Microbiological Studies of *Coli-aerogenes* Bacteria*

Part XIII. The Enzymic Oxidation of Glycollic Acid to Glyoxylic Acid

By Hideo KATAGIRI and Tatsurokuro TOCHIKURA

Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University

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The oxidation of glycollate to glyoxylate by molecular oxygen was found to be catalyzed by the dried cells of *coli-aerogenes* bacteria such as *E. coli* and *A. aerogenes*, and baker's yeast. The enzyme (glycollic oxidase) was ascertained to be contained in the bacterial cells of *coli-aerogenes* grown aerobically on various media such as glucose-ammonium salt, acetate-bouillon and citrate-bouillon. The bacterial cells with citritase and isocitritase were also found to contain glycollic acid oxidase. The cell-free extracts of *coli-aerogenes* were fractionated with ammonium sulfate for glycollic acid oxidase. The enzyme was resolved to yield an inactive apoenzyme which could be reactivated by the addition of FMN** or FAD. The enzyme was found to be strongly inhibited by cyanide. It was suggested that a glyoxylate $\rightleftharpoons$ glycollate system may function in the respiration of microorganisms.

INTRODUCTION

In the previous papers, the authors have studied the oxidative fermentations by *coli-aerogenes* bacteria of carbohydrates and organic acids which belong to the terminal respiration, and have demonstrated that these bacteria are capable of inducing α -ketoglutaric acid-fermentation under aerobic conditions^{1-6,10,11}. Further investigations on the bacterial dissimilation of C₆-tricarboxylic acids have led the authors to elucidate the physiological relationship among citritase, isocitritase, isocitric dehydrogenase and glutamic dehydrogenase^{5,7-9}. In the course of

investigations on the metabolism of C₂-acids in *coli-aerogenes* bacteria, it has also been found by the authors that an enzyme catalyzing the reduction of glyoxylic acid to glycollic acid by reduced pyridine nucleotides is contained in microorganisms¹². The present paper deals with the oxidation of glycollic acid to glyoxylic acid by microorganisms such as *coli-aerogenes* and baker's yeast. The function of glyoxylate $\rightleftharpoons$ glycollate system in plant tissues has been

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** The following abbreviations are used: FMN=riboflavin-5'-phosphate (flavin mononucleotide); FAD=flavin adenine dinucleotide; TPN (DPN)=tri(di) phosphopyridine nucleotides; TPN⁺ and TPNH=oxidized and reduced triphosphopyridine nucleotide, respectively; DPN⁺ and DPNH=oxidized and reduced diphosphopyridine nucleotide, respectively; m (μ) M=m (μ) mole (g).

1) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 210 (1957).

2) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 215 (1957).

3) H. Katagiri, T. Tochikura and K. Imai, This Bulletin, **21**, 346 (1957).

4) H. Katagiri and T. Tochikura, This Bulletin, **21**, 351 (1957).

5) H. Katagiri and T. Tochikura, This Bulletin, **22**, 143 (1958).

6) H. Katagiri, T. Tochikura and Y. Suzuki, This Bulletin, **23**, 322 (1959).

7) H. Katagiri and T. Tochikura, This Bulletin, **23**, 475 (1959).

8) H. Katagiri and T. Tochikura, This Bulletin, **23**, 482 (1959).

9) H. Katagiri and T. Tochikura, This Bulletin, **23**, 489 (1959).

10) H. Katagiri and T. Tochikura, This Bulletin, **24**, 182 (1960).

11) H. Katagiri and T. Tochikura, This Bulletin, **24**, 188 (1960).

12) H. Katagiri and T. Tochikura, This Bulletin, **23**, 558 (1959);

24, 351 (1960).

13) C.O. Clagett, N.E. Tolbert and R.H. Burris, *J. Biol. Chem.*,

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14) N.E. Tolbert, C.O. Clagett and R.H. Burris, *J. Biol. Chem.*,

181, 905 (1949).

15) N.E. Tolbert and R.H. Burris, *J. Biol. Chem.*, **186**, 791

(1950).

16) I. Zelitch and S. Ochoa, *J. Biol. Chem.*, **201**, 707 (1953).

17) H. Chiba, F. Kawai and S. Ueda, *Bull. Res. Inst. Food*

Sci., Kyoto University, No. 15, 89 (1954).

extensively studied by several workers¹³⁻¹⁸. The enzymic oxidation of glycollate by mammalian tissues was also reported by Dohan¹⁹ and Kun et al.^{20,21}.

