Microbiological Studies of *Coli-aerogenes* Bacteria. (XIV)

Competition between Glyoxylic Reductase and Glutamic Dehydrogenase*

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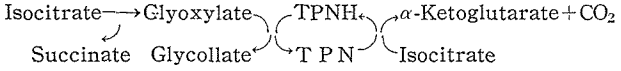
The anaerobic degradation of citrate was investigated with the dialyzed cell-free extracts of *coli-aerogenes*. Both α-ketoglutarate and glycollate were obtained in good yields when the bacterial cell-free extracts were anaerobically incubated in the presence of TPN*** but in the absence of NH_4Cl. Remarkable production of glutamate took place in the presence of both TPN and NH_4^+. At high concentration of citrate, however, the cell extracts were able to produce α-ketoglutarate and glycollate in addition to glutamate even in the presence of NH_4^+. The mode of anaerobic degradation of citrate was observed to depend upon TPN concentration and pH value of reaction media. Glyoxylic reductase was also found in a cell of baker's yeast. Thus, experimental results obtained in the present paper, indicated the possibility of a competiton between isocitric-glutamic dehydrogenase system and isocitritase-isocitric dehydrogenase-glyoxylic reductase system.

INTRODUCTION

In the previous papers[1~5], the authors have studied the mode of bacterial metabolism of C_6-tricarboxylic acids and have pointed out that isocitritase, catalyzing the cleavage of citrate (isocitrate) to glyoxylate plus succinate, may play an important role in microbial respiration. Further investigations have demonstrated the occurrence of pyridine nucleotide-linked glyoxylic acid reductase in microorganisms[6,7]. Experimental results have shown that the formation of α-ketoglutarate with citrate is the result of a coupling reaction between isocitric dehydrogenase and glyoxylic reductase.



It has also been found that the oxidation of glycollate to glyoxylate is catalyzed by a riboflavin phosphate-linked oxidase isolated from microorganisms including *coli-aerogenes* bacteria. Thus, the glyoxylic reductase-glycollic oxidase system may provide an effective means of transferring hydrogen from reduced pyridine nucleotides to molecular oxygen according to the following reactions :

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* Part XIII, *Bull. Agr. Chem. Soc. Japan*[8].

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*** The following abbreviations were used ; TPN=triphosphopryidine nucleotide ; TPN and TPNH=oxidized and reduced triphosphopyridine nucleotides, respectively; DPN and DPNH=oxidized and reduced diphosphopyridine nucleotides, respectively.

$$\text{Substrate} + \text{TPN}^+(\text{DPN}^+) \longrightarrow \text{oxidation-product} + \text{TPNH(DPNH)} + \text{H}^+$$
$$\text{TPNH(DPNH)} + \text{H}^+ + \text{glyoxylate} \longrightarrow \text{TPN}^+(\text{DPN}^+) + \text{glycollate}$$
$$\text{Glycollate} \xrightarrow{\text{oxygen}} \text{Glyoxylate}$$
$$\text{Sum :} \quad \text{Substrate} \xrightarrow{\text{oxygen}} \text{oxidation-product}$$

The function of glyoxylate-glycollate system in plant respiration has already been indicated by other workers[9~12]. The present paper deals with the physiological relationship among isocitric dehydrogenase, glyoxylic reductase and glutamic dehydrogenase.

EXPERIMENTAL AND RESULTS

Methods. Analytical procedure was performed by the methods mentioned in the previous papers[4~7].

Chemicals.——Triphosphopyridine nucleotide (TPN) was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan; baker's yeast from Oriental yeast Co., Ltd., Osaka, Japan.

Microorganisms and enzyme preparations. Bacteria——Two strains of *coli-aerogenes* bacteria (*Escherichia coli*, strain G-2 and *Aerobacter aerogenes*, strain B-2) were grown at 30°C for 20-30 hours on a shaker with the following media : glucose medium ; 2% glucose, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.1% $(NH_4)_2HPO_4$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, 5 mg% $FeSO_4 \cdot H_2O$ and 1.5% $CaCO_3$: citrate or acetate medium ; 2.5% either Na-citrate or Na-acetate, 1% bouillon and 0.3% $(NH_4)_2SO_4$. After cultivation, the bacterial cells were disintegrated by treatment with 10 kc Raytheon oscillator in the similar way as described previously. Cell debris was removed by a centrifuge and the resulting cell-free extracts were dialyzed against $2 \times 10^{-2} M$ phosphate buffer, pH 7.4, for 20-30 hours at 5°C.

