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L-Glutamate Fermentation with Acetic Acid by an Oleic Acid-requiring Mutant

(II) Inhibitory Factors against the Extracellular Accumulation of L-Glutamate

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Abstract

The effects of substrates (glucose and acetate), L-glutamate, which is an end product of fermentation, and sodium chloride, which raises the osmotic pressure on the L-glutamate fermentation using an oleic acid-requiring mutant, were investigated, and the L-glutamate yield was found to be reduced in the presence of these substances. The mode of action of these substances differed and was able to be classified into two types. One type includes glucose, L-glutamate, and sodium chloride; the other includes acetate.

The inhibition of L-glutamate synthesis by the former group was interpreted as a result of the shift of metabolism from L-glutamate synthesis to a complete oxidation system of the substrate, probably due to an indirect action which would bring about a change in the transport activity of L-glutamate across the membrane caused by the increase in osmotic pressure.

On the contrary, a direct inhibitory effect on a biosynthetic process of L-glutamate was presumed to be the nature of the acetate effect.

Introduction

The previous paper[1] revealed that an oleic acid-requiring mutant of *Brevibacterium thiogenitalis* produced a large amount of L-glutamate from acetate.

It is well known that L-glutamate fermentation is markedly affected by various chemical and physical conditions such as medium components, temperature, pH, oxygen, and so on.[2~7] However, there are only a few reports concerning the effect of substrate, osmotic pressure, and L-glutamate accumulation on this fermentation.[3,8]

During studies on L-glutamate fermentation with acetate, the yield was found to be reduced in the presence not only of acetate but also of L-glutamate. Although such facts also had been observed in the case where glucose was used as a substrate, there were definite differences between glucose and acetate in their mode of action.

The present report deals with the changes in biochemical aspects induced by acetate as compared with glucose, L-glutamate, and sodium chloride.

Materials and Methods

1. Organism *B. thiogenitalis* D-248, an oleic acid-requiring mutant, was used throughout this study.

2. Cultivation The standard conditions for shake culture are described in Table 1. Desired materials were added to the basal medium and the detailed conditions are described in the appropriate table or figure.

Table 1. Standard conditions for shaking culture.

Component \ Kind of medium	Seed	Main	
		Glucose medium*	Acetate medium**
Glucose	2.0%	12.0%	—
Sodium acetate	—	—	1.37 (initial) %
Urea	0.5	0.5	0.05
Corn steep liquor	1.0	0.5	0.15
$(NH_4)_2SO_4$	—	—	0.1
KH_2PO_4	0.1	0.1	0.1
$MgSO_4 \cdot 7H_2O$	0.04	0.04	0.04
$MnSO_4 \cdot 4\text{-}6H_2O$	—	—	0.001
$CaCl_2 \cdot 2H_2O$	—	—	0.005
Thiamine·HCl	—	100 $\mu g/l$	100 $\mu g/l$
Biotin	—	30 $\mu g/l$	30 $\mu g/l$
Oleic acid	300 mg/l	175 mg/l	175 mg/l
Phenol red	—	10 mg/l	10 mg/l
Temperature	28°C	32°C	32°C
Volume/200 ml flask	20 ml	30 ml	50 ml
Revolutions per minute	200	200	200
Inoculum size	one loopful	5%	5%
Culture time	18 hr	48 hr	48 hr
pH	7.0 (start)	7.0~8.5	7.0~8.5

* Baffled conical flasks were used. The pH was adjusted with urea when it fell below 7.0.

** Baffled conical flasks without closure were used.

Glacial acetic acid corresponding to 0.4% relative to the initial liquid volume was added every hour after the initial acetate had been consumed. Total acetic acid added 35 times amounted to 15%. The amounts of acetic acid were expressed as weight per volume percentages based on the initial volume. The pH was kept within 7.0 to 8.5 with ammonia water.