EXPERIMENTAL AND RESULTS

Methods. Analytical procedure was performed by the methods mentioned in the previous paper¹².

Chemicals. Glycollic acid and riboflavin-5'-phosphate were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo); flavin adenine dinucleotide from Wako Pure Chem. Ind. Co., Ltd. (Osaka); baker's yeast from Oriental Yeast Co., Ltd. (Osaka).

Oxidation of Glycollate to Glyoxylate by Dried Cells of *Coli-aerogenes* Bacteria and Baker's Yeast.

In the previous papers^{7-9,12}, the following important facts were reported. When citric acid was added to either cell-free extracts of *coli-aerogenes* or the ammonium sulfate-precipitated protein of the bacteria, the formation of three kinds of keto-acids including glyoxylic, pyruvic and α -ketoglutaric acids occurred under the anaerobic conditions. There was a progressive increase with time in the amounts of α -ketoglutarate and a decrease in the amounts of glyoxylate under such conditions that a large quantity of triphosphopyridine nucleotide was present. Finally, an enzyme which catalyzed the reduction of glyoxylate to glycollate by either reduced diphospho- or triphosphopyridine nucleotide was detected in the bacterial cells such as *E. coli*, *A. aerogenes* and *Ps. fluorescens*.

Thus, it is now of interest to ascertain whether or not glycollate would be oxidized by the bacteria of *coli-aerogenes*. Experiments were at first carried out with the dried cells of *A. aerogenes* (strain B-2). The organism was grown on a shaker for 1 day at 30°C in a medium containing 2% Na-citrate, 1% bouillon and 0.4% yeast extract, and harvested by a centrifuge. The washed cells were dried with an electric fan at room temperature for 4~5 hours. The reaction mixture contained 4 mM phosphate buffer, pH 7.4, 4 mM semicarbazide, the requisite amount of Na-glycollate, 200 mg dried cells of *A. aerogenes*, and water to a final volume of 40 ml; 4 hours' incubation on a shaker at 30°C (see Table I). It was observed that when the dried cells of *A. aerogenes*

TABLE I. OXIDATION OF GLYCOLLATE BY DRIED CELL OF STRAIN B-2 OF *A. Aerogenes* GROWN AEROBICALLY ON CITRATE

Vessel No.	I	II	III*
Glycollate added (μ M)	0	1000.0	1000.0
Glyoxylate formed (μ M)	0	13.5	0

* Incubation after heat-treatment for 5 minutes at 90°C

(strain B-2) were aerobically incubated with glycollate in the presence of semicarbazide, a considerable amount of a keto-acid appeared in the medium. This keto-acid was identified as glyoxylic acid by chromatographic behaviour of the 2,4-dinitrophenylhydrazone. Subsequently, the oxidation of glycollate to glyoxylate was investigated with other strains of *coli-aerogenes* and a commercial baker's yeast. Baker's yeast was dried in the same manner as in the bacteria mentioned above. The bacterial cells used here were as follows: (1) strain G-2 of *E. coli* grown aerobically for 20 hours at 30°C on 1% each Na-acetate and bouillon; (2) the same strain of *E. coli* with a high potency of inducing α -ketoglutarate-fermentation, grown on the same glucose-ammonia medium as previously reported³⁻⁵; (3) strain B-T of *coli-aerogenes* with a high activity of citritase, grown on a citrate medium in the same manner as previously reported⁹.

Reaction mixtures contained 4 mM phosphate buffer, pH 7.4, 4 mM semicarbazide, 1000 μ M Na-glycollate, the stated amounts of dried cells, and water to a final volume of 40 ml; 3~5 hours' incubation on a shaker at 30°C. From the results shown in Table II, it has been found that not only the dried cells of *coli-aerogenes* grown on the organic acid-containing media but those grown on a glucose medium can also catalyze the oxidation of glycollate to glyoxylate by molecular oxygen. This oxidation was further ascertained to occur by a dried cell of baker's yeast. No oxidation of glycollate was ever observed with a heated cell (see Table I).

