Studies on the Microbiological Production of Ketonic and Amino Acids

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The bacteria of *coli-aerogenes* were found to produce a large amount of α-ketoglutaric acid as a major oxidation-product of carbohydrates under suitable conditions. α-Ketoglutaric acid-fermentation was demonstrated to be greatly affected by the varied factors. The mode of the bacterial respiration was studied and discussed. The process for producing glutamic and ketonic acids was also studied with the transaminase of various microorganisms.

INTRODUCTION

It is a well known fact that ketonic acids play a very important role in the metabolisms of microorganisms. Several investigators have reported the production of α-ketoglutaric acid from simple carbohydrates by various kinds of bacteria[1-13]. α-Ketoglutaric acid is obtained in good yields of 40-60% from glucose by the action of some bacterial strains. This oxidative fermentation has been termed "α-ketoglutaric acid-fermentation" in Japan. Great interest has recently been evinced in various parts of the world relating to the production of amino acids by enzymic or fermentative process. L-Glutamic acid has been reported to be produced from α-ketoglutaric acid by microorganisms (two-stage process)[14,15,44]. Another method for producing glutamic acid from simple carbohydrates and nitrogen substances has been studied[16-19] and in Japan, this amino acid is in part being produced by a one-stage fermentation process using a biotin-requiring strain of Micrococcus[18,19]. The present paper deals with the production of α-ketonic acids by fermentation of *coli-aerogenes* bacteria and also the preparations of L-glutamic and ketonic acids with the transaminases of various microorganisms.

EXPERIMENTAL AND RESULTS

Analytical Methods

Lactic acid was determined by the method of Friedemann *et al.*[20] and ethanol by Kolthoff's method[21]. Acetic acid and acetoin were estimated by the methods of Friedemann[22] and Van Niel[23], respectively. Ketonic acids were identified by paper-partition chromatography of the 2, 4-dinitrophenylhydrazones[24,25] and determined by modified methods of Carvallini *et al.*[24] and Friedemann *et al.*[26] Citric acid was determined according to the method

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of Kuratomi *et al.*[27] Amino acids were estimated by paper-partition chromatography[28] or with decarboxylase preparations[29][30]. Sugar determination was carried out in the usual manner by the Bertrand's method. Aeration efficiency was determined by the oxidation of sulfite in the presence of copper according to the mthod of Cooper *et al.*[31]

α-Ketoglutaric Acid-Fermentation

Conversion of the lactic acid-fermentation to α-ketoglutaric acid-fermentation. The bacteria of the genus *Escherichia* and the genus *Aerobacter* which are gram-negative, rod-shaped cells without endospores, ferment glucose and lactose; such as species *Escherichia coli, Escherichia freundii, Escherichia intermedia, Aerobacter aerogenes* or *Aerobacter choacae* considered to belong mainly to intestinal microorganisms. Moreover, it is a well known fact that these bacteria produce lactic, acetic, formic and succinic acids, ethylalcohol, acetylmethylcarbinol (acetoin), 2 : 3 butyleneglycol (butandiol), CO_2 and H_2 by the anaerobic fermentation of carbohydrates. The main anaerobic fermentation of glucose by the bacteria is given according to Harden[32] as follows :

$$2C_6H_{12}O_6+H_2O \longrightarrow 2CH_3\ CHOH\ COOH+CH_3\ COOH+C_2H_5OH+2CO_2+2H_2$$

In general, it is believed that the fermentation-products of carbohydrates by *coli-aerogens* bacteria vary with the type of their pyruvic acid metabolism. On the other hand, various carbon compounds such as glucose, pyruvate and acetate are recognized to be oxidized to CO_2 and H_2O by way of the bacterial respiration system as follows :

$$C_6H_{12}O_6+6O_2 \longrightarrow 6CO_2+6H_2O$$
$$CH_3COOH+2O_2 \longrightarrow 2CO_2+2H_2O$$
$$CH_3CO\ COOH+2.5O_2 \longrightarrow 3CO_2+2H_2O$$

In the course of investigating carbohydrate metabolisms, the authors found that by aerobic cultivation of *coli-aerogenes* bacteria by replacement of conventional anaerobic cultivation, remarkable changes took place in the ultimate fermentation-products, in other words, these bacteria produced a large amount of α-ketoglutaric acid from glucose in growing culture under aerobic conditions[40-42][53][54]. The bacteria of *coli-aerogenes* were grown in the media containing 2-5% glucose, 0.05-0.1% $(NH_4)_2HPO_4$, requisite amounts of $(NH_4)_2SO_4$, 0.1% KH_2PO_4 or K_2HPO_4, 0.5% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$ in water, to which sterilized $CaCO_3$ in an amount of 75% of glucose used was added at the time of inoculation. One hundred ml of the medium was dispensed in a 500 ml-shaking flask and sterilized. The inoculum was prepared by suspending a loop of stock culture in 5 ml of bouillon and incubated at 37°C for 20-40 hours under statical conditions. About 0.4-1.0 ml of this bouillon was used as the inoculum. After inoculation, the flasks were placed on a shaker of 110-120 r.p.m and incubated at 28°C for 5-6 days. Experiments under static conditions were performed for the sake of comparison. The results of experiments are shown in Tables 1 and 2. It will be seen that lactic acid-fermentation which occurs anaerobically by the action of *E. coli,* is converted into α-ketoglutaric acid-

Table 1. Fermentation products of *E. coli* under static conditions.

	Glucose consumed	Lactic acid formed	Acetic acid formed	Pyruvic acid formed	Ethanol formed
mg/*dl*	2000.0	900.0	205.7	20.0	128.0
% on consumed glucose		45.0	10.3	1.0	6.4

Incubation for 6 days at 28°C.

Table 2. Fermentation products by shaking culture.

Glucose used (%)	2.0	4.8
$(NH_4)_2HPO_4$ used (%)	0.05	0.1
$(NH_4)_2SO_4$ used (%)	0	0.1
Incubation for 5 days at 28°C		
Glucose consumed (%)	100.0	100.0
Lactic acid formed (%)	0	0
Acetic acid formed (%)	0	0
α-Ketoglutaric acid formed (%)	40.0	45.0
Pyruvic acid formed (%)	0	0

fermentation under aerobic conditions.

Isolation of α-ketoglutaric acid from cultivated media. A portion of the fermented liquor was filtered through the pulp of filter paper in order to remove bacterial cells and residual $CaCO_3$. The filtrate was treated with decolorizing carbon at 50°C and the transparent liquor was concentrated to a small volume under reduced pressure at 35°C. After being kept overnight, fine needle crystals of calcium α-ketoglutarate were obtained which were difficulty soluble in water. The calcium salt of α-ketoglutaric acid was suspended in warm water, acidified by H_2SO_4 or HCl and extracted with ether. Crystals of α-ketoglutaric acid were obtained from the ether extract. After recrystallization, a crystal which melted at 115-116°C was obtained. This crystal was verified by mixed melting point test with synthetic α-ketoglutaric acid. Moreover, α-ketoglutaric acid was isolated as 2, 4-dintrophenylhydrazone from the calcium salt. Recrystallization of 2, 4-dinitrophenylhydrazone was performed as follows: The hydrazone was dissolved in ethylacetate and then extracted with 10% Na_2CO_3 solution. When the carbonate extract was acidified by conc. HCl, a pale yellow crystalline precipitate was formed, and the precipitates were then crystallized from ethanol and water. After further recrystallization from ethylacetate, the crystals were dried in a desiccator over $CaCl_2$. Paper chromatography of this crystal showed only one spot corresponding to 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid. The melting point of this crystal was 220°C (decomp.), not depressed by the admixture with an authentic specimen. Results of analysis were as follows:

Found : C, 40.75 ; H, 3.06

Calcd. for $C_{11}H_{10}O_8N_4$; C, 40.49 ; H, 3.07

Production of acetic, pyruvic and α-ketoglutaric acids by shaking cultures of various strains of coli-aerogenes bacteria. Subsequently, the oxidative fermentation of glucose by shaking culture was investigated with various strains of *coli-aerogenes* bacteria. As it will be seen from Tables 3 and 4,

Table 3. Oxidation products with various species of *coli-aerogenes* types.

Strains of *coli-aerogenes*	*E. coli neapolitana*	*E. coli communior*	*Bact. friedlander*	*B. acidi lactici*	*Aerobacter aerogenes* B-1	*Aerobacter aerogenes* B-2
Glucose consumed (%)	100.0	100.0	100.0	87.3	88.6	90.0
Products (% on consumed glucose)						
Acetic acid	12.8	4.2	0	0	0	10.6
α-Ketoglutaric acid	19.7	29.3	44.4	36.7	29.6	24.2
Pyruvic acid	0	0	0	0	3.1	2.8
Acetoin	0	0	0	0	2.4	3.2

Incubation for 5 days at 28°C with the medium containing 2% glucose, 0.05% $(NH_4)_2HPO_4$ and other mineral salts.

Table 4. Production of acetic, pyruvic and α-ketoglutaric acids by *coli-aerogenes* types.

