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**BIOCHEMICAL STUDIES ON  
SOYBEAN ROOT NODULAR BACTERIA**  
*Rhizobium japonicum*

By

**Ko SAWAI\***

**CONTENTS**

Introduction . . . . .	322
Chapter A. The presence of the TCA cycle and enzymic studies on its individual reaction . . . . .	325
Part 1. The presence of the TCA cycle . . . . .	325
Part 2. The oxidation of $\alpha$ -ketoglutaric acid by cell free extracts and its properties . . . . .	328
Part 3. The oxidation of succinic acid by a crude enzyme preparation . . . . .	332
Part 4. The oxidation of lactic acid . . . . .	338
Part 5. The oxidation of malic acid . . . . .	342
Part 6. The presence of fumarase and isocitric acid dehydrogenase . . . . .	347
Chapter B. The action of bacteria upon certain amino acids . . . . .	350
Part 1. The enzymic degradation of L-cysteine . . . . .	351
Part 2. Behavior toward aspartic acid . . . . .	358
Part 3. Deamination of several amino acids by the action of cell suspensions . . . . .	362
General discussion . . . . .	364
Conclusion . . . . .	366
Bibliography . . . . .	368

**Introduction**

HELLRIEGEL and WILFARTH<sup>(1)</sup> presented the first experimental proof that a large quantity of molecular nitrogen was fixed by the microorganisms living in leguminous root nodules. On the other hand, BEIJERINCK<sup>(2)</sup> succeeded in the isolation of these bacteria from nodules and obtained them in pure cultures. Either of these two studies might be considered as the most valuable one on biological nitrogen fixation in the latter half of the nineteenth century. Since then, many investigations have been carried out in this field. These researches

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may be divided into three main groups according to the scientific contents of the published papers :

1. Physiological and chemical studies on symbiosis.
  - (1) Chemical components of root nodule<sup>(2,4,5)</sup>.
  - (2) Cytological studies on nodule<sup>(6,7)</sup>.
  - (3) Relation between bacterial species and host plant<sup>(8,30)</sup>.
  - (4) Application as bacterial fertilizers<sup>(11)</sup>.
2. Various studies on bacteria themselves.
  - (1) Chemical components of bacterial cell<sup>(9,5)</sup>.
  - (2) Morphological studies on bacteria<sup>(10)</sup>.
  - (3) Studies on the respiration of bacteria<sup>(20-25)</sup>.
  - (4) Relation between bacterial growth and the constituents of media<sup>(12-14)</sup>.
3. Studies on the mechanisms of nitrogen fixation.

This subject closely relates to the present paper, and so the author wishes to explain several studies in detail. Concerning this problem, there are famous studies made by VIRTANEN and LAINE<sup>(15)</sup>, BURRIS and WILSON<sup>(16)</sup>, and CASTLE<sup>(17)</sup>. In spite of their efforts, the mechanism how molecular nitrogen is activated and ammonia is formed from it has not been clearly demonstrated. Recently, however, CASTLE<sup>(18)</sup> succeeded in obtaining preparations of cell-free extracts which had the ability of nitrogen fixation. Such an active preparation has not been obtained by any other investigator. Thus, it is expected that his study plays an important part as a key to solve these mechanisms. All the above studies have been made by the use of non-symbiotic microorganisms as the experimental material, for instance, *Clostridium pasteurianum*<sup>(26)</sup>, *Azotobacter vinelandii*<sup>(27)</sup>, *Nostoc muscorum*<sup>(28)</sup>, and so on. In the case of symbiosis it is impossible to demonstrate nitrogen fixation by a microorganism which is separated from its host plant. Some investigators suggest that the symbiotic organism supplies its host plant with nitrogen compounds and is given a certain kind of energy source by it, but at present a suitable method to distinguish between available energy for microorganism and that available for its host plant is not known. Thus, WILSON, et al.<sup>(19)</sup> stated that symbiotic nitrogen fixation might be more complicated and more difficult to understand than non-symbiotic fixation. Hence it would be rather reasonable and unavoidable in the present state of progress of investigation to consider the mechanism of the nitrogen fixation in symbiosis on the basis of the knowledge obtained from the investigations of the non-symbiotic fixation.

Reading through all the above-mentioned studies from the standpoint of

biochemistry, the author is led to think the following warrant consideration.

1. Bacteria live in specific circumstances, in other words, in nodules containing a kind of hemoprotein. Besides this, no hemoglobin homologue has ever been found in plants. Therefore KEILIN<sup>(28)</sup> considered this protein as an essential catalyst in the assimilation of atmospheric nitrogen. This view is supported by the fact that the fixation of nitrogen is inhibited by carbon monoxide, but any metabolic significances other than this inhibitory effect are not known.

2. By means of KJELDAHL method and tracer experiments  $N^{13}$  or  $N^{15}$  was used in nitrogen fixation by non-symbiotic bacteria, but not by symbiotic bacteria without their host plant.

3. Culture media containing comparatively large amounts of nitrogen compounds such as bouillon or broth are not suitable for the growth of bacteria.

So far as the author knows, there have not been many biochemical studies of symbiotic bacteria. BURK<sup>(29)</sup> reported that the respiration velocity of *Azotobacter*, a non-symbiotic bacteria, was much higher than that of other bacteria. In connection with the investigation of the respiration of *Rhizobium* species the existence of dehydrogenase<sup>(20-22)</sup>, catalase<sup>(23)</sup>, peroxidase<sup>(24)</sup>, and indophenoloxidase<sup>(25)</sup> in this microorganism was detected qualitatively in the experiments in which the cell suspension was used as an enzyme material. Perhaps, this may be due to the higher enzymic instability of cell-free preparations of *Rhizobium* species.

Now, the amount of nitrogen fixed by bacteria is nearly about  $10^7$  tons per year in entire world and this quantity is more than five times as great as the amount of nitrogen added to the soil as nitrogenous fertilizer. This fact is thought to indicate that biological nitrogen fixation plays a most important role in the practical questions of agriculture. However, there has been little progress in understanding the biochemistry of this process, as is stated above. From this standpoint, it is thought that investigations to find the biochemical and physiological properties of bacteria themselves are fundamental and important.

In the present investigation, the metabolic behavior of *Rhizobium japonicum* toward some organic acids and amino acids were studied with resting cells or cell-free preparations. Such an investigation seems to be most useful not only for detecting the individual reaction operating on the TCA cycle, but for making clear the process where amino acids are utilized by the above bacteria. The author wishes to report several new results about bacterial properties obtained from these experiments.

## Chapter A. The presence of the TCA cycle and the enzymic studies on its individual reaction

### Part 1. The presence of TCA cycle

ALLISON et al.<sup>(31)</sup> assumed that oxygen transfer from nodular tissue to *Rhizobium* cells would be controlled by certain factors, for instance, the partial pressure of oxygen. Although these bacteria are aerobic, they are forced to exist against the unfavorable condition of a small partial pressure of oxygen. As was described in the introduction, it is of importance to know how to build their respiratory systems in such a specific circumstance. This part deals with fundamental studies to find, know, and ascertain whether *Rhizobium japonicum* has a complete TCA cycle system or not.

### Experimental results and discussion

Effects of several organic acids on bacterial growth.

The following two methods were adopted. One is macroscopic observation of the colony and the other is nephelometry. In the former, *Rhizobium japonicum* was inoculated on the agar medium shown in Table 1 and incubated at 30°C for 3 days. Colonies were observed every day, and the results are shown in Table 2. In the latter, a loop of inoculum was transferred into 50 ml. of the culture solution (its composition was the same as in Table 1 except agar omitted), and incubated at 30°C for 3 days with intermittent shaking : the flasks

TABLE 1. Composition of Culture Medium

KNO <sub>3</sub>	0.2 g	FeCl <sub>3</sub> ·6H <sub>2</sub> O	trace
NH <sub>4</sub> Cl	0.2 g	MnSO <sub>4</sub> ·4H <sub>2</sub> O	trace
KH <sub>2</sub> PO <sub>4</sub>	0.5 g	*each organic acid	2 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	0.2 g	agar	25 g
NaCl	0.1 g	water up to	1,000 ml

The medium was adjusted to pH 6.5 with NaOH.

\* 17 organic acids were used, as was shown in table 2.

were shaken for 5 hr., allowed to stand for 15 hr., and successively the procedure of 10 hr. Shaking and 15 hr. standing were repeated twice. The turbidity of the culture solution on the third day was measured by use of ERMA's N-type Nephelometer. The data are presented in Table 2.

Effect of organic acids on reduction of methylene blue and oxygen consumption.

*Rhizobium japonicum* was grown on the following agar medium : KH<sub>2</sub>PO<sub>4</sub>,

0.5 g ;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.2 g ;  $\text{NaCl}$ , 0.1 g ;  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , trace ;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , trace ; mono-sodium glutamate, 0.5 g ; mannitol, 10 g ; agar, 25 g ; water, 1,000 ml. ; final pH, 6.5. After incubation at  $30^\circ\text{C}$  for 4 days, the cells were harvested, washed three times with 0.8%  $\text{NaCl}$  solution and suspended into a suitable amount of water. Nitrogen concentration of the suspension was determined by the KJELDAHL method.

Decolorization of methylene blue was estimated in Thunberg tubes. The reaction mixture was as follows : washed cell suspension, 1 ml. ; M/5 phosphate buffer (pH, 6.5), 1 ml ; each organic acid (sodium salt), 1 ml. ( $100 \mu\text{M}$ ) ; methylene blue, 0.2 ml. ( $8 \mu\text{g}$ ) ; paraffin liquid, 1 ml. The reaction temperature was  $38^\circ\text{C}$  and the pressure was 10 mm Hg.

TABLE 2. Behavior of *Rhizobium japonicum* Toward Various Organic Acids

Organic acids added	Growth		Time of decolorization min./mg N	*Oxygen uptake ( $\mu\text{l}/\text{mg N}/\text{hr.}$ )
	by macroscopical measurement	by turbidity (%)		
**Blank	±	3.0	Ca. 26	0
Formate	+	5.9	" 21	43.6
Acetate	±	4.3	" 12	58.2
Butyrate	—	4.7	" 28	48.2
Valerate	+	5.6	" 19	52.2
Isovalerate	±	6.0	" 30	53.9
Lactate	++++	29.0	" 18	101.9
Pyruvate	++++	21.0	" 10	75.7
Citrate	+++	27.0	" 24	55.5
Cisaconitate	+++	60.9	" 22	43.5
DL-isocitric acid lactone	+++	34.0	" 22	51.4
Succinate	++++	55.0	" 15	185.9
Fumarate	++++	65.6	" 14	98.5
Malate	+++	29.8	" 15	99.5
$\alpha$ -Ketoglutarate	++++	53.9	" 14	186.3
Glutarate	+++	50.0	" 19	143.4
Tartarate	++++	45.0	" 16	31.7
Adipate	+++	39.0	" 24	47.3
Glucose	+++	10.9	" 30	17.9

\* Each value of this column was obtained from the deduction of endogenous oxygen uptake.

\*\* Blank test was carried out without any organic acid.

Oxygen absorption was determined manometrically. The reaction mixture in this experiment was as follows; washed cell suspension, 1 ml.; M/5 phosphate buffer (pH, 6.5), 1 ml. in the main chamber; each organic acid (sodium salt), 1 ml. (100  $\mu$ M) in the side arm; 15% KOH, 0.2 ml. in the center well. The reaction temperature was 38°C and the gas phase was air. The results of these experiments are shown in Table 2.

From these experimental results, it was found that the data by macroscopical observation were generally similar to the values for turbidity. Namely, *Rhizobium japonicum* could grow well in the culture media containing each intermediate on the TCA cycle, adipate, glutarate or tartarate, but less on addition of formate and other acids. The decolorization time of methylene blue in the presence of organic acids increased in the following order: pyruvate, acetate, fumarate,  $\alpha$ -ketoglutarate, succinate and other acids. KONISHI et al.<sup>(20)</sup> observed the same results in their studies on the decolorization of methylene blue by *Rhizobium trifolii* or by *Rhizobium meliloti*. The values of oxygen uptake decreased in the following order:  $\alpha$ -kotoglutarate, succinate, glutarate, lactate, malate, fumarate, pyruvate and other acids. From these results, it was known that the larger consumption of oxygen was generally observed with the organic acids which were responsible for faster decolorization, in other words, each component of the TCA cycle. There was clear relationship between bacterial growth and the reduction of methylene blue. These facts may be due to the permeability of cell wall or to bacterial adaptation, and is certainly not explained by the results in Table 2.

When glucose was used as substrate for the comparison with other acids, both oxygen uptake and the decolorization time of methylene blue showed the lowest value, but bacterial growth was observed to some degree.

It was assumed that TCA cycle might exist in this organism, and the presence of glyoxylic acid cycle<sup>(32)</sup> was uncertain, because methylene blue was reduced rapidly by acetate, while it was somewhat useful for growth.

### Summary

1. Lower fatty acids such as formate, acetate, butyrate, valerate and isovalerate were not available for bacterial growth. On the other hand, intermediates on TCA cycle, glutarate, tartarate and adipate were good carbon sources.

2. Methylene blue was reduced rapidly by cell suspensions in the presence of tartarate, and each intermediate on TCA cycle, except citrate, cisaconitate or DL-isocitrate, but was not reduced by it in the presence of other acids.

3. A higher level of oxygen uptake was observed with some intermediates

on TCA cycle, and lower levels, with citrate, cisaconitate, DL-isocitrate and other acids.

4. In order to compare with organic acids, glucose was employed as supplement.
5. It was assumed that TCA cycle might exist in *Rhizobium japonicum*.

### Part 2. The oxidation of $\alpha$ -ketoglutaric acid by cell-free extracts and its properties

LINDSTROM<sup>(33)</sup> pointed out that  $\alpha$ -ketoglutaric acid oxidase from *Azotobacter vinelandii* was a complex system in which deacylase, DPN and coenzyme A participated. Using cell-free preparations from *Escherichia coli* or *Streptococcus faecalis*, GUNSALUS<sup>(34)</sup> obtained similar results. It has been generally thought that there may be no difference between the properties of bacterial enzyme preparations and those of animal tissue. This part of the present investigation deals with some properties of  $\alpha$ -ketoglutaric acid oxidase preparations obtained from *Rhizobium japonicum*.

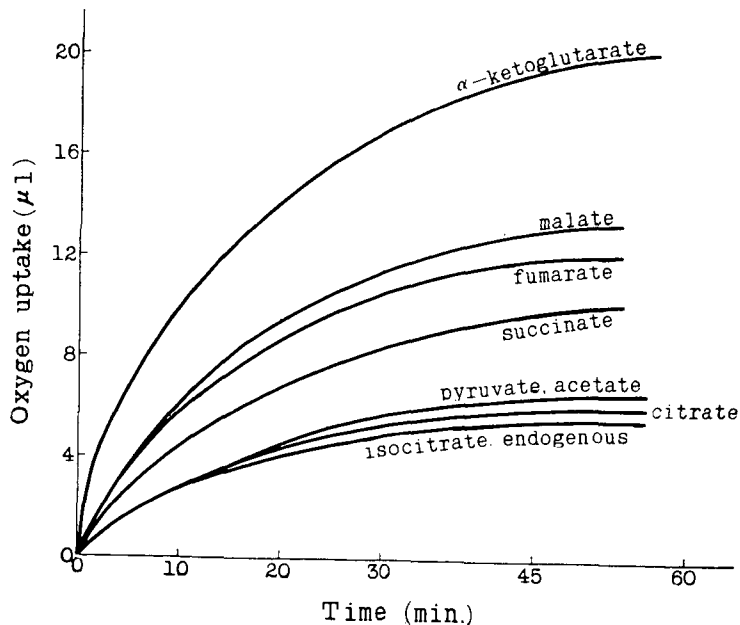


Fig. 1. Oxygen Uptake by the Extracts.

