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Excretion of Glyoxylate, Pyruvate and α-Ketoglutarate during Oxidation of Acetate

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Oxidations of several organic acids were investigated with the whole cells of *coli-aerogenes* grown aerobically on either citrate or acetate-medium. A considerable amount of α-ketoglutarate was obtained from acetate and lactate, when the washed cells were aerobically incubated in the absence of semicarbazide. This C_5-ketonic acid accumulated only in a very small amount during the oxidation of citrate by a citrate-grown cell. When the whole cells oxdized acetate in the presence of semicarbazide, a remarkable excretion of either pyruvate or glyoxylate occurred, under which conditions the accumulation of α-ketoglutarate was extremely decreased. The yields of pyruvate and glyoxylate from acetate were found to vary with the kinds of the bacterial strains. The oxidatons of both acetate and glycollate were also investigated with the dried cells. Mechanisms for microbial oxidation of acetate were propounded and discussed.

INTRODUCTION

In the previous papers[1~5], the authors have studied the chemical pathway of α-ketoglutaric acid-fermentation in *coli-aerogenes* bacteria. The mechanism for the bacterial respiration has also been studied, and it has been indicated that an enzyme, isocitritase catalyzing the degradation of isocitrate to glyoxylate and succinate may play an important role in the terminal oxidation of substrate-carbon[1,6,7]. Further investigations have revealed that a glyoxylic acid reductase-glycollic acid oxidase system may function in the respiration of microorganisms[8~10]. The present paper deals with the excretion of ketonic acids including pyruvate, glyoxylate and α-ketoglutarate occurring during the oxidation of substrates, especially acetate, by the whole cells of *coli-aerogenes*.

EXPERIMENTAL AND RESULTS

Methods. The organisms used : *Escherichia coli* (strain G-2) ; *Aerobacter aerogenes* (strain B-2) ; *Aerobacter cloacae* (strain A-c). These organisms were grown on a shaker at 30°C for 20-26 hours in the media containing 2.0% either Na-citrate or Na-acetate, 1.0% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl. Washed cells : Cells were harvested by a centrifuge, washed with and suspended in distilled water. Dried cells : Washed cells were dried by an electric fan at room temperature for 5-7 hours.

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Analytical procedures were performed by methods mentioned in the previous paper[9].

Oxidation of several organic acids by washed cells. The washed cells of *coli-aerogenes* grown on either citrate or acetate were incubated with several substrates. The typical results of experiments are shown in Tables 1 and 2. α-Ketoglutarate was obtained in a remarkable amount as the oxidation-products of substrates such as; acetate, lactate, pyruvate and C_4-dicarboxylic acids.

Table 1. Oxidation of acetate and citrate by washed cells of strain B-2 of *A. aerogenes* grown on citrate.

Vessel No.	I	II	III	IV		V
Substrates added	Acetate	Acetate	Citrate	Citrate plus acetate		DL-Lactate plus acetate
Inhibitors (*M*)	0	Semicarbazide 1×10^{-1}	0	0	α, α′-Dipyridyl 1×10^{-3}	0
Products (μmoles):						
Glyoxylate	0	8.5	0	0	0	0
Pyruvate	0	1.5	0	0	0	0
α-Ketoglutarate	17.8	3.2	3.5	21.0	3.0	78.5

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles $MgSO_4$, 2.0 m moles substrates (Na-salts), the indicated amounts of inhibitors and 110 mg (as dry weight) washed cells of *A. aerogenes*; total volume, 20 ml; 6 hours' incubation at 30°C on a shaker.

Table 2. Oxidation of acetate and citrate by washed cells of strain A-c of *A. cloacae* grown on citrate.

Vessel No.	I	II	III	IV	V
Substrates added	Acetate	Acetate	Acetate	Citrate	Citrate
Conditions	Anaerobically	Aerobically			
Semicarbazide added (*M*)	1×10^{-1}	0	1×10^{-1}	0	1×10^{-1}
Products (μmoles):					
Glyoxylate	0	0	3.0	0	1.5
Pyruvate	0	0	36.8	0	3.7
α-Ketoglutarate	0	35.7	5.8	2.5	2.1

Each flask contained 1.5 m moles phosphate buffer, pH 7.4 40 μmoles $MgSO_4$, 2.0 m moles substrates (Na-salts), the indicated amounts of semicarbazide and 185 mg (as dry weight) washed cells of *A. cloacae*; total volume, 20 ml; aerobically, on a shaker; anaerobically, Thunberg tube in vacuo; 4 hours' incubation at 30°C.