Organisms	*E. coli* 25E	*E. coli* 26S	*E. coli* 2E	*Bact. succinicum*
Glucose consumed (%)	100	80.0	71.6	90.0
Products (% on consumed glucose)				
Acetic acid	13.0	31.2		14.6
Pyruvic acid	0	6.6	16.6	41.4
α-Ketoglutaric acid	20.0	9.5	19.5	7.5

Cultivated for 6 days at 30°C on the medium containing 2.0% glucose, 0.05% $(NH_4)_2HPO_4$ and other mineral salts.

various strains of bacteria of *coli-aerogenes* types accumulated α-ketoglutaric acid as the oxidation-product of their fermentation, although remarkable difference was pointed out among the yields of α-ketoglutaric acid, since some strains of the bacteria produced none of the metabolic intermediates besides α-ketoglutaric acid, while acetic or pyruvic acid and acetoin were produced by other strains of the bacteria in addition to α-ketoglutaric acid. Resently Asai, Aida and Sugisaki[6] reported that *Bact. succinicum* produced a considerable amount of α-ketoglutaric acid and a small quantity of pyruvic acid in the course of oxidation of glucose, the maximum yields of α-ketoglutaric and pyruvic acids were 13.6% and 2.5% on glucose used, respectively. Table 4 shows that a high yield (41.4% on glucose consumed) of pyruvic acid was obtained by *Bact. succinicum* which belonged to *coli-aerogenes* bacteria and was isolated by Sakaguchi and Tada[33] in 1938. In other words, it is apparent

that pyruvic acid-fermentation occurs by the shaking culture of *Bact. succinicum*. Thus, many forms of oxidative fermentation of glucose exist in the metabolisms of the bacteria of *coli-aerogenes* types, as given below :

1) α-Ketoglutaric acid fermentation.
2) Acetic, pyruvic and α-ketoglutaric acids fermentation.
3) Acetic and α-ketoglutaric acids fermentation.
4) Acetoin and α-ketoglutaric acid fermentation.
5) Pyruvic acid fermentation.
6) Complete oxidation to CO_2 and H_2O.

It is worth to note that a lower yield of α-ketoglutaric acid was generally observed when the production of acetic acid was increasing.

Lockwood *et al.*[1] observed that 2-ketogluconic acid was produced in the early stage of fermentation of glucose by *Ps. fluorescens* and that 2-ketogluconic acid was then metabolized to α-ketoglutaric acid by the organism. As for *Serratia marsescens*, similar results were obtained by Asai and Aida *et al.*[5)10)] On the contrary, it was demonstrated by the authors that 2-ketogluconic acid could not be an intermediate product of α-ketoglutaric acid with *E. coli*, since, 2-ketogluconic acid was never detected at any stage of the fermentation. It was also found by the authors that a remarkable amount of α-ketoglutaric acid was already produced in the early stage (within 14 hours' culture) of fermentation of glucose by the bacteria of coli-aerogenes types.

Effect of nitrogen source upon production of α-ketoglutaric acid by *E. coli*. In the experiments shown in Tables 5 and 6, relations of the kind and the

Table 5. Influence of $(NH_4)_2HPO_4$ on the production of α-ketoglutaric acid.

Conc. of $(NH_4)_2HPO_4$ (%)	0.05	0.10	0.20	0.30
Glucose consumed (%)	70.0	100.0	100.0	100.0
α-Ketoglutaric acid produced (% on consumed glucose)	40.0	45.0	12.5	Trace
Bacterial cells formed (relative value)	1	2	6	7

Cultivated for 5 days at 30°C on 2% glucose medium.

Table 6. Influence of peptone on the production of α-ketoglutaric acid.

Conc. of peptone (%)	0.05	0.10	0.50	1.00	2.00
Glucose consumed (%)	50.9	78.2	85.2	90.0	100.0
α-Ketoglutaric acid produced (mg/dl)	528.2	777.2	204.4	152.4	Trace

Cultivated for 5 days at 28°C on 2% glucose medium.

amount of source of nitrogen to α-ketoglutaric acid-fermentation were investigated with 2% glucose-media. It will be seen in Tables 5 and 6 that the concentrations of nitrogen substances would be more important for the production of α-ketoglutaric acid than the kinds of nitrogen. At the low concentration of

nitrogen, the rate of fermentation was slow but α-ketoglutaric acid was obtained in high yields. On the other hand, at the high concentration of nitrogen, the rate of fermentation was observed to be much more rapid, and enormous growth of bacteria had taken place but the yields of α-ketoglutaric acid were found to be low or nil.

Effect of vitamin B_1 on pyruvic and α-ketoglutaric acids-fermentation. It was anticipated that vitamin B_1 would reveal a noticeable effect on the decarboxylation of pyruvic and α-ketoglutaric acids by the bacteria of *coli-aerogenes*, since vitamin B_1 was already ascertained to be a principal component of coenzymes relating to pyruvic and α-ketoglutaric oxidases. It can be seen

Table 7. Effect of vitamin B_1 on the production of pyruvic acid.

Exp. No.	1	2	3	4
Addition of B_1 (mg/dl)	0	0.5	1.0	3.0
Glucose consumed (%)	88.3	76.2	77.0	77.0
Acetic acid produced (mg/dl)	263.0	478.4	499.2	499.2
Pyruvic acid produced (mg/dl)	747.0	103.5	103.5	100.8
α-Ketoglutaric acid produced (mg/dl)	136.6	197.3	134.2	197.3

Oxidative fermentation of 2% glucose media by *Bact. succinicum*.

in Table 7 that when pyruvic acid-fermentation bacteria were cultivated aerobically at 30°C in the presence of B_1 with 2% glucose medium, the production of pyruvic acid was diminished remarkably and the production of acetic acid was very much accelerated, while any noticeable effect of vitamin B_1 was

Table 8. Influence of the concentration of glucose on the production of α-ketoglutaric acid.

Bacteria used	(strain No.)	Time of incubation (hours)	Initial conc. of glucose (%)	Glucose consumed (g)	α-Ketoglutaric acid produced (% on consumed glucose)
Escherichia species	2C	84	5.0	4.94	42.9
	2C	46	5.0	4.50	42.3
	B24	60	5.0	4.70	43.6
	B25	60	6.0	5.58	39.8
	B24	60	5.0	4.70	44.7
	B24	120	8.0	7.61	48.8
	B25	120	8.0	6.48	51.5
	2C	140	9.0	8.33	51.0
	7E	115	9.0	9.00	33.2
	8E	115	9.0	8.73	34.4
	9E	115	9.0	9.00	41.8
Aerobacter cloacae	AC	110	7.5	7.00	42.4

never observed on α-ketoglutaric acid-fermentation.

Influence of concentration of glucose on production of α-ketoglutaric acid. The oxidative fermentation of the bacteria of *coli-aerogenes* types were carried out at 28-32°C with the media containing various concentrations (5-9 %) of glucose, 0.10-0.15% $(NH_4)_2HPO_4$, 0.10-0.20% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl and $CaCO_3$ corresponding to 75% of glucose used. The results of experiments are shown in Table 8. The facts that high yields (about 55-40% on consumed glucose) of α-ketoglutaric acid are obtained with such media of simple composition containing 5-9% glucose and some other mineral matters by about 45-140 hours' incubation will indicate the possibility to industrialize the production of α-ketoglutaric acid by fermentation. α-Ketoglutaric acid is readily obtained from the fermented liquor as its calcium salt when initial amount of glucose exceeds 4% concentration.

The yield of α-ketoglutaric acid may be given according to the following equation, based on the experimental results mentioned above.

$$3C_6H_{12}O_6 + 10O_2 \longrightarrow 2C_5H_6O_5 + 8CO_2 + 12H_2O$$

Molar yield; about 0.667

(% on cosumed glucose; about 54)

Production of α-ketoglutaric acid from various carbohydrates by *E. coli*. *E. coli* (G-2 strain) was grown on a shaker for 4-5 days with the media (100 ml) containing 3-5% various carbohydrates, 0.05-0.10% $(NH_4)_2HPO_4$, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.2% NaCl and 0.05% $MgSO_4 \cdot 7H_2O$ in tap water, to which sterilized $CaCO_3$ corresponding to 75% of carbon-sources tested was added at the time of inoculation. The results of experiments are shown in Table 9, from which it will be seen that α-ketoglutaric acid was produced with all carbohydrates tested; maltose, lactose, sucrose, Koji ext. D-glucose, D-fructose, D-mannose, D-mannitol, D-galactose, L-rhamnose, D-xylose, L-arabinose

Table 9. Production of α-ketoglutaric acid from various carbohydrates by *E. coli*.

Carbon-sources (%)		Time of incubation (days)	α-Ketoglutaric acid found (g/100ml)	Pyruvic acid found
Maltose	5.0	5	0.98	−
Lactose	5.0	5	0.99	+
Sucrose	5.0	4	0.49	+
Koji ext.	4.8 (as glucose)	4	1.25	−
D-Glucose	5.0	4	2.29	−
D-Fructose	5.0	4	2.15	−
D-Mannose	5.0	4	1.55	−
D-Mannit	4.0	4	0.58	−
D-Galactose	4.0	4	0.54	+
L-Rhamnose	5.0	5	0.88	−
D-Xylose	4.0	4	0.48	++
L-Arabinose	4.0	4	0.65	++
Glycerol	3.0	4	0.57	−

and glycerol, but remarkable difference was pointed out among the yields of of α-ketoglutaric acid, high yields being obtained with glucose, fructose and mannose.

Production of pyruvic acid from glucose. The oxidative fermentation of glucose by *coli-aerogenes* actually gives rise to a large amount of pyruvic acid in the following cases. Firstly, in the case of some strains of *coli-aerogenes*, pyruvic acid is obtained in a high yield of about 10-40% on consumed glucose. The authors gave them the term "bacteria inducing pyruvic acid-fermentation". Secondly, in the case of the others, pyruvic acid is obtainable as a main fermentation-product under such conditions that the oxidation of glucose proceeds in the presence of arsenic compounds including Na-arsenite or Na-arsenate. α-Ketoglutaric acid-fermentation takes place in the absence of the inhibitors. This effect of arsenic compounds is shown in Tables 10 and 11.