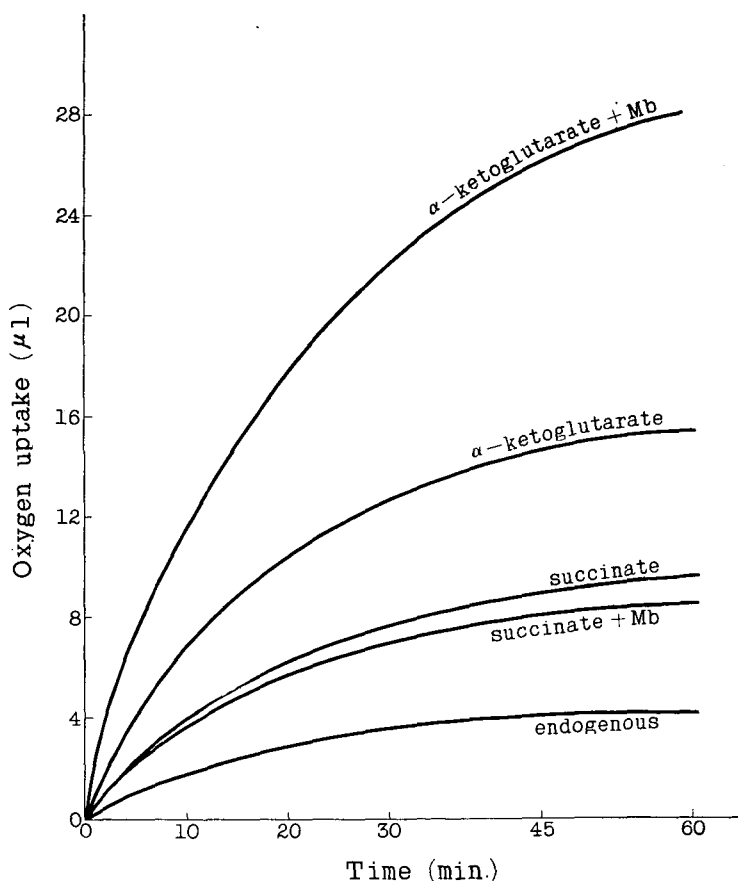
Main chamber: enzyme preparation, 1 ml. ( $N \approx 0.5$  mg);  
 M/5 phosphate buffer (pH, 7.0), 0.5 ml.  
 Side arm: each organic acid (sodium salt), 0.5 ml. (10  $\mu$ M).  
 Center well: 15% KOH, 0.2 ml.  
 Reaction temperature, 38°C.



### Experimental Results and Discussion

*Rhizobium japonicum* was grown in the mannitol-glutamate culture solution (as described in part 1), and its washed cell suspension was prepared by the usual method. By means of EISENBERG's method<sup>(35)</sup>, a dried cell preparation was obtained from the washed cell suspension and then a cell free extract was prepared from the dried cell preparation according to the method of LINDSTROM<sup>(33)</sup>. Oxygen uptake was determined by means of a Warburg manometer.

As is shown in Fig. 1,  $\alpha$ -ketoglutarate, malate, fumarate and succinate were oxidized by the extract without the addition of any kind of cofactor, but citrate, acetate, isocitrate and pyruvate were oxidized much less. As the extracts



**Fig. 2.** Effects of Methylene Blue.

The same conditions as in Fig. 1, except the addition of methylene blue, 0.2 ml. (200  $r$ ) in main chamber.

contained succinic dehydrogenase, besides  $\alpha$ -ketoglutaric oxidase, it is inadequate to determine the rate of  $\alpha$ -ketoglutaric acid oxidation without any control for succinic dehydrogenase action. This difficulty was overcome by the addition of methylene blue and malonate to reaction mixtures. As is shown in Fig. 2 and Fig. 3, the stimulating effect of methylene blue as electron acceptor was observed only toward  $\alpha$ -ketoglutarate, and malonate completely inhibited only the activity of succinic dehydrogenase in the concentration indicated in the text upon the addition of methylene blue. On the other hand, it was found that while sodium azide did not inhibit the oxidation of  $\alpha$ -ketoglutarate, potassium fluoride inhibited it completely.

To identify the oxidation product of  $\alpha$ -ketoglutarate, the reaction mixtures containing enzyme preparation,  $\alpha$ -ketoglutarate, methylene blue and malonate

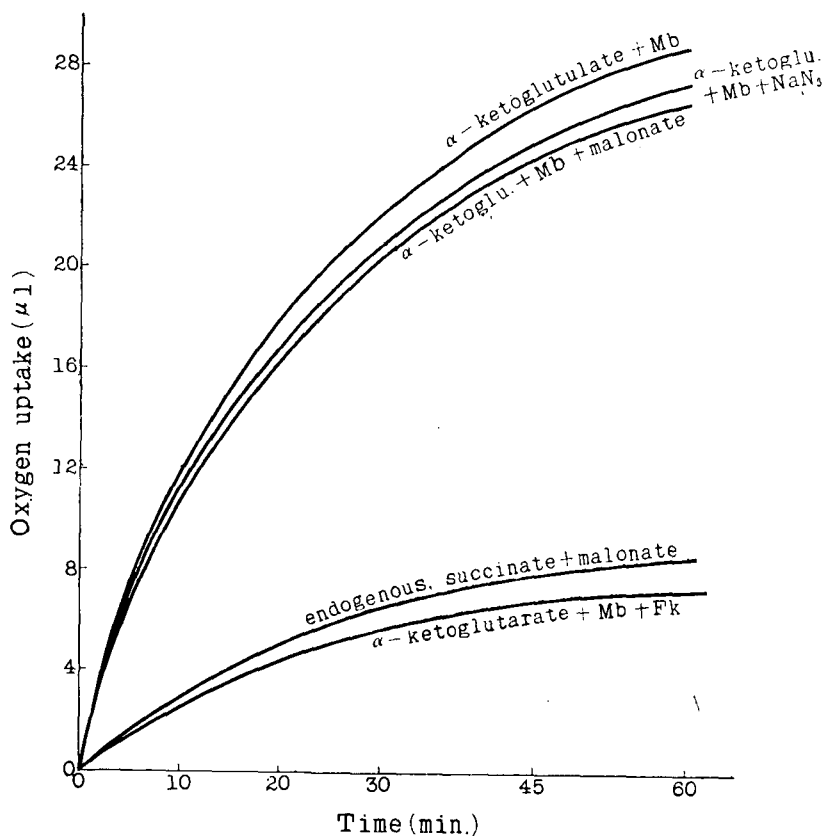


Fig. 3. Effects of Inhibitors

The same conditions as in Fig. 1, except the addition of  $\text{NaN}_3$ , 0.5 ml (4.9 mg); FK, 0.5 ml. (11.8 mg) or sodium malonate, 0.5 m (40 mg) in main chamber.

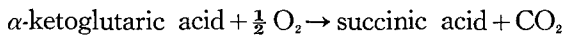
were acidified with hydrochloric acid after one hour's incubation, and extracted with ether. After evaporation of ether, a crystalline residue was obtained. It was dissolved in a small amount of water, and developed by paper chromatography<sup>(36)</sup>. As is shown in Table 3, paper chromatogram of the residue showed three spots. One of them, RF=0.12, was an unknown spot, which was detected in the reaction mixture without addition of substrate. Therefore it will be a spot of an unknown acidic substance which does not participate in the reaction. The second, RF=0.53, might be due to both  $\alpha$ -ketoglutaric acid and malonic acid, and the third, RF=0.68, was in good agreement with that of pure succinic acid. The residue was recrystallized repeatedly with water, and a white crystalline material, m. p. 180°C, was obtained. This substance showed no depression in the mixed melting point determination with the pure succinic acid, and the results of elementary analysis showed values corresponding to pure succinic acid, as is shown Table 3.

TABLE 3. Identification of Succinic Acid

	sample	pure substance
RF-values	0.12	0.52 : $\alpha$ -ketoglutaric acid
	0.53	0.54 : malonic acid
	0.68	0.69 : succinic acid
elementary analysis of spot of RF=0.68	H : 4.85% C : 40.70%	H : 5.12% C : 40.68% succinic acid
m. p. of spot of RF=0.68	180°	184° : succinic acid

solvent of paper-chromatography; phenol : water : formic acid = 60 : 20 : 1

After reaction for one hour, oxygen uptake and carbon dioxide evolution were determined with a WARBURG manometer. A volume of 9.6  $\mu$ l of oxygen was taken up and 23.1  $\mu$ l of carbon dioxide were produced. From these results, following formula will be introduced: O<sub>2</sub> : CO<sub>2</sub> =  $\frac{1}{2}$  moles : 1 mole. Thus the  $\alpha$ -ketoglutaric oxidase preparation of *Rhizobium japonicum* was found to catalyze following reaction :



The effects of several co-factors upon enzymic action were examined. The results are shown in Table 4. Manganese ion and DPN had stimulating effects on oxygen uptake, but magnesium ion, cytochrome c and ATP did not. LOCKWOOD et al.<sup>(37)</sup> recognized for the first time that  $\alpha$ -ketoglutaric acid was one of the main products of glucose metabolism by *Pseudomonas fluorescens*,

TABLE 4. Effects of Co-factors Upon the Oxidation of  $\alpha$ -Ketoglutaric Acid (K. G. A.)

oxygen uptake ( $\mu$ /hr.)							
endogenous	K.G.A.	K.G.A. + Mb	K.G.A. + Mb + Mg	K.G.A. + Mb + Mn	K.G.A. + Mb + DPN	K.G.A. + Mb + Cytochrome c	K.G.A. + Mb + ATP
1.1	4.3	18.2	18.9	20.1	23.5	1.90	17.0

main chamber: enzyme solution, 1 ml. ( $N \approx 0.2$  mg); M/5 phosphate buffer (pH, 8.0) 0.5 ml; Mb, 0.2 ml (500  $\tau$ );  $MgSO_4$ , 0.2 ml (20  $\mu$ M);  $MnSO_4$ , 0.2 ml (20  $\mu$ M); DPN, 0.2 ml. (2  $\mu$ M); cytochrome c, 0.2 ml (10  $\mu$ g); ATP, 0.2 ml (30  $\mu$ M).

side arm:  $\alpha$ -ketoglutarate, 0.5 ml (30  $\mu$ M).

center well: 15% KOH, 0.2 ml.

and that it was also one of the most important acids on the TCA cycle pathway. As mentioned above,  $\alpha$ -ketoglutaric oxidase present in animal tissues and in two or three microorganisms is a complex enzyme system in which several co-factors, such as DPN, co-carboxylase, and lipoic acid are involved. This enzyme catalyzes the oxidative decarboxylation of  $\alpha$ -ketoglutaric acid, and it has been thought that the succinyl-coenzyme A is an intermediate in its reaction. The results of the present investigation are consistent with the above information. Therefore it seemed that  $\alpha$ -ketoglutaric oxidase of *Rhizobium japonicum* would have the same properties as those of the oxidase obtained from other enzyme sources.

### Summary

1. The  $\alpha$ -ketoglutaric oxidase system of *Rhizobium japonicum* was extracted by the method of LINDOSTROM<sup>(33)</sup>.
2. The oxidation of  $\alpha$ -ketoglutaric acid was stimulated by the addition of methylene blue.
3. Sodium azide did not significantly inhibit the reaction, but potassium fluoride completely inhibited it.
4. The enzyme preparation catalyzed the following reaction:
 
$$\alpha\text{-ketoglutaric acid} + \frac{1}{2} O_2 \rightarrow \text{succinic acid} + CO_2$$
5. Both manganese ion and DPN stimulated the oxidation of  $\alpha$ -ketoglutaric acid, but magnesium ion, cytochrome c and ATP did not.

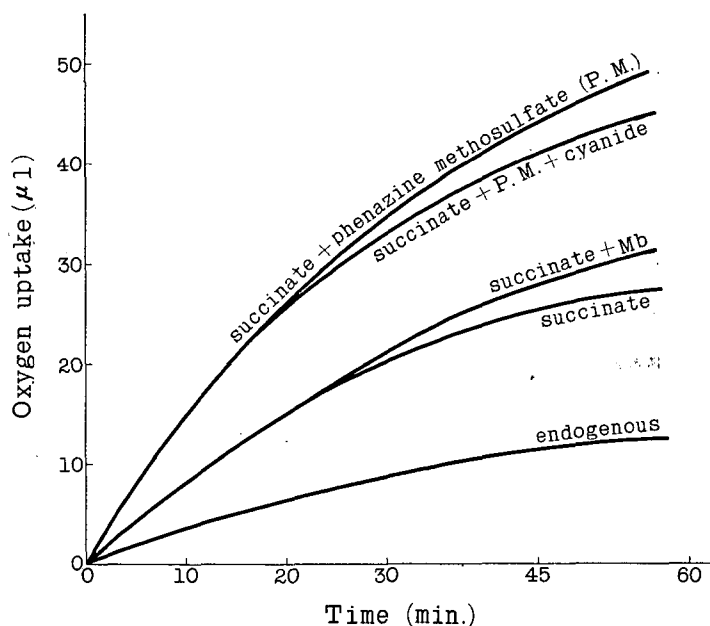
### Part 3. The oxidation of succinic acid by crude enzyme preparation

As described in the preceding section, the enzyme solution prepared by

the method of LINDOSTROM<sup>(39)</sup> showed a weak activity upon the oxidation of succinic acid, and it was not stimulated by the addition of methylene blue. In this section, results obtained by the use of dried cell preparation<sup>(36)</sup> as crude enzyme, that is, effects of accelerator or inhibitor to the reaction, isolation and identification of the reaction products and so on are described.

### Experimental results and discussion

The succinic acid oxidase activity of the dried cell preparation was measured both in the presence and the absence of methylene blue, but no marked difference was observed between the two activity curves, as is shown in Fig. 4. These results should be interpreted to mean that cytochrome b might exist in lower concentration in the preparation, because methylene blue succeeds cytochrome b as electron acceptor, if the reaction takes place according to the following scheme of KEILIN et al.<sup>(38)</sup>.