Dried cells of baker's yeast——Pressed baker's yeast was dried by an electric fan at room temperature for 8 hours.

Cell-free extracts of baker's yeast——Pressed baker's yeast was incubated for one hour at 37°C in the presence of ethylacetate. After incubation, cells were collected by a centrifuge, ground for one hour with powdered glass and thereafter suspended in 0.25 M phosphate buffer, the pH being adjusted to 8.6 with 5% ammonia solution. After being kept for one week at 5°C, the suspension was centrifuged and the precipitate was discarded. The supernatant fluids were dialyzed against distilled water at 5° C for 40 hours.

Anaerobic degradation of citrate by *coli-aerogenes*. Investigations were at first directed towards the enzymic degradation of citrate by *coli-aerogenes* in the presence and absence of ammonium chloride. Tables 1-3 show the results of experiments carried out with the dialyzed cell-free extracts of the bacteria. Glycollate and α-ketoglutarate were produced in good yields from citrate when the cell-free extracts were anaerobically incubated under such conditions that TPN was present and NH_4^+ absent. The addition of NH_4^+ brought about the remarkable production of glutamate. However, it was found that considerable amounts of α-ketoglutarate and glycollate were produced even in the presence of both

Table 1. Anaerobic degradation of citrate by dialyzed cell-free extracts of *E. coli* grown on acetate.

Vessel No.	I	II	III
TPN added (γ/ml)	0	40	40
NH_4Cl added (μmoles)	0	0	100.0
Glyoxylate found (μmoles)	3.2	0	0
α-Ketoglutarate found (μmoles)	0	37.5	12.9
Glycollate found (μmoles)	Trace	37.9	11.1
Glutamate found (μmoles)	0	0	48.2

Reaction mixture contained 400 μmoles phosphate buffer, pH 7.4, 10 μmoles $MgSO_4$, 100 μmoles Na-citrate, 2.5 ml (80 mg protein) of the dialyzed cell extracts of *E. coli*, G-2 strain grown on acetate; total volume 6.7 ml. Thunberg tube in vacuo at 37°C for 6 hours.

Table 2. Influence of NH_4^+ upon fermentation of citrate by dialyzed cell-free extracts of *E. coli* grown on glucose.

Vessel No.	I	II	III	IV
Citrate added (μmoles) (M)	100.0 1.6×10^{-2}	100.0 1.6×10^{-2}	600.0 1×10^{-1}	600.0 1×10^{-1}
NH_4Cl added (μmoles)	0	100.0	0	100.0
α-Ketoglutarate found (μmoles)	11.1	0	15.0	4.5
Glutamate found (μmoles)	0	40.2	0	22.5
Glycollate found (μmoles)	16.5	Trace	20.3	

Reaction mixture contained 500 μmoles phosphate buffer, pH 7.4, 10 μmoles $MgSO_4$, 26 γ/ml TPN, requisite amounts of Na-citrate and NH_4Cl, and 2.5 ml (80 mg protein) of the dialyzed cell extract of *E. coli*, G-2 strain, grown on glucose; total volume, 6 ml. Thunberg tube in vacuo at 37°C for 5 hours.

Table 3. Anaerobic degradation of citrate by dialyzed cell-free extracts of *A. aerogenes* grown on acetate.

Vessel No.	I	II	III
TPN added (γ/ml)	0	22	22
Citrate added (μmoles) (M)	600.0 9×10^{-2}	100.0 1.5×10^{-2}	600.0 9×10^{-2}
Glyoxylate found (μmoles)	14.3	0	0
α-Ketoglutarate found (μmoles)	0	8.3	30.8
Glutamate found (μmoles)	0	73.0	58.8

Reaction mixture contained 500 μmoles phosphate buffer, pH 7.4, 10 μmoles $MgSO_4$, 100 μmoles NH_4Cl, 2.5 ml dialyzed cell-free extracts of *A. aerogenes*, B-2 strain, grown on acetate, and the requisite amounts of TPN and Na-citrate; total volume, 6.7 ml. Thunberg tube in vacuo. Incubation at 37°C for 5 hours.