The yield of pyruvic acid may be given according to the following equation.

Table 10. Influence of arsenic compounds on oxidation of glucose in growing culture of *E. coli*.

Addition	Na-arsenite		Na-arsenate	
	0	4×10^{-3} *M*	0	2.4×10^{-3} *M*
Products (molar yield)				
α-Ketoglutaric acid	0.47	0.30	0.61	0.22
Pyruvic acid	0	0.51	0	0.87
Acetic acid	0	0.09	0	0

Arsenic compounds were added after 1 day's fermentation.

Table 11. Influence of Na-arsenate upon oxidation of glucose by washed cells of *E. coli*.

Vessel No.	Addition of Na-arsenate (μ*M*)	Glucose consumed (μ*M*)	α-Ketoglutaric acid produced (molar yield)	Pyruvic acid produced (molar yield)
1a	0	842	0.71	0
1b	484	1000	0.27	0.70
1c	648	1000	0.24	0.80
1d	970	1000	0.20	0.93
2a	0	1000	0.76	0
2b	162	1000	0.57	0.27
2c	162	1000	0.45	0.56
2d	970	835	0.37	0.66

Reaction mixtures (7.5 ml) contained 0.5-1.0 m*M* phosphate buffer (pH : 6.2), 10 μ*M* $MgSO_4$, 150 mg $CaCO_3$, 1 m*M* glucose, requisite amount of Na-arsenite and 43-51 mg washed cells of *E. coli* grown aerobically on a glucose medium ; Incubation for 14 hours at 30°C.

$$C_6H_{12}O_6+8/3\ O_2 \xrightarrow{+As} 4/3\ C_3H_4O_3+2CO_2+10/3\ H_2O$$

On the whole, the amounts of increasing pyruvate are observed to be nearly equivalent to those of decreasing α-ketoglutarate. These facts indicate that the formation of pyruvate may actually be an intermediate stage in α-ketoglutarate-fermentation by the bacteria.

Enfluence of Ba^{++} on the production of α-ketoglutaric acid from glucose. Several workers have studied the influence of metallic cations on the formation and decomposition of citric acid in various biochemical systems. Citric acid is found in considerable quantities as the oxidation-product of acetic acid by the yeast, principally only in the presence of such metallic cations as Mg^{++}, Ba^{++}, Mn^{++} and Co^{++}[34-36]. Kalnitsky have found that $BaCl_2$ and $MgCl_2$ inhibit the utilization of citrate by the kidney cortex homogenates of rabbit[37]. It is generally believed that the metallic cations mentioned above inhibit the metabolism of citric acid, that is to say, the tricarboxylic acid cycle is inhibited by the presence of cations capable of forming insoluble citric salts (strontium, barium) or of forming complex citric salts (magnesium, cobalt). If α-ketoglutaric acid-fermentation by the bacteria of *coli-aerogenes* proceeded by way of the conventional tricarboxylic acid cycle, the presence of metallic cations should bring about the striking inhibition on the oxidative fermentation. In the present paper, α-ketoglutaric acid-fermentation of glucose by *E. coli* was investigated in the presence and absence of Ba^{++}. The growth-medium (100 ml) was composed of 7.0% glucose, 0.1% $(NH_4)_2HPO_4$, 0.1% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.2% NaCl, and the incubation with strain G-2 of *E. coli* was carried out on a shaker at 30°C for 4 days. The effect of Ba^{++} was examined with 6% $BaCO_3$ in place of 4% $CaCO_3$ as a neutralizing agent. The results of experiments are shown in Table 12, from

Table 12. Production of α-ketoglutaric acid from glucose in presence of barium carbonate or calcium cabonate.

Carbonate added (%)	Barium carbonate 6.0	Calcium carbonate 4.0
Glucose consumed (g/dl)	5.14	6.32
α-Ketoglutaric acid produced (g/dl)	3.00	3.08
(% on consumed glucose)	58.3	48.7

which it was ascertained that when strain G-2 of *E. coli* was aerobically grown on a glucose-medium, the production of α-ketoglutaric acid proceeded with a readiness even in the presence of $BaCO_3$, and higher yields of this ketonic acid on cosumed glucose was obtained in the presence of $BaCO_3$ than in the presence of $CaCO_3$. Thus, it has been suggested that the α-ketoglutarate-producing system of *E. coli* may be of a different type from that of yeast. The authors have also found an interesting fact that Ba-α-ketoglutarate is readily

soluble in water ; the Ba-salt in fermented broth is soluble even in about 3.5% concentration of the corresponding free acid, while the Ca-salt dissolves only in about 0.7-1.3% concentration. This process with barium carbonate as a neutralizing agent may be of practical importance for producing soluble salt of α-ketoglutaric acid on an industrial scale. The influence of barium carbonate on the metabolism of glucose in *E. coli* was also investigated with a growth-media containing a large amount of ammonium nitrogen such as ammonium chloride. It was observed that when *E. coli* was aerobically grown with a glucose-medium under high concentrations of nitrogen-source, the presence of $BaCO_3$ brought about an increase in the amount of free amino acids such as glutamic acid in the external media, revealing an inhibiting effect on the synthesis of bacterial cells.

Chemical pathway of α-ketoglutaric acid-fermentation. α-Ketoglutaric acid was observed to be produced without any accumulation of pyruvic acid in the course of oxidative fermentation of glucose by the washed cells of some strains of *E. coli* grown on a glucose-medium[43]. It has furthermore been found that the bacterial oxidation of pyruvic acid gives rise to a large quantity of α-ketoglutaric acid when the washed cells of *coli-aerogenes* grown on various carbon-sources are incubated aerobically with a Na-pyruvate solution containing phosphate buffer and $MgSO_4$. The yield of α-ketoglutaric acid amounted to about 0.5-0.7 mole per two moles of consumed pyruvic acid under suitable conditions[43)44)]. This conversion of pyruvic acid into α-ketoglutaric acid was also observed with the enzyme-preparations including the dried cells, the dry-ice treated cells or the cell-free extracts of *coli-aerogenes*[45]. These

Table 13. Production of α-ketoglutaric acid from pyruvic and lactic acids by *E. coli* grown aerobically on glucose-medium.

Bacterial preparations	Cells used (mg)	Time of incubation (hours)	Substrate consumed (m*M*)	α-Ketoglutaric acid produced (m*M*)	(molar yield)
Washed cells	148	13	Pyruvic acid 4,000	1.335	0.334
Washed cells	145	8	Pyruvic acid 2,000	0.593	0.296
Dried cells	195	14	Pyruvic acid 4,000	0.815	0.204
Cell-free exts.	1000	13	Pyruvic acid 1,600	0.165	0.103
Washed cells	150	7	D(−) Lactic acid 2,675	0.345	0.129

Reaction mixtures contained 5-10 m*M* phosphate buffer (pH : 6.0), 50-100μ*M* $MgSO_4$, requisite amounts of Na-salts of substrates and bacterial preparations of *E. coli* (strain G-2), and water to 30-50 ml ; Incubation on a shaker at 30°C.

Growth-medium : 2% glucose, 0.1% $(NH_4)_2HPO_4$, 0.3% $(NH_4)_2SO_4$, 0.1% K_2HPO_4 or KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% NaCl, 5 mg % $FeSO_4 \cdot 7H_2O$ and 1% $CaCO_3$; 1-2 days' incubation on a shaker at 30°C.

results are shown in Table 13.

In general, it is recognized by many researchers that *coli-aerogenes* bacteria oxidize pyruvic acid to form acetic acid and CO_2 according to the following equation :

$$CH_3 \cdot CO \cdot COOH + 1/2\ O_2 \longrightarrow CH_3 \cdot COOH + CO_2$$

From the results shown in Table 13, however, it has been demonstrated by the authors that the bacterial oxidation of pyruvic acid gives rise to α-ketoglutaric acid as the main product according to the following equation in which the synthesis of the carbon chain would necessarily be involved.

$$2CH_3 \cdot CO \cdot COOH + O_2 \longrightarrow HOOC \cdot CO \cdot (CH_2)_2 \cdot COOH + CO_2 + H_2O$$

This indicates that pyruvic acid is a potent precursor in α-ketoglutaric acid fermentation. The bacteria of *coli-aerogenes* types are well known to produce lactic acid as their major end-product of anaerobic fermentation. Thus, it is of interest to ascertain what kind of oxidation of lactic acid would take place with the bacteria. Experiments were instituted on the growing culture containing glucose or glucose plus lactate as the sources of carbon, $(NH_4)_2HPO_4$ and $(NH_4)_2SO_4$ as the sources of nitrogen. The oxidation of lactic acid was also carried out with the washed cells of *E. coli*.

Table 14. Production of α-ketoglutaric acid from glucose or glucose plus lactic acid by growing culture of *E. coli* (strain G-2).

Source of carbon			Time of cultivation (days)	Consumption of glucose (%)	α-Ketoglutaric acid produced (mg/dl)
Conc. of glucose (%)	Conc. of lactic acid (%)	Optical form of free acid			
2.00	0		6	100.0	Trace
2.00	4.00	DL-	6	100.0	988.0
2.00	4.00	D(−)-	6	100.0	877.0
4.00	0		10	100.0	1395.0
4.00	4.00	DL-	10	100.0	3294.0

Nitrogen-source : 0.1% $(NH_4)_2HPO_4$ plus 0.1% $(NH_4)_2SO_4$.

