Microbiological Studies of *Coli-aerogenes* Bacteria Part VII. Degradation of Citrate

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The cell-free extracts of *coli-aerogenes* produced glyoxylate and succinate in addition to pyruvate and α -ketoglutarate when incubation was carried out with a citrate-medium. The fresh cell-extracts possessed a very poor ability to produce α -ketoglutarate from citrate, but the addition of coenzyme II (TPN) to the reaction mixtures brought about an increase in the yield of α -ketoglutarate. The degradation of citrate by the intact (washed), dried and ground cells of the bacteria also resulted in an accumulation of glyoxylate in the external medium in which semicarbazide was present. It was suggested that the concentration of coenzyme II within the bacterial cells was relatively low, so that coenzyme II-dependent isocitric dehydrogenase was not operative at such a high rate enough to effect the complete removal of citrate. The bacterial cells revealed a high potency of producing α -ketoglutarate from pyruvate or succinate under aerobic conditions. It was indicated that the major breakdown of citrate by the cells did not proceed by way of isocitric- α -ketoglutaric dehydrogenase system, but occurred by way of splitting-reactions.

INTRODUCTION

Recent studies concerning the metabolisms of tricarboxylic acids in microorganisms performed by many workers have revealed an interesting fact that various organisms can effect a C₂ plus C₄ cleavage of citrate, in addition to the formation of α -ketoglutarate by way of the Krebs cycle. The cleavage of tricarboxylic acids into C₂ and C₄ fragments proceeds according to the following reactions:

- (1) Citrate $\rightarrow$ oxalacetate + acetate (citritase)¹⁻¹¹⁾,
- (2) Citrate $\rightleftharpoons$ isocitrate $\rightarrow$ glyoxylate + succinate (isocitritase)¹²⁻¹⁸⁾.

In the previous papers of this series, the authors have demonstrated that the bacteria of *coli-aerogenes* possess an exceedingly high potency of producing α -ketoglutaric acid from varied carbonaceous substances including glucose, pyruvate, lactate and C₄-dicarboxylic acids such as succinate, fumarate, malate and oxalacetate¹⁹⁻²⁴⁾. Moreover, it has been found that the washed and treated cells of the bacteria are capable

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of oxidizing the tricarboxylic acids, but little formation of α -ketoglutaric acid is observed, while these dried cells accumulate glyoxylate during the oxidation of citrate in the presence of added trapping agents such as semicarbazide. This indicates that the major oxidation of citrate by the bacteria is accompanied with a cleavage-reaction (glyoxylate plus succinate formation), although a part of oxidation may proceed by way of a isocitrate- α -ketoglutarate-succinate system²⁴. Thus, the authors have concluded that the major production of α -ketoglutaric acid from the varied substances, such as glucose, C₃- and C₄- acids, by *coli-aerogenes* does not proceed by way of the conventional tricarboxylic acid cycle, but takes place by way of a certain reaction in which pyruvate and acetate are involved^{23,24}.

In the preceding paper²⁵, the authors have also investigated the effects of various antibiotics on the respiration and α -ketoglutaric acid-fermentation in the bacteria, and, the present paper deals with the mechanism of the degradation of citrate caused by the bacteria.

EXPERIMENTAL AND RESULTS

Microorganisms: Three strains of the bacteria, i.e. *Escherichia coli* (strain G-2), *Aerobacter aerogenes* (strain B-2), *coli-aerogenes* (strain B-T), were used throughout the experiments.

Methods: Analytical procedure was performed by the methods mentioned in the previous paper¹⁹⁻²⁵. Triphosphopyridine nucleotide (TPN) was obtained from the Tokyo Kasei Co. Ltd.