3. Preparation of resting cells and cell free extracts The cells harvested from the culture broth were washed twice with 0.5% saline, suspended in M/15 phosphate buffer (pH 7.5), and subjected to starvation by shaking on a rotary shaker for 3 hr at 28°C. The cells were then harvested and suspended again in M/15 phosphate buffer (pH 7.5) at a concentration of 10% (wet base) of cells. The starved cells were disrupted through ultrasonic treatment at 20 KC for 30 min with cooling, followed by centrifugation at 16,000×g for 30 min under refrigeration. The supernatant was used as an enzyme preparation.

4. L-Glutamate synthesis by resting cells The experimental conditions are shown in Table 2.

5. Assay methods of enzyme activities Glutamate and isocitrate dehydrogenase were determined according to the spectrophotometric method by which absorption changes of NADP and NADPH at 340 mμ were measured. Isocitrate lyase was

Table 2. Conditions for reaction by resting cells.

Basal glucose mixture		Basal acetate mixture	
Glucose	5.0%	NH_4-acetate	0.4%*
Urea	0.5%	$MgSO_4 \cdot 7H_2O$	0.01%
$MgSO_4 \cdot 7H_2O$	0.01%	Phenol red	10 μg/ml
Phenol red	10 μg/ml	Resting cell suspension	10 ml
Resting cell suspension	10 ml		
Total	30 ml	Total	30 ml

* Glacial acetic acid corresponding to 0.4% relative to the initial liquid volume was added every hour from the start of the reaction. Total acetic acid added 10 times amounted to 4%.

Reaction was carried out for 10 hours. The resting cell suspension contained about 30 mg/ml of dry cells. Other reaction conditions were the same as those in Table 1, unless stated otherwise.

determined by the method of Kimura, Tanaka, and Kinoshita.[9] α-Ketoglutarate decarboxylase was determined by the colorimetric measurement of ferrocyanide produced from ferricyanide by oxidative decarboxylation of α-ketoglutarate.[10] Relative activities of enzymes were calculated based on their specific activities which were represented as units per mg protein, where protein in cell free extracts was estimated by the method of Lowry *et al.*[11]

6. Permeability of L-glutamate For the measurement of efflux ratios of L-glutamate, a modified method of Yoshii *et al.*[12] was applied. The cells harvested from 50 ml of the culture broth by centrifugation were washed twice with 2 M sodium chloride. Intracellular L-glutamate was represented as that extracted by boiling the cells for ten minutes. Efflux ratios were calculated as follows: efflux ratio $(\%) = \frac{A-B}{A} \times 100$, where A is the intracellular L-glutamate after washed twice with 2 M sodium chloride and B is intracellular L-glutamate after two washings with water.

7. Other determinations Bacterial growth was tentatively indicated by optical density at 590 mμ. L-Glutamate was assayed by a turbidometric method using *Leuconostoc mesenteroides*. O_2 uptake and CO_2 evolution were determined by the manometric method. Acetate was estimated as reported previously.[1] Residual sugar was estimated by the method of Somogyi-Nelson.[13]

Results

1. Effect of substrates Substrate inhibition is often noticed in enzymic reactions and fermentations. A marked effect of substrate was also observed in the L-glutamate fermentation as shown in Fig. 1. In the glucose medium the bacterial growth was stimulated in parallel with the increase in glucose concentration, while L-glutamate production was reduced in contrast to the bacterial growth. In the acetate medium, however, the increase in the initial concentration of acetate resulted in the reduction of both bacterial growth and L-glutamate production.

A similar relation between substrate concentration and L-glutamate yield as that mentioned above was also demonstrated in the resting cell system (Fig. 2). Namely, either substrate apparently had an inhibitory action on L-glutamate production.

In L-glutamate fermentation, it is generally accepted that there is a close relationship between L-glutamate production and its efflux. The effect of glucose and