Tables 13 and 14 show that *coli-aerogenes* bacteria possess an ability to synthesize α-ketoglutaric acid from lactic acid in good yields under aerobic conditions. The production of α-ketoglutaric acid by *coli-aerogenes* is moreover demonstrated with C_4-dicarboxylic acids of the tricarboxylic acid cycle. In this case, it is observed that the yields of α-ketoglutaric acid with C_4-dicarboxylic acids are not higher than that with pyruvic acid[46]. The bacteria of *coli-aerogenes* are capable of producing a small amount of α-ketoglutaric acid from acetic acid as a sole source of carbon under aerobic conditions. It should, however, be noted that much higher yield of this C_5-ketonic acid is obtained in the case of acetate-grown cells than in the case of glucose-grown cells. In view of the facts that C_3- or C_4-acids are potent precursors in the bacterial

Table 15. Influence of addition of acetate on production of α-ketoglutarate from C_3- or C_4-acids by *E. coli* grown aerobically on glucose-medium.

Bacterial cells	Time of incubation (hours)	C_3- or C_4-acids added (m*M*)		Acetic acid added (m*M*)	α-Ketoglutaric acid produced (m*M*)
245	15	Pyruvic acid	2.000	0	0.400
245	15		2.000	2.000	0.795
245	15		0	2.000	Trace
110	15	D(−)-Lactic acid	2.500	2.000	0.616
110	15		2.500	0	0.200
185	12	Oxalacetic acid	1.500	2.000	0.500
185	12		1.500	0	0.142

Reaction mixtures contained 5 m*M* phosphate buffer (pH : 6.0–6.2), 100 μ*M* $MgSO_4$, stated amounts of washed cells of *E. coli* (strain G-2) and Na-salts of substrates and water to 40–50 ml ; Incubation on a shaker at 30°C.

Table 16. Influence of addition-time of acetic acid on production of α-ketoglutaric acid from C_3- or C_4-acids by *E. coli* grown aerobically on glucose-medium.

Bacterial cells (mg)	Addition of C_3- or C_4-acids (m*M*)		Addition of acetic acid (m*M*)	α-Ketoglutaric acid produced (m*M*)
138	Pyruvate	3.000	3.000 (added before incubation)	1.160
138	Pyruvate	3.000	3.000 (added after 8 hours' incubation)	0.826
200	D(−)-Lactic acid	2.500	3.000 (added before incubation)	0.450
200	D(−)-Lactic acid	2.500	3.000 (added after 8 hours' incubation)	0.200
133	Succinate	2.000	2.000 (added before incubation)	0.653
133	Succinate	2.000	2.000 (added after 8 hours' incubation)	0.358

Reaction mixtures contained 5 m*M* phosphate buffer (pH : 6.2), 100 μ*M* $MgSO_4$, stated amounts of washed cells of *E. coli* (strain G-2) and Na-salts of substrates and water to 40–60 ml. Incubation for 21 hours on a shaker at 30°C.

production of α-ketoglutaric acid and are also oxidized to acetic acid, it is of interest to learn whether the yield of α-ketoglutaric acid from C_3- or C_4-acids would result in an increase in the presence of externally added acetate. From the results of experiments shown in Tables 15 and 16, it has been found that higher yields of α-ketoglutaric acid are obtainable when the oxidations of C_3- or C_4-acids by the cells of *E. coli* proceed in the presence of externally added acetate than when in its absence.

Recently, a number of studies on the microbial formation of α-ketoglutarate have been carried out and it has frequently been argued that this ketonic acid might be formed from citric acid by way of the conventional Krebs cycle. The authors have investigated the degradation of C_6-tricarboxylic acids by

Table 17. Production of α-ketoglutarate from pyruvic and tricarboxylic acids by washed cells of *A. aerogenes*.

Exp. No.	I	II	III	IV	V
Sources of carbon with which *A. aerogenes* was grown	Citrate	Citrate	Citrate	Citrate	Glucose
Substrates against which washed cells were tested	Citrate	cis-Aconitate	iso-Citrate	Pyruvate	Pyruvate
Bacterial cells (mg)	165	155	155	100	150
Substrates (m*M*) initial	1.068	0.690	1.000	4.000	4.000
Substrates (m*M*) consumed	1.068			4.000	4.000
α-Ketoglutarate produced (m*M*)	0.049	Trace	0	0.800	0.640
(molar yield)	0.046			0.200	0.160

Reaction mixtures contained 5–10 m*M* phosphate buffer (pH : 6.0) 100 μ*M* $MgSO_4$, requisite amounts of Na-salts of substrates and washed cells of *A. aerogenes* (strain B-2) grown aerobically on either glucose-ammonium or Na-citrate-peptone medium ; total volume 30–40 ml. Incubation for 10–15 hours on a shaker at 30°C.

Table 18. Aerobic degradation of pyruvate plus acetate and citrate by dry- and ground dry- cells of *E. coli* grown aerobically on glucose-ammonium medium.

Substrates added (μ*M*)	Pyruvate 1500.0 plus acetate 1000.0	Citrate 500.0	Citrate 500.0	Citrate 500.0
Bacterial cells of *E. coli* (mg)	Dry-cells 300	Dry-cells 300	Dry-cells 300	Ground dry-cells 800
Semicarbazide added (m*M*)	0	4.5	0	4.5
Glyoxylate found (μ*M*)	0	14.8	0	35.5
Pyruvate found (μ*M*)	200.0 (consumed 1300.0)	4.4	0	25.4
α-Ketoglutarate found (μ*M*)	90.6	3.5	Trace	Trace

Reaction mixtures contained 5 m*M* phosphate buffer (pH : 6.3–7.4), 100 μ*M* $MgSO_4$, requisite amounts of bacterial cells (strain G-2), Na-salts of substrates and semicarbazide ; total volume 50 ml. Incubation for 5 hours on a shaker at 30°C.

Table 19. Oxidation of several substrates by bacterial cells of *A. aerogenes* grown aerobically on citrate-peptone media.

Substrates added (μ*M*)	Citrate 1500.0	Pyruvate 3000.0 plus acetate 2000.0 Washed cells	Citrate 1500.0	Citrate 1000.0
Bacterial cells (mg as dry matter)	100	100	270	Dry-cells 430
Semicarbazide (m*M*)	0	0	4.5	3.3
Time of incubation (hours)	13	13	6	5
Pyruvate found (μ*M*)	0	0	8.1	Trace
Glyoxylate found (μ*M*)	0	0	5.1	237.0
α-Ketoglutarate found	Trace	510.5	9.9	Trace

Reaction mixtures contained 5–10 m*M* phosphate buffer (pH : 6.2–7.3), 50 μ*M* $MgSO_4$, requisite amounts of Na-salts of substrates and the bacterial cells of *A. aerogenes* (strain B-2) and semicarbazide ; total volume 45–50 ml. Incubation at 30°C on a shake.

Table 20. Influence of addition of coenzyme II upon anaerobic degradation of citrate by cell extract of *coli-aerogenes*.

Enzyme source	With cell ext. obtained from 60 mg cells of *E. coli* (G-2) grown aerobically on glucose		With cell ext. obtained from 20 mg of *A. aerogenes* (B-2) grown aerobically on citrate-bouillon	
Na-citrate added (μM)	100.0		70.0	
Coenzyme II added (γ/ml)	0	25	0	15
Time of incubation (min.)	240	240	150	150
Glyoxylate found (μM)	5.3	Trace	9.8	Trace
α-Ketoglutarate found (μM)	1.0	12.0	Trace	11.8

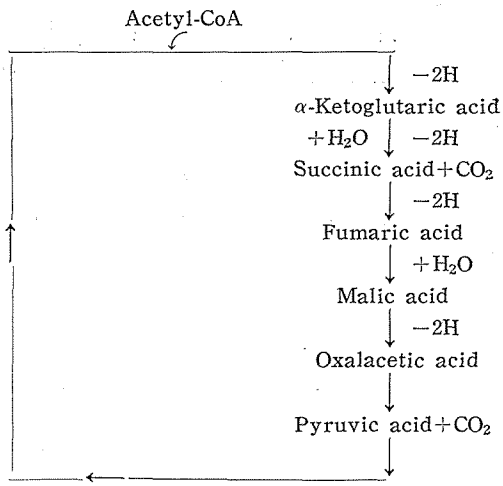
Reaction mixtures contained 500 μM phosphate buffer, pH 7.5, 15 μM $MgSO_4$ and indicated additions ; total volume 5 ml. Incubation at 37°C anaerobically.

coli-aerogenes under various conditions and the following facts have been elucidated (Tables 17-20)[46)49)50)].

(1) The intact cells of *A. aerogenes* grown on a citrate-medium are capable of oxidizing C_6-tricarboxylic acids with a readiness, but there is a very small amount of α-ketoglutarate to be observed, while under the same conditions the oxidative fermentation of pyruvate gives rise to a large amount of this C_5-ketonic acid. (2) Citric acid undergoes oxidative degradation by the treated (dried or ground) cells of *E. coli* grown on a glucose-medium, but there is not any noticeable amount of α-ketoglutaric acid as its degradation-product. (3) A large amount of glyoxylate is excreted in the external media, when not only the dried and ground cells of *coli-aerogenes*, but the intact (washed) cells of *A. aerogenes* are also incubated in the presence of citrate and a trapping agent such as semicarbazide. (4) Isocitritase is contained in the various cells of *coli-aerogenes* obtained from the following growth-media : bouillon-, peptone-, glucose plus ammonium nitrogen-, acetate plus bouillon-, succinate plus bouillon-, and citrate plus bouillon (peptone)-media. (5) The fresh cell extracts of *coli-aerogenes* reveal a very poor potency of producing α-ketoglutarate from citrate, whereas, the addition of coenzyme II(TPN) to the reaction mixtures brings about an increase in the formation of α-ketoglutarate from citrate. It should be noted that C_6-tricarboxylic acids is degradated to glyoxylic and succinic acids by the intact (washed) or the dried cell which is an organized system, that is, in which a directive function of enzyme exists just as it is.