TPN and NH_4^+, although there was an increase in the yield of glutamate against decreaing yields of both α-ketoglutarate and glycollate. The amounts of products were also observed to vary with the kinds of organisms. These facts elucidates that the anaerobic degradation of citrate takes place by the two kinds of coupling reactions; (a) isocitric dehydrogenase-glutamic dehydrogenase system and (b)

isocitritase-isocitric dehydrogenase-glyoxylic reductase system. The reactions are given according to the following equations :

(I) Citrate (isocitrate) + $TPN^+ \longrightarrow$ α-ketoglutarate + CO_2 + TPNH + H^+
α-Ketoglutarte + TPNH + H^+ + $NH_4^+ \longrightarrow$ glutamate + TPN^+

Citrate + $NH_4^+ \longrightarrow$ glutamate + CO_2

(II) Citrate (isocitrate) $\longrightarrow$ glyoxylate + succinate
Citrate (isocitrate) + $TPN^+ \longrightarrow$ α-ketoglutarate + CO_2 + TPNH + H^+
Glyoxylate + TPNH + $H^+ \longrightarrow$ glycollate + TPN^+

2 Citrate $\longrightarrow$ α-ketoglutarate + CO_2 + glycollate + succinate

Effect of substrate concentration. The concentration of citrate produced a striking effect on the yields of glutamate and α-ketoglutarate (glycollate). At the low concentration of citrate, glutamate was obtained in good yields. At its high concentration, however, there was a remarkable increase in the yields of α-ketoglutarate and glycollate. A typical result is shown in Fig. 1. It is interesting to

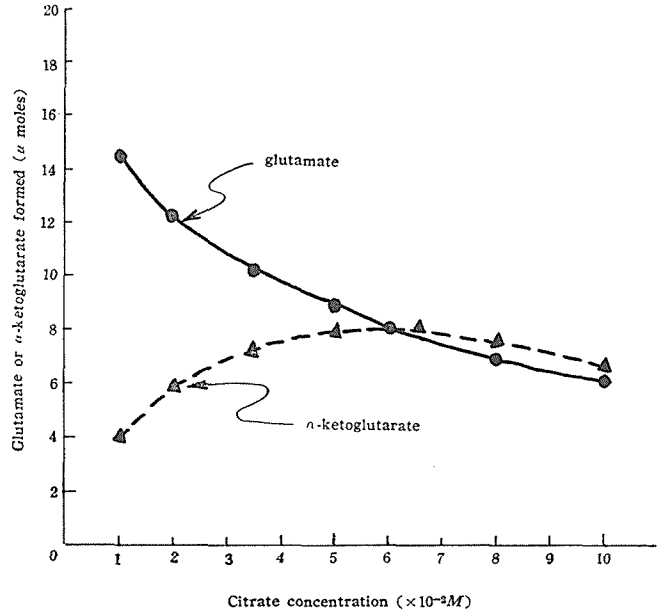


Fig. 1. Influence of various concentration of citrate upon the formation of glutamate and α-ketoglutarate.
Reaction mixture contained 500 μmoles phosphate buffer, pH 7.4, 10 μmoles $MgSO_4$, 25 γ/ml TPN, 100 μmoles ($2\times10^{-2}M$) NH_4Cl, the requisite amount (1×10^{-2}—$1\times10^{-1}M$) of Na-citrate as indicated and 1 ml (10 mg as protein) of dialyzed cell-free ext. of *A. aerogenes* grown on citrate ; total volume, 5 ml ; incubation anaerobically at 37°C for one hour.

note that larger amounts of glutamate are obtained at a low level of citrate than at its high level. It appears that citrate in the range of about $1\times10^{-2}M$ to $1\times10^{-1}M$ does not greatly affect the following enzyme ; isocitritase, isocitric dehydrogenase and glyoxylic reductase. Thus, it may be indicated that bacterial glutamic dehydrogenase is considerably inhibited by high concentration of citrate.