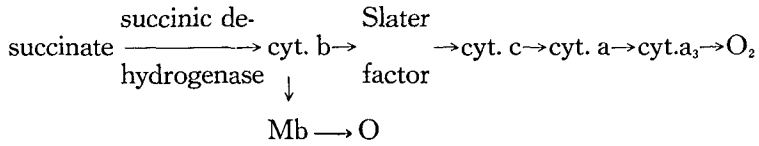


**Fig. 4.** Effects of dyes on succinic dehydrogenase activity.

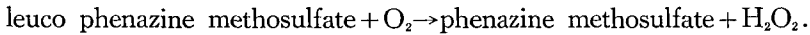
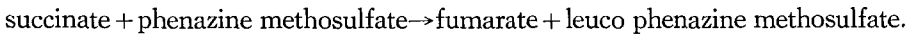
Main chamber: enzyme preparation, 15 mg ( $N \approx 1.5$  mg; M/5 phosphate buffer (pH, 7.5), 0.5 ml; 0.01 M cyanide, 0.3 ml. (adjusted by HCl to pH 9.0.

Side arm: sodium succinate (0.1 mM), 0.3 ml; phenazine methosulfate (1%), 0.2 ml or methylene blue (300  $\tau$ ), 0.2 ml.

Reaction temperature, 38°C.



This presumption is supported by the fact that oxygen uptake in the presence of phenazine methosulfate did not always decrease simultaneously with the addition of cyanide by which cytochrome oxidase system was inhibited. Meanwhile, SINGER et al.<sup>(39)</sup> reported that phenazine methosulfate was as effective as methylene blue as an electron acceptor for the action of succinic dehydrogenase, and that it might react directly and maximally with succinic dehydrogenase as follows :



When synthetic phenazine methosulfate<sup>(40)</sup> was added to the reaction mixture, a remarkable increase of oxygen consumption was observed. Therefore it seemed that the preparation contained succinic dehydrogenase, but not a heavy metal electron transferring system, such as cytochrome oxidase. In the presence of phenazine methosulfate, oxygen consumption was completely inhibited with malonate, but not with cyclohexanol, within 30 minutes of reaction (Fig. 5).

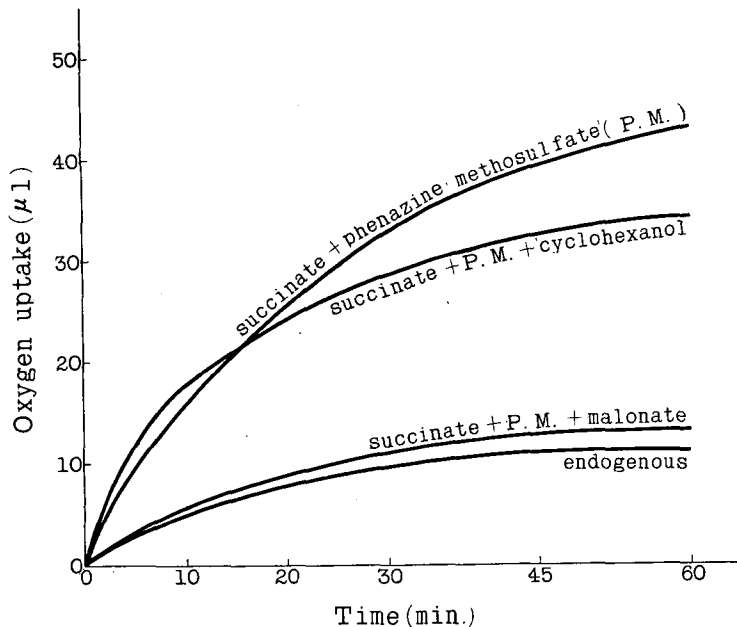


Fig. 5. Effect of Inhibitors on the Oxidation of Succinic Acid.

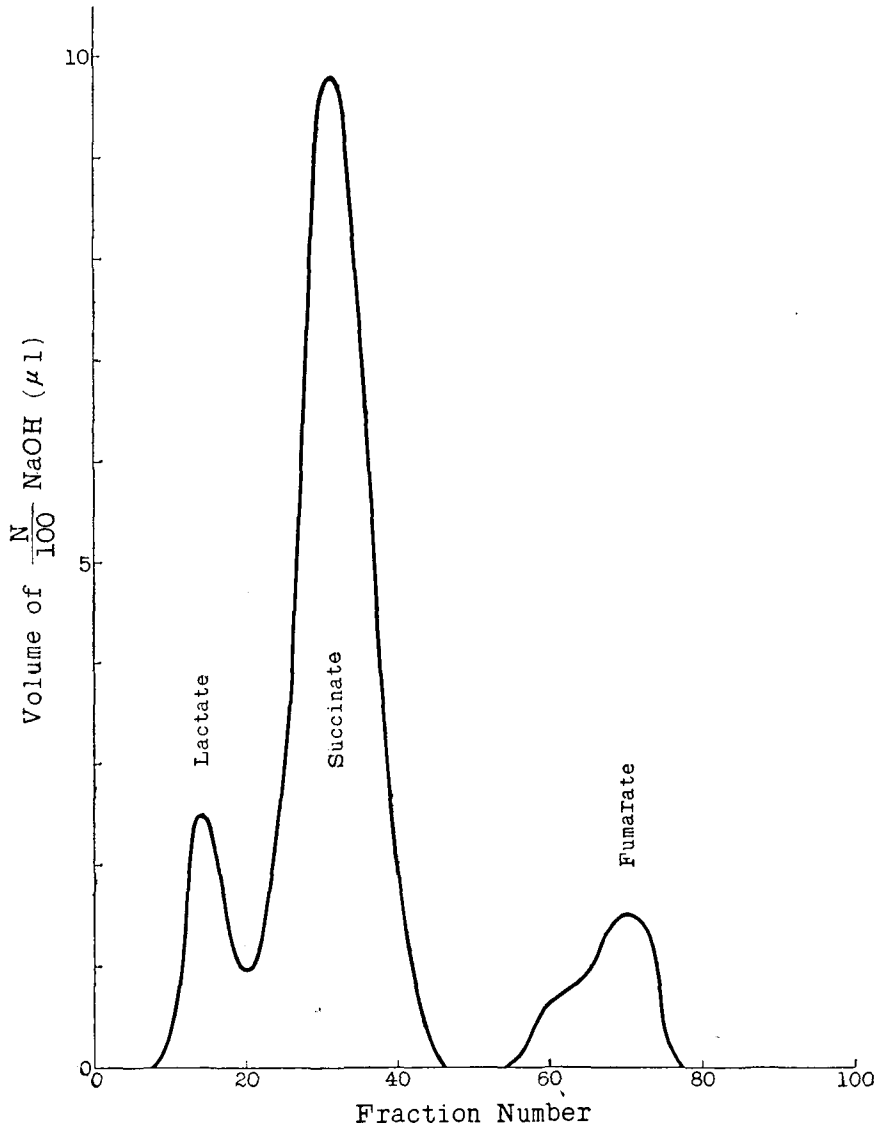


Fig. 6. Column Chromatography of Reaction Products.

However, in the cyclohexanol system, oxygen uptake decreased somewhat successively after 30 minutes' reaction. This may be due to the fact that side reaction catalyzed by other oxidase except succinic dehydrogenase, for instance, malic oxidase and pyruvic oxidase are controlled by the addition of cyclohexanol. In other words, further oxidation of fumaric acid produced from succinic acid would be probably inhibited in the presence of cyclohexanol, which is a fumarase inhibitor. Therefore the amount of oxygen uptake observed in the presence of cyclohexanol will express true value of oxygen liberated by succinic acid dehydrogenase action. As in the second experiments, isolation and identification of reaction products were carried out. The components of the reaction mixture and the experimental conditions were just the same as in the presence of phenazine methosulfate in Fig. 4. After incubation for one hour, the reaction mixture was centrifuged for 10 minutes at 8,000 r.p.m. The supernatant was acidified with hydrochloric acid, and extracted continuously with ether for 20 hours. The ether extract was evaporated, and the aqueous solution of the residue was subjected to paperchromatography<sup>(36)</sup>. The paperchromatogram showed four spots and their RF-values were 0.80, 0.60, 0.39 and 0.20, respectively. The same incubation experiment was repeated several times. The residues from ether extract were combined together, dissolved in 5 ml. of water and dropped into a column (diameter, 1 cm; height, 15 cm) packed with Dowex 1 (formated form, ca. 200 mesh)<sup>(41)</sup>. Successive development was carried out with formic acid in the following order: 25 ml. of 0.1 N; 25 ml. of 1 N; 55 ml. of 3 N; 115 ml. of 5 N; and 115 ml. of 6 N. Every 2 ml. of effluent collected by the fraction collector was evaporated in vacuo and the solution of the residue in 1 ml. of water was subjected to titration with N/100 sodium hydroxide. The results are shown in Fig. 6. Fractions 10 to 19, fractions 20 to 40 and fractions 50 to 75 contained acidic substances. Respective eluates from fractions 14 to 18, 19 to 31, 32 to 38 and 58 to 70 were mixed together. This mixed solution was evaporated in vacuo, acidified with hydrochloric acid and extracted with ether. After the ether was evaporated, the residue was dissolved in a small amount of water, and examined by paperchromatography<sup>(36)</sup>. The results are shown in Table 5. POTTER et al.<sup>(41)</sup> observed that lactic, succinic, malic and fumaric acid were contained in fractions 19 to 20, 25 to 30, 31 to 32 and 80 to 90, respectively. From their findings and the present results of column- or paper-chromatography, it was assumed or presumed that fractions 14 to 18 (RF=0.76) would contain lactic acid, fractions 19 to 31 or 32 to 38 (RF=0.44 or 0.67) contain the mixture of succinic and malic acid and the last fractions 58 to 70 (RF=0.21 and 0.63) contain fumaric acid together with a small amount of unknown acidic substance. To identify these substances, the following



TABLE 5. RF-values of Every Fraction

fraction number	RP	
14 ~ 18	0.76	
19 ~ 31	0.44	0.66
32 ~ 38	0.44 (trace)	0.67
58 ~ 70	0.21 (trace)	0.63
pure substance		
malic acid	0.44	
succinic acid	0.66	
fumaric acid	0.63	
lactic acid	0.73	

experiments were carried out. The fractions 14 to 18 indicated purple color by the treatment with p-hydroxydiphenyl which was used for qualitative color test upon  $\alpha$ -hydroxy acid. When the fractions 14 to 18 were also treated with zinc carbonate, a small quantity of zinc salt was obtained. Its crystalline form was identical with that of pure zinc lactate. The white crystalline substance obtained from the fractions 32 to 38 was recrystallized with water, and it was found by microscopic observation that this crystal was a mixture of two substances. One sublimated at about 110°C and the other melted at about 145°C. The former seemed to be succinic acid and the latter, malic acid. The substance from the fractions 58 to 70 was recrystallized repeatedly with water and its m. p. was microscopically measured. This crystal sublimated at 190°C, melted at 280°C in a sealed tube and did not show a temperature depression in the mixed melting point determination with pure fumaric acid. Therefore succinic acid was assumed to be converted to fumaric acid by the dried cell preparation of *Rhizobium japonicum*, which was likely to catalyze further degradation of fumaric acid to malic or lactic acid.

### Summary

1. The succinic acid dehydrogenase activity of a dried cell preparation was small. As methylene blue did not show the stimulating effect for the oxygen uptake, it was assumed that cytochrome b existed either in lower concentration or in a nearly inactivated form.

2. By addition of phenazine methosulfate, oxygen uptake was increased. Therefore it is a good or effective electron acceptor for the action of succinic acid dehydrogenase.

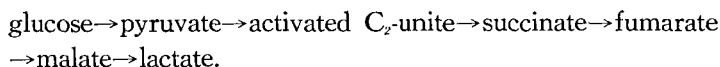
3. Oxygen uptake shown by the dried cell preparation was completely inhibited by addition of malonate (Fig. 5).

4. After 30 minutes' reaction, oxygen consumption decreased in the presence of cyclohexanol (Fig. 5). From this fact, it was assumed that cyclohexanol did not inhibit succinic acid dehydrogenase activity, but inhibited further oxidation processes of fumaric acid formed and accumulated in the reaction.

5. Fumaric acid was identified as the reaction product, while the formation of lactic acid should be expected from fumaric acid.

#### Part 4. The oxidation of lactic acid

It is well known that certain kinds of microorganisms, for instance, *Rhizopus oryzae*<sup>(42)</sup> or *Rhizopus nigricans*<sup>(43)</sup>, are able to produce only L-lactic acid from glucose under aerobic conditions, but the metabolic pathway of glucose in the above case was not clearly established. On this problem, CARSON et al.<sup>(43)</sup> presented the scheme described below:



Such a pathway will closely relate to the TCA cycle. Meanwhile, it was described in part 3 that there would be the similar pathway in dried cell preparation of *Rhizobium japonicum*, that is, succinate  $\rightarrow$  fumarate  $\rightarrow$  malate  $\rightarrow$  lactate. Therefore this part deals with the oxidation of lactic acid by the use of acetone powder of *Rhizobium japonicum* in connection with the above subject.

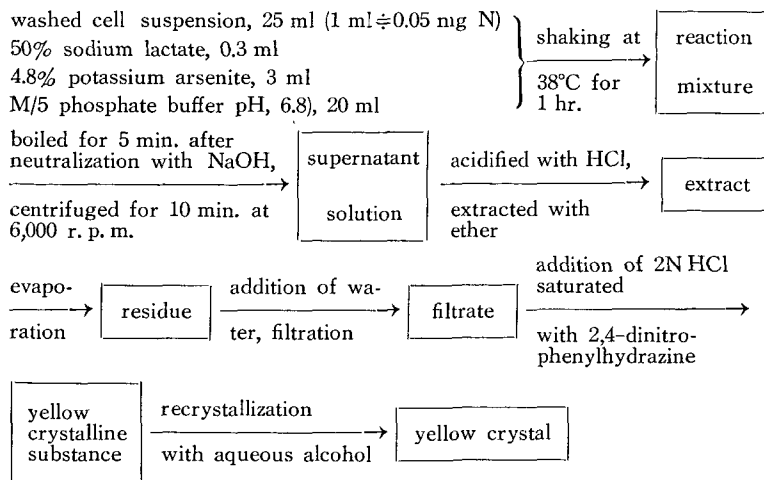
#### Experimental results and discussion

Presence of lactic acid dehydrogenase in washed cell suspension.

As was described previously, *Rhizobium japonicum* was grown in the mannitol-glutamate culture medium, and after 5 days' incubation at 30°C, its washed cell suspension was obtained by the usual method. The formation of pyruvic acid from lactic acid by the suspension was demonstrated. The reaction mixture and experimental procedure are shown in Table 6.

In either case of two experiments which were carried out with the use of the boiled suspension or in the absence of potassium arsenite, respectively, no derivative of 2,4-dinitrophenylhydrazine was obtained. At the same time, paper-chromatography of hydroxamic acid was employed to determine whether or not acetic acid was formed in this reaction. The formation of acetic acid was not detected either in the presence or the absence of potassium arsenite. The yellow

TABLE 6. Reaction Mixture and Identification Procedure of Pyruvic acid from Lactic acid



crystals obtained as in Table 6 showed following properties. These results indicate the existence of lactic acid dehydrogenase in *Rhizobium japonicum*.

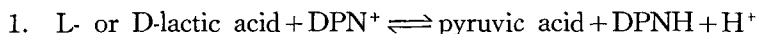
	m. p.	mixed m. p.	*RF	**RF	analytical values of N
sample	210° 211°		0.33		19.98%
pyruvic acid 2,4-dinitrophenylhydrazone	215°	211° 212°	0.33	0.34	20.88%

\* solvent: N-butanol saturated with 3% ammonia (v/v).

