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Glutathione Production by Immobilized *Saccharomyces cerevisiae* Cells Containing an ATP Regeneration System

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Summary. Whole cells of *Saccharomyces cerevisiae* were immobilized in polyacrylamide gel. Consuming glucose, the immobilized cells produced glutathione from its constituent amino acids, and glutathione produced was excreted out of the gels. The conditions for immobilization of the yeast cells and for glutathione production were studied. Based on these data, the properties and the feasibility of the glycolytic pathway as an ATP regeneration system were discussed in reference to glutathione production.

Introduction

Recent advances in the immobilization and stabilization of enzymes have greatly enhanced their potential for use as highly specific catalysts in large scale industrial processes. However, up to now, industrial application of these enzymes has been limited almost entirely to the catalysis of degradation reactions and simple transformations, and the application of enzymes to complicated synthetic reaction processes has not been done. The barrier preventing the development of economically feasible processes of multistep enzymatic syntheses is the lack of adequate regeneration and recycle systems of cofactors such as adenosine-5′-triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD), which are essentially required for many biosynthetic reactions.

To overcome this barrier, we have been studying the construction of ATP regeneration systems for the production of glutathione, and, as shown in our previous paper (Murata et al. 1978), our attention was focused on the continuous production of glutathione using *Saccharomyces cerevisiae* cells immobilized in polyacrylamide gel. The reactor system employed for glutathione production consisted of two sequential enzyme processes. One was a glutathione synthetic process catalyzed by γ-glutamylcysteine synthetase (E.C. 6.3.2.2), and glutathione synthetase (E.C. 6.3.2.3), and the other was an ATP regeneration process catalyzed by the enzymes involved in the glycolytic pathway. The immobilized *S. cerevisiae* cells posessing the two enzyme systems mentioned above produced glutathione from its constituent amino acids consuming glucose and converting it to ethanol and carbon dioxide. Glutathione was also found to be continuously produced by a column packed with immobilized *S. cerevisiae* cells. These results clearly show that the glutathione synthetic process and the ATP regeneration process were coupled in the polyacrylamide gel lattice and the glycolytic pathway is feasible as an ATP regeneration system for glutathione production.

To improve further the productivity of glutathione using immobilized *S. cerevisiae* cells, we studied the conditions for glutathione production.

Materials and Methods

Chemicals

NAD was purchased from Oriental Yeast Co., Ltd., Tokyo, Japan. Other chemicals were all analytical grade reagents.

Cultivation and Immobilization of S. cerevisiae Cells

S. cerevisiae IFO 2044 was cultivated in 100 ml of medium containing 0.5% glucose, 1.0% peptone, 1.0% yeast extract, 0.5% meat extract and 0.5% NaCl (pH 6.0) at 30 °C for 20 h with reciprocal shaking. After cooling the broth to 0 °C, cells were harvested by centrifugation, and resuspended in 0.85% cold saline solution. The cell suspension was centrifuged at 10,400 ×g for 10 min, and weighed.

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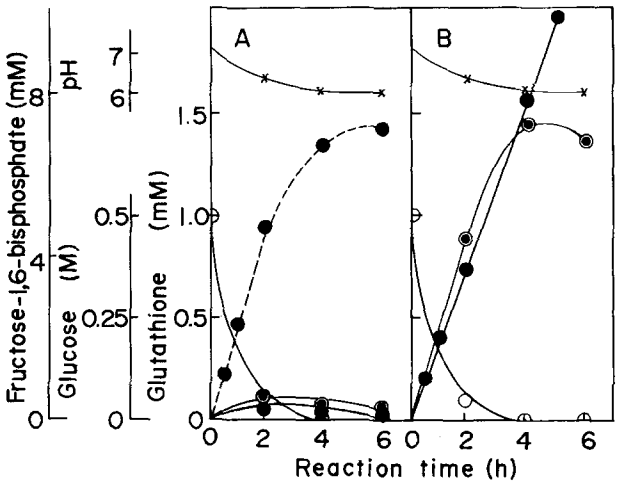