Table 4. Influence of pH of medium upon anaerobic degradation of citrate by dialyzed cell-free extracts of *A. aerogenes* grown on citrate.

Vessel No.	I	II	III	IV
pH of medium	6.0	6.0	7.6	7.6
Citrate added (μmoles)	200.0	1000.0	200.0	1000.0
α-Ketoglutarate found (μmoles)	8.5	7.1	8.0	16.5
Glutamate found (μmoles)	12.2	13.6	85.8	53.0

Reaction mixture contained 1 m mole phosphate buffer, pH 6.0–7.6, 20 μmoles $MgSO_4$, 200 μmoles NH_4Cl, 18 γ/ml TPN, 2 ml (20 mg as protein) of the dialyzed cell extracts of *A. aerogenes*, B-2 strain, and the indicated amounts of Na-citrate; total volume, 10 ml. Thunberg tude in vacuo; 2 hours' incubation 37°C.

Table 5. Influence of TPN concentration upon anaerobic degradation of citrate by dialyzed cell-free extracts of *A. aerogenes* grown on citrate.

Vessel No.	I	II	III	IV	V	VI
TPN added (γ/ml)	0	2	5	20	100	180
Glyoxylate found (μmoles)	7.0	3.8	2.8	0.8	0.4	0
Glutamate found (μmoles)	0	2.0	5.1	17.8	32.2	40.8
α-Ketoglutarate found (μmoles)	0	3.1	6.4	10.1	6.8	5.9

Reaction mixture contained 250 μmoles phosphate buffer, pH 7.4, 5 μmoles $MgSO_4$, 100 μmoles NH_4Cl, 250 μmoles of Na-citrate, requisite amounts of TPN and 0.5 ml of dialyzed cell extracts of *A. aerogenes*, B-2 strain grown on citrate; total volume, 2.5 ml. Thunberg tube in vacuo; 3 hours' incubation at 37°C.

On the other hand, ammonium chloride of 2×10^{-2} to 1×10^{-1} *M* had no remarkable effect on the yield of glutamate, when the cell-free extract of B-2 strain was incubated with citrate of 1×10^{-1} *M*.

Table 4 shows the effect of pH on the degradation of citrate by B-2 strain. It will be seen that higher yield of glutamate is obtained in alkaline media than in acidic media, when incubations are carried out with phosphate buffer.

Effect of TPN concentration. As will be seen in Table 5, increasing TPN concentration resulted in the higer yield of glutamate, the yield being 5.1μmoles at 5 γ/ml TPN and 40.8μmoles at its concentration of 180 γ/ml. On the other hand, the maximal yield of α-ketoglutarate was obtained with as low a concentration as 20 γ/ml TPN.

Anaerobic degradation of citrate by baker's yeast. Investigations on the bacterial metabolism mentioned above, have led the authors to clarify the mode of degradation of citrate by baker's yeast. The occurrence of isocitritase in baker's yeast has recently been reported by Olson[13]. Table 6 shows the results of experiments carried out with the dried cells of baker's yeast. Remarkable production of both α-ketoglutarate and glycollate took place, when the dried cells were anaerobically incubated with citrate in the absence of semicarbazide. However, in the presence of semicarbazide, a large amount of glyoxylate was obtained in place of α-ketoglutarate. Experiments were furthermore carried out with the cell-free extracts. The results are shown in Table 7. When the cell extracts

Table 6. Anaerobic degradation of citrate by dried cells of baker's yeast.

Vessel No.	I	II	III
Citrate added (μmoles)	0 (Endogenous)	2000.0	2000.0
Semicarbazide added (M)	5×10^{-2}	5×10^{-2}	0
α-Ketoglutarate found (μmoles)	0	45.5	168.1
Glyoxylate found (μmoles)	0	410.0	Trace
Glycollate found (μmoles)	Trace		89.5

Reaction mixture contained 2.5 m moles phosphate buffer pH 7.5, 100 μmoles $MgSO_4$, 3 g dried cells of baker's yeast and additions as indicated in the table; total volume, 50 ml; 6 hours' incubation at 37°C. Thunberg tube in vacuo.

