OXIDATION OF GLYCOLIC ACID BY *PENICILLIUM CHRYSOGENUM*¹

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Glycolic and glyoxylic acids have been implicated in an increasing number of metabolic reactions. The oxidation of glycolate to glyoxylate by plant and animal tissues has been explored by Zelitch and Ochoa (1953) and Kun, Dechary, and Pitot, (1954); the purified glycolic acid oxidase was shown to be a flavoprotein in each instance. Palleroni and Doudoroff (1957), have reported studies on the metabolism of D-arabinose by *Pseudomonas saccharophila* and showed that glycolic acid accumulated as one of the products of oxidation in both growing cultures and cell-free systems. Jayasuria (1954) reported that glycolic acid was used as a carbon source for the synthesis of citrate by an oxalate decomposing bacterium. The importance of glyoxylate in the synthesis of C₄-dicarboxylic acids has been elucidated by Kornberg (1957) and Wong and Ajl (1957).

The formation of glycine by amination of glyoxylate or transamination of glyoxylate with amino acids has not been shown definitely in molds but has been considered an attractive possibility (Olson, 1954). Transamination of glyoxylate with several amino acids has been shown in pseudomonad extracts by Campbell (1956). This paper reports a study of glycolic acid metabolism in extracts of *Penicillium chrysogenum*.

MATERIALS AND METHODS

The medium used for growth of *P. chrysogenum* strain Q176 was a modification of a defined medium (Stone and Farrell, 1946): glucose, 20 g; acetic acid (glacial), 10 g; NH₄NO₃, 10 g; KH₂PO₄, 4 g; MgSO₄, 1 g; FeSO₄, 0.4 g; MnSO₄, 0.04 g; ZnSO₄, 0.002 g; sodium citrate, 2.0 g;

H₂O, 1 liter. The pH was adjusted to 6.0 to 6.2 with KOH and dispensed into 3-liter Fernbach flasks, 300 ml per flask. The flasks were sterilized for 15 min at 120 C, inoculated with 2 ml of spore suspension, and placed on a shaker with a stroke of 2.75 inches at 80 cycles per min. The temperature of incubation was 24 to 25 C.

Cells produced after 50 hr incubation were washed with distilled water and cold potassium phosphate buffer (0.1 M, pH 7.0). Nonspecific endogenous respiratory activity was reduced by shaking the washed cells in 0.05 M phosphate buffer at pH 7.0 for 5 to 6 hr at 24 to 25 C.

Crude cell-free extracts were obtained by grinding 10 g washed cells in a chilled mortar with an equal weight of sand. The finely ground paste was extracted with 20 ml of 0.05 M phosphate buffer, pH 7.4, for 30 min, and centrifuged for 10 min at 14,000 × *g*. The supernatant was used directly for metabolic experiments or subjected to the purification described below. All of the steps in the preparation of cell-free extracts were carried out at temperatures below 5 C.

Crude extracts of *P. chrysogenum* contained free amino acids, malic acid, and small amounts of pyruvate and α-ketoglutarate which interfered significantly with the measurement of certain enzymatic reactions, especially when the product was not present in high concentration. Partial purification of the crude extract was achieved by dialysis against 0.05 M phosphate buffer, pH 7.4 to 7.6, for 5 to 6 hr.

Removal of nucleic acids was accomplished by adding protamine sulfate (20 mg dissolved in 5 ml of 0.05 M phosphate buffer, pH 6.3) to 20 ml of crude extract adjusted to pH 6.5. The insoluble precipitate was centrifuged and discarded. Crystalline ammonium sulfate was added slowly to the supernatant to 65 to 70 per cent of saturation. The protein precipitate was centrifuged at 4,600 × *g* for 10 min and was completely soluble when taken up in 0.05 M phosphate buffer. Most of the residual ammonium sulfate was removed by dialysis of the protein solution for 5 hr. The

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resulting enzyme preparation was free of noticeable endogenous respiratory activity.

Glycolic acid (Fisher Scientific Company) was recrystallized four times from ethyl ether and dried over concentrated H_2SO_4 . The melting point and the neutralization equivalent compared closely to the theoretical values. Glycolic acid-2- C^{14} was obtained from Isotope Specialties Company of Berkeley, California. Sodium glyoxylate (Bios Laboratories, New York) was recrystallized by the method of Radin and Metzler (1955) and its purity determined by the method of Long (1942). All substrates used were checked for purity by paper chromatography.

Diphosphopyridine nucleotide (50 to 55 per cent, DPN) was obtained from Schwarz Laboratories, Inc., New York; triphosphopyridine nucleotide (80 per cent, TPN) and flavin adenine dinucleotide (FAD) from Sigma Chemical Company, St. Louis; flavin mononucleotide (FMN) from General Biochemicals, Inc., Chagrin Falls, Ohio. Each of these cofactors was dissolved in water equivalent to one mg per ml and adjusted to pH 6.5 with NaOH.