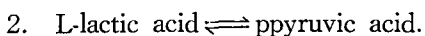
\*\* RF of the mixture of sample and pure derivative.

Behavior of acetone powder from *Rhizobium japonicum* toward the oxidation of lactic acid.

It has been generally known that three types of lactic acid dehydrogenase occur in various kinds of microorganisms. All of them catalyze the conversion of lactate to pyruvate, but differ from each other in their requirements of cofactors.

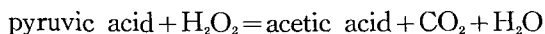
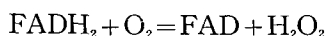
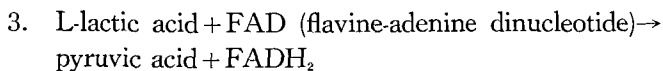


$\text{DPN}^+$  or  $\text{TPN}^+$  as cofactor is essential in this reaction. Enzyme sources are *Leuconostoc mesenteroides*<sup>(44)</sup> and *L. arabinosus*<sup>(45)</sup> and other microorganisms.



$\text{DPN}^+$  or  $\text{TPN}^+$  is not necessary in this reaction. The electron transfer

system linked with this reaction is not clear. APPLEBY et al.<sup>(46)</sup> suggest that flavine-mononucleotide (FMN) acts as the direct acceptor of electrons from lactic acid, and that electrons are transferred from reduced FMN to protoheme which is a component of lactic acid dehydrogenase. Enzyme sources are bakers' yeast,<sup>(46)</sup> *Bacterium tularensis*<sup>(47)</sup>, etc..



DPN<sup>+</sup> or TPN<sup>+</sup> is not necessary. Lactate is oxidized anaerobically to pyruvate, and in this case, methylene blue is able to act as electron acceptor<sup>(48)</sup>. Aerobically, acetate, carbon dioxide and water are formed as final reaction products. Enzyme sources are *Mycobacterium phlei*<sup>(48)</sup>, *M. tuberculosis*<sup>(49)</sup>, etc..

The present experiments were carried out to find to which of the above three types lactic acid dehydrogenase of *Rhizobium japonicum* belongs. Oxygen uptake and carbon dioxide evolution were measured manometrically. The reaction mixture was as follows: enzyme solution, 1 ml. (acetone powder

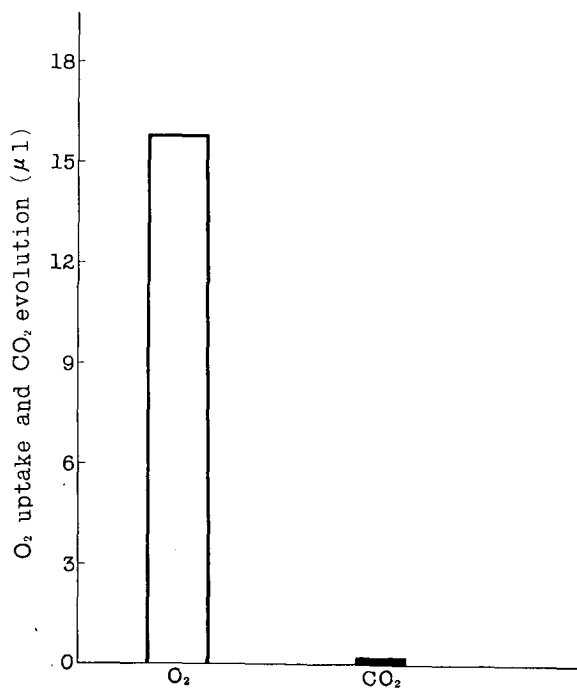


Fig. 7. Change of Gas Pressure After One Hour's Incubation.

40 mg  $\pm$  0.2 mg N); M/5 phosphate buffer (pH 7.5), 1 ml.; M/10 sodium lactate, 0.5 ml. The results obtained after one hour's incubation at 38°C are shown in Fig. 7. Carbon dioxide evolution was not observed under aerobic conditions. It is assumed that lactic acid dehydrogenase in acetone powder is at least not the enzyme of type 3. To examine the effects of cofactors, 0.2 ml. of methylene blue (100  $\mu$ ), 0.2 ml. of DPN (300  $\mu$ ) and 0.2 ml. of cytochrome c (2 mg) were added respectively to the above reaction mixture, and the results are shown in Fig. 8. The effect of methylene blue was negligible, and cytochrome c did not stimulate the oxygen uptake.

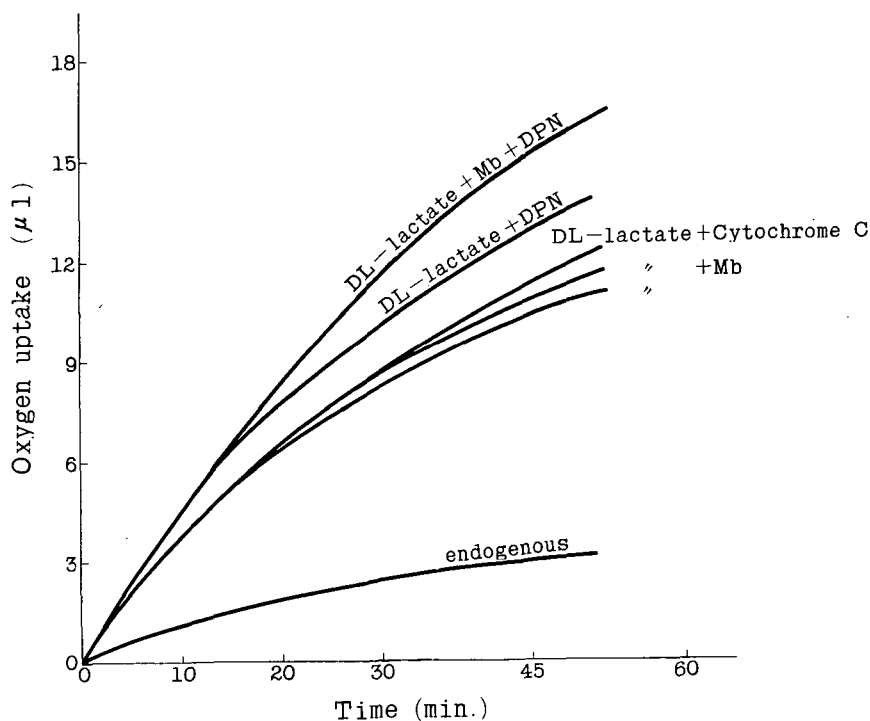
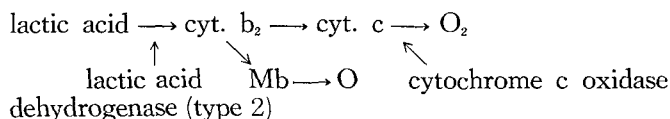


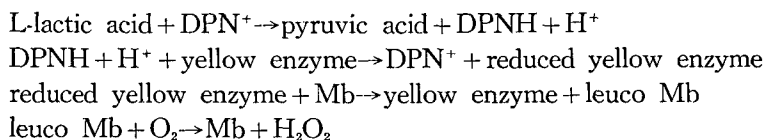
Fig. 8. Effects of Cofactors on Lactic Acid Dehydrogenase.

APPLEBY et al.<sup>(46)</sup> presumed that the electron transfer system linked with the enzyme of type 2 would be written as follows:



If lactic acid dehydrogenase (type 2), cytochrome  $b_2$ , cytochrome c and

cytochrom c oxidase do exist in acetone powder, the reaction must be stimulated by the addition of methylen blue or cytochrome c according to this scheme. On the other hand, it is thought that the acetone powder may contain the cytochrome system or yellow enzyme, because the solution obtained from acetone powder by extraction with buffer solution (5 ml. of KCl (0.02M) + 5 ml. of  $\text{KHCO}_3$  (0.002 M); pH, 8.0) was reddish brown in color. From these discussions, it is likely that the lactic acid dehydrogenase in acetone powder may not belong to the enzyme of type 2. Meanwhile, oxygen uptake was somewhat increased by addition of DPN and still more, by the simultaneous addition of DPN and methylene blue. From these results, it was assumed that the lactic acid dehydrogenase of *Rhizobium japonicum* was similar to the enzyme of type 1. In this case, electrons will be probably transferred according to STRAUB's scheme<sup>(50)</sup> which was derived from his studies on muscle lactic acid dehydrogenase as follows:



Besides *Rhizobium japonicum*, soy bean leaves<sup>(51)</sup>, gonococci<sup>(52)</sup>, yeast<sup>(53)</sup>, etc. have been reported to serve as sources of lactic acid dehydrogenase requiring  $\text{DPN}^+$ .

### Summary

1. The cell suspension of *Rhizobium japonicum* catalyzed the conversion of DL-lactate to pyruvate.
2. Under aerobic conditions, acetone powder from *Rhizobium japonicum* was able to oxidize DL-lactic acid, but its decarboxylation did not take place.
3. Oxygen uptake was obviously increased only in the experiment in which  $\text{DPN}^+$  and methylene blue were added simultaneously.
4. It was assumed that  $\text{DPN}^+$  might be required for the action of lactic acid dehydrogenase of *Rhizobium japonicum*.

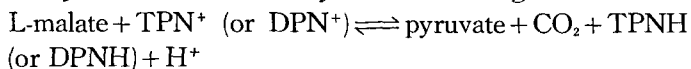
### Part 5. The oxidation of malic acid

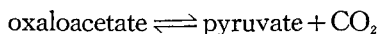
The following two kinds of enzyme which attack malic acid are known:

1. Malic acid dehydrogenase<sup>(54)</sup>.



2. Malic enzyme. This can catalyze the following two reactions<sup>(55), (56)</sup>.



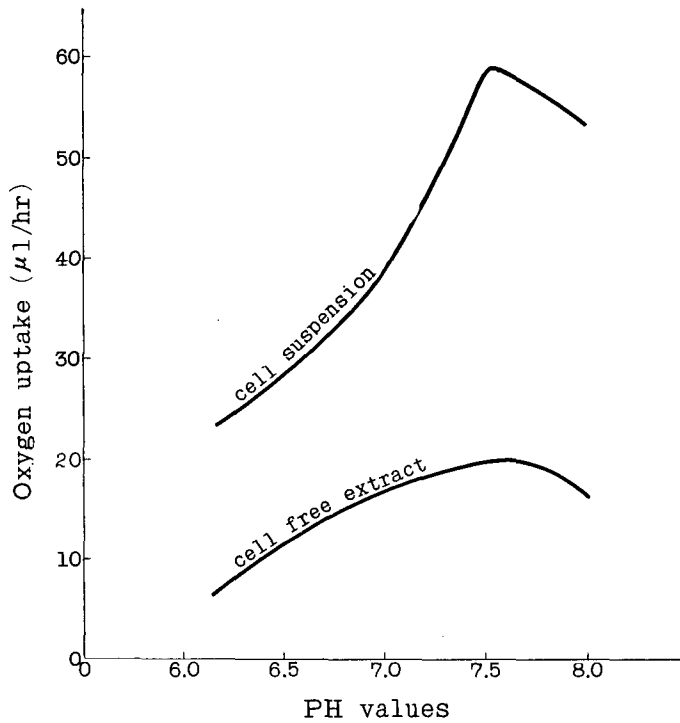


The malic enzyme has many properties in common with malic dehydrogenase. Namely, DPN<sup>+</sup> <sup>(57)</sup> is required for both enzymic reactions, and the presence of Mn<sup>++</sup> <sup>(58), (59)</sup> is indispensable in either enzymic reaction. Then, the dissociation constants of enzyme-substrate and enzyme-Mn<sup>++</sup> complex were nearly of the same value <sup>(59)</sup>. Namely, for malic enzyme-pyruvate complex, dissociation constant,  $9.8 \times 10^{-3}$  M/L was determined: for malic acid dehydrogenase-pyruvate complex,  $10 \times 10^{-3}$  M/L; for malic enzyme-Mn<sup>++</sup> complex,  $2.9 \times 10^{-4}$  M/L; for malic acid dehydrogenase-Mn<sup>++</sup> complex,  $3 \times 10^{-3}$  M/L. Moreover, the ratio of both activities was found to be constant during the purification procedure. On the other hand, there were the following differences between the properties of two enzymes. Malic enzyme had its optimal pH in the basic range, while for dehydrogenase it was in the acidic range: for instance, the optimal pH of malic enzyme from pigeon liver <sup>(59)</sup> was 7.5, and that of dehydrogenase was 4.5. In addition, the malic enzyme could catalyze dismutation of pyruvic acid in the presence of lactic acid dehydrogenase, but it was not the case with malic acid dehydrogenase. Taking into account these points, the present part deals with the examination of the effect of Mn<sup>++</sup>, determination of optimal pH, oxygen uptake and carbon dioxide evolution, identification of reaction products, and reduction of DPN<sup>+</sup>.

### Experimental results and discussion

After *Rhizobium japonicum* was grown in the mannitol-glutamate culture medium for three days at 30°C, a cell suspension was prepared by the ordinary method as described in part 1. The same volume of M/15 phosphate buffer was added to the present suspension and the resulting mixture was ground with sand in a mortar at 0°C. The supernatant obtained by centrifugation was used as the crude enzyme solution. Oxygen uptake by cell suspension or cell free extract was determined in the range of pH 6.0 to 8.0. The results are shown in Fig. 9. It was observed that the optimal pH was near 7.5 in both cases. As the pH value decreased the oxygen consumption decreased. Therefore it was assumed that oxygen uptake would be due to the action of the malic enzyme. Although oxygen uptake was demonstrated by both the cell suspension and cell free extract in the pH range tested, it was not shown by the dried cell preparation obtained by the same method as mentioned in part 2. Therefore the malic acid oxidizing enzyme in *Rhizobium japonicum* appeared to be inactivated by drying treatment.

By the use of the cell suspension, the ratio of oxygen uptake to carbon



**Fig. 9.** Effects of pH Values.

Reaction mixture: cell suspension, 1 ml. ( $N \doteq 0.15$  mg) or cell free extract, 1 ml. ( $N \doteq 0.20$  mg); M/5 phosphate buffer, 1 ml; sodium malate ( $50 \mu\text{M}$ ), 0.5 ml.

**TABLE 7.** Oxygen Uptake and Carbon Dioxide Evolution ( $\mu\text{L/hr}$ ).

		molar ratio
O <sub>2</sub>	109.2	1
CO <sub>2</sub>	331.8	3

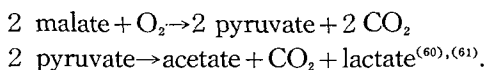
Reaction mixture: cell suspension, 1 ml ( $N \doteq 0.7$  mg); M/5 phosphate buffer (pH 7.5), 1 ml; sodium malate ( $50 \mu\text{M}$ ), 0.5 ml.

dioxide evolution was measured by a WARBURG manometer, and the results are presented in Table 7.