However, the formation of α-ketoglutarate was strongly inhibited in the presence of semicarbazide or α,α′-dipyridyl. On the other hand, the cells formed a large quantity of pyruvate from C_4-dicarboxylic acids such as succinate when incubated aerobically in the presence of semicarbazide.

Oxidation of acetate. In the previous papers of this series, the effect of semicarbazide has been investigated on both the anaerobic and aerobic degradation of C_6-tricarboxylic acids by the bacteria. In the present paper, the bacterial oxidation of acetate was carried out under the presence or absence of semicarbazida. From the results of experiment shown in Tables 1-3, it has been found

Table 3. Oxidation of acetate by washed cells of *coli-aerogenes* grown on acetate.

Bacteria used	A-c strain of *A. cloacae*		G-2 strain of *E. coli*	
Semicarbazide added (*M*)	0	1×10^{-1}	0	1×10^{-1}
Products (μmoles):				
α-Ketoglutarate	50.2	Trace	54.5	Trace
Glyoxylate	0	2.9	0	3.5
Pyruvate	0	12.5	0	2.0

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles $MgSO_4$, 1.5 m moles Na-acetate, indicated amounts of semicarbazide and 100 mg (as dry weight) washed cells of the bacteria; total volume 15 ml; 4 hours' incubation at 30°C on shaker.

that the washed cells are able to excrete three kinds of ketonic acids including glyoxylate, pyruvate and α-ketoglutarate when they oxidize acetate in the presence of semicarbazide. Under anaerobic conditions, little or no formation of ketonic acids took place, as shown in Table 2. No ketonic acids were also formed in a control without acetate.

Paper chromatography of the 2 : 4-dinitrophenylhydrazones showed that α-ketoglutarate was the sole ketonic acid produced during acetate oxldation in the absence of added semicarbazide, while a considerable amount of either pyruvate or glyoxylate was obtainable in the presence of the trapping agent, under which conditions α-ketoglutarate was strikingly decreased. The amounts of pyruvate and glyoxylate excreted were moreover observed to vary with the kinds of the bacterial strains : using B-2 strain of *A. aerogenes*, glyoxylate was detected in higher yield, whereas using A-c strain of *A. cloacae*, pyruvate detected in much higher yield. Table 4 shows the oxidation of acetate and

Table 4. Oxidation of acetate and glycollate by dried cells of *coli-aerogenes*.

Bacterial strains and growth-media	Dried cells of B-2 strain of *A. aerogenes* grown on citrate			Dried cells G-2 strain of *E. coli* grown on acetate		
Substrates added	0 (Endogenous)	Acetate	Glycollate	0 (Endogenous)	Acetate	Glycollate
Products (μmoles):						
Glyoxylate	Trace	11.5	9.8	Trace	4.5	22.8
Pyruvate	Trace	7.5	Trace	Trace	3.3	Trace
α-Ketoglutarate	Trace	Trace	Trace	Trace	Trace	Trace

Each flask ccntained 2 m moles phosphate buffer, pH 7.4, 50 μmoles $MgSO_4$, 2 m moles semicarbazide, 200 mg dried cells of indicated bacteria and 500 μmoles substrates (Na-salts); total volume, 20 ml; 4 hours' incubation at 30°C on shaker.

glycollate by the dried cells. It will be seen that the dried cell preparations possess the ability of producing glyoxylate and pyruvate from acetate.

The preparations also oxidized glycollate to yield glyoxylate under same conditions, indicating that glycollic oxidase was contained in the preparation. Properties of the bacterial glycollic oxidase have already been reported in the previous papers[8,10].