The Thunberg technique, described by Umbreit, Burris, and Stauffer (1957), was used for rapid assay of glycolate and other dehydrogenase activity with 2,6-dichlorophenolindophenol as the electron acceptor. The dye solution was prepared according to the procedure of Godzeski and Stone (1955). Standard manometric techniques were used to determine oxygen uptake. Carbon dioxide was determined by the "direct method" in the absence of alkali (Umbreit et al., 1957).

Glycolic acid was determined by the method of Calkins (1943), but the reagent used was chromotropic acid (4,5-dihydroxy-2,7-naphthalene-disulfonic acid). The method gave reproducible results in the presence of purified enzyme but not with crude extracts.

The method described by Brummond and Burris (1953) was used for the separation of 2,4-dinitrophenylhydrazine derivatives of keto acids. The hydrazones eluted from the silica column with chloroform-butanol solvent mixtures were concentrated by extraction with 10 per cent Na_2CO_3 , acidified and extracted with a small amount of ethyl acetate. The ethyl acetate extract was spotted on paper strips and chromatographed in *n*-butanol saturated with 3 per cent NH_4OH . Routine identifications were made

by comparison with known samples of keto acid hydrazones.

Organic acids were identified on paper chromatograms by the method of Buch and Montgomery (1952) using amyl alcohol equilibrated against 5 M formic acid as the developing solvent. Amino acids were separated and identified by comparison of R_F values with known samples using water-saturated phenol or *n*-propanol-water (4:1) mixture.

RESULTS

When glycolic acid was incubated with crude extracts of *P. chrysogenum* in Warburg vessels, a small amount was oxidized although the amount of glyoxylate recovered was much less than expected. The total amount of keto acids produced was essentially equivalent to the moles of glycolate oxidized. When the reaction was carried out in an excess of hydrazine sulfate only glyoxylate was recovered (table 1).

The activity of the ammonium sulfate fraction prepared from the crude extracts was measured by the reduction of dichlorophenolindophenol anaerobically in Thunberg tubes. The preparation contained an active glycolate dehydrogenase that was not significantly affected by the addition of flavin mononucleotide, flavin adenine dinucleotide, DPN and TPN, singly or in combination as shown in table 2. Essentially the same slight stimulation of glycolate dehydrogenase was obtained with flavin mononucleotide or flavin adenine dinucleotide as was found with DPN. Besides activity on glycolic and lactic

TABLE 1
Oxidation of glycolic acid by crude extracts of *Penicillium chrysogenum* strain Q176

Time	Glycolate Used	α -Keto Acids Formed	Identity on Paper Chromatograms
<i>min</i>	μ moles	μ moles	
<i>A. No additions</i>			
10	0.68	0.37	—
60	1.61	1.09	Glyoxylate, pyruvate, α -ketoglutarate
<i>B. Hydrazine sulfate, 100 μmoles</i>			
10	0.28	0.38	—
30	1.05	1.07	Glyoxylate
60	1.75	1.71	Glyoxylate

Flask contents: 2 ml extract, 10 μ moles glycolic acid, 1 ml of 0.05 M phosphate buffer, pH 7.6.

TABLE 2

Activity of glycolic acid dehydrogenase prepared by ammonium sulfate fractionation

Substrate	Cofactor	Time of Dye Reduction <i>min</i>
None.....	None	>60
None.....	DPN or TPN	>60
Glycolate.....	None	6
Glycolate.....	DPN*	4
Glycolate.....	TPN*	5
Lactate.....	None, DPN, or TPN	7
Malate.....	None, DPN, or TPN	>60
L-Glutamate..	None or TPN	>60
D-Isocitrate...	None	>60
D-Isocitrate...	TPN	5

System: dichlorophenolindophenol, 0.5 ml; 0.05 M phosphate buffer (pH 7.0), 1 ml; cofactor, 0.1 mg; substrate, 1 μ mole; H₂O to make 3.5 ml. Side arm contained 0.4 ml extract, 10.5 mg protein/ml. Tubes incubated 30 C for 1 hr.

* Comparable reaction times were obtained with the addition of flavin mononucleotide or flavin adenine dinucleotide.

acids, the preparation also contained an isocitric acid dehydrogenase which required TPN.

Several attempts were made to find a glyoxylate reductase in both crude and dialyzed extracts using the methods described by Zelitch (1955) with plant enzyme, but without success. When glyoxylate was added to crude extracts, it disappeared rapidly although there was no significant oxygen uptake and carbon dioxide evolution. Both the dialyzed extracts and the ammonium sulfate fractions contained considerable transaminase activity as shown in table 3. The equilibrium of the glyoxylate-alanine reaction favors the accumulation of more product (carbonyl) than in the other reactions tested. The relative intensity of spots of glycine shown by paper chromatography corresponded to the amount of glyoxylate used by the reaction. No significant nonenzymatic transamination between glyoxylate and amino acids could be detected under these conditions. There was no evidence of marked deaminase activity for amino acids in the dialyzed extracts, except for action on serine to give pyruvic acid.