The amount of carbon dioxide evolved was considerably greater than that of oxygen consumed, and the molar ratio was about 1 : 3. This ratio may



be caused by dismutation of pyruvic acid, because, as was described in part 4, lactic acid dehydrogenase was also present in the cell suspension of *Rhizobium japonicum*. As was described in the present part, it was known that malic enzyme could catalyze the following reaction in the presence of lactic acid dehydrogenase :



In the above reaction, the ratio of oxygen uptake to carbon dioxide evolution is 1 : 3. It was shown in part 2 that lactic acid was produced from malic acid by the action of the cell suspension. From these discussions, it is assumed that dismutation of pyruvic acid may take place according to the above scheme.

The effects of various kinds of substances on oxygen uptake were examined with the cell suspension. As is shown in Table 8, methylene blue, potassium ion and chlor ion did not affect the oxidation, but magnesium ion and arsenious ion acted as inhibitors, and manganese ion as a stimulator. These results were not in complete agreement with the findings of some investigators. Namely, oxidation of malic acid was stimulated by addition of methylene blue according to GREEN<sup>(62)</sup>, while TAKAHASHI<sup>(63)</sup> pointed out that arsenious ion inhibited this reaction. In addition to these, many different results on effect of

TABLE 8. Effects of Several Substances for the Oxidation of Malic Acid

oxygen uptake ( $\mu$ l) for 1 hr. at 38°C									
control	methylene blue M/L	MgSO <sub>4</sub> mM/L			K <sub>3</sub> AsO <sub>3</sub> mM/L			KCLm M/L	MnSO <sub>4</sub> mM/L
	10	5	10	20	5	10	20	10	10
150	148	124	110	97	97	85	67	151	172

reaction mixture; cell suspension, 1ml (N $\approx$ 0.7 mg); M/5 phosphate buffer (pH 7.5), 1 ml; sodium malate (50  $\mu$ M), 0.5 ml; water (control), 0.2 ml; each sample solution, 0.2 ml.

magnesium ion have been reported, but its function is not yet clarified. The experimental conditions to identify reaction products were the same as shown in Table 6 except for the addition of 10 ml. of 5% malate solution as the substrate in place of lactate. Yellow crystals of 2,4-dinitrophenylhydrazone that separated out from the reaction mixture were recrystallized with aqueous alcohol. Its m.p. was 213°C, and showed no depression in the mixed melting point determination with pure pyruvic acid 2,4-dinitrophenylhydrazone. This hydrazone showed tow spots (RF, 0.34 and 0.50) on a paperchromatogram. One of them (RF, 0.50) may be due to an isomer of pyruvic acid 2,4-dinitrophenyl-

hydrazone<sup>(64)</sup>, because these two spots were often observed on the paperchromatograms of pure pyruvic acid 2,4-dinitrophenylhydrazone. Further experiments to identify acetate and oxaloacetate were carried out with the reaction mixture in the presence of arsenite, but they were not detected.

Reduction of DPN<sup>+</sup> by cell free extracts was examined by means of a BECKMAN spectrophotometer. The cell free extract was fractionated with ammonium sulfate at the saturation point, 0.25 and the separated precipitate was suspended in M/20 phosphate buffer (pH, 7.1) and used as enzyme A. The supernatant solution was further saturated with ammonium sulfate to 0.5

TABLE 9. Enzyme Activities of Fraction A and B

	activity/mg of protein
fraction A	1008.9
fraction B	813.0

and the suspension of the resulting precipitate in M/20 phosphate buffer (pH, 7.1) was used as enzyme B. The protein content of the two enzyme fractions was measured by the method of NEILANDS et al.<sup>(65)</sup>. DPN was prepared from baker's yeast according to the method of LEPAGE<sup>(66)</sup>. Its purity determined by LEPAGE's method<sup>(67)</sup> was about 10%. One ml. of sodium malate (1 M), 1 ml. of DPN solution (12.2 mg) and 13 ml. of M/20 phosphate buffer (pH, 7.1) were mixed. Immediately after 3 ml. of this mixture was added to 0.1 ml. of enzyme A or enzyme B, the optical density was measured at 340 m $\mu$  every 15 sec. Malic enzyme activity in enzyme A or enzyme B was calculated by the method of BACKER<sup>(68)</sup>. This method is based on the following definition: one unit is defined as a change in log  $I_0/I$  of 0.001 per minute. The increase in density, log  $I_0/I$ , between the reading at 15 and 45 seconds after mixing the solution, multiplied by 2, is taken as the enzyme activity per minute. The results are shown in Table 9. It was found that DPN<sup>+</sup> was reduced by either fraction and the activity of fraction A was superior to that of fraction B.

From these experimental results, the breakdown of malic acid by *Rhizobium japonicum* might be assumed to lead to the formation of pyruvate by the action of malic enzyme during the initial stage, followed by the probable dismutation of pyruvate during the later stage.

### Summary

1. The optimal pH of enzyme participating in the oxidation of malic acid was about 7.5.

2. In the experiment added with potassium arsenite, pyruvic acid was detected and identified as a reaction product as its 2,4-dinitrophenylhydrazone.

3. When malic acid was attacked by cell suspensions, consumption of one mole of oxygen and evolution of three moles of carbon dioxide were observed.

4. Manganese ion stimulated the oxygen uptake, while arsenious ion inhibited it.

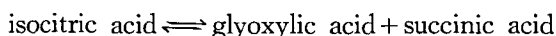
5. Reduction of DPN<sup>+</sup> was observed in the enzyme reaction.

6. It was assumed that malic enzyme might probably be responsible for this reaction. The presence of malic acid dehydrogenase in *Rhizobium japonicum* was uncertain.

#### Part 6. The presence of fumarase and isocitric acid dehydrogenase

Fumarase, which is widely distributed not only in animals<sup>(69)</sup> and plants<sup>(70)</sup>, but also in microorganisms<sup>(71)</sup> has been extensively investigated among various kinds of enzyme participating in the TCA cycle. It has been obtained from animal tissue in a crystalline form<sup>(69)</sup>, and its physical and chemical properties had been examined, isoelectric point, pH 5.0~5.4<sup>(72)</sup>, molecular weight, 200,000<sup>(72)</sup>. It is most stable when kept in glycine buffer of pH 9.0. Bacterial fumarase has not been obtained in crystalline form, but it is thought to have the same properties as animal fumarase. In addition, it was found from the investigations of *Azotobacter vinelandii*, *Mycobacterium avium*, *Proteus vulgaris* and *Pseudomonas fluorescens* that fumarase was ordinarily present in the soluble fraction of cells, and not in mitochondria.

Isocitric acid is decomposed by the actions of two enzymes, isocitritase and isocitric acid dehydrogenase. The former catalyzes the following reaction and is activated by addition of Mg<sup>++</sup> and glutathione or cysteine<sup>(73)</sup>.

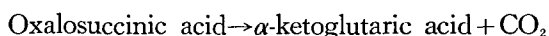


This enzyme was investigated by the use of *Escherichia coli*<sup>(74)</sup> and *Pseudomonas aeruginosa*<sup>(75)</sup>.

The latter catalyzes the following reaction and is activated by the addition of TPN<sup>+</sup>, and inhibited by PO<sup>---</sup><sup>(76)</sup>.



Oxalosuccinic acid produced in this reaction is unstable, and immediately converts to  $\alpha$ -ketoglutaric acid under decarboxylation.



It has not been shown whether or not this reaction is enzymic. This

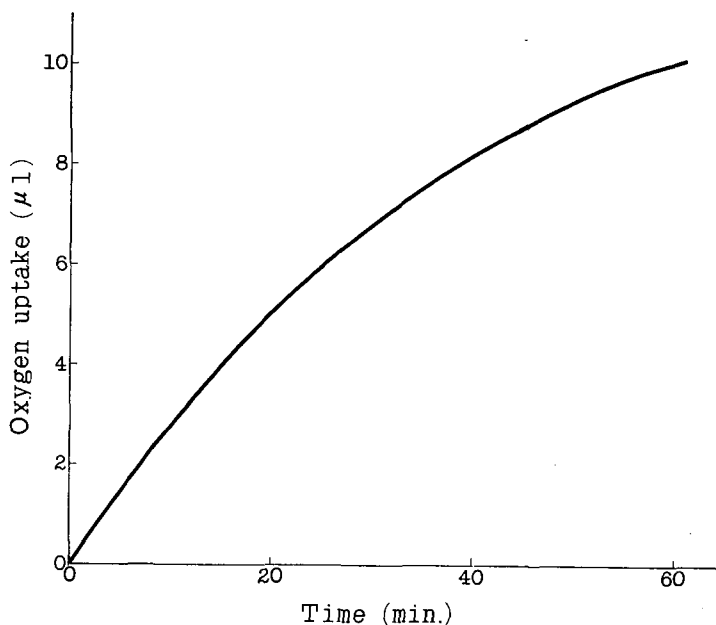
enzyme was investigated by the use of *Azotobacter vinelandii*<sup>(77)</sup> and *Pseudomonas fluorescens*<sup>(78)</sup>.

As is shown in Table 2, fumaric acid and isocitric acid were metabolized by the cell suspensions of *Rhizobium japonicum*. Therefore the existence of fumarase, isocitritase or isocitric acid dehydrogenase in this organism is suggested. These problems are dealt with in the present part of the study.

### Experimental results and discussion

In order to separate and identify the reaction product formed from fumaric acid by the dried cell preparations, the following experiment was carried out. Reaction mixture was as follows: dried cell preparations (20 mg) 10 ml; M/5 phosphate buffer (pH 7.0), 10 ml; sodium fumarate (20 mg), 10 ml. After one hour's incubation at 38°C in a shaker, the culture filtrate was made alkaline with sodium hydroxide, concentrated in vacuo, acidified with hydrochloric acid, and extracted with ether. After the ether was evaporated off, the residue was dissolved in a small quantity of water. A small part of this solution was subjected to paperchromatography<sup>(36)</sup>, and the other part, to column chromatography, which was essentially the same as mentioned in part 3, except for the use of Dowex 1 of 350 mesh. Two spots, showing RF=0.43 and 0.61, were observed on a paperchromatogram. The former RF corresponds to that of pure malic acid and the latter, to that of pure fumaric acid. The main part of above solution was poured into the column and successive elution was made with formic acid of different concentrations. By the use of a fraction collector, every 2 ml. of eluate were collected successively. In both fractions 39 to 55 and fractions 85 to 118, the acidic substances were detected. In the control experiment, the same chromatographic procedure was applied to the aqueous solution containing both 10 mg of malic acid and the same amount of fumaric acid. It was found that malic acid appeared in the range of fractions 40 to 54 and fumaric acid, in the range of fractions 90 to 105. Fractions 39 to 55 were collected together, and evaporated to dryness. A white crystalline substance was obtained, and recrystallized from water. Its m. p. was 101°~105°C and showed no depression in the mixed melting point determination with pure malic acid. These results seem to demonstrate the conversion of fumaric acid to malic acid, which is probably catalyzed by fumarase, and in addition, this fact would support the assumption in part 3.

As is shown in Table 2, the oxidation rate of isocitric acid by cell suspensions was not great. To extract isocitric acid oxidizing enzyme from the cells, two kinds of buffer solution, phosphate buffer of pH 7.2 and KCl-KHCO<sub>3</sub>,



**Fig. 10.** Isocitric Acid Oxidation.

Main chamber: 1 ml of cell free extract (1 ml = 0.04 mg N), 1 ml. of velonal acetate buffer solution (pH, 7.6).

Side arm: 1 ml. of sodium isocitrate (50 μM).

Center well: 0.2 ml. of 15 % KOH. Reaction temp., 38°C.

The figures showed the deduction of endogenous oxygen uptake.

buffer of pH 8.0, were employed. In the former case, isocitric acid oxidation was not demonstrated. This fact might be due to the inhibitory effects of phosphate ion. In the latter case, isocitric acid oxidation was obviously shown, but the enzymic activity was not so great as in cell suspensions. The results are shown in Fig. 10. Now, the reaction products were examined according to following procedure. The reaction mixture was dried in vacuo and the residue was acidified with hydrochloric acid and extracted with ether. After the ether was evaporated, the residue was dissolved in small amount of water and filtered. A small part of this filtrate was developed by paperchromatography with the following solvent: phenol: water: 90% formic acid = 6 g: 1 cc: 1 cc<sup>(36)</sup>. Three spots, 0.84, 0.60 and 0.54 were observed on the paperstrip. The spot of 0.84 might be due to an unknown acidic substance, 0.60 was identical with the spot of standard isocitric acid, and 0.54 was located in the same position as that of pure  $\alpha$ -ketoglutaric acid. 2,4-dinitrophenylhydrazone obtained from the reaction mixture was also applied to paperchromatography, and only

one spot (RF=0.32~0.35) was observed. This spot is nearly in the same position as that of pure  $\alpha$ -ketoglutaric acid 2,4-dinitrophenylhydrazone. From these results, it was assumed that isocitric acid dehydrogenase was present in *Rhizobium japonicum*, and that the presence of isocitritase was uncertain.

### Summary

1. The conversion of fumaric acid to malic acid in the presence of a dried cell preparation of *Rhizobium japonicum* was proved by isolation and identification of malic acid as reaction product. As a result of this experiment, it was suggested that the dried cell preparation contained fumarase.

2. The existence of isocitric acid dehydrogenase in this microorganism was shown by the following experimental results: a cell free extract was able to catalyze the oxidation of isocitric acid.  $\alpha$ -ketoglutaric acid was formed in this reaction, but succinic acid was not detected.

## Chapter B. The actions of bacteria upon certain amino acids

Many studies on the degradation of amino acids have been made by the use of various microorganisms. As is generally known, amino acids are principally or mainly decomposed through the reactions liberating ammonia, carbon dioxide or hydrosulfide, and at the same time producing  $\alpha$ -keto acids or amines, which are directly or indirectly introduced into the TCA cycle. In connection with the studies in chapter 1, it is important to examine the actions of *Rhizobium japonicum* upon amino acids.

VIRTANEN et al.<sup>(79)</sup> reported that  $\beta$ -alanine was formed from aspartic acid by the action of decarboxylase prepared from *Rhizobium trifolii*, and this reaction was re-examined quantitatively by BILLEN et al.<sup>(80)</sup>. JORDAN<sup>(81)</sup> was able to obtain a transaminase preparation from *Rhizobium* species, and studied following two systems,  $\alpha$ -ketoglutaric acid+alanine and  $\alpha$ -ketoglutaric acid+aspartic acid. ALMON<sup>(82)</sup> described the qualitative results of tyrosinase activity of several *Rhizobium* species. With the exception of the above studies, there are not any other studies reported in the literature on amino acid metabolism carried out with *Rhizobium* species.

In consideration of such limited information in this field, the present studies have been undertaken for the purpose of obtaining some knowledge about the biochemical behavior of this microorganism toward some amino acids, which are formed by the reaction between fixed nitrogen compound (ammonia) and  $\alpha$ -keto acids produced via the TCA cycle.