Subsequently, $^{14}CO_2$ was added to the reaction mixture and the incorporation of radioactivity into glyoxylate and pyruvate measured. Reaction mixture contained 2.5 mmoles phosphate buffer, pH 7.8, 40 μmoles $MgSO_4$, 500 μmoles Na-acetate,210 mg (as dry weight) washed cells of B-2 strain of *A. aerogenes* grown on acetate, 2 mmoles semicarbazide and 40 μmoles $NaH^{14}CO_3$-$Na_2{}^{14}CO_3$ (400,000 c.p.m.) ; total volume, 20 ml in 500 ml-shaking flask with a rubber plug ; incubation for 3 hours at 30°C on a shaaker.

After incubation, ketonic acids were obtained in the following yields : 3.0 μmoles of pyruvate and 3.5 μmoles of glyoxylate. However, little or no incorporation of $^{14}CO_2$ into these ketonic acids occurred. Thus, it appears that CO_2-fixation reaction is not concerned in the formation of both glyoxylate and pyruvate from acetate. Tables 5-7 show the effect of reagents on the oxidation of acetate. The yield of glyoxylate appeared to decrease in the presence of arsenite. Antibiotics including chloramphenicol and tetracycline pro-

Table 5. Effect of inhibitors on the formation of pyruvate and glyoxylate by washed cells of *A. aerogenes* grown on citrate.

Inhibitors added ($5\times10^{-3}M$)	0	NaF	$NaAsO_2$
Pyruvate found (μmoles)	1.4	1.9	3.2
Glyoxylate found (μmoles)	3.4	3.8	Trace

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles $MgSO_4$, 1.0 m mole Na-acetate, 1.2 m moles semicarbazide, 100 mg (as dry weight) washed cells of strain B-2 of *A. aerogenes* and inhibitors as indicated; total volume 20ml; 2 hours' incubation at 30°C on shaker.

Table 6. Effect of inhibitors on the oxidation of acetate by washed cells of strain G-2 of *E. coli* grown on acetate.

Inhibitors added (590 γ/ml)	0	Chloramphenicol	Tetracycline	Lederkyn*
Pyruvate found (μmoles)	1.56	1.39	2.23	1.70
Glyoxylate found (μmoles)	2.11	1.56	1.85	1.58

* Lederkyn=3-sulfanil amido-6-methoxypyridazine

Each flask contained 1.5 m moles phosphate buffer, pH 7.4, 40 μmoles $MgSO_4$, 2.0 m moles Na-acetate, 2 m moles semicarbazide, 95mg (as dry weight) washed cells of *E. coli* and inhibitors as indicated; total volume 17 ml; 5hours' incubation at 30°C on shaker.

Table 7. Inhibiting effect of antibiotics on the formation of α-ketoglutarate from acetate by washed cells of *E. coli* grown on acetate.

Antibiotics added (500 γ/ml)	0	Chloramphenicol	Tetracycline
α-Ketoglutarate found (μmoles)	48.9	15.7	14.3

Each flask contained 1.5 m moles phosphate buffer, pH, 7.4, 40 μmoles $MgSO_4$, 2.0 m moles Na-acetate, antibiotics as indicated and 100 mg (as dry weight) washed cells of strain G-2 of *E. coli* ; total volume, 15 ml; 3 hours' incubation in the absence of semicarbazide at 30°C on shaker.

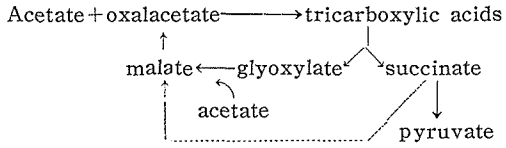
duced no remarkable inhibion on the formation of both glyoxylate and pyruvate but probuced a striking inhibition on the formation of α-ketoglutarate.

