was made to 3.0 ml. Hydroxypyruvate used was 0.2 μM. Tartronic semialdehyde was generated in situ from glyoxylate by the addition of magnesium chloride, 10.0 μM, and TPP, 0.15 mg at the time shown by the arrow.*

RESULTS

Glycolate utilization by growing and resting cells. Inasmuch as the possibility existed that glycolic acid was catabolized by an intramolecular oxidation-reduction, determinations of the end products of glycolate utilization were made. Carbon-balance studies with growing cultures and manometric determinations of glycolate utilization in resting cells were made. Table 1 contains the results of a carbon-balance study. Glycolic acid was determined by the method of Calkins (1953) at the time of inoculation and again 24 hr later. CO_2 release was determined by measuring the weight increase of tared, Ascarite absorption tubes. All cultures were aerated with CO_2-free air; at the end of the experiments, the pH of the medium was lowered to 3.0 with 6.0 N H_2SO_4 to release dissolved carbon dioxide. Glycolic acid was catabolized completely to carbon dioxide.

Table 1 also contains the results obtained in manometric experiments with resting cells, using glycolic acid as a substrate. It should be noted that the amounts of oxygen taken up and carbon dioxide evolved are stoichiometric. These data complement those of the carbon balance, and lend strong support to the contention that glycolic acid is catabolized to carbon dioxide.

TABLE 2. *Chromatographic indentification of the products from the carboligase and glyceric acid dehydrogenase reactions**

Sample	Solvent 1	Solvent 2
Glyceric acid	0.38	0.54
Glycolic acid	0.50	0.49
Reaction mixture	0.36, 0.49	0.55, 0.50
Reaction plus glyceric acid	0.36, 0.50	0.55, 0.50

* Solvent 1: *n*-butanol–acetic acid–water; solvent 2: *n*-butanol–formic acid–water (see Materials and Methods). Results are expressed as R_F values.

Glyceric dehydrogenase. Krakow et al. (1959, 1961) presented evidence that tartronic semialdehyde was the product formed from glyoxylic acid by *E. coli* extracts, and that the glyceric dehydrogenase acted upon tartronic semialdehyde and not upon the tautomeric compound, hydroxypyruvate. The specificity of the *E. coli* glyceric dehydrogenase was reinvestigated, in

TABLE 3. *Stoichiometry of the glyceric dehydrogenase reaction*

Reaction	Absorbance change at 340 mμ	Reaction product	
		Calculated*	Found
		μmoles	μmoles
Glyoxylate reduction to glycolate	−0.097	0.047	0.052
TSA† reduction to glycerate	−0.443	0.215	0.213

* Calculated using 6.22×10^6 cm²/mole as extinction coefficient for DPNH (Horecker and Kornberg, 1948).

† Tartronic semialdehyde.

view of the report (Stafford et al., 1954) of a glyceric dehydrogenase specific for hydroxypyruvate in plants. In addition, further information was needed on the action of cell extracts on glyoxylate in the presence of reduced DPN.

DPNH is not oxidized to any great extent in the presence of hydroxypyruvate (Fig. 1). When glyoxylate is added, to serve as substrate for the production of tartronic semialdehyde, no oxidation is observed. It is apparent, however, that the addition of TPP and $MgCl_2$ to the glyoxylate mixture brings about an instant and marked increase in the oxidation of DPNH.

The product formed by cell extracts in the presence of DPNH, glyoxylate, TPP, and $MgCl_2$ is predominantly glyceric acid (Tables 2 and 3). Of 0.6 μmoles of glyoxylate added to the reaction mixture, 0.426 μmoles was recovered as glyceric acid (1.0 μmole of glycerate being equivalent to 2.0 μmoles of glyoxylate), a recovery of 72%. The total change in A_{340}, due to reduction of glyoxylate to glycolate, was calculated from the rate of A_{340} decrease before addition of the cofactors, TPP and $MgCl_2$. The change in absorption due to the reduction of tartronic semialdehyde was taken as the difference between that of glyoxylate and the total change.

Glyceric kinase. In a typical experiment determining the presence of a glyceric kinase, there was release of titrable hydrogen only in the complete system. The absence of glycerate, ATP, or enzyme rendered the system inactive. Potassium fluoride was added initially to cause inhibition of ATP enzyme activity by the fluoride ion. It was found, subsequently, that the phosphorylation rate was markedly increased by the potassium ion itself. The product resulting from the phosphorylation reaction, 3-phosphoglyceric acid, was identified chromatographically in two different solvent systems (Table 4). No trace of 2-phosphoglyceric acid was noted.