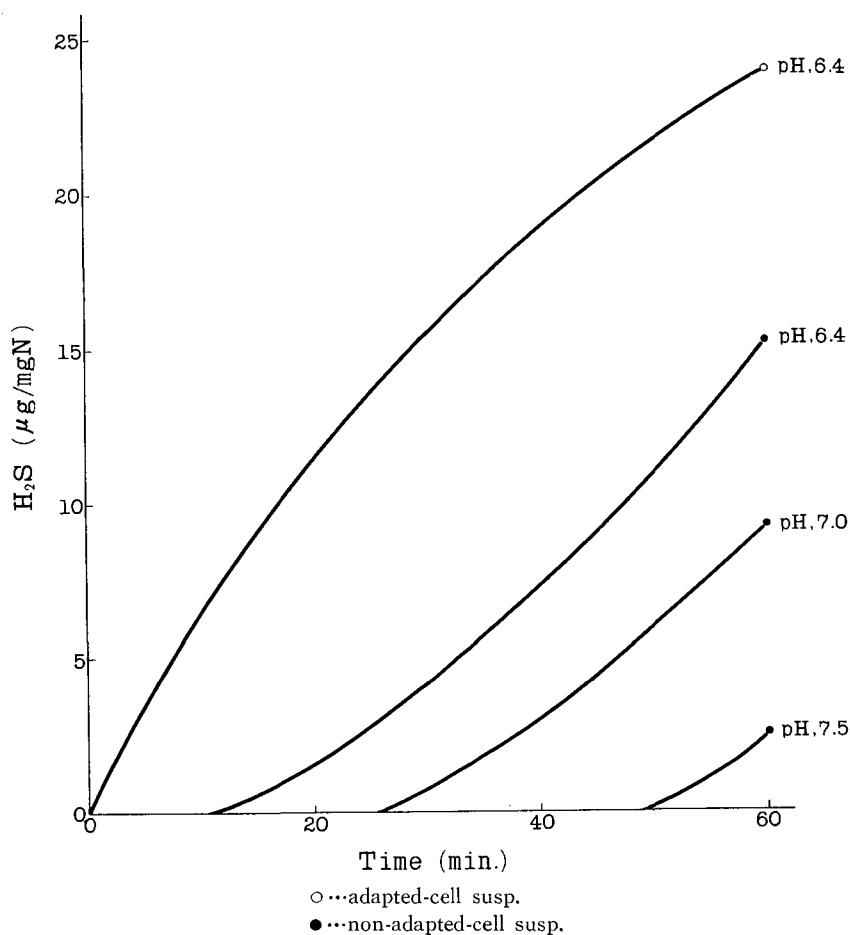
### Part 1. The enzymic degradation of L-cysteine

It has been known for a long time that cysteine is decomposed by different kinds of microorganism, for instance, *Escherichia coli*<sup>(83), (84)</sup>, *Proteus vulgaris*<sup>(85), (86)</sup> and *Bacillus subtilis*<sup>(87)</sup>. SUDA et al.<sup>(88)</sup> reported that pyridoxal phosphate was required in the degradation of cysteine, and that the evolution of ammonia took place before the liberation of hydrogensulfide. On the contrary, FROMAGEOT<sup>(84)</sup> obtained the reverse results to SUDA's findings<sup>(88)</sup>, that is, hydrogensulfide was liberated before the evolution of ammonia. According to MIWATANI et al.<sup>(89)</sup>, L-cysteine is also decomposed by the action of methioninase, liberating ammonia, hydrogensulfide and pyruvate. Therefore, it appears that cysteine may be decomposed by different mechanisms, being dependent upon the kinds of bacteria. It was found in the author's experiment that L-cysteine was decomposed by the action of enzyme preparations obtained from L-cysteine-adapted *Rhizobium japonicum*. Some experimental results on the properties of this enzyme are described in the present part.

#### Experimental results and discussion

**Culture test.** The composition of the culture medium was as follows: potassium phosphate (monobasic), 10 mg; magnesium sulfate, 4 mg; sodium chloride, 2 mg; ferric chloride, trace; manganese sulfate, trace; L-cysteine hydrochloride, 20 mg; distill. water, 20 ml. The pH of the culture solution was adjusted to ca. 7.0 with sodium hydroxide. A loop of inoculum was transferred into every 20 ml. of sterilized media and the culture flasks were kept at 30°C. After two days' incubation, hydrogensulfide vapour evolved from the flasks and after four days the culture solution was remarkably turbid as a result of bacterial growth. From these facts, it seemed that L-cysteine may be metabolized by *Rhizobium japonicum* and utilized as energy and a nitrogen source for its growth.

**Adaptation experiments.** Another culture solution was used in this experiment, that is, mannitol (0.4 g) and L-aspartic acid (10 mg) were added to the above medium in place of L-cysteine hydrochloride. After inoculation and four days' incubation at 30°C, cells were harvested, washed three times with 0.5% sodium chloride and suspended in water. The nitrogen content of resulting cell suspensions was about 0.57~0.65 mg/ml. (by micro KJELDAHL method). Composition of reaction mixture was as follows; cell suspension, 2 ml; L-cysteine hydrochloride (0.1%), 2 ml; M/5 phosphate buffer (pH 6.4), 2 ml. After one hour's incubation at 40°C under aeration, the reaction was stopped by the addition of 5N-H<sub>2</sub>SO<sub>4</sub> (1 ml). Hydrogensulfide was determined according to the



**Fig. 11.** The Formation of Hydrogensulfide from L-Cysteine.

method of ALMY<sup>(90)</sup>. The data obtained are shown in Fig. 11. It was found that hydrogensulfide was not produced immediately at the beginning of reaction, but after lag period, and that the length of lag time was dependent upon the pH of the reaction mixture. On the other hand, in the experiments carried out by using the so-called cysteine-adapted cell suspension prepared from bacteria which were grown on the medium containing L-cysteine hydrochloride, hydrogensulfide was formed immediately at the initial stage of reaction. The data in this case are shown in Fig. 12. From these experimental results, it may be considered that *Rhizobium japonicum* is able to produce adaptively L-cysteine desulfhydrase under aerobic condition. DESNUELLE<sup>(91)</sup> reported that similar



results were obtained in his investigations in which *Escherichia coli* was employed.

Enzyme activity of acetone powder or cell free extract. L-cysteine-adapted *Rhizobium japonicum* was obtained according to OHIGASHI et al.<sup>(85)</sup> and its acetone powder was prepared by the usual method. A loop of this powder was put on the BEIJERINCK culture medium (2 g of mannitol + 0.02 g of potassium phosphate + 2 g of agar + 100 ml of water) and kept at 30°C. In this case bacterial growth was not observed. This fact shows that there were not any intact cells in the acetone powder. Fifty mg of acetone powder, 2 ml of M/5 phosphate buffer and 2 ml of 0.1% L-cysteine hydrochloride were mixed and incubated under the same experimental conditions as described above. As is shown in Fig. 12, hydrogensulfide evolution was affected by different pH values and it showed maximal effect at pH 7.3. In this case, hydrogensulfid evolution increased according to a S-curve. Therefore it seems that the desulfydration of L-cysteine must be coupled with other reactions such as deamination or decarboxylation. To ascertain this point, the following experiments were carried

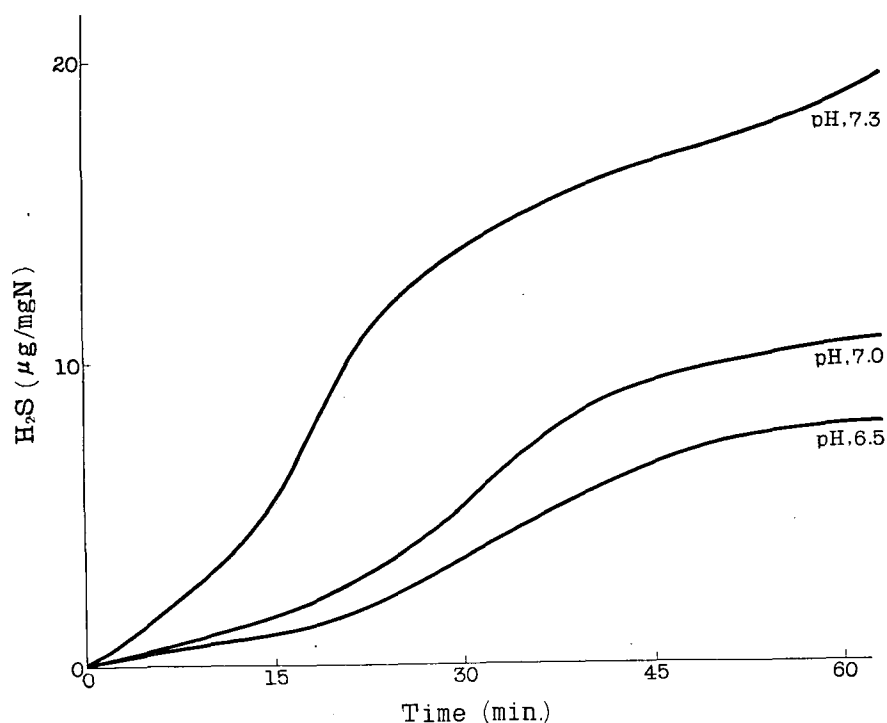


Fig. 12. The Amounts of Hydrogensulfide Produced by Acetone Powder of Adapted-cell at Different pH.

out by using cell free extract. Cell free extracts were prepared from acetone powder according to the procedures in Table 9.

To isolate and identify the product from the reaction mixture, enzyme A (10 mg) was incubated at 40°C in the presence of 1 ml of M/5 phosphate buffer (pH, 7.3) and 1 ml of L-cysteine hydrochloride (1%) under aeration. After one hour's reaction the mixtures were collected together and filtered. When 2N-hydrochloric acid solution with 2,4-dinitrophenylhydrazine was added to the filtrate, a small amount of 2,4-dinitrophenylhydrazone was obtained and recrystallized from aqueous alcohol. This yellow crystalline derivative had a m.p. of 218°C, and showed no depression in the mixed melting point determination with pure pyruvic acid 2,4-dinitrophenylhydrazone. The RF of this crystalline material on paperchromatogram was 0.38, when phenol saturated with 3% ammonia was used as the solvent, and that of pyruvic acid 2,4-dinitrophenylhydrazone developed with the same solvent was 0.37. These results indicated

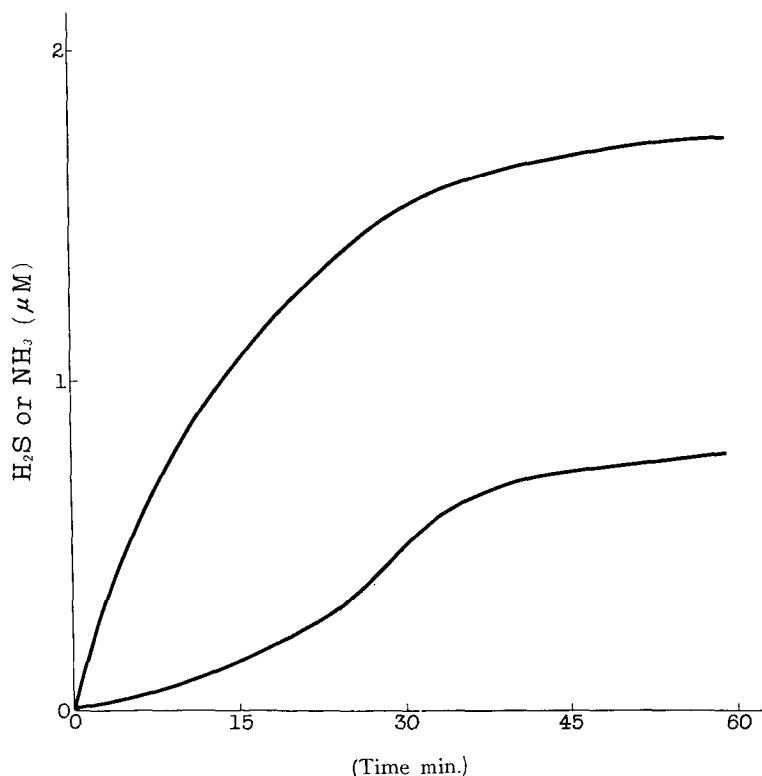


Fig. 13. Amounts of NH<sub>3</sub> and H<sub>2</sub>S Formed from Cysteine by Enzyme A. (Reaction temp., 40°C; pH, 7.3).

that pyruvic acid was one of the reaction products in the degradation of L-cysteine. Hence it is naturally considered that cysteine must undergo deamination besides desulfhydration in order to convert to pyruvic acid. Therefore the determination of ammonia<sup>(92)</sup>, hydrogensulfide and pyruvic acid<sup>(93)</sup> formed in the reaction were carried out, but pyruvic acid was not detectable owing to its small amounts. The results are shown in Fig. 13. The formation of hydrogensulfide was not so rapid as that of ammonia at the initial stage of the reaction, and after one hour's reaction the amount of the former was less than half that of the latter. From these reactions, it is unlikely that one enzyme catalyzes simultaneously both deamination and desulfhydration of cysteine. In other words, deamination may take place in the initial stage of the degradation process of cysteine, and then the resulting intermediate will be converted to pyruvic acid by desulfhydration. Therefore hydrogensulfide formation from cysteine probably does not occur without its deamination. This assumption is supported by the anaerobic experiments, as shown in Fig. 14. Hydrogensulfide formation under anaerobic conditions was negligible, compared with its production