Table 7. Anaerobic degradation of citrate by cell-free extracts of baker's yeast.

Addition of TPN (γ/ml)	0	40
Glyoxylate found (μmoles)	7.6	Trace
α-Ketoglutarate found (μmoles)	Trace	11.6
Glycollate found (μmoles)	Trace	15.1
Succinate found (μmoles)	43.0	44.5

Reaction mixture contained 380 μmoles phosphate buffer, pH 7.4, 10 μmoles $MgSO_4$, 150 μmoles Na-citrate, 5 ml dialyzed cell extract of baker's yeast and the indicated amount of TPN; total volume, 10 ml; 5 hours' incubation at 37°C; Thunberg tube in vacuo.

Table 8. Anaerobic degradation of citrate by dialyzed cell-free extract of baker's yeast.

Citrate added (M)	4×10^{-2}	7×10^{-2}	1×10^{-1}
Glutamate found (μmoles)	16.2	13.2	11.7
Glycollate found (μmoles)			7.9

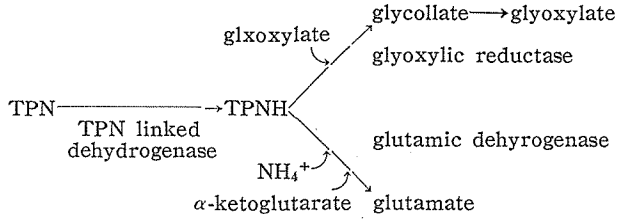
Reaction mixture contained 500 μmoles phosphate buffer, pH 7.4, 200 μmoles NH_4Cl, 10 μmoles $MgSO_4$, 10 γ/ml TPN, indicated amounts of Na-citrate and 2.5 ml cell extracts of yeast; total volume, 10ml; 3 hours' incubation at 37°C, Thunberg tube in vacuo.

were anaerodically incubated with citrate in the presence of added TPN, α-ketoglutarate and glycollate were produced in addition to succinate and carbon dioxide. From these observations, it has now been indicated that baker's yeast metabolizes citrate to succinate, carbon dioxide, glycollate and α-ketoglutarate by the combined action of aconitase, isocitric dehydrogenase, isocitritase and glyoxylic reductase. On the other hand, the presence of NH_4^+ brought about the remarkable production of glutamate, as shown in Table 8.

DISCUSSION

The experimental results mentioned above, may indicate the possibility of a competition between a reduction of glyoxylate to glycollate and a reductive aminaton of α-ketoglutarate to glutamate. This is of great interest in connection with the directive function of enzymes. The mode of the metabolism of citrate

may be determined by the following factors : (1) the relative amounts of (a) isocitric dehydrogenase and isocitritase ; (b) glyoxylic reductase and glutamic dehydrogenase ; (2) affinities of glyoxylic reductase and glutamic dehydrogenase for reduced pyridine nucleotides ; (3) relative concentrations of substrates, pyridine nucleotides and of reaction products.



As regards the oxidation of citrate to α-ketoglutarate, it may be suggested that either glyoxylate or glycollate is acting catalytically as a hydrogen carrier :

$$\text{Citrate (isocitrate)} + TPN^+ \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + TPNH + H^+ \quad \text{(isocitric dehydrogenase)}$$

$$\text{Glyoxylate} + TPNH + H^+ \longrightarrow \text{glycollate} + TPN^+ \quad \text{(glyoxylic reductase)}$$

$$\text{Glycollate} + O \longrightarrow \text{glyoxylate} + H_2O \quad \text{(glycollic oxidase)}$$

$$\text{net result} \quad \text{Citrate} + O \longrightarrow \alpha\text{-ketoglutarate} + CO_2 + H_2O$$

Citrate metabolism of this type is greatly influenced by the various factors as mentoned above. In the case of the bacterial cells of *coli-aerogenes*, it appears that some part of citrate is oxidized to α-ketoglutarate according to this reaction system. In the previous papers, the authors have already studied the mode of the degradation of citrate by the bacteria.

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