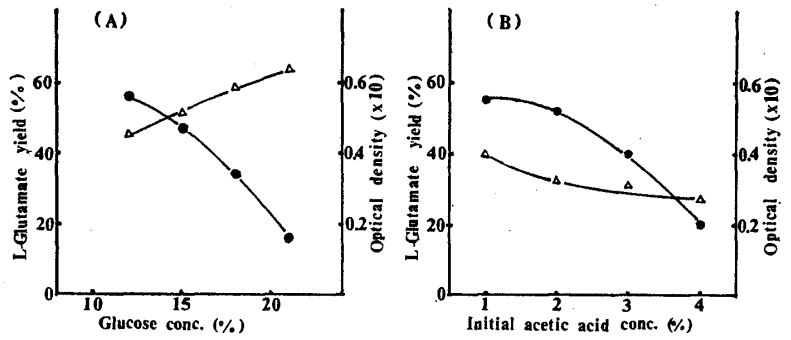

Fig. 1. Effect of substrate concentration on the bacterial growth and L-glutamate yield.
(A) Glucose medium (B) Acetate medium
●—●; L-Glutamate yield △—△; Optical density
Cell concentration was expressed as the optical density at 590 mμ.

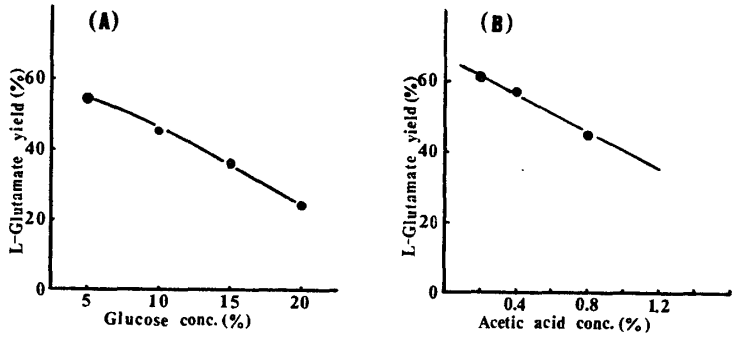

Fig. 2. Effect of substrate concentration on the L-glutamate yield using resting cells.
(A) The substrate for growth and reaction was glucose.
(B) The substrate for growth and reaction was acetate.
In Fig. 2(A), reaction conditions were the same as those in Table 2, except that the glucose concentrations were varied.
In case of (B), indicated amounts of acetic acid were added every time at even intervals until total acetic acid amounted to 4% for 10 hours after the start of the reaction, where the initial concentration was also as indicated in the abscissa. Namely, 0.2% means that the initial concentration of acetic acid was 0.2% and that acetic acid corresponding to 0.2% relative to the initial liquid volume was added every half-hour. Total acetic acid added 19 times amounted to 4%. Other conditions were the same as those in Table 2.

acetate was examined in the cultivation system (Table 3). In the glucose medium, the increase in glucose concentration brought on a concomitant increase in the intracellular L-glutamate and a remarkable decrease in the efflux ratio. A contrary finding was, however, obtained in the acetate medium where the increase in acetate concentration reduced the intracellular content of L-glutamate and did not substantially affect the efflux ratio, notwithstanding the fact that the increase in acetate concentration reduced the L-glutamate formation markedly as shown in Figs. 1 and 2. This finding was one of the most outstanding differences in the effects of glucose and acetate on the biochemical behavior of L-glutamate-producing cells.

To examine the effect of acetic acid and glucose on the efflux of L-glutamate, a washing test by the solutions of these substances was made (Table 4). The inhibitory effect of a high level of glucose on the efflux was clearly demonstrated as expected. However, in 1.5% acetic acid which depressed L-glutamate productivity to one half of that in 0.5% acetic acid, the reduction of L-glutamate efflux

Table 3. Effect of the substrate concentration of the medium on the efflux of L-glutamate.

Glucose-grown cells			Acetate-grown cells		
Substrate conc. (%)	Intracellular L-GA* (mg/g DCW**)	Efflux ratio (%)	Substrate conc.***(%)	Intracellular L-GA* (mg/g DCW**)	Efflux ratio (%)
12	5.7	93.0	1	18.9	98.0
21	9.5	61.1	2	15.4	97.5
			3	14.3	98.3

* L-GA, L-glutamic acid. ** DCW, dry cell weight.
*** Initial substrate concentration as acetic acid.

Table 4. Effect of the concentration of acetate and glucose on the efflux of L-glutamate.