Fig. 1A and B. Glutathione production by intact cells (**A**) and immobilized cells (**B**). Reaction conditions were the same as described in Materials and Methods. In case of intact cells, glutathione in cells (---•---) and in the extracellular medium (—•—) were determined. Symbols: (•), glutathione; (○), glucose; (⊙), fructose-1,6-bisphosphate; (x), pH

Table 1. Effect of culture time on glutathione-producing activity of intact and immobilized cells

Culture time (h)	Growth (mg of cells/ml of broth)	Glutathione-producing activity[a] of	
		Intact cells	Immobilized cells
16	15.5	1.97	2.26 (0.80)[b]
24	18.4	2.12	2.40 (0.85)
40	17.3	1.64	1.84 (0.65)
64	15.4	0.99	1.11 (0.39)

[a] μmole of glutathione produced/g (wet wt.)-cells/h
[b] μmole of glutathione produced/ml-gel/h

S. cerevisiae cells were grown on the medium described in Materials and Methods for several hours. In case of intact cells, activity was determined by extracting glutathione from the cells as described in Materials and Methods

For immobilization in polyacrylamide gel, harvested cells (28 g wet wt.) were suspended in 15 ml of 30% KCl solution. To this cell suspension, 15 ml of 33.5% acrylamide monomer solution containing 2.0% N, N'-methylenebisacrylamide, 5.0 ml of 5.0% β-dimethylamino-propionitryl and 6.0 ml of 6.5% potassium persulfate were successively added at 0 °C. The mixture was slowly warmed to 20 °C and allowed to stand until a gel was formed. The resulting stiff gel was cut into cubic (2 x 2 x 2 mm) granules and washed with saline solution. By this method, 80 ml of gel was obtained containing 0.35 g (wet wt.) cells per ml of gel.

Assay of Glutathione-Producing Activity

Immobilized cells (8 ml) or intact cells (2.8 g wet wt.) were incubated in 20 ml of a mixture containing 0.5 M glucose, 0.01 M $MgCl_2$, 0.02 M L-glutamate, 0.02 M L-cysteine, 0.02 M glycine and 0.1 M potassium phosphate buffer (ph 7.0) at 30 °C for several hours with shaking. At a prescribed time, 0.5 ml of reaction mixture was removed and the concentrations of reactants and products were determined. Glutathione was determined by the method of Tietze (1969). Fructose-1,6-bisphosphate (FBP) and glucose were determined by the methods of Roe and Papadopoulos (1954) and Hugget and Nixon (1957), respectively.

Extraction of Glutathione from Cells

The 0.5 ml sample was heated in a boiling water for 1 min, chilled immediately in ice water and centrifuged to remove cell debris. Using the clear supernatant, glutathione formed by intact cells was determined. Temperature and time for extraction and recovery experiments of glutathione (data not shown) showed that this extraction method was adequate to extract glutathione from the cells.

Results

Glutathione Production by Intact and Immobilized Cells

The patterns of glutathione production by intact cells and by immobilized cells are shown in Fig. 1. As is well known, (Fig. 1A), glutathione produced by intact cells was accumulated in the cells and was not excreted. To estimate the glutathione-producing activity of intact cells, heat treatment of cells was necessary for glutathione extraction. Not only the accumulation of glutathione, but also the extracellular accumulation of FBP, a key metabolite of the glycolytic pathway, was not detected. On the other hand, in the case of immobilized cells (Fig. 1B), glutathione produced was excreted into the medium from the gel, and a considerable amount of FBP was also found extracellularly.

Conditions for Immobilization. Table 1 shows the effect of culture time on glutathione-producing activity of intact cells and immobilized cells. The most favorable glutathione-producing activity was obtained by immobilizing cells grown for 24 h.