Figure 2 illustrates the time course followed by the kinase reaction. Table 5 shows the stoichiometry of the reaction, as calculated from the titration data and analyses of acid-labile phosphate. Although 9.1 μmoles of ATP were added to the reaction mixture, more than the expected 9.1 μmoles of acid-labile phosphate were obtained. It is assumed that this higher value was due to partial hydrolysis of ADP.

Oxidation of tricarboxylic acid cycle intermediates by resting cells. Manometric studies of the oxidation by resting cells of intermediates in the tricarboxylic acid cycle were made. The data in Table 6 were obtained from the maximal oxidation rates for those substrates and are given as Q_{O_2} (μliters of O_2 uptake/hr per mg dry wt of cells). These data show that all intermediates except citrate were oxidized at a rate sufficient to account for glycolate oxidation. Citrate, however, has been shown to penetrate cell walls with difficulty (Wheat et al., 1956) and its relatively slow rate of oxidation is probably a function of the rate of entry into the cell. Because of the rapid oxidation shown here, and because oxidation occurred without appreciable lag, it was concluded that the tricarboxylic acid could serve as the terminal oxidation pathway in glycolate-adapted *E. coli*.

TABLE 4. *Chromatography of glyceric kinase reaction products*

Sample	Descending P constants*	Ascending R_F value
	Solvent 1†	Solvent 2
3-Phosphoglyceric acid	118	0.35
Reaction mixture	126, 22	0.36, 0.15
Reaction plus 3-phosphoglycerate	126, 22	0.36, 0.15
ATP	23	0.13

* P constant $= \frac{\text{distance of compound}}{\text{distance of inorganic p}} \times 100.$

† Solvent 1: Methyl Cellosolve–methylethyl ketone–3 N NH_4OH (7:2:3); solvent 2: methanol –NH_4OH–H_2O (6:1:30); Bandurski and Axelrod, 1951).

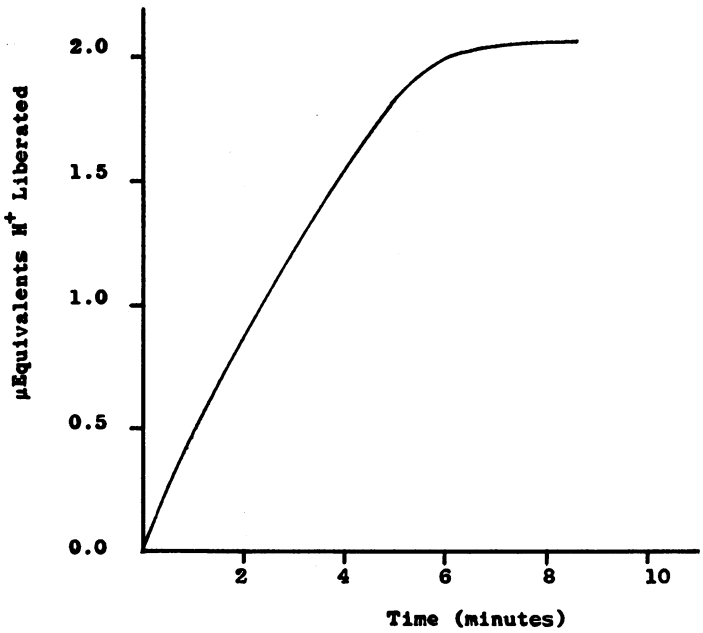

FIG. 2. *Titration of the glyceric kinase reaction. Reaction mixture:* DL-*glyceric, 5.3 μM; $MgCl_2$, 1.0 μM; KF, 100 μM; enzyme extract, 1.56 mg of protein. All components adjusted to pH 7.4 and reaction started by adding 9.1 μM ATP. The pH was maintained at 7.4 by addition of 0.05* N *NaOH. Temperature, 30 C.*

TABLE 5. *Stoichiometry of the glyceric kinase reaction*

Time	DL-Gycerate	Acid-labile phosphate*	H^+ produced
min	*μmoles*	*μmoles*	*μmoles*
0	5.8	15.72	0
8	3.10	13.54	2.13
Used	2.7	2.18	

* After 7 min of hydrolysis at 100 C in 1 N HCl.