Glycolic acid-2-C¹⁴ was used in an attempt to follow the pathway of glycolate metabolism in crude extracts. Paper strips spotted with depro-

TABLE 3

Transamination of sodium glyoxylate and amino acids in dialyzed extracts of *Penicillium chrysogenum* strain Q176

Amino Acid Additions	Keto Acids Recovered		
	Pyruvate	Glyoxylate	α -Ketoglutarate
	<i>μmoles</i>		
None.....	1.16	8.62	0.62
DL-Aspartate...	3.20	7.84	0.56*
L-Glutamate...	0.58	5.80	3.80
L-Alanine.....	6.12	3.30	0.56

Reaction mixture: 1 ml dialyzed extract; 1 ml 0.1 M phosphate buffer, pH 7.4; 20 μ moles amino acid; 10 μ moles sodium glyoxylate; flask contents, 3 ml. The mixture was incubated for 60 min at 30 C.

* Calculated as α -ketoglutarate although chromatograms of the hydrazine revealed a small amount of oxalacetate.

teinized reaction mixture were developed in *n*-pentanol equilibrated against 5 M formic acid. These strips showed only two areas of radioactivity. One of these was located at the origin and the other had an *R_F* value equivalent to that of glycolic acid. Radioactive material which remained at the origin proved to be an amino acid fraction. A sample of the reaction mixture was passed through a column of Duolite C-20 cation exchange resin with distilled water. The amino acids retained on the resin were eluted with both normal and 6 N HCl, neutralized, and rechromatographed. The only radioactivity detected on the chromatogram coincided with the spot for glycine when the area was eluted from the paper and rechromatographed. The anion fraction showed no C¹⁴-labeled organic acids other than glycolic acid.

DISCUSSION

In studies on the metabolism of acetate in extracts of *P. chrysogenum*, glycolic acid was occasionally identified colorimetrically. Unfortunately, this experimental observation could not be consistently repeated. Several workers, including Nord and Vitucci (1947) and Weinhouse (1951) have concluded that acetate is oxidized directly to glycolate. Recently Bolcato, Boschetti, and Montoya (1956) have implicated glycolic acid as an intermediate in the direct oxidation of

acetate by *Escherichia coli*. Wong and Ajl (1957), however, believe that the results of Bolcato can be explained by known mechanisms involving C₆-intermediates. Judging from the results of Olson (1954) and Heberling, Berky, and Stone (1959), it seems most likely that the appearance of glycolic acid from *P. chrysogenum* is via the pathway of isocitric and glyoxylic acids. It is necessary to make clear, however, that the possibility of the direct oxidation of acetate to glycolic acid still has not been ruled out.

The oxidation of glycolate to glyoxylate was clearly demonstrated in extracts of *P. chrysogenum*. No cofactor requirement could be shown by either dialysis or fractionation of the enzyme with ammonium sulfate. Kornberg (1957) has shown that glyoxylate arising from the non-oxidative cleavage of isocitrate can condense with acetyl coenzyme (CoA) to yield malic acid. Heberling et al. (1959) working with *P. chrysogenum* extracts have demonstrated the synthesis of isocitrate from glyoxylate and succinate in a nitrogen atmosphere. The reaction is reversible and would likely make available some glyoxylate for reduction to glycolic acid. Although this reduction was not demonstrated in extracts of *Penicillium*, it has been recognized in tobacco leaf extracts by Zelitch (1955) who found that glyoxylate was reduced by DPNH formed during glucose oxidation.

The glyoxylate cycle provides an alternate pathway for the oxidation of acetate as well as a possible additional source of C₄-acids. In addition, glyoxylate provides a supply of C₂-keto acid for transamination to glycine. Based on the fact that extracts of *P. chrysogenum* split isocitrate to glyoxylate and succinate (Olson, 1954) and the ease with which glyoxylate reacts with either glutamate or alanine, it seems plausible that during the active oxidation phase of growth the isocitratase reaction may provide C₄-skeletons for aspartic acid and C₂-units for glycine. An excess of glyoxylic acid not used for glycine would also be available for condensation with acetyl-CoA to give more C₄-acids. It is not so likely that during aerobic growth there would be sufficient DPNH available to reduce an appreciable amount of glyoxylate to glycolic acid. Hence the appearance of glycolate may well be transitory and not often observed in well aerated cultures.

SUMMARY

Cell-free extracts of *Penicillium chrysogenum* strain Q176 were able to oxidize glycolate to glyoxylate. The oxidation was stimulated only slightly by the addition of flavin and pyridine nucleotide cofactors. Dialyzed preparations of fractions precipitated with ammonium sulfate appeared to retain any necessary cofactors. In crude extracts, glyoxylate disappeared very rapidly with the appearance of glycine and equivalent amounts of α -ketoglutarate plus pyruvate. Glycine was the only labeled compound that could be detected as a product of glycolate-C¹⁴ metabolism.

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