Oxidation of Glycollate to Glyoxylate by Cell-Free Extracts of *Coli-aerogenes*. A cell-free extract of *coli-aerogenes* was prepared from a sonically treated cell in the same manner as previously reported. The results of experiments with the dialyzed cell extract are shown in Table III, in which incubations were carried out on a shaker for 3~5 hours at 30°C with the media (15~30 ml) containing 1~3 mM phosphate buffer, pH 7.4, 1~2.8 mM semicarbazide, 200~400 μ M Na-glycollate and 5 ml dialyzed cell extracts (obtained from 100 mg bacterial cells). It will be seen that the dialyzed cell extracts of *coli-aerogens* possess an ability to oxidize glycollate to glyoxylate. This may indicate that the oxidation of

18) N.A. Frigerio and H.A. Harbury, *J. Biol. Chem.*, **231**, 135 (1958).

19) J.S. Dohan, *J. Biol. Chem.*, **135**, 793 (1940).

20) E. Kun, *J. Biol. Chem.*, **194**, 603 (1952).

21) E. Kun, J.M. Dechary and H.C. Pitot, *J. Biol. Chem.*, **210**, 269 (1954).

TABLE II. FORMATION OF GLYOXYLATE FROM GLYCOLLATE BY DRIED CELLS OF *Coli-Aerogenes* BACTERIA AND BAKER'S YEAST

Organisms	Strain G-2 of <i>E. coli</i>		Strain B-T of <i>coli-aerogenes</i>	
	grown on acetate	grown on glucose	grown on citrate	Baker's yeast
Dried cells used (mg)	180	120	300	1500
Time of incubation (hours)	4	5	5	3
Glyoxylate formed (μ M)	20.9	9.5	27.6	21.7

TABLE III. OXIDATION OF GLYCOLLATE TO GLYOXYLATE BY DIALYZED CELL EXTRACT OF *Coli-Aerogenes*

Enzyme source	A cell ext. of <i>E. coli</i> (strain G-2) grown on acetate	A cell ext. of <i>A. aerogenes</i> (strain B-2) grown on citrate	A cell ext. of <i>coli-aerogenes</i> (strain B-T) with citritase
Reaction mixtures (ml)	30	20	15
Time of incubation (hours)	4	5	3
Glyoxylate formed (μ M)	9.5	6.5	4.5

TABLE IV. OXIDATION OF GLYCOLLATE BY SALT-FRACTION OF CELL EXTRACT OF *Coli-Aerogenes*

Organisms used	Ammonium sulfate saturation fraction of cell extracts	Glyoxylate formed (μ M)
Strain G-2 of <i>E. coli</i> grown on acetate	0 ~0.35 0.3~50.70 (corresponded to 60 mg cells)	1.8 0 (3 hours' incubation)
Strain B-2 of <i>A. aerogenes</i> grown citrate	0 ~0.40 0.40~0.70 (corresponded to 100 mg cells)	1.9 0 (5 hours' incubation)

TABLE V. EFFECT OF ADDITION OF FMN ON OXIDATION OF GLYCOLLATE BY DIALYZED CELL EXTRACT AND SALT-PRECIPITATES OBTAINED FROM ACETATE-GROWN *E. Coli*

Enzyme preparations	FMN added (M/l)	Glyoxylate formed (μ M)
3 ml dialyzed cell extract (corresponded to 90 mg cells of strain G-2)	0 0 1×10^{-4}	5.3 0* 5.4
3 ml ammonium sulfate saturation fraction (corresponded to 70 mg cells of strain G-2)	0 ~0.35 0 ~0.35 0.35~0.70 0.35~0.70	2.0 2.0 0 0
	1×10^{-4}	0

* Incubation after heat-treatment for 5 minutes at 90°C.

Reaction mixture contained 1m M phosphate buffer, pH 7.4, 1 mM semicarbazide, 200 μ M Na-glycollate and the indicated amounts of enzyme preparations and FMN: total volume 10 ml; 3 hours' incubation on a shaker at 30°C.

glycollate to glyoxylate is catalyzed by an oxidase in which DPN or TPN is not involved. The formation of glyoxylate was never observed in the absence of molecular oxygen.

Ammonium Sulfate Fractionation of Cell Extract.