Degradation of Citrate by Cell-Free Extract of *Coli-aerogenes* Bacteria. In the previous paper²⁴, it has been ascertained that the dried cells of *A. aerogenes* (strain B-2) grown on citrate or those of *E. coli* (strain

G-2) grown on glucose-medium, accumulate glyoxylate in the media as a sort of degraded product of citrate in the presence of externally added trapping agents such as semicarbazide²⁴. In the present paper, the authors at first, investigated the degradation of citrate by the cell-free extracts of *coli-aerogenes*.

E. coli was grown on a shaker at 30°C for 2 days in a medium containing 2% glucose, 0.1% (NH₄)₂HPO₄, 0.3% (NH₄)₂SO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 5 mg% FeSO₄·7H₂O, 0.2% NaCl and 1% CaCO₃. In the case of *A. aerogenes* (B-2), the following medium was employed: 3% Na-citrate, 1% each chrysalis extract and peptone, 0.2% NaCl, 0.3% (NH₄)₂SO₄, 0.1% KH₂PO₄ and 0.05% MgSO₄·7H₂O (citrate medium). After cultivation, the bacterial cells were harvested with a centrifuge and washed once with distilled water. The cells were suspended to a level of 20~30 mg of dry weight per ml of distilled water and then disintegrated by treatment with a 10 kc. Raytheon oscillator for 15~25 minutes. The cell debris was removed by a centrifuge, and the cell-free extract was used without dialysis in the experiments.

The reaction mixtures (10~25 ml) containing 1.7mM phosphate buffer (pH:7.3), 30 μ M MgSO₄, 200 μ M Na-citrate and 5~15 ml cell-free extracts, were dispensed in a Thunberg tube and incubated at 37°C under anaerobic conditions (in vacuo). The results are shown in Table I, from which it has been observed that glyoxylate and succinate are produced in addition to pyruvate and α -ketoglutarate when the cell-free extracts are anaerobically incubated in the presence of citrate.

Keto-acids observed in the experiments are considered to be produced by way of the following reactions:

TABLE I. ANAEROBIC DEGRADATION OF CITRATE BY CELL FREE EXTRACTS OF *Coli-aerogenes*

Bacterial strains	<i>E. coli</i> (G-2) grown aerobically on a glucose-medium	<i>A. aerogenes</i> (B-2) grown aerobically on a citrate-bouillon medium
Cell-free extracts (ml)	15.0	5.0
(mg Bacterial cells used)	(450)	(130)
Reaction mixtures (ml)	25	10
Time of incubation (hours)	4	2
Pyruvate found (μ M)	3.0	2.1
Glyoxylate found (μ M)	8.5	30.6
α -Ketoglutarate found (μ M)	3.0	Trace
Succinate found (μ M)	30.5	38.3

It is of interest to note that *coli-aerogenes* grown aerobically on either glucose or citrate, may possess the ability to degradate citrate to form acetate and oxalacetate, the latter subsequently being decomposed to pyruvate and CO₂.

Effect of Triphosphopyridine Nucleotide on Degradation of Citrate. It has already been demonstrated by Ajl et al. that the cell extracts obtained from the bacteria of genus *Escherichia* such as *E. coli* and *E. freundii* produce α -ketoglutarate from citrate or isocitrate.^{8,9,25)}

The present authors investigated the influence of addition of TPN on the degradation of citrate by cell-free extracts of *coli-aerogenes*. In order to obtain bacterial cells with a high citritase activity, strain B-T was cultured according to the method of Takahashi et al.¹¹⁾, who found that when *Bact. succinicum* grown aerobically on citrate-bouillon was again incubated in the presence of glucose and citrate under anaerobic conditions, the activity of citrate-splitting enzyme (citritase) was remarkably increased. In the present investigation, *coli-aerogenes* (B-T strain) was grown on a shaker at 30°C for 20 hours in a medium containing 3% Na-citrate, 1% bouillon, 0.1% KH₂PO₄, 0.3% (NH₄)₂SO₄, and 0.05% MgSO₄·7H₂O and thereafter incubated at 37°C for 15 hours in the presence of 1% glucose under static conditions (citrate-glucose grown cells). The cells obtained have been observed to degradate citrate to evolve CO₂ under anaerobic conditions. The cell extracts were

prepared with a sonic oscillator in the same manner as mentioned above. Incubations were carried out anaerobically at 37°C for 4 hours in either the presence or absence of addition of TPN with the reaction mixture (15 ml) containing 2.5 mM phosphate buffer (pH: 5.7~7.3), 60 μ M MgSO₄, 100 μ M Citrate and 3 ml fresh cell extracts (obtained from 30 mg cells). Table II shows