Washings		Intracellular L-GA* (mg/g DCW)	Efflux ratio** (%)
Ice water		<0.3	>98.7
Acetic acid	0.5%	0.7	96.9
	1.0	1.5	93.4
	1.5	3.5	84.6
Glucose	12%	0.4	98.2
	21	20.4	10.5
2 M NaCl		22.8	0

* Intracellular L-glutamate content was measured after the cells were washed twice. Cells grown on acetate medium were employed.

** Efflux ratio $(\%)=\frac{A-B}{A}\times 100$, where, A: intracellular L-glutamate after washing twice with 2 M sodium chloride; B: intracellular L-glutamate after washing twice with water and the test solutions.

was limited to a slight degree. The decrease of efflux ratios in glucose was presumed to be due to the osmotic pressure, which might change the membrane permeability.

The effect of substrate concentration was also investigated with respect to the activities of four enzymes related to the TCA cycle. As shown in Table 5, a noticeable result was obtained for α-ketoglutarate decarboxylase whose activity in 21% glucose-grown cells was about three times as great as that of 12% glucose-grown cells. On the other hand, the enzyme activities in acetate-grown cells were not substantially affected by the initial acetate concentration of the medium. The increase in α-ketoglutarate decarboxylase activity due to the high concentration of glucose might be related to the decrease in isocitrate lyase activity.

Further experiments were made on the effect of substrate concentration on the oxidative activities of cells which also had a close relation to L-glutamate

Table 5. Comparison of enzyme activities in a cell-free extract prepared from cells grown on various concentrations of substrate.

	Glucose		Acetate	
	12%	21%	1%*	3%*
Isocitrate dehydrogenase	100**	94	80	89
Glutamate dehydrogenase	100	98	89	68
Isocitrate lyase	100	38	423	385
α-Ketoglutarate decarboxylase	100	323	74	66

* As acetic acid. ** Relative activity.

productivity. Fig. 3 shows that 21% glucose-grown cells exhibited a higher oxidative activity for glucose than those grown on 12% glucose medium. In acetate-grown cells, however, the initial concentration of acetate slightly affected the oxidative activity for acetate when acetic acid was used at a concentration of 1% to 3%.

The results of the four experiments described above suggest that the mode of inhibitory action of glucose and acetate on the extracellular accumulation of L-glutamate was essentially different, that is, the inhibition of glucose was closely related to the decrease in the efflux of L-glutamate, an increase in intracellular L-glutamate and α-ketoglutarate decarboxylase activity, and a higher oxidative activity for the substrate, whereas that of acetate was substantially independent of such factors.

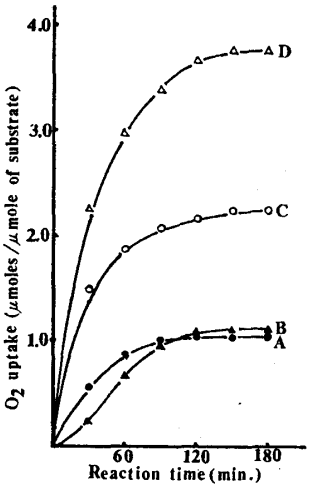

Fig. 3. Effect of substrate concentration on the oxidative activity of cells toward acetate or glucose.
Resting cells used for the estimation of oxidative activities were grown on the following media; A, acetate medium containing 1% acetic acid; B, acetate medium containing 3% acetic acid; C, 12% glucose medium; and D, 21% glucose medium.
Substrates used for the estimation of oxidative activities were as follows: A and B, acetate; C and D, glucose.
The RQ ratios of A to D were 0.898, 0.840, 0.818, and 0.912, respectively.

2. Effect of L-glutamate The effect of L-glutamate which is an end product in the fermentation was then investigated.

Evidence of L-glutamate inhibition against L-glutamate synthesis is illustrated in Fig. 4. An increase in L-glutamate concentration in the reaction mixture resulted in a remarkable reduction of L-glutamate yield. The same figure also shows that the inhibitory effect of L-glutamate was greater in the acetate mixture than in the glucose mixture. The relation between intracellular L-glutamate and its extracellular accumulation was very similar to the case of glucose, that is, addition of L-glutamate to the reaction mixture of resting cells resulted in a concomitant increase in intracellular L-glutamate (Fig. 5) and a significant decrease in L-glutamate yield (Fig. 6).