For the immobilization of cells grown for 24 h, the optimum acrylamide monomer concentration was found to be 80 mg/ml. At concentrations above 80 mg/ml, the monomer reduced the glutathione-producing activity of the immobilized cells. Thus, the monomer concentration for immobilization was 80 mg/ml throughout this study.

Conditions of Glutathione Production by Immobilized Cells

As Fig. 1B shows, the immobilized cells produced glutathione from the constituent amino acids while consuming glucose, and the glutathione produced was excreted

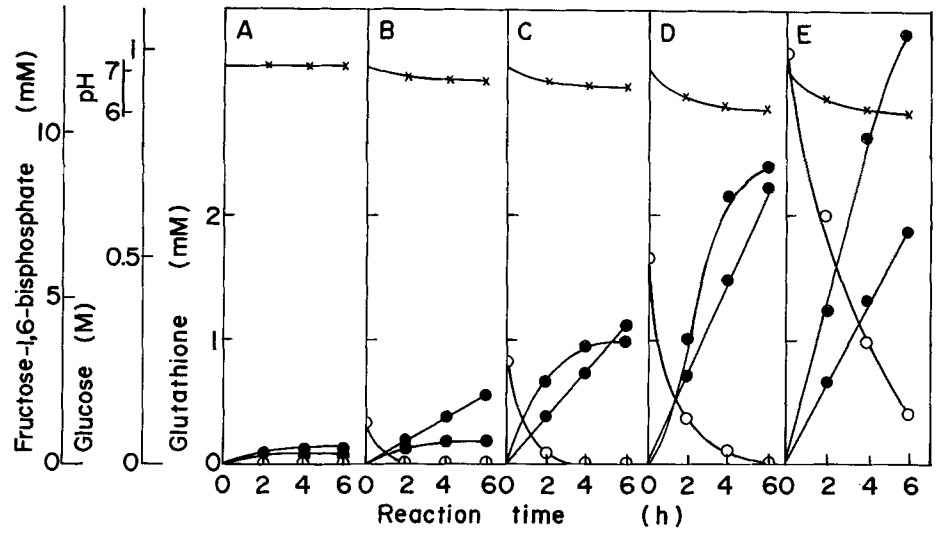

Fig. 2A–E
Effect of glucose concentration on glutathione production by immobilized cells. Reaction was carried out for several hours as described in Materials and Methods. Glucose concentrations were: 0 M (A); 0.13 M (B); 0.25 M (C); 0.5 M (D) and 1.0 M (E). Symbols: (•), glutathione; (○), glucose; (◎), fructose-1,6-bisphosphate; (×), pH

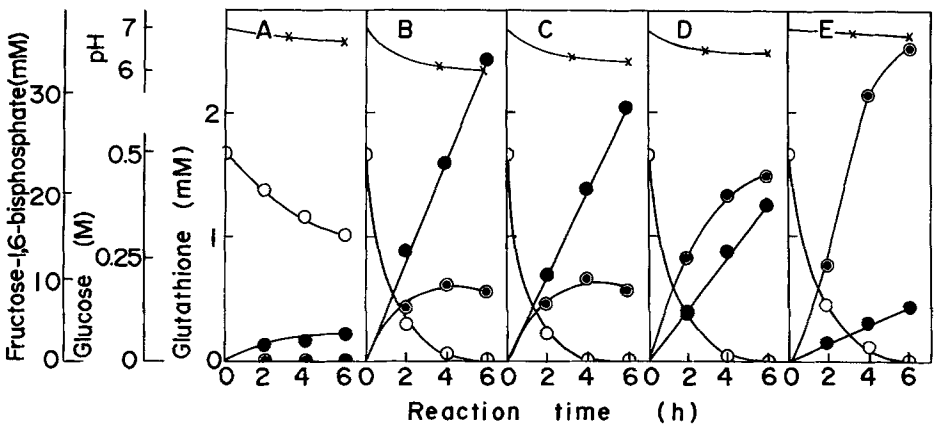

Fig. 3A–E
Effect of phosphate buffer concentration on glutathione production by immobilized cells. Reaction was carried out for several hours as described in Materials and Methods. Phosphate buffer concentrations used were: 0 M (A); 0.05 M (B); 0.1 M (C); 0.2 M (D); and 0.5 M (E). Symbols: (•), glutathione; (○), glucose; (◎), fructose-1,6-bisphosphate; (×), pH

into the medium. We next investigated the optimum conditions for glutathione production.