The cell-free extracts of *coli-aerogenes* were fractionated for glycollic acid oxidase, procedures being carried out at 0~5°C. Solid ammonium sulfate was added to the cell extracts to give the saturation concentration as indicated in Table IV, and the precipitates formed were collected with a centrifuge and dissolved in 0.05~0.1 M phosphate buffer (pH 7.4). The ammonium sulfate fractions were dialyzed against distilled water at 5°C for 20~40 hours. The results of experiments carried out with the salt-precipitates are given in Table IV, in which the reaction mixtures (10 ml) contained 1 mM phosphate buffer, pH 7.4, 1 mM semicarbazide, 200 μ M Na-glycollate and 3 ml (corresponding to 60~100 mg cells) enzyme of *coli-aerogenes*; 3~5 hours' incubation at 30°C on a shaker. From the data given in Table IV, it has been found that the salt 0.35 or 0.40 saturation fraction prepared from the acetate-grown or the citrate-grown cells of *coli-aerogenes* reveals an activity of glycollic acid oxidase, whereas, the salt 0.35~0.70 or 0.40~0.70 saturation fraction reveals no activity of the oxidase.

Prosthetic Group of Glycollic Acid Oxidase. It has been demonstrated by Zelitch and Ochoa¹⁶⁾ that glycollic acid oxidase of plant tissues is a flavoprotein with FMN as the prosthetic group. On the other hand, Kun et al.²¹⁾ have reported that glycollic acid oxidase of rat liver is resolved to yield an inactive apoenzyme which can be reactivated by FMN or FAD. These findings led the

authors to clarify the prosthetic group of glycollic acid oxidase of microorganisms. Table V shows the effect of addition of FMN to the dialyzed cell extract or the salt-precipitated protein of *coli-aerogenes*. Experiments were carried out in the same manner as mentioned above, except the addition of flavin nucleotide. It will be seen that the addition of FMN revealed no striking effect on the oxidation of glycollate by the dialyzed cell extract or the salt-precipitates. However, these enzyme preparations were soon found to be resolved by the method of Warburg and Christian²². An inactive apoenzyme was obtained from the ammonium sulfate 0.35 or the 0.40 saturation fraction of the cell extract of *E. coli* grown aerobically on the acetate-bouillon, according to the following manner. The salt-precipitates were again suspended in a 0.6 saturation solution of ammonium sulfate at 0°C. The suspension was acidified with 2 N HCl to pH 2.3~2.5. After stirring for 60~120 minutes at 0°C, the precipitated protein was centrifuged and the supernatant fluid discarded. The precipitates were washed with a saturated ammonium sulfate and dissolved in 0.05 M phosphate buffer (pH 7.4). The reaction mixture contained 600 μ M phosphate buffer, pH 7.0, 600 μ M semicarbazide, 500 μ M

TABLE VI. REACTIVATION OF GLYCOLLIC OXIDASE APOENZYME OF *E. Coli* BY FLAVIN NUCLEOTIDES

Flavin nucleotides added (M/l)	Glyoxylate formed (μ M/10ml)	
None	Trace	
Riboflavin-5'-phosphate	5 $\times 10^{-5}$	0.66
Flavin adenine dinucleotide	2.5 $\times 10^{-5}$	0.63

TABLE VII. INFLUENCE OF CONCENTRATION OF ADDED RIBOFLAVIN PHOSPHATE UPON REACTIVATION OF GLYCOLLIC OXIDASE APOENZYME OF *E. Coli*

Riboflavin-5'-phosphate added (M/l)	Glyoxylate formed (μ M/10 ml)
None	0.00
5.0 $\times 10^{-7}$	0.50
2.5 $\times 10^{-6}$	0.52
1.0 $\times 10^{-5}$	0.53
1.0 $\times 10^{-4}$	0.53

Experimental conditions, same as experiment shown in Table VI.

TABLE VIII. INHIBITING EFFECT OF CYANIDE ON OXIDATION OF GLYCOLLATE BY SALT 0.40 SATURATION FRACTION OF *E. Coli*

KCN added (M/l)	None	2.4 $\times 10^{-3}$	2.4 $\times 10^{-3}$	2.4 $\times 10^{-3}$
Flavin nucleotides added (M/l)	None	None	FMN 3.2 $\times 10^{-4}$	FAD 2.4 $\times 10^{-4}$
Glyoxylate formed (μ M)	4.1	1.2	1.2	1.5

Na-glycollate, the requisite amounts of flavin nucleotides and 5 ml apoenzyme; total volume 10 ml; 3 hours' incubation at 30°C on a shaker. From the results of experiments given in Table VI, it has been found that the acid-treated enzyme can not catalyze the oxidation of glycollate to glyoxylate unless FMN or FAD is added. The reactivation of this apoenzyme in the presence of varying amounts of FMN is also shown in Table VII. The results of experiments indicated that the K_m for FMN was approximately 3×10^{-7} M.