TABLE 6. *Oxidation of tricarboxylic acid cycle intermediates by resting-cell suspensions*

Substrate	Q_{O_2}
Pyruvate	25.0
Acetate	16.3
Citrate	2.0
α-Ketoglutarate	15.1
Succinate	24.8
Fumarate	29.4
Malate	30.6
Glycolate	4.8
Glyoxylate	3.1

TABLE 7. *Comparison of enzyme characteristic of specific oxidative pathways*

Enzymes	Specific activity
Isocitric dehydrogenase	0.375 μM TPN reduced
Isocitratase	0.013 μM glyoxylate formed
Condensing enzyme	0.280 μM acetyl CoA used
Malate synthetase	0.133 μM acetyl CoA used
Malic enzyme	0.093 μM TPNH oxidized

Specific activities of enzymes in cell-free extracts. The activities of certain enzymes which are important in the functioning of specific oxidation pathways were measured. Condensing enzyme and isocitric dehydrogenase, which are enzymes in the tricarboxylic acid cycle, were assayed, as were isocitratase and malate synthetase, enzymes of the glyoxylate shunt. Glyoxylic acid carboligase, glyceric dehydrogenase, and glyceric kinase, enzymes of the pathway from glyoxylate to acetyl coenzyme A (CoA), and the malic enzyme were also determined in these cell-free extracts. The specific activities of some of these enzymes, calculated as μmoles of substrate used

or μmoles of product formed per min per mg of extract protein, are compared in Table 7. The activities of these enzymes are in accord with the conclusion that the tricarboxylic acid cycle is the most probable route of terminal oxidation during glycolate catabolism by glycolate-adapted *E. coli* Crooks'.

DISCUSSION

A number of reports have suggested strongly the presence, in glycolate-adapted organisms, of a dehydrogenase which formed glyceric acid from a three-carbon intermediate in a more highly oxidized state, and a kinase catalyzing the formation of phosphoglyceric acid from which pyruvate and acetyl CoA could arise by pathways demonstrated previously in *E. coli*. Several reports (Kornberg and Gotto, 1959, 1961; Mann, 1957; Quayle and Keech, 1959) demonstrated the occurrence of glyceric acid during glycolate metabolism in microorganisms and plants. Kornberg and Gotto (1959) reported evidence for the formation of 1 mole of acetyl CoA and 2 moles of carbon dioxide from 2 moles of glycolate. A subsequent report (Kornberg and Gotto, 1961) showed that glycerate was converted to pyruvate in the presence of ATP. The presence of a possible three-carbon precursor of glycerate had been demonstrated by Krakow and Barkulis (1956) and Krakow et al. (1959, 1961) when they showed that the enzyme, glyoxylic acid carboligase, in extracts of glycolate-adapted *E. coli* converted 2 moles of glyoxylic acid to 1 mole each of carbon dioxide and tartronic semialdehyde.

The data in this paper indicate that the pathway suggested by the above reports is a likely pathway by which glycolate-adapted organisms metabolize glycolic acid. The enzymes glyoxylic acid carboligase (Krakow et al. 1959, 1961), glyceric acid dehydrogenase, and glyceric acid kinase, acting sequentially, would convert the two-carbon substrate to an intermediate which is an established participant in pathways known to exist in *E. coli*.

At the present time no evidence has been obtained concerning the glyceric acid isomer which is formed by the dehydrogenase, nor for the isomer for which the kinase is specific. It is apparent, however, from the stoichiometry of the glyceric kinase reaction (Table 5) that only one isomer is utilized by glycolate-adapted *E. coli*. A similar result was obtained in studies of glycerate oxidation by resting cells.

Consideration of the data presented on oxidation of tricarboxylic acid cycle intermediates and on the activity of key enzymes of the possible pathways leads to the conclusion that the tricarboxylic acid cycle is probably the major pathway of glycolate oxidation by the organism used in these experiments. Carbon-balance studies, using glycolate as a substrate for growing cells, and manometric determinations of oxidation of several substrates, show that these compounds are oxidized completely to carbon dioxide by glycolate-adapted *E. coli*. The relatively slow oxidation of glycolic acid (Table 6) is probably due to difficulty in penetrating the cell walls of *E. coli* since Krakow and Barkulis (1956) and Krakow et al. (1959) have shown that this compound is an intermediate in glycolate oxidation.