The levels of intracellular L-glutamate were very sensitive to the extracellular L-glutamate concentration when acetate was used as a substrate (Fig. 5), while the L-glutamate yield depended upon the intracellular L-glutamate to a greater extent when glucose was used as a substrate (Fig. 6).

The L-glutamate effect on the oxidative behavior of cells was also examined. The more the L-glutamate concentration was raised the more the oxidative activities and RQ values were increased (Fig. 7).

Although an apparent identity was observed for the inhibitory effect of glucose, acetate, and L-glutamate on L-glutamate production, the results obtained here sug-

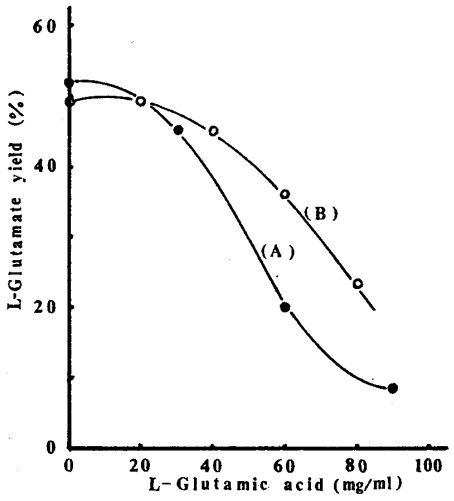

Fig. 4. Effect of added L-glutamate on L-glutamate yield in the reaction by resting cells.

The experiments were carried out by adding L-glutamate as shown in the abscissa to the reaction mixture.

The substrates in (A) and (B) were acetate and glucose, respectively.

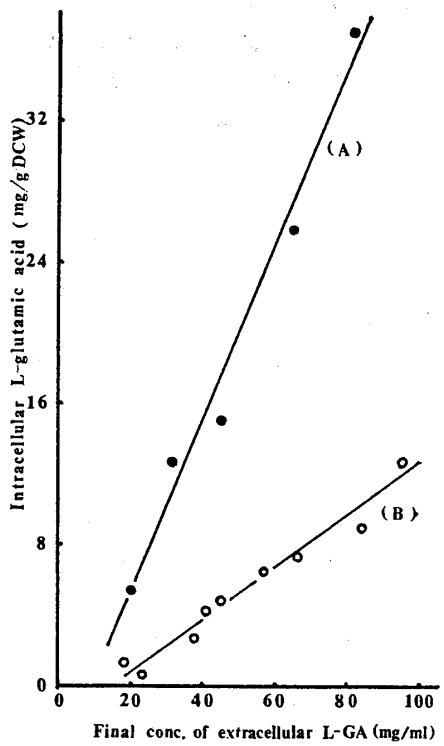

Fig. 5. Relation between extracellular and intracellular L-glutamate in the resting cell system.

L-Glutamic acid concentration in the abscissa is represented as a total of L-glutamic acid both added and produced.

The substrates in (A) and (B) were acetate and glucose, respectively.

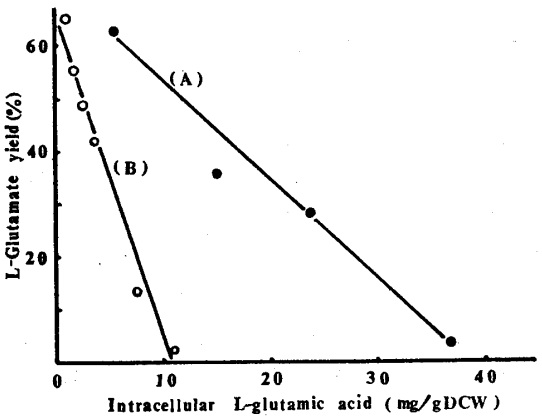

Fig. 6. Relation between intracellular L-glutamate and L-glutamate yield in the resting cell system.

The substrates in (A) and (B) were acetate and glucose, respectively.