DISCUSSION

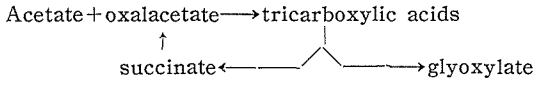
The authors' interest has been directed to the mechanisms for microbial oxidation of acetate. In the case of the organic acids-grown cells of *coli-aerogenes*, it may be considered that some portion of acetate is oxidized via ketonic acids including pyruvate, glyoxylate and α-ketoglutarate. Isocitritase has been demonstrated in the bacterial cells of *coli-aerogenes* grown with various carbon-sources, as already reported in the previous papers[1,6,7]. Thus, the excretion of both pyruvate and glyoxylate occurring during the bacterial oxidation of acetate, may be explained according to the following Reaction A, in which condensing enzyme[11], malate synthetase[12] and isocitritase are concerned.

Reaction system A :

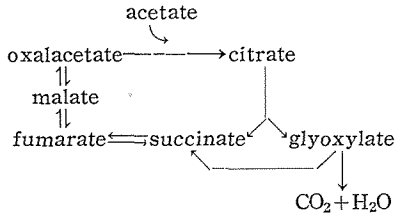
Pyruvate excretion by way of glyoxylate cycle



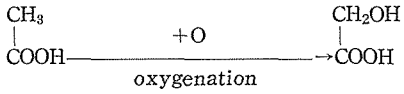
Glyoxylate excretion



The authors have recently found that the dialyzed cell-free extracts of *E. coli* were able to decompose glyoxylate under such conditions that triphosphopyridine nucleotide was absent, and succinate was anaerobically formed from glyoxylate[13]. Therefore, some portion of acetate may be oxidized to CO_2 and H_2O according to the following cycle.



On the other hand, the authors have presented the hypothesis that some portion of acetate may be metabolized to glycollate by way of an oxygenation reaction ; CH_3 of acetate being oxidized to CH_2OH by an oxygenase action.



The formation of either glyoxylate or pyruvate from acetate may also be explaind according to the following Reaction B.

Reaction system B :

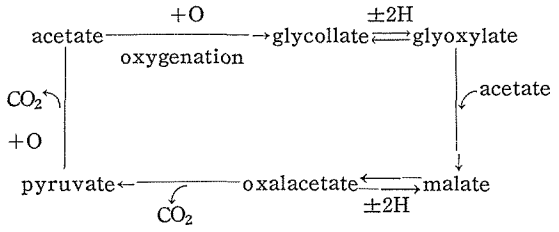
Glyoxylate excretion

$$\text{Acetate} \xrightarrow[\text{oxygenase}]{+O} \text{glycollate} \xrightarrow[\text{oxidase}]{-2H} \text{glyoxylate}$$

Pyruvate excretion

$$\text{Acetate} \longrightarrow \text{glycollate} \longrightarrow \text{glyoxylate} \xrightarrow{\text{acetate}} \text{malate} \longrightarrow \text{pyruvate}$$

Glycollic acid oxidase is already demonstrated in the bacterial cells of *coli-aerogenes*. This hypothesis implies that the terminal oxidation of two carbon fragments proceeds by way of the following cycle.



Sum ; $\text{Acetate} + 2O_2 \longrightarrow 2CO_2 + 2H_2O$

In this cyce, acetate is oxidized to CO_2 and H_2O by the combination of the following enzyme reactions : oxygenation of acetate to form glycollate, glycollic oxidase, malate synthetase, C_4-dicarboxylic acid system and pyruvic oxidase.

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REFERENCES

(1) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 22, 143 (1958).
(2) H. Katagiri, T. Tochikura and Y. Suzuki, *Bull. Agr. Chem. Soc. Japan*, 23, 322 (1659)
(3) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 24, 182 (1960).
(4) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 24, 188 (1960).
(5) H. Katagiri and T. Tochikura, This Bulletin, 38, 94 (1960)
(6) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 23, 475 (1959).
(7) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 23, 482 (1959).
(8) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 23, 558 (1959).
(9) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 24, 351 (1960).
(10) H. Katagiri and T. Tochikura, *Bull. Agr. Chem. Soc. Japan*, 24, 357 (1960).
(11) G. D. Novelli and F. Lipmann, *J. Biol. Chem.*, 182, 213 (1950).
(12) D. T. O. Wong and S. J. Ajl, *J. Am. Chem. Soc.*, 78, 3230 (1956).
(13) H. Katagiri and T. Tochikura, Unpublished.