Inhibitors and Activator. Several substances were tested for their ability to accelerate or to inhibit the oxidation of glycollate by the enzyme of *coli-aerogenes*. Glutathione and MgSO₄ had no effect on the action of the salt-precipitated enzymes. No inhibiting effect was observed with α, α' -dipyridyl, 2,4-dinitrophenol, NaN₃ and quinacrine. However, cyanide has been found to be a powerful inhibitor. The results of experiments are shown in Tables VIII and IX. Table VIII: reaction mixture (10 ml) contained 500 μ M phosphate buffer, pH 7.3, 1000 μ M semicarbazide, 400 μ M Na-glycollate, the requisite amounts of KCN and flavin nucleotides, and 4 ml of the salt 0.40 saturation fraction (obtained from 100 mg cells of acetate-grown *E. coli*); 4 hours' incubation at 30°C aerobically. Table IX: reaction mixture (10 ml) contained 500 μ M phosphate buffer, pH 7.3, 500 μ M semicarbazide, 500 μ M Na-glycollate, requisite amounts of KCN and FMN, and 5 ml acid-treated enzyme (obtained from 80 mg bacterial protein of acetate-grown *E. coli*); 3 hours' incubation at 30°C aerobically. It will be seen that the addition of flavin nucleotides to the enzyme can not afford protection against cyanide. It appears that this oxidase may be a metalloflavoprotein.

TABLE IX. INHIBITING EFFECT OF CYANIDE ON OXIDATION OF GLYCOLLATE BY ACID-TREATED ENZYME OF *E. Coli*

KCN added (M/l)	None	None	5.0 $\times 10^{-3}$
FMN added (M/l)	None	2.5 $\times 10^{-5}$	2.5 $\times 10^{-5}$
Glyoxylate formed (μ M)	0.5	2.5	0.4

DISCUSSION

Since two enzymes catalyzing glyoxylate $\rightleftharpoons$

22) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

glycollate system, i.e., glyoxylic acid reductase and glycollic acid oxidase are found in plant tissues, it has been considered that this system may play an important role in the respiration of green leaves^{16-18; 23, 24)}. In the presence of catalytic amounts of diphospho- or triphosphopyridine nucleotide and glyoxylic or glycollic acid, the glyoxylic reductase-glycollic oxidase system can act as a hydrogen transport system from reduced pyridine nucleotides to molecular oxygen.

In the previous papers^{5, 7-9)}, the authors have demonstrated that when the intact or the treated cells of *coli-aerogenes* bacteria are incubated with citric acid under either aerobic or anaerobic conditions, little formation of α -ketoglutaric acid is observed, while these cells accumulate glyoxylic acid during the degradation of citric acid in the presence of an added trapping agent such as semicarbazide. It has moreover been found by the authors that microorganisms such as *coli-aerogenes* and *Ps. fluorescens* possess abilities to metabolize a certain part of citric acid according to the following reactions¹²⁾. [A] Coupling reaction between isocitric acid dehydrogenase and glyoxylic acid reductase

23) I. Zelitch, *J. Biol. Chem.*, **201**, 719 (1953).

24) I. Zelitch, *J. Biol. Chem.*, **216**, 553 (1955).

+succinate+glycollate
[B] Enzymic oxidation of TPNH and DPNH by glyoxylate

In the present paper, another enzyme, glycollic oxidase, which catalyzes the oxidation of glycollate by molecular oxygen has been further detected in microorganisms including *coli-aerogenes* and baker's yeast.

The combination of Reactions 5 and 6 provides an effective means of transferring hydrogen from reduced pyridine nucleotides to molecular oxygen.

Therefore, it has now been suggested that some part of respiration in microorganisms may proceed by a combined system of Reactions 5 and 6. However, it appears that the major reoxidation of reduced pyridine nucleotides may proceed by way of the diaphorase-cytochrome system rather than by way of glyoxylic reductase-glycollic oxidase.

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