(1) Effect of Glucose Concentration. In our glutathione production system, glucose is essential to generate ATP; thus glucose concentration may influence the glutathione-producing activity of immobilized cells. Figure 2 shows the time course of glutathione production with various glucose concentrations. In the absence of glucose (A), little glutathione was produced. Glutathione production was increased with glucose concentrations up to 0.5 M. Similarly, FBP accumulation also increased according to the glucose concentration.

(2) Effect of Phosphate Concentration. The effect of phosphate buffer concentration on glutathione production is shown in Fig. 3. Glutathione production was efficient at low concentration of phosphate buffer, but was reduced in the absence of phosphate buffer (A). On the other hand, accumulation of FBP increased with phosphate concentration.

(3) Effect of $MgCl_2$ Concentration. The effect of $MgCl_2$ on glutathione-producing activity was investigated. The optimum $MgCl_2$ concentration was found to be 0.02 M.

(4) Effect of Initial pH. As shown in Fig. 2 the pH of the reaction mixtures decreased with increased glucose consumption; about one pH unit shift was observed for each 0.5 M glucose used. Hence, the effect of initial pH on glutathione production was investigated. The initial pH 6.5 to 7.0 seems to be favorable for the glutathione production. Starting the reaction in this pH range, the pH of reaction mixture decreased to about 6.0 after complete consumption of 0.5 M glucose.

(5) Effect of Amino Acids Concentrations. Table 2 shows the amino acid requirements for glutathione production. Although the glutathione-producing activity was considerably lowered in the absence of L-glutamate and glycine, these amino acids were not necessarily required for glutathione production by freshly prepared immobilized cells. This is presumably due to the L-glutamate

Table 2. Amino acid requirement for glutathione production with immobilized cells

Amino acid omitted	Glutathione-producing activity[a]
None	2.45 (0.87)[b]
L-glutamate	1.68 (0.58)
L-cysteine	0 (0)
Glycine	1.44 (0.41)
L-glutamate and glycine	0.96 (0.32)

[a] μmole of glutathione produced/g (wet wt.)-cells/h
[b] μmole of glutathione produced/ml-gel/h

Glutathione-producing activity was assayed under standard conditions as described in Materials and Methods with or without amino acid omittions

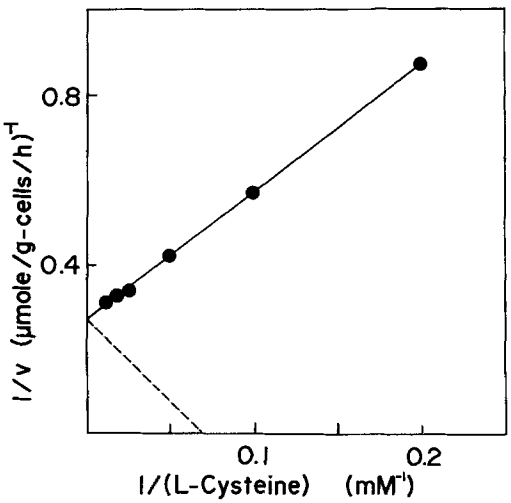

Fig. 4. Effect of varying concentration of L-cysteine on glutathione production by immobilized cells. Reaction was carried out under the same conditions as described in Materials and Methods with the exception of L-cysteine concentration. The data are presented as a double reciprocal plot of the rate of glutathione production as a function of L-cysteine concentration

or glycine carried over in the assay mixture by *S. cerevisiae* cells. On the other hand, L-cysteine was absolutely required for glutathione production and the Michaelis constant (Km) for L-cysteine was calculated to be 14.3 m*M* from the Lineweaver-Burk plot (Fig. 4). (Lineweaver and Burk 1934). By considering the Km for L-cysteine, the concentration of the three amino acids was set at 0.025 M each.