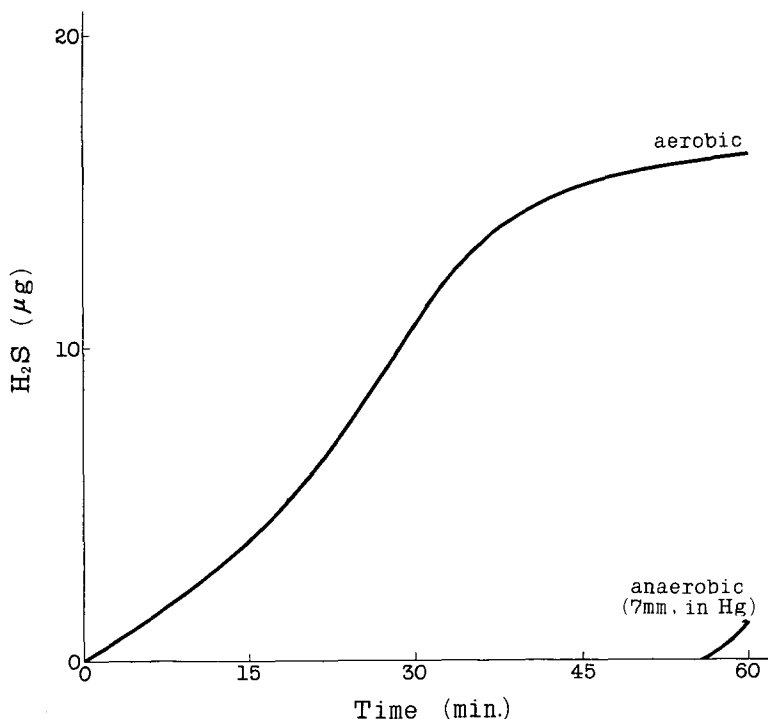
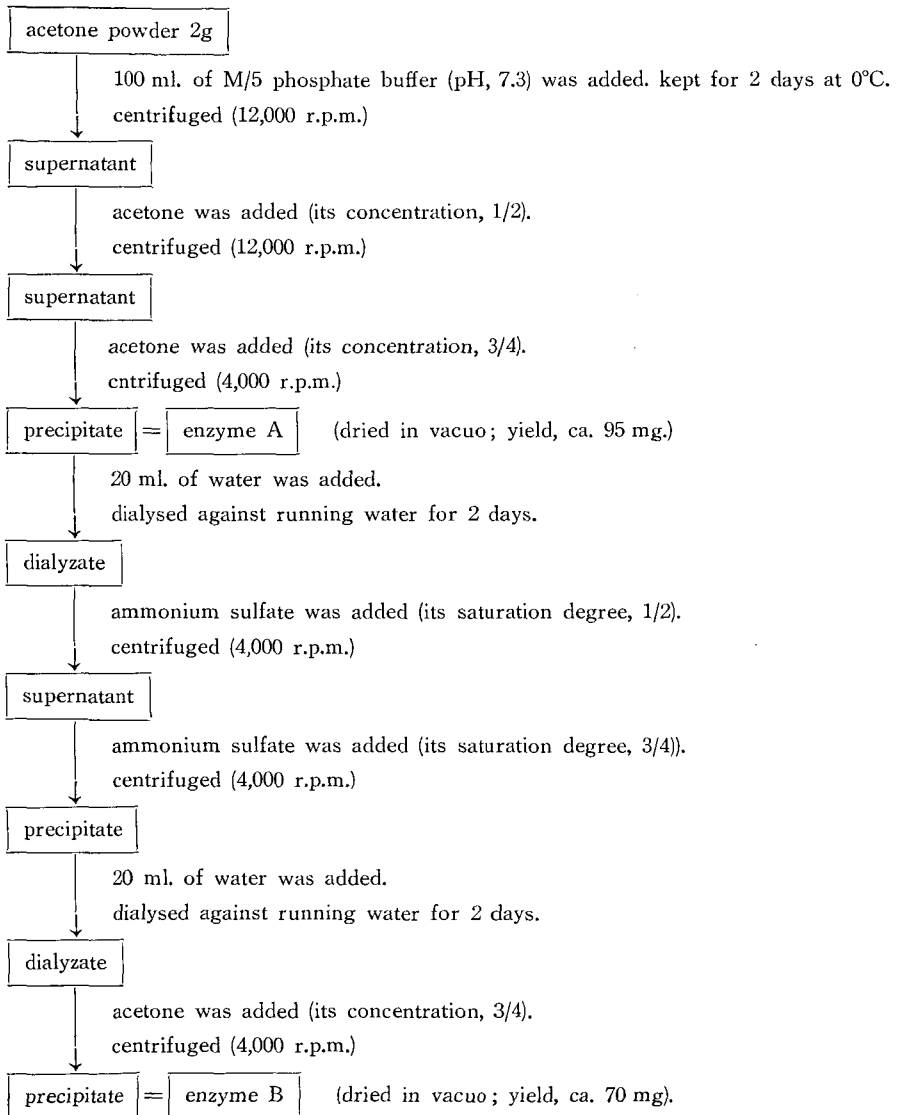


Fig. 14. H<sub>2</sub>S Formation Under Aerobic and Anaerobic Conditions. (Reaction temp., 40°C; pH, 7.3)

TABLE 10. Preparation of Cell-free Extract from Acetone Powder



under aerobic conditions. The degradation of cysteine to pyruvic acid may be preceded by oxidative deamination in advance. Moreover, as the pyruvic acid concentration in the reaction mixture is too small to be determined, it may be further oxidized by oxygen in the presence of pyruvic acid oxidase. If this is the case, oxygen will be utilized not only for the oxidative deamination of cysteine, but also to oxidize the pyruvic acid produced in the reaction. From these results, it is assumed that this enzyme preparation may be at least a mixture of L-cysteine-desulphhydrase, L-cysteine deaminase, and pyruvic acid oxidase. According to IMAMIYA et al.<sup>(87)</sup>, SUDA et al.<sup>(88)</sup> and FROMAGEOT<sup>(84)</sup>, it was presumed that same enzyme was able to catalyze both deamination and desulphhydration of L-cysteine. It seems that the present results differ from those previously reported. In summary, enzyme A contained two kinds of enzyme, cysteine deaminase and desulphhydrase, and catalyzed following reaction: L-cysteine  $\rightarrow$  (intermediate X + NH<sub>3</sub>  $\rightarrow$  intermediate Y + NH<sub>3</sub> + H<sub>2</sub>S)  $\rightarrow$  pyruvic acid + NH<sub>3</sub> + H<sub>2</sub>S.

The effects of inhibitors or activators for hydrogensulfide formation were examined and the results are shown in Tables 11 and 12. The composition of the reaction mixture in both experiments was as follows: 1 ml. of M/5 phosphate buffer (pH, 7.3), 1 ml. of 0.1% L-cysteine hydrochloride and 10 mg of enzyme A or 10 mg of enzyme B.

TABLE 11. Effects of Activators for Hydrogensulfide Formation by Enzyme B. (one hour's reaction at 40°C; pH 7.3)

addition	control H <sub>2</sub> O, 1.5 ml	pyridox- amine (250 r), 0.5 ml, H <sub>2</sub> O, 1ml	1.0 ml of ATP (500 r)					
			H <sub>2</sub> O, 0.5 ml	pyridox- amine (250 r), 0.5 ml	thiamine- HCl (250 r), 0.5 ml	pan- tho- tenic acid (250r), 0.5 ml	biotin (250 r), 0.5 ml	folic acid (250 r), 0.5 ml
H <sub>2</sub> S (g)	4.9	28.4	6.2	31.7	14.0	4.0	4.0	17.7

Every carbonyl-reagent among the tested inhibitors showed an inhibitory effect. In activation experiments, the most remarkable effect on hydrogensulfide formation was shown in the presence of both ATP and pyridoxamine. Positive effects were also observed to some degree in the presence of ATP and thiamine hydrochloride salt or ATP and folic acid. Concerning inhibitors or activators for cysteine desulphhydrase, the above results are inconsistent with other results, but the stimulating effect of thiamine hydrochloride salt or folic acid has not been previously reported.

TABLE 12. Effects of Inhibitors for Hydrogen-sulfide Formation by Enzyme A (One hour's reaction at 40°C; pH 7.3)

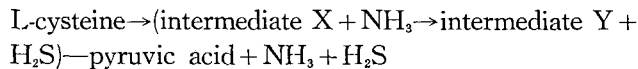
inhibitors	conc. of inhibitors	H <sub>2</sub> S formation (μg)	rate of inhibition (%)
control		15.8	
potassium arsenite	10 <sup>-5</sup> M	0	100
semicarbazide	"	5.7	64
potassium cyanide	"	0	100
monoiodoacetate	"	5.4	65
2,4-dinitrophenol	3×10 <sup>-3</sup> M	15.6	0
hydroxylamine	10 <sup>-3</sup> M	12.4	21

### Summary

1. By the action of a cell suspension of *Rhizobium japonicum* which is not adapted to L-cysteine, hydrogen sulfide was formed from L-cysteine after a lag time. On the contrary, hydrogen sulfide was rapidly formed from L-cysteine by a cell suspension of L-cysteine-adapted bacteria.

2. An enzyme preparation containing at least three kinds of enzyme, cysteine desulfhydrase, deaminase and pyruvic acid oxidase was prepared from cysteine-adapted *Rhizobium japonicum* and partially purified.

3. The reaction catalyzed by the enzyme preparation may be able to proceed in the presence of oxygen as follows:



It is assumed that oxygen probably participates in the deamination of L-cysteine and also in the oxidation of pyruvic acid.

4. The reaction was inhibited by each carbonyl-reagent.

5. Pyridoxamine seems to be a stimulator to L-cysteine desulfhydrase. Thiamine and folic acid were also shown to have a certain stimulating effect in the presence of ATP.

### Part 2. Behavior toward aspartic acid

Aspartic acid is one of the most important amino acids in the sense of the initial organic nitrogen compound produced by the reaction between oxaloacetic acid or fumaric acid and ammonia formed in biological nitrogen fixation. From the results of many investigations which were carried out by using different kinds of microorganism, the first steps of aspartic acid cleavage have been

summarized as follows :

1. aspartic acid  $\xrightarrow{\text{aspartase}}$  fumaric acid + ammonia<sup>(94)</sup>
2. aspartic acid  $\xrightarrow{\text{decarboxylase}}$   $\alpha$ -alanine or  $\beta$ -alanine + carbon dioxide<sup>(79,95)</sup>
3. aspartic acid +  $\alpha$ -keto acids  $\xrightarrow{\text{transaminase}}$  oxaloacetic acid + amino acids<sup>(96)</sup>

Taking into consideration these processes, the metabolic behavior of *Rhizobium japonicum* toward aspartic acid was studied.

### Experimental results and discussion

When *Rhizobium japonicum* was cultivated at 30°C for 4 days on the culture medium containing aspartic acid, glutamic acid or valine as a sole nitrogen source and mannitol besides inorganic salts shown in Table 1. The yield of bacterial cells (wet weight) obtained from the aspartic acid culture medium was usually about 1.5~2 times as much as that obtained from the glutamic acid or valine culture medium. Therefore aspartic acid seems to be the most useful or effective amino acid of the three for the growth of *Rhizobium japonicum*.

Cell suspensions were prepared by the usual method and each 20 ml was incubated at 37°C for one hr. in the presence of aspartic acid (50 mg), 20 ml of M/5 phosphate buffer (pH, 6.8) containing 0.9 g of potassium arsenite. The reaction mixture was treated by the same procedure as that in Table 6 and a small quantity of 2,4-dinitrophenylhydrazone was obtained. This hydrazone melted at 215°C and the mixed melting point with pure pyruvic acid 2,4-dinitrophenylhydrazone was not depressed. The RF of this hydrazone on paper-chromatogram (solvent; n-butanol: water: ethanol=5:4:1) was 0.35, which was identical with that of pyruvic acid 2,4-dinitrophenylhydrazone.

Although pyruvic acid was found or detected as a reaction product from aspartic acid by the action of the cell suspension, it is not certain which pathway of the above three processes participates predominantly in the formation of pyruvic acid, because there is a possibility that pyruvic acid is formed through any reaction in above scheme. Similar experiments were carried out by the use of a cell free extract prepared by the same procedure as that in part 5. The experimental conditions were as follows: 1 ml. of cell free extract, 1 ml. of M/5 phosphate buffer (pH, 6.8) and 1 ml. of aspartic acid (50 mM as sodium salt) were mixed in the center well of a WARBURG apparatus. The reaction temperature was 38°C. The carbon dioxide liberated after one hour's reaction

was about  $8 \mu\text{l}$ , while no oxygen uptake was detected. The resulting reaction mixture was made slightly basic with sodium hydroxide, dried in vacuo and the residue was extracted with 80% ethanol solution. After concentration of the ethanol extract, the aliquot was developed on paper with phenol saturated with water as the solvent and amino acids were identified by their color reaction with ninhydrin. Besides the spot of aspartic acid ( $\text{RF}=0.20$ ), only one faint spot ( $\text{RF}=0.69$ ) corresponding to the position of  $\beta$ -alanine, was recognized. From these results, it was presumed that decarboxylation might take place in this reaction, but that the  $\beta$ -decarboxylation of aspartic acid would not take place, judging from the results of paperchromatographic procedure in which  $\alpha$ -alanine was not detected. For the same reason, transamination would not take place.  $\alpha$ -Decarboxylation of aspartic acid would take place to slight degree, so that the biochemical significance of  $\alpha$ -decarboxylation seemed to be much less, because the color intensity of the  $\beta$ -alanine spot (probably) on paper was too weak, compared with the amount of carbon dioxide liberated. Therefore it was thought that aspartic acid was previously converted to fumaric acid by aspartase, and the carbon dioxide was formed by further oxidation of malic acid produced from fumaric acid, because this extract contained fumarase and malic enzyme as previously mentioned. If this was the case, oxygen uptake

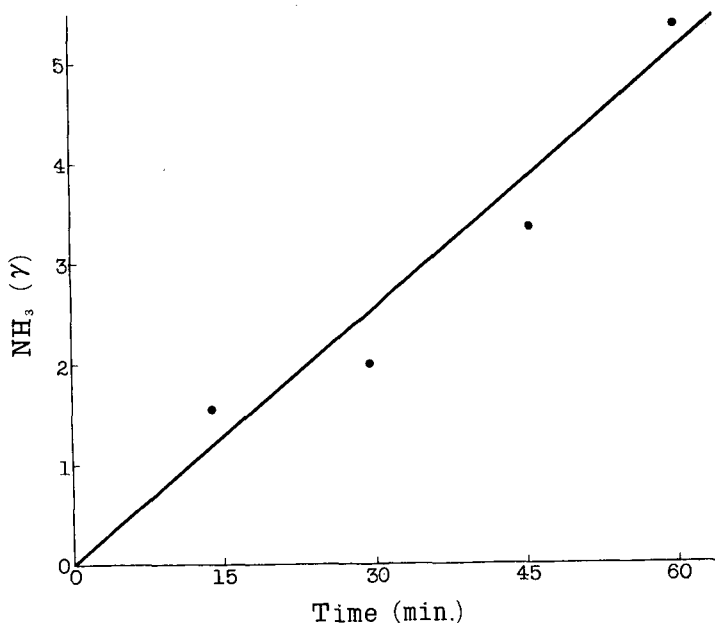
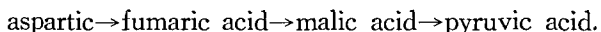


Fig. 15. Ammonia Formation by Cell Free Extract.



should be observed in proportion to carbon dioxide evolution, but the determination of oxygen uptake by the manometric method was unsatisfactory.

To examine aspartase activity of the cell free extract, ammonia formed from aspartic acid was determined by the method of CONWAY et al.<sup>(92)</sup>. The reaction mixture containing 10 ml. of cell free extract (1 ml  $\doteq$  0.10mg N), 10 ml. of M/10 phosphate buffer (pH, 6.8) and 10 ml. of aspartic acid (1.3 mg) was incubated at 37°C and 1 ml. of this mixture was subjected to ammonia determination at intervals of 15 minutes. The results are shown in Fig. 15. Ammonia formation was increased in proportion to the reaction time. In addition, the reaction mixture containing cyclohexanol after one hour's incubation was dried in vacuo, made acidic with hydrochloric acid, and extracted with ether. After the ether was evaporated, the residue was dissolved in a small quantity of water. A part of this aqueous solution was applied to paperchromatography (solvent; phenol: water: 90% formic acid = 15 g : 5 ml : 2 ml.). Two spots (RF = 0.74 and 0.59) were observed. Although the higher spot was faint, the longer one was intensive in color and its RF was identical with that of fumaric acid. From these results, it was concluded that aspartic acid was converted to ammonia and fumaric acid by the action of the cell extract of *Rhizobium japonicum*, and that the present extract might catalyze the next reactions:



### Summary

1. In the presence of potassium arsenite, pyruvic acid formed from aspartic acid by the cell suspension was identified as its 2,4-dinitrophenylhydrazone.