TABLE II. EFFECT OF ADDITION OF TPN ON ANAEROBIC DEGRADATION OF CITRATE BY CELL FREE EXTRACTS OBTAINED FROM CITRATE-GLUCOSE-GROWN CELLS OF *Coli-aerogenes* (B-T) STRAIN

Addition of TPN (γ /ml)	None	30	30
Initial pH	5.7	5.7	7.3
Keto- acids found after 4 hours' incubation			
Pyruvate (μ M)	7.2	5.7	5.5
Glyoxylate (μ M)	3.8	1.1	Trace
α -Ketoglutarate (μ M)	1.2	5.0	5.8

that considerable amounts of glyoxylate and pyruvate in addition to a small amount of α -ketoglutarate are produced during the anaerobic fermentation of citrate in the absence of added TPN, whereas an increasing amount of α -ketoglutarate against decreasing amount of glyoxylate is observed in its presence. Thus, it is evident that the bacterial strain employed here possesses an enzyme isocitric dehydrogenase dependent to triphosphopyridine nucleotide.

Degradation of Citrate by Intact (Washed) Cells of *A. aerogenes*. Table III shows the results of experiments with the intact (washed) cells of *A. aerogenes* grown on citrate medium in the same manner as already mentioned. The reaction mixtures contained 2~5 mM

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TABLE III. STATIC INCUBATION OF WASHED CELLS OF *A. Aerogenes* GROWN ON CITRATE-MEDIUM

Growth-conditions	<i>A. aerogenes</i> (B-2) grown aerobically for 1 day on 3% citrate-1% peptone	<i>A. aerogenes</i> (B-2) grown aerobically for 1 day on 3% citrate-1% peptone and thereafter incubated for 15 hours in presence of 1% glucose under static conditions		
		390	320	320
Washed cells (mg)	370	390	320	320
Citrate added (μ M)	1000.0	1000.0	1000.0	1000.0
Semicarbazide added (mM)	4.5	4.5	3.0	0
Total volume (ml)	50	50	35	35
Time of incubation (hours)	15	10	10	10
Glyoxylate found (μ M)	105.0	157.0	154.0	0
α -Ketoglutarate found (μ M)	Trace	Trace	Trace	Trace
Acetate found (μ M)			Trace	780.0

phosphate buffer (pH: 7.5~7.8), 50 μ M $MgSO_4$ and the amounts of citrate (Na-salt), semicarbazide and washed cells as indicated in Table III, and the reactions were carried out statically at 37°C. From Table III it will be seen that the intact cells of *A. aerogenes* (B-2) grown on citrate excrete a large amount of glyoxylate in the external medium as a degraded product of citrate under conditions where semicarbazide is present, whereas, acetate appears in place of glyoxylate in the external medium where the trapping agent is absent.