(6) Glutathione Production by Various Amounts of Immobilized Cells. Based on the data shown above, the optimum concentrations of reactants were determined to be as follows: 0.5 M glucose, 0.05 M potassium phosphate buffer (pH 7.0), 0.02 M $MgCl_2$, 0.025 M for each of the amino acids corresponding to twice that of the Km for L-cysteine. Figure 5 shows the time course of glutathione production by various amounts of immobilized cells under optimum conditions. The concentration of glutathione initially increased, reached a maximum, and then slowly declined. The glutathione-producing activity was almost proportional to the amount of immobilized cells, and the glutathione-producing activity of immobilized cells calculated from the data was 1.31 μmole/h/ml-gel.

Table 3 shows the comparison of glutathione-producing activities of intact cells and immobilized cells under optimum conditions. The glutathione-producing activity of immobilized cells was higher than that of intact cells, as was FBP accumulation.

Continuous Production of Glutathione by an Immobilized Cell Column

To produce glutathione continuously, immobilized cells were packed in a column and the substrate solution shown in the legend of Fig. 6 was passed upward through

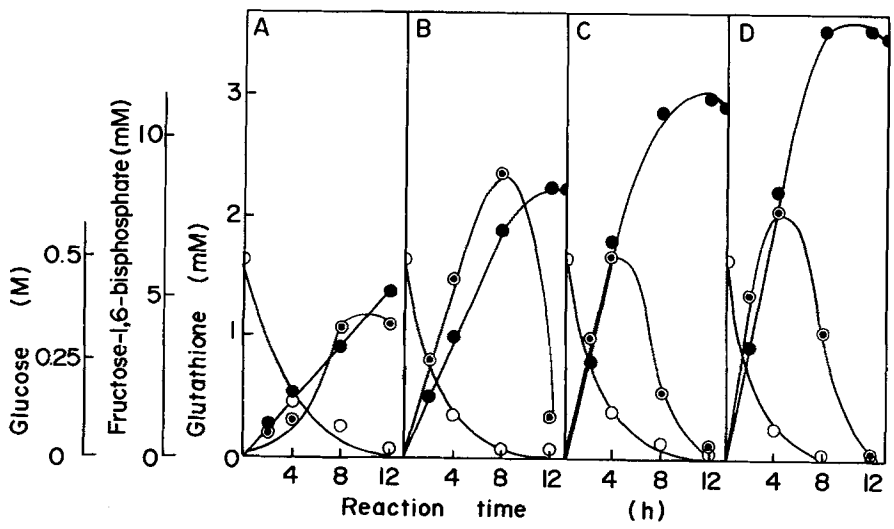

Fig. 5A–D
Time course of glutathione production with various amounts of immobilized cells. Reaction was carried out in 20 ml of mixture containing 0.5 M glucose, 0.02 M $MgCl_2$, 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, 0.05 M potassium phosphate buffer (pH 7.0) and 2.0 ml (A), 4.0 ml (B), 6.0 ml (C) or 8.0 ml (D) of immobilized cells. Other reaction conditions were the same as described in Materials and Methods. Symbols: (•), glutathione; (○), glucose; (⊙), fructose-1,6-bisphosphate

Table 3. Glutathione-producing activity and FBP-accumulating ability of intact and immobilized cells

Cell preparation	Glutathione-producing activity[a]	FBP-accumulation at 4 h reaction (m*M*)
Intact cells	3.16	0.21
Immobilized cells	3.71 (1.31)[b]	10.7