This may indicate that coenzyme II available for isocitric dehydrogenase within the cells exists only in a small amount, so that the coenzyme dependent enzyme is not operative at such a high rate sufficient to effect complete removal of C_6-tricarboxylic acids while another enzyme isocitritase which is independent upon the coenzyme is operative at a high rate.

From the results mentioned above, the authors have concluded that the major production of α-ketoglutarate from varied carbonaceous substances including sugars, pyruvate, lactate and C_4-dicarboxylic acids, by the bacteria of *coli-aerogenes* does not proceed by way of the conventional tricarboxylic acid



Scheme 1. α-Ketoglutaric acid cycle.

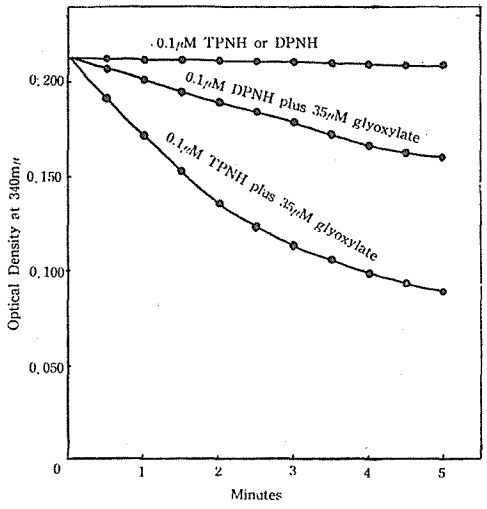


Fig. 1. Oxidation of TPNH and DPNH by glyoxylate in presence of glyoxylic acid reductase of *A. aerogenes*.

The reaction mixtures contained 250 μM phosphate buffer, pH 6.4, approx. 0.1 μM TPNH or DPNH, and 1 ml of the ammonium sulfate 0.4-0.7 saturation fraction (obtained from the cell extract of strain B-2 of *A. aerogenes* grown aerobically at 30°C for 20 hours on 1.5% Na-citrate plus 1% bouillon); the indicated amount of Na-glyoxylate was added at zero time; total volume, 3.0 ml. Oxidation of pyridine nucleotides was determined at room temperature spectrophotometrically at 340 mμ with a Beckman DU spectrophotometer, using 3 ml quartz cells, 1 cm light path.

cycle, but occurs by way of a certain reaction in which pyruvate and active acetate are concerned. This α-ketoglutaric acid-producing pathway has been termed "pyruvate-acetate reaction" by the authors. It has, moreover, been propounded by the authors that a new metabolic cycle might occur by the combination of C_4-dicarboxylic acid system and a pyruvate-acetate reaction. This cycle is termed the α-ketoglutaric acid cycle.

Consequently, it has strongly been suggested that the major oxidation of citrate by the bacterial cells of *coli-aerogenes* is accompanied with a cleavage-reaction (glyoxylate plus succinate formation), although a part of oxidation may proceed by way of the conventional tricarboxylic acid cycle, that is, isocitrate-α-ketoglutarate-succinate system. The authors have furthermore found the interesting fact that α-ketoglutaric acid is aerobically and anaerobically formed from C_6-tricarboxylic acids according to a coupling reaction between the microbial glyoxylic reductase and isocitric dehydrogenase, under such conditions that coenzyme II is present (Fig. 1)[52]. Glycollic oxidase, dependent upon flavin mononucleotide, has also been demonstrated in microorganisms such as *coli-aerogenes* bacteria and baker's yeast, and it has been pointed out that a glyoxylic reductase-glycollic oxidase system might function as a hydrogen carrier system in the respiration of microorganisms (Table 21)[52].

Table 21. Reactivation of glycollic acid oxidase apoenzyme of *E. coli* by flavin nucleotides.

Flavin nucleotides added (*M*/L)	Glyoxylate formed (μ*M*)
None	0.35
Riboflavin-5′-phosphate, 1×10^{-4}	2.45
Riboflavin adenine dinucleotide, 5×10^{-5}	2.36

Reaction mixtures contained 600 μ*M* phosphate buffer, pH 7.0, 500 μ*M* semicarbazide, 500 μ*M* Na-glycollate, 4 ml apoenzyme (prepared from strain G-2 of *E. coli* grown aerobically at 30°C for 20 hours on 1.5% Na-acetate plus 1% buillon) and the requisite amount of flavin nucleotides; total volume, 10 ml; 3 hours' incubation at 30°C on a shaker.

Koepsell investigated the oxidation of gluconic and 2-ketogluconic acids to α-ketoglutaric acid by *Pseudomonas fluorescens* and found that the oxidation of 2-ketogluconic acid was remarkably accelerated in the presence of a trace of inorganic iron such as ferrous ammonium sulfate[2]. The authors investigated the effect of αα′-dipyridyl or *o*-phenanthroline capable of forming Fe-complex compound upon α-ketglutarate-fermentation in the bacteria of *coli-aerogenes*[44)45)]. The production of α-ketoglutaric acid by the washed cells of *coli-aerogenes* from glucose was observed to be inhibited in the presence of αα′-dipyridyl. Table 22 shows the effect of αα′-dipyridyl and *o*-phenanthroline upon the oxidation of pyruvate by *coli-aerogenes*. It will be seen that both αα′-dipyridyl and *o*-phenanthroline extremely inhibt the bacterial synthesis of α-ketoglutarate from

Table 22. Influence of $\alpha\alpha'$-dipyridyl and *o*-phenanthroline on production of α-ketoglutaric acid from pyruvic acid by washed cells of *coli-aerogenes*.

(A) *E. coli*

Addition of inhibitiors (μM/40 ml)		$\alpha\alpha'$-Dipyridyl		*o*-Phenanthroline	
		0	30	0	25
Pyruvic acid (mM)	initial	3.500	3.500	4.000	4.000
	consumed	3.477	3.432	4.000	4.000
α-Ketoglutaric acid produced (mM)		0.927	0	0.815	0.142
Acetic acid produced (mM)		0	2.950	0	3.210

(B) *B. lactis aerogenes*

Addition of inhibitor (μM/25 ml)		*o*-Phenanthroline	
		0	25
Pyruvic acid (mM)	initial	4.000	4.000
	consumed	4.000	4.000
α-Ketoglutaric acid produced (mM)		0.660	Trace
Acetic acid produced (mM)		0.900	1.880
Acetoin produced (mM)		Trace	0.340

Reaction mixtures (25-40 ml) contained 1-5 mM phosphate buffer (pH : 6.0), 60-100 μM $MgSO_4$, 3.5-4.0 mM Na-pyruvate, requisite amount of inhibitors and 80-140 mg washed cells of *coli-aerogenes* grown aerobically on a glucose-medium ; incubation for 14 hours at 30°C.

pyruvate, while the consumption of pyruvate is not greatly affected and acetate or acetoin can be produced even in the presence of these inhibitors. In the case of the oxidation of pyruvic acid by *E. coli*, acetic acid-fermentation occurs by the addition of these inhibitors.

$$CH_3\cdot CO\cdot COOH + O \xrightarrow{\substack{\alpha\alpha'\text{-dipyridyl or} \\ o\text{-phenanthroline}}} CH_3\cdot COOH + CO_2$$

It may thus be concluded that inorganic iron is a kind of cofactor of the enzyme system relating to the synthesis of α-ketoglutarate from pyruvate. The authors have also studied the effect of various antibiotics including dihydrostreptomycin, chloramphenicol and tetracyclines upon the carbon-metablism of *coli-aerogenes*, and it has been found that α-ketoglutaric acid-fermentation is greatly influenced by the addition of these antibiotics[47)48)].

Influence of air-supply upon oxidation of carbonaceous substances by *coli-aerogenes*. The mode of metabolism of glucose in the bacteria of *coli-aerogenes* has already been known to depend upon the amount of available oxygen present in the media. Anaerobically, the synthesis of the bacterial cells occurs at their minimum levels and most of glucose-carbon is converted into lactate, formate, acetate, succinate, pyruvate, ethanol, acetoin, 2 : 3-butandiol, hydron gas and carbon dioxide. Aerobically, most of glucose-carbon is converted into α-ketoglutarate, carbon dioxide and the bacterial cells. Tables 23

Table 23. Degradation of glucose by washed cells of *E. coli* under static or shaking conditions.

Conditions of incubation	Static	Shaking at high efficiency of aeration (on a shaker)
Glucose consumed (m*M*)	2.500	2.500
Pyruvate found (m*M*)	Trace	0
α-Ketoglutarate found (m*M*)	0	1.520
Lactate found (m*M*)	1.985	0
Acetic acid found (m*M*)	1.270	0

Reaction mixtures (25 ml) contained 2 m*M* phosphate buffer (pH : 6.2), 30 μM $MgSO_4$, 400 mg $CaCO_3$, 2.5 m*M* glucose and 140 mg (as dry matter) washed cells of *E. coli* grown aerobically on a glucose-medium : static incubation in a 100 ml-flask or shaking in a 500 ml-flask on the shaker. Incubation for 14 hours at 30°C.