On the other hand, the oxidations of varied substrates were investigated with the intact cells of *A. aerogenes*. Incubation mixtures (50 ml) contained 5~10 mM Phosphate buffer (pH: 6.2), 50 μ M $MgSO_4$ and the amounts of substrates (Na-salts) and washed cells of *A. aerogenes* grown in the conditions as indicated in Table IV; the reactions were carried out at 30°C on the shaker. The results are shown in Table IV, from which it is observed that a remarkable excretion of α -ketoglutarate takes place in the external medium if the intact cells of *A. aerogenes* are aerobically incubated in the presence of pyruvate plus acetate or succinate plus pyruvate, while little or no excretion of α -ketoglutarate occurs in the presence of citrate without semicarbazide. These results appear to indicate that the major oxidation of citrate by the intact cells of *A. aerogenes* does not proceed by way of the conventional tricarboxylic acid cycle (citrate $\rightarrow$ α -ketoglutarate $\rightarrow$ succinate), but occurs by way of a splitting reaction in which isocitritase is concerned. It should

now be noted that the oxidations of C_3 and C_4 -acids give rise to a large amount of α -ketoglutaric acid, that is, the oxidations of these substrates proceed toward the formation of α -ketoglutarate. Accordingly, it has strongly been suggested that the mode of the major oxidation of C_3 - and C_4 -acids including pyruvate, lactate, succinate, fumarate, malate and oxalacetate may be of a different type from that of tricarboxylic acids.

Degradation of Citrate by Dry- and Ground Dry-cells of *Coli-aerogenes*. The results mentioned above were again tested with both dry- and ground dry-cells of *coli-aerogenes*. The cells of *E. coli* (G-2 strain) grown on a glucose-medium and those of *A. aerogenes* (B-2 strain) and *coli-aerogenes* (B-T strain) grown on a citrate-medium were dried by an electric fan at room temperature for 5 hours. Ground cells of the bacteria were prepared by the methods mentioned in the previous paper²⁴. Both anaerobic and aerobic degradations of citrate by these treated cells were carried out at 30°C in the presence and absence of externally added semicarbazide.

In both cases, the oxidation of pyruvate plus acetate by the treated cells was tested for the sake of comparison. The results are shown in Tables V and VI, from which it has been ascertained that a remarkable accumulation of glyoxylate takes place in addition to the formation of pyruvate when the dry- and ground dry-cells (250~800 mg) of *coli-aerogenes* are incubated with the

TABLE IV. OXIDATION OF VARIED SUBSTRATES BY WASHED CELLS OF *A. aerogenes* GROWN ON CITRATE-MEDIUM

Growth-conditions	<i>A. aerogenes</i> (B-2) grown aerobically for 2 days on 3% citrate-1% peptone		<i>A. aerogenes</i> (B-2) grown aerobically for 2 days on 3% citrate-1% bouillon -0.5% yeast extract		<i>A. aerogenes</i> (B-2) grown aerobically for 1 day on 3% citrate-1% bouillon and thereafter incubated statically for 15 hours in presence of 1% glucose	
	Citrate	Pyruvate	Citrate	Pyruvate	Pyruvate	Citrate
Substrates against which washed cells were tested (μ M)	1500.0	3000.0 plus acetate 2000.0	1500.0	2000.0 plus acetate 1000.0	2000.0 plus succinate 1000.0	1500.0
Bacterial cells (mg)	100	100	270	270	320	320
Semicarbazide added (mM/50 ml)	0	0	4.5	0	0	0
Time of incubation (hours)	13	13	6	6	5	5
Consumption of substrates (%)	100	100		100	100	100
Pyruvate found (μ M)	0	0	8.1	0	0	0
Glyoxylate found (μ M)	0	0	5.1	0	0	0
α -Ketoglutarate found (μ M)	Trace	510.5	9.9	202.5	555.6	50.3

TABLE V. AEROBIC DEGRADATION OF PYRUVATE PLUS ACETATE AND CITRATE BY DRY—AND GROUND DRY—CELLS OF *E. coli* (G-2) GROWN AEROBICALLY ON GLUCOSE-AMMONIUM MEDIUM