A similar difficulty in penetrating the cell wall is shown in the case of citric acid as a substrate for resting cells (Table 6). It has been shown previously that such behavior is known to occur with citric acid, and it probably also occurs here, especially since all the other tricarboxylic acid cycle intermediates were oxidized completely and without lag by resting cells. This oxidative pattern points to the presence of a functioning citric acid cycle in glycolate-adapted *E. coli*.

It is still possible, however, that the oxidation of the tricarboxylic acid cycle intermediates may have occurred by way of the glyoxylate shunt or the dicarboxylic acid cycle of Kornberg. Assays of the specific activities of certain enzymes in cell-free extracts of the glycolate-adapted organism showed that they were present and operating at rates commensurate with the oxidation of glycolate by way of the tricarboxylic acid cycle. The enzyme isocitratase is a characteristic and

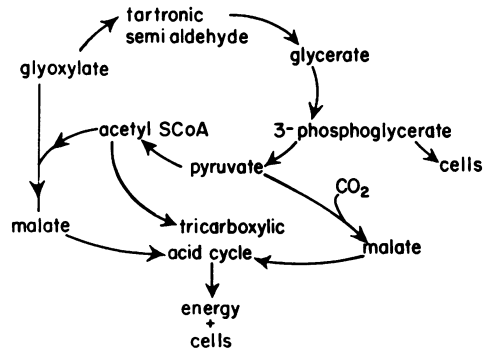

FIG. 3. *Mechanism for terminal oxidation of glyoxylate.*

necessary component of the glyoxylate shunt. In extracts of glycolate-adapted *E. coli*, isocitratase was found at a relatively low activity (1/35 the activity of isocitric dehydrogenase). It seems likely, then, that any isocitric acid present in the organism would be oxidized by isocitric dehydrogenase and continue through the tricarboxylic acid cycle, rather than be acted upon by isocitratase. Under these circumstances, the possible role of the glyoxylate shunt would be, at best, a synthetic pathway rather than a route of terminal oxidation.

The dicarboxylic acid cycle has been proposed to account for the metabolism of an *E. coli* mutant which lacked the condensing enzyme. Although the presence of an active condensing enzyme in glycolate-adapted *E. coli* would not exclude a dicarboxylic acid pathway, it appears unlikely that this is the major pathway of terminal oxidation; the combined activities of carboligase and condensing enzyme are great enough to limit substrate for malate synthetase.

It is apparent, of course, that the assays of enzyme activities in cell-free extracts may not give a true picture of conditions in the intact organism. Final proof of the relative importance of the pathways studied must rest upon experiments with the intact organism. The data presented here, however, are indicative that the major pathway of glycolate oxidation could be the tricarboxylic acid cycle.

A pathway for the catabolism of glycolate in glycolate-adapted *E. coli*, postulated on the basis of the data presented in this paper, is given in Fig. 3. A similar pathway has also been postulated by Kornberg and Gotto (1961) for *Pseudomonas ovalis* Chester. This pathway provides for the entrance of glycolate into the tricarboxylic acid cycle to meet the energy requirements of the organism. The synthesis of 3-phosphoglycerate by the organism studied also provides for the net incorporation of carbon from the two-carbon substrate glycolic acid into the growing cell.

ACKNOWLEDGMENTS

This work was supported by grant E-1032 from the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service, and in part by National Science Foundation grant G9114.

LITERATURE CITED

BACHRACH, U. 1957. The degradation of glycine by *Pseudomonas aeruginosa*. Biochem. J. **66**:559–561.

BANDURSKI, R. S., AND B. AXELROD. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. **193**:405–410.

BARNABAS, J., AND G. V. JOSHI. 1955. Phenol-indo-2,6-dichlorophenol as a spray indicator. Anal. Chem. **27**:443–444.

BARTLETT, G. R. 1959. Colorimetric assay methods for free and phosphorylated glyceric acids. J. Biol. Chem. **234**:469–471.

BLACK, S., AND N. G. WRIGHT. 1956. Enzymatic formation of glyceryl and phosphoglycerul methylthiol esters. J. Biol. Chem. **221**:171–180.

CALKINS, V. 1953. Microdetermination of glycolic and oxalic acids. Ind. Eng. Chem., Anal. Ed. **15**:762–763.

DIXON, G. H., AND H. L. KORNBERG. 1959. Assay methods for key enzymes of the glyoxylate cycle. Biochem. J. **72**:3P.