Table 24. Degradation of pyruvate by washed cells of *E. coli* under static or shaking conditions.

Conditions of incubation	Static	Shaking at high efficiency of aeration (on a shaker)
Pyruvate consumed (m*M*)	4.000	4.000
α-Ketoglutarate found (m*M*)	0	1.185
Acetate found (m*M*)	3.125	0
Formate found (m*M*)	Trace	0
Lactate found (m*M*)	0.135	0

Reaction mixtures (30 ml) contained 10 m*M* phosphate bufter (pH : 6.2), 100 μM $MgSO_4$, 4 m*M* Na-pyruvate, and 400 mg washed cells of *E. coli* grown aerobically on a glucose-medium ; Static incubation in a 100 ml-flask or shaking in a 500 ml-flask. Incubation for 7 hours at 30°C.

and 24 show the influence of the amount of available oxygen upon the mode of degradation of glucose or pyruvate by the washed cells of *E. coli*. The authors also investigated the bacterial metabolisms of several substrates under various oxygen-levels. Experiments were instituted on the growing cultures of *E.coli*. The organism was grown aerobically by bubbling a stream of sterile air into the cultural media by a compressed air cylinder, and the incubation was carried out with or without an agitator. Incubation on a shaker was taken for the sake of comparison. The results of experiments are shown in Table 25. It has been found that when *E. coli* is aerobically grown by bubbling a gentle stream of air into the media, a remarkable production of acetate occurs, the yield being about 24-33% on consumed glucose, but α-ketoglutarate is produced only in a very small amount. It is ascertained that *coli-aerogenes* are capable of inducing α-ketoglutarate-fermentation only under high levels of available oxygen, that is, at effective aeration rates of about 0.7-3.0 m*M* oxy-

Table 25. Oxidative fermentation of glucose in growing culture of *E. coli* under various aerobic conditions.

Method of aeration	Bubbling a stream of air without agitation		Agitation and bubbling a stream of air		Incubation on a shaker at 120 r.p.m.	
Effective aeration (m*M* oxygen per L. per min.)	0.08–0.11		3.0		0.9–1.3	
Volume of media (ml)	1000		8000		100	
Exp. No.	I	II	III	IV	V	VI
Initial conc. of glucose (%)	2.0	5.0	3.0	4.5	2.0	5.0
Conc. of $(NH_4)_2HPO_4$ (%)	0.10	0.10	0.10	0.10	0.05	0.10
Conc. of $(NH_4)_2SO_4$ (%)	0	0.10	0.10	0.10	0	0.10
Time of incubation (hours)	48	96	40	50	120	96
Consumption of glucose (%)	100	100	100	100	100	1000
Products found (% on consumed glucose)						
Acetate	32.5	31.5	0	0	0	0
Pyruvate	1.5	Trace	0	0	0	0
Lactate	15.7		0	0	0	0
α-Ketoglutarate	Trace	2.3	52.2	54.5	42.3	46.3

gen per liter per minute. Experiments were furthermore carried out with the conventional Warburg technique. The aeration efficiency of the Warburg apparatus employed here was about 0.10–0.35 m*M* oxygen per liter per minute under shaking conditions. *E. coli* was grown on a shaker for 24 hours at 30°C with a medium containing glucose and other mineral salts as already mentioned, collected by a centrifuge and washed with distilled water. The results of experiments are shown in Tables 26 and 27, from which it will be seen that when the shaking incubations of *E. coli* were carried out with the conventional Warburg apparatus, the organism oxidized substrates such as glucose, pyruvate and acetate to form carbon dioxide and water with a great readiness,

Table 26. Oxidation of glucose by washed cells of *E. coli*. With Warburg manometer under shaking conditions.

Glucose consumed (μ*M*)	O_2 absorbed (μ*M*)	CO_2 evolved (μ*M*)	α-Ketoglutaric acid found (μ*M*)
10.0	42.5	41.5	0
10.0	46.5	43.0	0
20.0	90.0	88.5	0
50.0	170.5		0.6

Reaction mixtures (2.5 ml) contained 100–500 μ*M* phosphate buffer (pH : 5.6), 4 μ*M* $MgSO_4$, 10–50 μ*M* glucose and 10–40 mg washed cells of *E. coli* grown aerobically on a glucose-medium, in the centre well, 0.2 ml H_2O or 15% KOH. Incubation for 2–6 hours at 30°C in air.

Table 27. Oxidation of pyruvate and acetate by washed cells of *E. coli*. With Warburg manometer under shaking conditions.

Substrates added (μM)	consumed (μM)	O_2 absorbed (μM)	CO_2 evolved (μM)	α-Ketoglutaric acid found (μM)
Na-Pyruvate				
10.0	10.0	20.5	21.0	Trace
20.0	20.0	31.1	30.5	0.1
60.0	60.0	87.5		1.5
Na-Acetate				
10.0		17.5	17.5	0
20.0		34.0	33.5	0

Reaction mixtures (2.5 ml) contained 100–500 μM phosphate buffer (pH : 6.0), 4 μM $MgSO_4$, indicated amounts of substrates and 5–40 mg washed cells of *E. coli* grown aerobically on a glucose-medium, in the centre well, 0.2 ml H_2O or 15% KOH. Incubation for 2–6 hours at 30°C in air.

but there was little or no formation of α-ketoglutarate. It was moreover ascertained that the bacterial oxidation of pyruvate with the Warburg apparatus brought about the accumulation of acetic acid during the early stage of fermentation, although this C_2-acid was oxidized to carbon dioxide and water at the end period of incubation. Investigations have also been directed towards the static incubation of *coli-aerogenes*. Tables 28 and 29 show the results of experiments carried out with the washed cells of the bacteria grown aerobically for 2 days at 30°C on a glucose-medium containing ammonium nitrogen and other mineral salts as already mentioned. The degradations of C_6-tricarboxylic

Table 28. Oxidation of C_3- and C_4-acids by washed cells of *E. coli*. With Warburg manometer under static or shaking conditions.

Conditions of incubation	Static		Shaking at very low-efficiency of aeration			
Substrates added (μM)	DL-Lactate 166.0	DL-Malate 200.0	Pyruvate 100.0	Pyruvate 100.0	DL-Lactate 166.0	DL-Malate 200.0
O_2 absorbed (μM)	55.6	30.5	93.0	93.0	67.4	41.0
CO_2 evolved (μM)	50.0	79.5		141.0	85.0	108.0
Acetate found (μM)	32.3		55.0	59.7	48.4	
Pyruvate found (μM)	Trace	0	0	0	0	0
α-Ketoglutarate found (μM)	0	0	0	0	0	0

Reaction mixtures (2.5 ml) contained 500 μM phosphate buffer (pH : 6.0), 2 μM $MgSO_4$, requisite amounts of Na-salts of substrates and 10–20 mg washed cells of *E. coli* grown aerobically on a glucose-medium, and in the centre well, 0.2 ml H_2O or 15% KOH. Incubation for 6–8 hours at 37°C in air.

Table 29. Oxidation of several substrates by washed cells of *E. coli* under static conditions.

Substrates added	Pyruvate	Acetate	Succinate	Fumarate
O_2 absorbed (μM)	35	21	41	36
CO_2 evolved (μM)	48	22	53	53
α-Ketoglutarate found (μM)	0	0	0	0

Reaction mixtures (2.5 ml) contained 300 μM phosphate buffer (pH: 6.0), 2 μM $MgSO_4$, 20 μM Na-salts of indicated substrates and 20 mg washed cells of *E. coli* grown aerobically on a glucose medium, and in the centre well, 0.2 ml H_2O or 15% KOH. Incubation for 7 hours at 37°C in air under static conditions.

Table 30. Oxidation of C_6-tricarboxylic acids by washed cells of *A. aerogenes* grown aerobically on a citrate-peptone medium. With Warburg-manometer.

	Substrates added (μM)	O_2 absorbed (μM)	Ketonic acid formed (μM as α-ketoglutrate)
Under shaking conitions ; 7.5 mg cells. Incubation for 2–7 hours at 30°C.	Citrate		
	10.0	29.4	0.9
	5.0	15.0	Trace
	cis-Aconitate		
	40.0	66.8	2.1
	20.0	43.0	1.2
Under static conditions ; 14 mg cells. Incubation for 2 hours at 30°C.	Citrate		
	20.0	5.0	0

Warburg vessel contained 150–300 μM phosphate buffer, 4 μM $MgSO_4$, requisite amounts of Na-salts of substrates and bacterial cells as indicated, in the centre well 0.2 ml 15% KOH ; total volume 2.5 ml ; gas phase, in air.

acids by washed cells of citrate-grown *A. aerogenes* were also investigated with the Warburg manometer under static or shaking conditions and the results are shown in Table 30. It has been found that *coli-aerogenes* bacteria are capable of oxidizing various substrates including acetate, pyruvate, lactate, C_4-dicarboxylic and C_6-tricarboxylic acids even under extremely low levels of available oxygen such as static conditions. The influence of a small amount of available oxygen (low efficiency of aeration) upon the bacterial production of α-ketoglutaric acid would, therefore, be explained as follows: both the oxidation of carbohydrates, C_3-acids such as pyruvic and lactic acids, and C_4-dicarboxylic acids to acetic acid and the complete oxidation of acetic acid into carbon dioxide and water can occur, with an extreme readiness, even under the low levels of available oxygen, so that the presence of a small amount of oxygen causes the deficiency in the precursors in α-ketoglutarate-synthesizing reaction. Moreover, it appears that α-ketoglutarate-synthesizing enzyme system

may be active under the high levels of oxygen. Consequently, it may be indicated by the authors that the terminal respiration in microorganisms does not proceed by way of the sole cycle, but the direction of aerobic respiration is, indeed, changeable according to variation of the environmental oxygen-level. Based on the experimental results mentioned above, it may also be suggested that the following respiratory systems: the conventional tricarboxylic acid cycle, the cleavage-system of C_6-tricarboxylic acids and the dicarboxylic acid cycle are operative even under low levels of oxygen, while α-ketoglutaric acid cycle is operative only under the high level of oxygen. Thus, experimental results hitherto mentioned may throw some light on the problem of the evolution of respiration-pattern in living organisms.