Substrates added (μM)	Pyruvate plus acetate 1500.0	1000.0	Citrate 500.0	Citrate 500.0	Citrate 500.0
Bacterial cells of <i>E. coli</i> (mg)	Dry-cells 300		Dry-cells 300	Dry-cells 300	Ground Dry-cells 800
Semicarbazide added (mM)	0		4.5	0	4.5
Reaction mixtures (initial pH)	6.3		7.2	7.2	7.4
(ml)	50		50	50	50
Time of incubation (hours)	5		5	5	5
Glyoxylate found (μM)	0		14.8	0	35.5
Pyruvate found (μM)	200.0		4.4	0	25.4
	(consumed 1300.0)				
α -Ketoglutarate found (μM)	90.6		3.5	Trace	Trace

TABLE VI. DEGRADATION OF CITRATE BY GROUND DRY—CELLS OF *Coli-aerogenes*

Growth conditions	<i>Coli-aerogenes</i> (B-T) grown aerobically for 1 day on 3% citrate- 2% peptone medium		<i>Coli-aerogenes</i> (B-T) grown aerobically for 1 day on 3% citrate- 1% peptone and there- after incubated stati- cally for 15 hours in presence of glucose		<i>A. aerogenes</i> (B-2) grown aerobically for 1 day on 3% citrate -0.5% peptone		
	320	320	250	250	430	430	430
Citrate added (μM)	500.0	500.0	500.0	500.0	1000.0	1000.0	1000.0
Semicarbazide added (mM)	2.5	2.5	2.2	2.2	3.3	3.3	0
Reaction mixtures { (initial pH)	7.5	7.5	7.5	7.5	7.3	7.3	7.3
{ (ml)	30	30	25	25	45	45	45
Time of incubation (hours)	5	5	6	6	5	5	5
	Shaking	Static	Shaking	Static	Shaking	Static	Static
Pyruvate found (μM)	20.5	25.3	65.0	68.5	Trace	Trace	Trace
Glyoxylate found (μM)	76.5	102.0	68.0	72.0	237.0	216.5	Trace
α -Ketoglutarate found (μM)	Trace	Trace	Trace	Trace	Trace	Trace	Trace

media (25~50 ml) containing 2.5~5.0 mM phosphate buffer (pH: 6.3~7.5), 50~100 μM MgSO_4 , 2.2~4.5 mM semicarbazide and 500~1000 μM citrate under either aerobic or anaerobic conditions. In the case of the treated cells of strain B-T, the preparations have been ascertained to contain citritase (citrate $\rightarrow$ oxalacetate + acetate), and therefore it has strongly been suggested that pyruvate detected during the degradation of citrate by the preparations may be derived from oxalacetate. Tables V and VI also show that the degradation of citrate by the treated cells results in yielding only a very small amount of α -ketoglutarate even under conditions in which a trapping agent such as semicarbazide is present. Thus, it has been concluded that a considerable portion of citrate is metabolized to glyoxylate and succinate or to oxalacetate and acetate by the action

of isocitritase and citritase respectively, rather than to succinate via the isocitric- α -ketoglutaric dehydrogenase system. It is already known that the following reactions proceed at a very slow rate: the oxidation of citrate by *coli-aerogenes* grown on a glucose-medium, and also the oxidation of α -ketoglutarate by the bacteria grown on either glucose- or citrate-medium.

Occurrence of Isocitritase in *Coli-aerogenes* Grown under Various Conditions. It has been reported by Smith and Gunsalus that when *E. coli* is grown with glucose as the source of energy, the bacterial cells formed are devoid of isocitritase, whereas, activity of the enzyme is high when the organism is grown aerobically with a medium containing acetic acid as a source of energy¹⁷⁾.

However, from the experiments described in the pre-

ceding and in the present papers, it will be clear that *E. coli* grown aerobically on a glucose-medium actually possesses isocitritase, and it thus appears that the low activity of isocitritase corresponds to a low potency of metabolizing (oxidizing) citrate under these conditions.