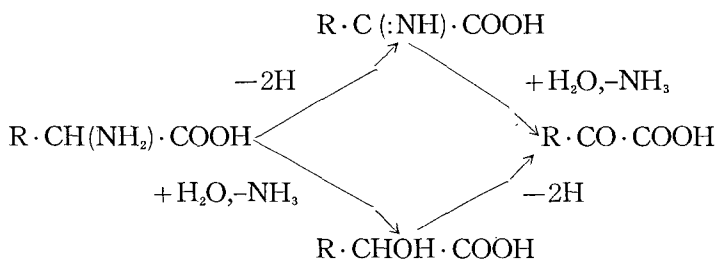
2. Carbon dioxide was liberated from aspartic acid by the action of a cell free extract, but  $\beta$ -alanine was only detected in negligible amount while none of the other amino acids were detected. Although the presence of aspartic acid  $\alpha$ -decarboxylase in *Rhizobium leguminosarium* or *Rhizobium trifolii*<sup>(93)</sup> is reported, the existence of this enzyme in *Rhizobium japonicum* is uncertain from the present experiments.

3. Aspartic acid was attacked by the cell free extract, and it was found that ammonia and fumaric acid were produced in presence of cyclohexanol.

4. It is assumed that aspartic acid may be metabolized by this bacteria according to the following reactions: aspartic acid  $\rightarrow$  fumaric acid  $\rightarrow$  malic acid  $\rightarrow$  pyruvic acid, and that carbon dioxide evolution may be due the oxidation of malic acid.

### Part 3. Deamination of several amino acids by the action of cell suspensions

It is generally known that the first step in the breakdown of amino acids in the microorganism is frequently their deamination. According to many investigators<sup>(107-111)</sup> the existence of D- or L-amino acid oxidase is known in various microorganisms. The deamination of the amino acids to keto acids proceeds either through the corresponding imino acids or through  $\alpha$ -hydroxy acids as intermediates.



While up to now imino acids had been considered as intermediates<sup>(113,114)</sup>, it was shown<sup>(115)</sup> that the L- $\alpha$ -hydroxy acids are oxidized to keto acids by the L-amino acid oxidase of rat kidneys.

As described in the previous part of this chapter, cysteine was broken by oxidative deamination and aspartic acid deaminated by aspartase independent of the presence of oxygen. In relation to these experimental results, the bacterial behaviour toward several amino acids were examined.

#### Experimental Results and Discussion

The reaction mixture was as follows: 2 ml. of each amino acid (150 mg), 25 ml. of cell suspension, 20 ml. of M/5 phosphate buffer (pH, 6.5) and 3 ml. of 4.8% potassium arsenite. After one hour's reaction at 37°C the reaction mixture was treated with the same procedure as mentioned in Table 6, that is, keto acids produced by the reaction were separated as their 2,4-dinitrophenylhydrazone derivatives. The quantity of each derivative was very small and its R<sub>f</sub> on paperchromatogram is shown in Table 13.

When DL- $\alpha$ -amino-n-butyric acid was used as substrate, it was found that three kinds of carbonyl compounds were formed from this amino acid by the action of the cell suspension. Their 2,4-dinitrophenylhydrazone derivatives showed three spots on paperchromatogram and their R<sub>F</sub> values were 0.91, 0.73 and 0.54, which corresponds to those of pyruvic acid 2,4-dinitrophenylhydrazone. Although these derivatives could not be separated from one another,

TABLE 13. RF Values Of 2,4-Dinitrophenylhydrazone Derivative

amino acids added as substrate	RF				
DL- $\alpha$ -amino-n-butyric acid	0.91	0.73	0.54		
DL- $\beta$ -amino-n-butyric acid					
$\gamma$ -amino butyric acid	0.68				
DL-threonine	0.93	0.73	0.65	0.39	0.31
DL-valine	0.82		0.54		

solvent ; butanol : water : ethanol=5 : 4 : 1<sup>(64)</sup>.

their presence leaves no doubt as to the ability to produce keto acids from this amino acid.

Using DL- $\beta$ -amino-n-butyric acid as substrate, no 2,4-dinitrophenylhydrazone was obtained. Therefore it is thought that the amino group at the  $\beta$ -position of this amino acid may not be attacked by this bacteria.

When  $\gamma$ -amino butyric acid was used as the substrate, 2,4-dinitrophenylhydrazone, showing one spot (RF, 0.68), was obtained in small quantity. This melted at about 180°~182°C under carbonization, but it has not yet been successfully identified.

In the case of DL-threonine, a mixture of 2,4-dinitrophenylhydrazone, showing five spots on paperchromatogram, was obtained. The derivative which showed RF=0.93 was separated from the other substances by the paperchromatographic method. The spot locating at the range of 0.90 to 0.95 was cut into strips, extracted by alcohol and purified by recrystallization from aqueous alcohol (50%). Yellow needle crystals were obtained which melted at 168°C, and which were nearly identical with those of 2,4-dinitrophenylhydrazone of  $\alpha$ -ketovaleric acid. The results of an elementary analysis was as follows:

Sample, 0.534 mg	N, 18.72%
Calcd. for C <sub>11</sub> H <sub>12</sub> O <sub>6</sub> N <sub>4</sub>	N, 18.91%

These results provide evidence indicating the formation of  $\alpha$ -ketovaleric acid from DL-threonine by *Rhizobium japonicum*, but the mechanism of this keto acid formation from DL-threonine is not known. The other derivatives were not obtained in pure state owing to their small amounts.

In the case of DL-valine, a 2,4-dinitrophenylhydrazone mixture showing two spots on paperchromatogram, was obtained. The spot (RF=0.54) was faint and corresponded to that of pyruvic acid 2,4-dinitrophenylhydrazone. The other spot (RF=0.82) was cut off from the paper and extracted with ethanol.

The ethanol extract was concentrated, and then diluted with water to about 50%. A yellow crystalline substance separated out. After repeated recrystallization from aqueous alcohol, its melting point was about 193°C, which was nearly identical with that of 2,4-dinitrophenylhydrazone of  $\alpha$ -ketoisovaleric acid. This melting point did not show a depression in the mixed melting point determination with pure 2,4-dinitrophenylhydrazone of  $\alpha$ -ketoisovaleric acid. Therefore it was found that DL-valine was converted to the corresponding  $\alpha$ -keto acid by *Rhizobium japonicum*.

### Summary

1. From the experimental results that keto acids were formed from both DL- $\alpha$ -amino-n-butyric acid and  $\gamma$ -amino butyric acid by the action of the cell suspension, it is assumed for the first time that the breakdown of these amino acids may be due to their deamination.

2. DL- $\beta$ -amino-n-butyric acid was not deaminated by the cell suspension.

3.  $\alpha$ -Ketovaleric acid and four other keto acids were formed from DL-threonine. It is probable that  $\alpha$ -ketovaleric acid may be an indirect and second reaction product of DL-threonine.

4.  $\alpha$ -Keto isovaleric acid was formed from DL-valine. This fact shows that amino acid oxidase is present in *Rhizobium japonicum*.

### General discussion

Since KREBS<sup>(97)</sup> pointed out the presence of the TCA cycle in pigeon heart muscle for the first time, many investigators have been working hard with this problem and now it is clearly recognized at present that this cycle plays a most important role in the metabolism of carbohydrates, amino acids and fatty acids by animal and plant cells<sup>(98)</sup>. It is further recognized that the energy liberated from the reactions of this cycle is transferred to produce energy-rich phosphate bonds as demonstrated by the studies of OCHOA<sup>(99)</sup>, SCHNEIDER<sup>(100)</sup> and LIPMANN<sup>(101)</sup>.

In animal tissues, one of the most functional and biochemically significant characteristics of the TCA cycle appears to consist undoubtedly in the production of energy, but this is not necessarily the case with bacterial cells: this cycle in bacteria may serve to produce intermediates for synthetic processes and not be concerned so much with the production of energy, as was described by KREBS<sup>(102)</sup>. Although many experiments have been conducted to find the existence of the TCA cycle in microorganisms, any decisive findings have not been obtained owing to their complex metabolic behavior<sup>(103)</sup>. Namely, it is

known that *Pseudomonas fluorescens* completely lacks the TCA cycle and in this case pyruvate is directly formed from glucose via 2-ketogluconate<sup>(104)</sup>. CROOK<sup>(105)</sup> reported the presence of the DCA cycle system (dicarboxylic acid cycle) in bacteria, but it is not yet clear whether this is main pathway of carbohydrate breakdown or not. The presence of the glyoxylic acid cycle<sup>(32)</sup> connecting with the TCA cycle was demonstrated in *Pseudomonas fluorescens*, and some other side pathways derived from the TCA cycle were also reported.

The metabolic behavior of microorganisms which are observed in connection with their respiration systems are so different from each other according to their species, that it is not appropriate to draw a hasty conclusion that *Rhizobium japonicum* has TCA cycle, on the basis of the fact that every organic acid in it is metabolized by this bacteria (Table 2). Therefore individual reaction in it should be studied enzymatically for the purpose of finding out its presence in *Rhizobium japonicum*.

On one hand, ammonia synthesis from gaseous nitrogen and hydrogen is in need of an energy supply. It is naturally considered that energy may be also required for biological nitrogen fixation as in the case of chemical synthesis of ammonia. From the viewpoint of thermodynamics, nitrogen is extremely stable and inactive on account of its triple bond, so that it will not react with hydrogen unless it undergoes the activation in the initial stage of the reaction. Such activation energy may be supplied from the respiration energy which is closely related to the TCA cycle. The present paper is a fundamental study to explain the role of the TCA cycle which may play an important part of energy supplier for nitrogen assimilation, and deals with the metabolism of certain amino acids which have close relation to this cycle.

In this paper, it was shown that cell suspensions or crude enzyme preparations obtained from *Rhizobium japonicum* were able to catalyze following reactions:

1. isocitrat —————→  $\alpha$ -ketoglutarate
2.  $\alpha$ -ketoglutarate —————→ succinate
3. succinate —————→ fumarate
4. fumarate —————→ malate
5. malate —————→ pyruvate
6. lactate —————→ pyruvate

and these data were not contradictory with many results published up to now. But preparations of aconitase and oxalosuccinic decarboxylase were not successfully obtained from *Rhizobium japonicum*, in spite of the fact that several methods were employed. For instance, the extract of the cell with phosphate buffer solution which was obtained after the mechanical destruction of the cell wall at 0°C did not show any enzymatic activity corresponding to each of the

two enzymes just mentioned. Probably this fact is not due to the lack of these enzymes in *Rhizobium japonicum*, but rather to the inactivation of the enzymes during the separation procedures or to the very low activity which may make it difficult to carry on the usual analytical methods. Such a presumption is introduced from the experimental facts that the present microorganism could grow in the culture medium supplemented with each of citrate and cisaconitate as a sole carbon source and that these two organic acids were oxidized to a great degree by the cell suspensions.

The presence of malic dehydrogenase is uncertain at present, because the oxidation of malate took place at pH, 7.5, pyruvate was formed as an oxidation product and oxaloacetate was not detected by the cyanohydrin method<sup>(106)</sup>. From these experimental results, it is assumed that the TCA cycle might most probably exist in *Rhizobium japonicum*, although the reaction due to the condensing enzyme has not been studied.

As was shown previously, the cell free extract of *Rhizobium japonicum* was able to catalyze the conversion of aspartate and cysteine into fumarate and pyruvate respectively, and these two reaction products would be fated to be oxidized via TCA cycle. Now, attention must be paid to the fact that  $\alpha$ - or  $\beta$ -decarboxylation of aspartate was not observed. Although satisfactory results were not obtained, some experiments showed that amino butyric acid, threonine and valine might be subjected to deamination but not decarboxylation by this bacteria. The physiological significance to living things of amino acid decarboxylation is not clear, but some investigators<sup>(112)</sup> have suggested that amino acid decarboxylation may be some kind of excretory function of nitrogen compound in microorganisms. If such suggestion is adopted, *Rhizobium japonicum*, which does not perform amino acid decarboxylation, may be thought to have high ability of utilizing completely amino acids as nitrogen and carbon sources by means of deamination.

### Conclusion

All organic acids in the TCA cycle were useful or effective for the growth of *Rhizobium japonicum*, and are metabolized by this bacteria in proportion to the values of oxygen uptake and to the decolorization time of methylene blue. As the presence of the TCA cycle in this organism was assumed from the above results, several enzymatic studies were carried out to confirm its existence.

By the action of cell free preparations on  $\alpha$ -ketoglutaric acid, succinic acid was produced. In this case the uptake of one mole of oxygen and the evolution

of two moles of carbon dioxide were observed. Therefore this reaction seems to be oxidative decarboxylation in which coenzyme A seems to participate.

The crude enzyme preparations of *Rhizobium japonicum* catalyzed the conversion of succinic acid to fumaric acid in this reaction. Methylene blue did not have an effect as an electron acceptor, while phenazine methosulfate was remarkably effective. It is assumed then that electrons split off through the reaction would be directly transferred to phenazine methosulfate and not via cytochrome b, and the resulting leuco phenazine methosulfate would be oxidized by molecular oxygen.

Fumarase action of the dried cell preparation was detected by the formation of malic acid from fumaric acid.

Lactic acid was oxidized to pyruvic acid by acetone powder of this bacteria. In this reaction, liberation of carbon dioxide or formation of acetic acid were not observed. Therefore the lactic acid dehydrogenase present was thought to be the same as that of muscle.

Malic acid degradation was maximal at pH, 7.5. In this reaction, pyruvic acid was detected as a reaction product, but oxaloacetic acid was not. The uptake of one mole of oxygen and the evolution of three moles of carbon dioxide were shown by the use of cell suspensions. This fact should mean that pyruvic acid formed from malic acid might be subjected to further conversions by intact cells. These conversions would include dismutation of pyruvic acid as one of the possible pathways. Moreover, the enzyme involved in the oxidation of malic acid was assumed to be malic enzyme.

The presence of isocitric acid dehydrogenase was shown by values of oxygen uptake and identification of  $\alpha$ -ketoglutaric acid as reaction product.

From these results, the presence of the TCA cycle in *Rhizobium japonicum* was most probably certain, and in addition considerable information on its respiration system was obtained.

L-cysteine desulphydrase was formed by bacteria adapted to L-cysteine, and was partially purified as the cell-free preparations contained both L-cysteine deaminase and desulphydrase. L-cysteine was deaminated by this preparation in advance, and successively the deaminated intermediate was desulhydrated.

Oxygen which was essential in the reaction might be employed in oxidative deamination of L-cysteine and oxidation of pyruvic acid due to occurrence of desulhydratation at the same time. Desulhydratation was inhibited by carbonyl reagents and stimulated to greater degree by pyridoxamine and to some degree by thiamine and folic acid in the presence of ATP.

The presence of aspartase was detected in this bacteria, but  $\alpha$ - or  $\beta$ -decarboxylase of aspartic acid was not. Thus, aspartic acid may be broken

down according to following scheme : aspartic acid→fumaric acid→malic acid→pyruvic acid.

On the behavior of this bacteria toward amino acids some discussion was presented. That is, every tested amino acid, DL- $\alpha$ -amino-n-butyric acid,  $\gamma$ -amino-butyric acid, DL-threonine and DL-valine, were attacked by *Rhizobium japonicum* and the formation of keto acids was observed. From these results, it is assumed that amino acid oxidase is present in this bacteria.

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