Production of L-Glutamic and α-Ketonic Acids by Microbial Transaminase

In the previous paper[44], the preparation of L-glutamic acid with microbial transaminases has been reported. According to this enzymic procedure, L-glutamic acid can be produced from hardly soluble amino-acids such as leucine, isoleucine, tyrosine, phenylalanine, valine, methionine, *etc.* and α-ketoglutarate. These hardly soluble amino-acids are produced abundantly as by-products in the commercial production of L-glutamic acid by hydrolysis of various proteins. They have relatively low solubility in water and are separated as crystals abundantly in the process of production of L-glutamic acid. However, these hardly soluble amino-acids have not been fully utilized heretofore and are generally used only as manure. On the other hand, α-ketoglutaric acid has become recently to be produced abundantly from varied carbonaceous substances by oxidative fermentation. The authors have demonstrated that hardly soluble amino-acids are suitable as the starting material in a process for producing L-glutamic acid by microbial transaminases.

The authors have, moreover, investigated the distribution and the kinds of transaminase of microorganisms, and pointed out the following facts:[55] (1) The occurrence of transamination reaction between α-ketoglutarate and aspartate has been demonstrated with the cells of a variety of microorganisms including yeast (*Saccharomyces cerevisiae*), *Mucor* (*M. racemosus, M. Rouxii, M. spinosus*), *Rhizopus* (*R. tonkinensis, R. chinensis*), *Aspergillus oryzae, Penicillium chrysogenum, Neurospora sitophila* and *Monilia candida.* However, there was an extremely low activity of transamination between α-ketoglutarate and hardly soluble amino-acids such as leucine and isoleucine to be observed. (2) Some strains of Actinomyces species were able to produce L-glutamate from α-ketoglutarate with aspartate or hardly soluble amino-acids such as leucine, isoleucine, phenylalanine and valine. (3) By use of *Flavobacterium, Achromobacter, Gaffkya, Pseudomonas, Escherichia* and *Aerobacter species,* a series of transaminase has been demonstrated which produces glutamate from α-ketoglutarate with the various amino donors such as aspartate, valine, leucine, isoleucine, tryptophan, tyrosine, phenylalanine and methionine. (4) In general, microbial cells with a high activity of glutamate-leucine transaminase have

been observed to reveal a high activity of transaminase between α-ketoglutarate and a variety of amino-acids including isoleucine, valine, methionine, phenylalanine and aspartate. On the other hand, a number of strains of microorganisms has been found which is able to catalyze glutamate-aspartate transamination but unable to catalyze glutamate-hardly soluble amino-acids transamination.

In the present paper, enzymic procedures are described for the preparations of L-glutamic acid and α-keto analogues of other amino-acids by microbial transamination.

Masuo and Wakisaka[14] reported the production of L-glutamic acid from α-ketoglutaric acid and alanine by the transaminase of *Bact. ketoglutaricum.* Recently, Aida and Asai have reported the preparation of L-tryptophan[38] and phenylalanine[39] by microbial transaminase.

Transamination catalyzed by various microorganisms. Experiments were at first carried out with the washed cells of Flavobacterium. The organism was grown at 30°C for 20 hours on a shaker with a medium containing 2% peptone and 0.2% NaCl. After cultivation, the bacterial cells were harvested with a centrifuge and washed once with distilled water. The washed cells (30 mg as dry basis) were anaerobically incubated at 37°C for 50 hours in the presence of toluol with the media containing 500 μM phosphate buffer pH 7.5, requisite amounts of Na-α-ketoglutarate and various amino-acids and water to make a final volume of 25 ml. The results of experiments are shown in

Table 31. α-Ketoglutarate-amino acids transamination by *Flavobacterium fuscum.*

Amino-donors (mM)		Na-α-Ketoglutarate added (mM)	L-Glutamic acid found (mM)
L-Leucine	1.25	0.70	0.50
L-Isoleucine	1.25	0.70	0.50
L-Phenylalanine	1.25	0.70	0.45
DL-Valine	1.25	0.70	0.45
L-Aspartate	0.63	0.35	0.27
L-Methionine	1.25	0.35	0.17
L-Tyrosine	0.63	0.35	0.15
DL-Tryptophan	0.63	0.35	0.11

Table 31, from which it has been found that the washed cells of *Flavobacterium fuscum* possess a high potency of catalyzing transamination reactions between α-ketoglutarate and a number of amino-acids including leucine, isoleucine, valine, methionine, phenylalanine, tyrosine, tryptophan and aspartate. It has furthermore been ascertained that the cultivated medium (peptone medium) as it is can be used as the source of transaminase without separating the organism from the aerobic cultivated medium. The occurrence of transami-

nation reactions between α-ketoglutarate and other amino-acids has also been demonstrated with several microorganisms. The results of experiments are shown in Tables 32-36. Microorganisms examined include *Flavobacterium*, *Achromobacter*, *Alkaligenes*, *Pseudomonas*, *Aerobacter* and *Escherichia* species.

Table 32. Production of L-glutamic acid from α-ketoglutarate and L-leucine by washed cells of some species of bacteria.

Species of bacteria	*A. aerogenes*	*Alkaligenes faecalis*	*Pseudomonas fluorescens*
Glutamic acid produced (g)	0.68	0.83	0.78

Reaction mixtures (pH: 7.6) contained 2.5 g L-leucine, 1.5 g (as free acid) Na-α-ketoglutarate, 50 mg washed cells and water to 50 ml. Incubation for 60 hours at 37°C in presence of toluol.

Table 33. Transamination with Ca-α-ketoglutarate.

Bacterial strain	*Escherichia coli* grown on glucose-ammonium nitrogen	*Flavobacterium fuscum* grown on peptone
Washed cells used (mg as dry matter)	350	50
pH	6.5	6.8
L-Glutamic acid found (mg)	233	270

Reaction mixtures contained 1000 mg L-leucine, 440 mg α-ketoglutaric acid (used as Ca-salt) and indicated amounts of bacterial cells, total volume, 100 ml; 65 hours' incubation statically at 37°C in the presence of toluol.

Table 34. Production of L-glutamic acid by transaminase of *Achromobacter* species.

With *Achromobacter butyri* :

	L-Leucine	L-Phenylalanine	DL-Methionine
Amino acids added (g)	1.00	0.83	1.49
α-Ketoglutaric acid added (g)	1.00	0.48	0.48
A. butyri cultivation medium (ml)	10	10	10
Water for making the total volume to 60 ml			
L-Glutamic acid produced by 25 hours' incubation at 37°C	0.37	0.17	0.19

With *Achromobacter candicans* :

	L-Leucine	L-Phenylalanine	DL-Methionine
Amino acids added (g)	1.00	0.83	1.49
α-Ketoglutaric acid added (g)	1.00	0.48	0.48
Water for making the total volume to 60 ml			
L-Glutamic acid produced by 25 hours' incubation at 37°C	0.45	0.19	0.24

Table 35. Production of L-glutamic acid from leucine and Na-α-ketoglutarate by *Flavobacterium* species.

L-leucine added (g)	α-Ketoglutaric acid added (g)	Cultivation media of bacteria (ml)	Total volume (ml)	Addition of toluol	Time of incubation (hours)	L-Glutamic acid produced (mg/ml)
1.00	0.90	7	50	+	50	10.3
2.50	2.10	20	50	+	15	13.6
6.10	6.10	80	100	+	16	23.9
4.00	3.50	20	100	+	65	16.5
1.60	1.60	10	30	+	25	16.5
2.00	1.60	15	50	−	60	18.7
2.50	1.50	15	50	−	60	19.0

pH ; 7.5-8.2 ; Temp, 37°C.

Table 36. Activity of Transaminase of several strains of bacteria.

Time of transamination (hours)	L-Glutamic acid produced by 1000 mg of bacterial cells grown aerobically at 28-30°C for 20 hours (g)	Yield of L-glutamic acid (% on α-ketoglutaric acid)
20-80	8-40	50-80

Some species of microorganisms mentioned here are found widely in sea water, water, air, soil, and various organic materials and are generally known to be harmful microorganisms pertaining to putrefaction. However, it has now been found that they can be utilized as useful microorganisms for the new process for the commercial production of L-glutamic acid.

Amino-donor. In the commercial production of L-glutamic acid by hydrolysis of protein, any one or the mixture of hardly soluble amino-acids are obtainable easily and abundantly as by-products. It is matter of significant practical value that the microorganisms mentioned above possess a high potency of producing L-glutamic acid from α-ketoglutaric acid with hardly soluble amino-acids, and the mixtures of amino-acids can also be advantageously used as a starting material for the transamination reaction.

Effect of temperature. Since the optimum temperature for the growth of of most of microorganisms as already mentioned is about 25-33°C, it is necessary to produce the microbial transaminase at the temperature of this range. It is, however, preferable to use relatively high temperature of the range from 37 to 45°C for the enzymic synthesis of L-glutamic acid, employing the cultivation medium of microorganisms as the source of transaminase in order to raise the reaction rate.