Subsequently, the authors have examined the activity of isocitritase in *coli-aerogenes* obtained under various growth-conditions. The results are shown in Table VII, in which experiments were carried out with various preparations of the bacteria such as dry- and ground-cells, and cell-free extracts in the same manner as already mentioned. It can be seen that isocitritase exists in all of the bacterial cells examined, the growth-media

used being as follows: bouillon-, peptone-, glucose-, acetate plus bouillon-, succinate plus bouillon-, and citrate plus bouillon (peptone)-media. It should be noted that *coli-aerogenes* grown anaerobically on a citrate plus bouillon-medium possesses considerable activity of isocitritase.

SUMMARY

1) Glyoxylate and succinate were formed in addition to pyruvate and α -ketoglutarate, when the cell free extracts of *coli-aerogenes* grown on either glucose or citrate were incubated in the presence of citrate.

TABLE VII. OCCURRENCE OF ISOCITRITASE IN *Coli-aerogenes* BACTERIA

Bacterial strains (Strain No.)	Growth-media	Enzyme preparations	Semicarbazide added (μ M/ml)	Glyoxylate formed by 100 mg cells per hour at 30-37°C (μ M)
<i>E. coli</i> (G-2)	{ 2% Glucose- NH ₃ -N aerobically	Dried cells	50~100	0.6~3.0
		Cell-free extracts	0	0.3
<i>A. aerogenes</i> (B-2)	{ 3% Citrate- 0.5% peptone aerobically	Cell-free extracts	0	4.0
		Dried cells	75	10.0
<i>A. aerogenes</i> (B-2)	{ 1% Peptone- 1% meat exts. aerobically	Dried cells	75	5.0
<i>Coli-aerogenes</i> (B-T)	{ 1% Peptone- 1% meat exts. aerobically	Sonically disintegrated cells	0	5.7
<i>Coli-aerogenes</i> (B-T)	{ 2% Peptone aerobically	Dried cells	75	3.0
<i>Coli-aerogenes</i> (B-T)	{ 2% Succinate- 1% peptone aerobically	Cell-free extracts	0	2.2
<i>Coli-aerogenes</i> (B-T)	{ 1% Acetate- bouillon aerobically	Cell-free extracts	0	10.0
<i>Coli-aerogenes</i> (B-T)	{ 3% Citrate- bouillon aerobically	Cell-free extracts	0	4.5
<i>Coli-aerogenes</i> (B-T)	{ 3% Citrate- bouillon statically	Cell-free extracts	0	2.0

2) The fresh cell extracts of *coli-aerogenes* revealed a very poor potency of producing α -ketoglutarate with citrate, whereas, the addition of coenzyme II (TPN) to the reaction mixtures resulted in an increase in formation of α -ketoglutarate from citrate.

3) It was found with *coli-aerogenes* that isocitritase existed in the various cells obtained from the following growth-media: bouillon-, peptone-, glucose-, acetate plus bouillon- succinate plus bouillon- and citrate plus bouillon (Peptone)- media.

4) Isocitritase was demonstrable, even when *coli-aerogenes* was anaerobically grown on a citrate-bouillon medium.

5) A large amount of glyoxylate was excreted in the external media, when not only the dried and ground cells of *coli-aerogenes*, but the intact (washed) cells of *A. aerogenes* were also incubated in the presence of citrate and a trapping agent

such as semicarbazide.

6) Isocitric dehydrogenase existed in the cells of *coli-aerogenes*.

7) From the results obtained in the previous and present papers, it was strongly suggested that the intracellular concentration of coenzyme II of *coli-aerogenes* might be relatively low, so that coenzyme II-dependent isocitric dehydrogenase could not be operative at such a high rate sufficient to effect the complete removal of citrate.

8) It was indicated that the major degradation of C_6 -tricarboxylic acids by *coli-aerogenes* took place by way of splitting reactions rather than depending on the isocitric- α -ketoglutaric dehydrogenase system.

9) With both C_3 - and C_4 -acids, α -ketoglutarate was again ascertained to be obtainable in a high yield.