FRISELL, W. R., L. A. MEECH, AND C. G. MACKENZIE. 1954. A simplified photometric analysis for serine and formaldehyde. J. Biol. Chem. **207**:709–716.

HORECKER, B. L., AND A. KORNBERG. 1948. The extinction coefficients of the reduced band of pyridine nucleotides. J. Biol. Chem. **175**: 385–390.

ICHIHARA, A., AND D. M. GREENBERG. 1957. Studies on the purification and properties of D-glyceric acid kinase of liver. J. Biol. Chem. **225**:949–958.

KALNITSKY, G., AND E. S. G. BARRON. 1947. The effect of fluoroacetate on the metabolism of yeast and bacteria. J. Biol. Chem. **170**:83–95.

KORNBERG, H. L., AND A. M. GOTTO. 1959. Biosynthesis of cell constituents from C_2 compounds: formation of malate from glycolate by *Pseudomonas ovalis* Chester. Nature **183**:1791–1793.

KORNBERG, H. L., AND A. M. GOTTO. 1961. The metabolism of C_2 compounds in micro-organisms. 6. Synthesis of cell constituents from glycolate by *Pseudomonas* sp. Biochem. J. **78**:69–82.

KORNBERG, H. L., AND N. B. MADSEN. 1958. The metabolism of C_2 compounds in micro-organisms. Biochem. J. **68**:549–557.

KORNBERG, H. L., AND J. R. SADLER. 1960. Microbial oxidation of glycolyate via a dicarboxylic acid cycle. Nature **185**:153–155.

KRAKOW, G., AND S. S. BARKULIS. 1956. Conversion of glyoxylate to hydroxypyruvate by extracts of *Escherichia coli*. Biochim. et Biophys. Acta **21**:593–594.

KRAKOW, G., J. A. HAYASHI, AND S. S. BARKULIS. 1959. Tartronic semialdehyde: Initial product of a glyoxylic acid-utilizing reaction in extracts of *Escherichia coli*. Federation Proc. **16**:256.

KRAKOW, G., S. S. BARKULIS, AND J. A. HAYASHI. 1961. Glyoxylic acid carboligase: An enzyme present in glycolate grown *Escherichia coli*. J. Bacteriol. **81**:509–518.

LOWRY, O. H., AND J. A. LOPEZ. 1946. The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem. **162**:421–428.

MANN, J. D. 1957. The metabolism of glycolic acid in tobacco leaves. Thesis, University of Wisconsin, Madison.

MORTIMER, D. C. 1952. Paper chromatographic separation of some biologically important phosphate esters. Can. J. Chem. **30**:653–660.

OCHOA, S., A. H. MEHLER, AND A. KORNBERG. 1948. Biosynthesis of dicarboxylic acids by CO_2 fixation. I. Isolation and properties of an enzyme decarboxylation of L-malic acid. J. Biol. Chem. **174**:979–1000.

QUAYLE, J. R., AND D. B. KEECH. 1959. Formation of glycerate from oxalate by *Pseudomonas oxalaticus* grown on oxalate. Nature **183**: 1794–1795.

SCHWARTZ, M., AND T. C. MEYERS. 1958. Simple microtitrimetric constant-pH method for accurate enzyme analysis apparatus. Anal. Chem. **30**:1150–1151.

STAFFORD, H. A., A. MAGALDI, AND B. VENNESLAND. 1954. The enzymatic reduction of hydroxypyruvic acid to D-glyceric acid in higher plants. J. Biol. Chem. **207**:621–629.

STEPKA, W. 1952. Paper chromatography, p. 25–62. *In* A. C. Corcoran [ed.], Methods in medical research, vol. 5. The Yearbook Publishers, Inc., Chicago.

SWIM, H. E., AND L. O. KRAMPITZ. 1954. Acetic acid oxidation by *Escherichia coli*: Evidence for the occurrence of a tricarboxylic acid cycle. J. Bacteriol. **67**:419–425.

UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1949. Manometric techniques and tissue metabolism, 2nd ed., Burgess Publishing Co., Minneapolis.

WHEAT, R. W., J. RUST, JR., AND S. J. AJL. 1956. Distribution of the tricarboxylic acid cycle enzymes in extracts of *Escherichia coli*. J. Cellular Comp. Physiol. **47**:317–339.