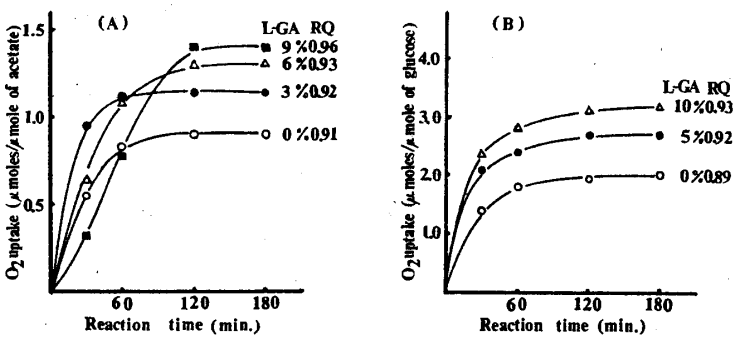

Fig. 7. Effect of L-glutamate concentration on the oxidative metabolism of acetate and glucose.

(A) Oxidation of acetate by acetate-grown cells.

(B) Oxidation of glucose by glucose-grown cells.

gest that the mode of action was different, i.e., the reduction of L-glutamate productivity by glucose and L-glutamate might be mainly attributable to the increase in osmotic pressure which might change membrane permeability and that by acetate, attributable to its direct action on the biosynthetic pathway of L-glutamate.

3. Effect of sodium chloride To investigate whether the inhibition of L-glutamate production by glucose and L-glutamate was attributable to osmotic pressure, sodium chloride was employed as an enhancing agent of osmotic pressure in the subsequent experiments. Fig. 8 represents the effect of sodium chloride on the bacterial growth and L-glutamate production during cultivation in the glucose and the acetate media. In both cases, the yield of L-glutamate was reduced in parallel with the increase in the concentration of sodium chloride but the bacterial growth was stimulated though it was depressed at a concentration above 3% of sodium chloride. A similar finding was observed for L-glutamate formation when the resting cell system was used (Fig. 9).

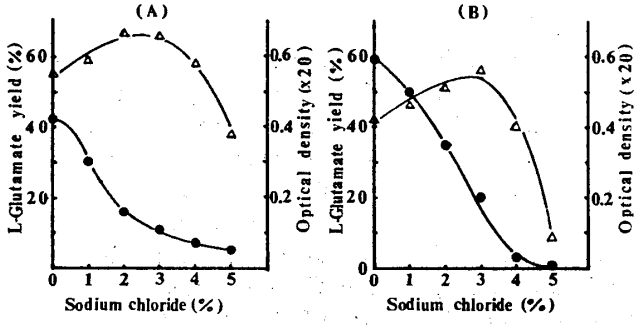

Fig. 8. Effect of sodium chloride on the bacterial growth and L-glutamate yield.
(A) Cultivation in glucose medium.
(B) Cultivation in acetate medium.
●—●: L-Glutamate yield.
△—△: Optical density.
Cell concentration was expressed as the optical density at 590 mμ.

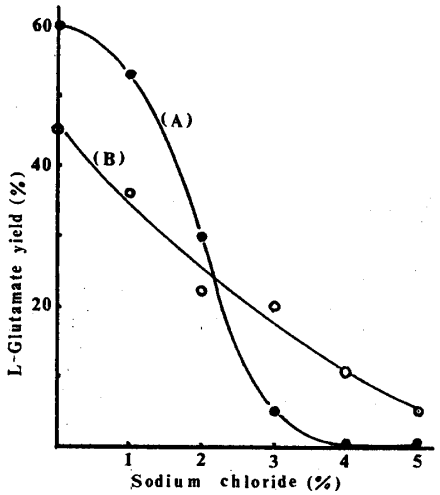

Fig. 9. Effect of sodium chloride on L-glutamate yield in a resting cell system.
The substrates for growth and reaction in (A) and (B) were acetate and glucose, respectively.

Yoshii *et al.*[12] reported that the efflux of L-glutamate was depressed remarkably by a high concentration of sodium chloride. Sodium chloride effect on the efflux of L-glutamate in the present strain is shown in Table 6. The inhibition of the efflux was clearly demonstrated by the addition of sodium chloride as well as glucose.