Effect of pH. Glutamic decarboxylase, an enzyme catalyzing the decarboxylation of glutamic acid to γ-aminobutyric acid are generally active when the reaction medium is weakly acid, while transaminase, enzyme catalyzing

the transamination between α-ketoglutarate and amino-acids reveal high activity in the range of pH from about 7.0 to 8.0. The solubility of hardly soluble amino-acids such as leucine, isoleucine etc. is higher in the side of weak alkaline than in the side of weak acid. Accordingly, it is ascertained that the range of pH of about 7.5-8.0 is most appropriate for the transamination reaction. The value of pH generally varies as the enzymic reaction proceeds and therefore so-called buffer solution is important. However, it has been established by the authors that there is no necessity of adding specially buffer solution such as phosphate solution to reaction liquid in the synthesizing reaction of L-glutamic acid. The adjustment of pH may be completely attained with NaOH, $NaHCO_3$ and the like.

Effect of NaCl. Amino-acids which are obtained as by-product in the process of the production of glutamic acid by hydrolysis of proteins, contain generally a substantial amount of sodium chloride. Since some strains of the microorganisms (*Flavobacterium, Achromobacter, Pseudomonas*) as mentioned above well resist to sodium chloride, the transamination reaction is not affected adversely by the presence of sodium chloride in the reaction liquid.

Source of transaminase. It is a well known fact that the living cells of microorganisms contain transaminase but at the same time other enzymes which are related to the metabolism (degradation) of amino-acids; such as amino-acid deaminase, amino-acid decarboxylase and amino-acid racemase. It has, however, been observed that by adding a small amount of toxic agent such as toluol, this tendency of degradation of L-glutamic acid by living cells can be effectively prevented. The addition of the toxic agent has also an advantage in preventing the invasion of miscellaneous harmful organisms. It has been ascertained by the authors that the microbial transaminase is not inhibited practically at all by the toxic agent to cells such as toluol. The addition of the toxic agent has significant practical advantage in that it eliminates entirely the necessity of troublesome and complicated operations such as drying, dehydration by solvent, freezing, sonic treatment and the like.

Table 37. Bacterial decomposition of L-glutamic acid under acidic conditions, (pH, 4.7).

Bacteria used	*Escherichia coli*	*Flavobacterium fuscum*
Residual L-glutamic acid (mg/dl)	79	150

Reaction mixture contained 10 ml washed cell-suspension of same concentration and 150 mg L-glutamic acid. Incubation for 5 hours at 37°C under static conditions.

The authors also selected some strains of microorganisms which could produce transaminase useful for the production of L-glutamic acid but produced little harmful enzymes relating to the degradation of glutamic acid. From the results shown in Table 37, it has been found that glutamic decarboxylase is contained in a cell of *E. coli*, but not contained in a cell of *Flavobacterium* species. By use of specific microorganisms, glutamic acid is obtainable in

good yield even under such conditions that the toxic agent such as toluol is absent. Some species of yeast may also be utilized as source of transaminase. Transamination reactions between α-ketoglutarate and several amino-acids have been observed with shoyu-brewing yeast such as *Zygosaccharomyces soya* and *Zygosaccharomyces major*. The results are shown in Table 38. In the previous paper[51], the authors have suggested that aspartase or the combined system of aspartase and transaminase might play a principal role in the assimilation of ammonium nitrogen in *coli-aerogenes*.

Table 38. Production of glutamic acid from α-ketoglutarate and several amino-acids by *Zygosaccharomyces soya*.

	L-Aspartic acid	DL-Valine	L-Isoleucine	L-Phenylalanine
Amino-acids added (mg)	78	69	65	83
Glutamic acid produced (mg)	32.2	21.1	21.0	22.0
	DL-Tryptophan	L-Leucine	L-Methionine	L-Tyrosine
Amino-acids added (mg)	70	65	70	40
Glutamic acid produced (mg)	10.0	25.5	18.0	10.0

Reaction mixtures (10 ml) contained 40 mg α-ketoglutaric acid, indicated amounts of amino-donors and 20 mg washed cells of yeast grown aerobically on a 3% Koji ext-medium containing 3% NaCl. Incubation for 4 days at 37°C in presence of toluol.

Preparation of α-keto analogues of amino-acids other than glutamic acid. The enzymic transamination between α-ketoglutaric acid and other amino-acids gives rise to glutamic acid and various ketonic acids other than α-ketoglutaric acid. The analogues (α-ketonic acids) of leucine and isoleucine are produced according to the following reactions.

$(CH_3)_2CHCH_2CH(NH_2)COOH$
leucine
or
$CH_3CH_2CH(CH_3)CH(NH_2)COOH$
isoleucine

$+\alpha$-ketoglutarate $\underset{\text{transaminase}}{\rightleftharpoons}$

$(CH_3)_2CHCH_2COCOOH$
α-keto-isocaproic acid
or
$CH_3CH_2CH(CH_3)COCOOH$
α-keto-β-methyl valeric acid

$+$glutamic acid

The reaction mixture after α-ketoglutarate-leucine (isoleucine) transamination was made acid and materials produced by heating were removed. The pH of the liquor was adjusted to about 6.0 and remaining hardly soluble amino acid which had been precipitated by concentration under reduced pressure was removed. Then the pH of the liquid was adjusted to 3.0-3.1 (near isoelectric point of L-glutamic acid) and the liquid was concentrated under reduced pressure according to conventional method to crystalize out L-glutamic

acid, which was then separated as crystals. The mother liquor was concentrated, made strongly acid. The separation of α-keto analogues of leucine or isoleucine may be effected by distillation under reduced pressure.

α-Keto-isocaproic or α-keto-β-methyl valeric acid was determined according to the method of Friedemann *et al.*,[26] using reaction with 2, 4-dinitrophenylhydrazine (incubation of the sample and 0.1% hydrazine solution for 3 minutes at 25°C), extraction of hydrazone by toluol, re-extraction by 10% Na_2CO_3 solution, addition of 4*N* NaOH and photoelectric determination of color density. Table 39 shows the production of L-glutamic acid and keto analogues from α-ketoglutaric acid and leucine or isoleucine by *Flavobacterium fuscum*.

Table 39. Production of L-glutamic and ketonic acids by transaminase of *Flavobacterium* species.

Starting materials (m*M*)		Products (m*M*)	
L-Leucine 38.2 α-Ketoglutarate 18.5		L-Glutamic acid 13.3	α-Keto-iso caproic acid 12.1
L-Isoleucine 38.2 α-Ketoglutarate 36.3		L-Glutamic acid 18.0	α-Keto-β-methylvaleric acid 15.1

SUMMARY

(1) During investigations on the metabolisms of glucose in the bacteria of *coli-aerogenes*, it was found that these bacteria accumulated a large amount of α-ketoglutaric acid under aerobic conditions. This indicated that lactic acid-fermentation was converted into α-ketoglutaric acid-fermentation. This ketonic-acid-fermentation also occurred when *coli-aerogenes* were aerobically incubated with various carbohydrates as a source of carbon.

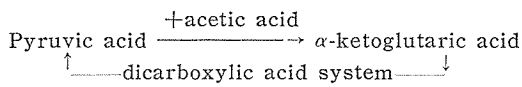
(2) The various types of oxidative fermentation of carbohydrates were described as follows: (i) α-ketoglutaric acid-fermentation; (ii) acetic, pyruvic and α-ketoglutaric acids-fermentation; (iii) pyruvic acid-fermentation; (iv) acetic (acetoin) and α-ketoglutaric acids-fermentation; (v) complete oxidation to CO_2 and H_2O. α-Ketoglutaric acid was obtained in a good yield (55-40%) from glucose in case of α-ketoglutaric acid-fermentation.

(3) The mode of the oxidative fermentation of glucose was ascertained to be greatly affected by the varied chemical factors.

(4)It was demonstrated that pyruvic acid was a potent precursor in α-ketoglutaric acid-fermentation.

(5) α-Ketoglutaric acid-fermentation was found to occur with various organic acids (C_2-, C_3-, C_4-acids) except C_6-tricarboxylic-acids.

(6) The chemical pathway of α-ketoglutarate-fermentation was studied and it was propounded that a cyclic mechanism might occur, according to which the oxidation of pyruvate or acetate proceeded, as given belows:



α-Ketoglutaric acid cycle

(7) The mode of the bacterial respiration was studied and it was elucidated that the direction of oxidative metabolisms of substrate-carbon was changeable according to variation of the environmental oxygen-level.

(8) The bacteria of *coli-aerogenes* were demonstrated to contain isocitritase and the mode of the bacterial degradation of C_6-tricarboxylic acids was studied.

(9) Two enzymes, glyoxylic reductase and glycollic oxidase were found in microorganisms and it was suggested that a glyoxylate $\rightleftarrows$ glycollate system might function in the respiration of microorganisms.

(10) Process for producing L-glutamic acid with microbial transaminase was studied.

(11) The distribution of transaminase in microorganisms was studied and some strains of bacteria including *Flavobacterium*, *Alkaligenes*, *Achromobacter*, *Pseudomonas* and *coli-aerogenes* were demonstrated to possess a high potency of producing L-glutamic acid from α-ketoglutaric acid with hardly soluble amino-acids such as leucine, isoleucine, tyrosine, phenylalanine, valine and methionine.

(12) Various factors influencing the production of L-glutamic acid were studied.

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