Finally, the oxidative effect of sodium chloride was studied. As shown in Fig. 10, cells grown on 3% sodium chloride plus 12% glucose or 1% acetic acid plus 3% sodium chloride exhibited a higher oxidative activity for glucose or acetate than those grown on the medium without sodium chloride.

Table 6. Effect of sodium chloride on the efflux of L-glutamate.

Washings	Glucose grown cells		Acetate grorwn cells	
	Intracellular L-GA (mg/g DCW)	Efflux ratio (%)	Intracellular L-GA (mg/g DCW)	Efflux ratio (%)
Water	0.8	88.6	0.3	98.4
1% NaCl	2.5	64.3	3.1	83.6
2% NaCl	3.6	48.6	5.6	76.4
3% NaCl	4.9	30.0	6.8	64.0
4% NaCl	5.8	17.1	18.9	0
5% NaCl	7.1	0	18.9	0
2 M NaCl	7.0	0	18.9	0

Intracellular L-glutamate and efflux ratios were expressed by the same method as in Table 4.

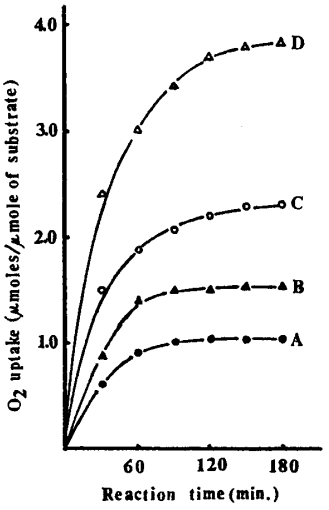

Fig. 10. Effect of sodium chloride on the oxidative activity of cells toward acetate or glucose.

Resting cells used for the estimation of oxidative activities were grown on the following media; A, acetate medium containing 1% acetic acid; B, acetate medium containing 1% acetic acid supplemented with 3% sodium chloride; C, 12% glucose medium; D, 12% glucose medium supplemented with 3% sodium chloride.

Substrates used for the estimation of oxidative activities were as follows; A, B, acetate; C, D, glucose.

The RQ ratios of A to D were 0.898, 0.930, 0.818, and 0.920, respectively.

Discussion

The present report reveals that glucose, acetate, L-glutamate, which was an end product of the fermentation, and sodium chloride, which raises osmotic pressure, had striking effects on L-glutamate fermentation using an oleic acid-requiring mutant. The results suggest that the mode of action of the four substances is not the same but is able to be classified into two types.

One is the type of glucose, L-glutamate, and sodium chloride, and the other, of acetate.

Addition of the former greatly reduced L-glutamate formation and gave rise to an increase in intracellular L-glutamate and oxidative activity toward substrates.

Glucose and sodium chloride also stimulated the bacterial growth and inhibited the efflux of L-glutamate. Their effects on L-glutamate production, levels of intracellular L-glutamate, and oxidative activity toward substrates were noticed in both cultivation and resting cell systems.

Acetate strongly reduced the L-glutamate productivity as did glucose, L-glutamate and sodium chloride. However, significant differences were observed between the latter group and acetate, which did not substantially affect the oxidative activity, L-glutamate efflux, and intracellular L-glutamate but inhibited the bacterial growth. In addition, when the cultivation was carried out at a higher level of glucose

(21%), acetate (3%), or sodium chloride (3%), the amount of by-products accumulated, such as α-ketoglutarate, aspartate, and succinate, were virtually equal to those in the control cultivation so far examined. From the above, inhibition of L-glutamate synthesis by glucose or L-glutamate might be interpreted as the result of a shift of metabolism from L-glutamate synthesis to a complete oxidation system of the substrate probably due to an indirect action which would bring about a change in the transport activity of L-glutamate across the membrane caused by the increase in osmotic pressure in view of a hypothetical mechanism of extracellular accumulation of L-glutamate.

On the contrary, a direct inhibitory effect on a biosynthetic process of L-glutamate was presumed for the acetate effect, since it did not produce significant changes in some biochemical characteristics in spite of the marked inhibition of L-glutamate synthesis as mentioned before.

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