[a] μmole of glutathione produced/g (wet wt.)-cells/h
[b] μmole of glutathione produced/ml-gel/h

Glutathione-producing activity was assayed by incubating the intact cells (2.8 g wet wt.) of immobilized cells (8 ml) in the 20 ml of reaction mixture containing 0.5 M glucose, 0.02 M $MgCl_2$, 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, and 0.05 M potassium phosphate buffer (pH 7.0) at 30 °C with shaking

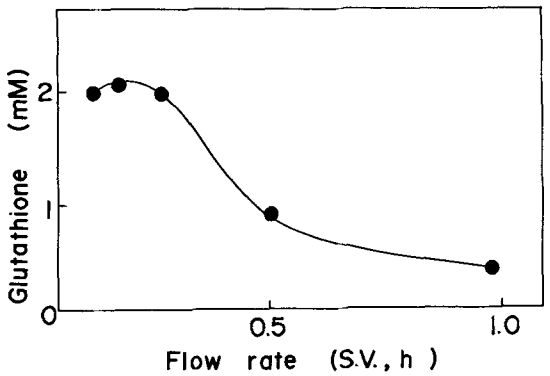

Fig. 6. Effect of flow rates of substrate solution on glutathione production. Ten milliliter of immobilized cells were packed in a column (1.0 cm x 10 cm) and substrate solution containing 0.5 M glucose, 0.02 M $MgCl_2$, 0.025 M L-glutamate, 0.025 M L-cysteine, 0.025 M glycine, 0.05 M potassium phosphate buffer (pH 7.0) and 0.5 mM NAD was passed through this column at various flow rates at 30 °C. Glutathione in the effluent was determined

this column. Figure 6 shows the effect of flow rates (space velocity, S.V.) of substrate solution on glutathione production. A flow rate of S.V. = 0.15 to 0.2 h^{-1} gave the maximum amount of glutathione in the effluent. From the industrial standpoint, the useful-life time of glutathione-producing activity is one of the important factors in evaluating the feasibility of the glutathione production system. Therefore, to determine operational stability, the substrate solution was continuously supplied at S.V. = 0.17 h^{-1} and 30 °C. As a result, about 2.0 m*M* of glutathione was continuously produced by this column, and the half-life of this column was calculated to be 26 days.

To isolate the glutathione, continuous production was carried out for 5 days. The pH of the effluent (about 200 ml containing 131 mg glutathione) was brought to 3.0 with 5 M H_2SO_4 and the solution was passed through a column packed with cation exchange resin (Diaion PK-228 H^+). The absorbed glutathione was eluted with 0.5 M NH_4OH and pH of the eluate was adjusted to 4.5 with 5 M H_2SO_4. The resultant solution was absorbed onto anion exchange resin (Duolite A-2, CH_3COO^-) and eluted with 0.5 M H_2SO_4. The eluate was concentrated and glutathione was crystalized from 50% ethanol with a yield of 73%.

Discussion

To produce glutathione efficiently, an ATP supply is indispensable, since this tripeptide is biosynthesized via ATP-requiring reactions catalyzed by γ-glutamylcysteine synthetase and glutathione synthetase. Thus, a glutathione synthetic process was coupled with the glycolytic pathway as an ATP regeneration system, and this glutathione production system was found to be feasible (Murata et al. 1978). To improve this system further, optimum conditions for glutathione production were determined, and the glutathione-producing activity of immobilized cells (Table 3) was increased by about 50% exceeding the activity previously reported (Murata et al. 1978). By using the immobilized *S. cerevisiae* cell column, we could attain continuous production of glutathione at a higher amount than that of previous method (Murata et al. 1978). The characteristics of this glutathione production system may be summarized as follows.

By using the immobilized yeast cells with polyacrylamide gel, glutathione was excreted from the cells. As shown in Fig. 1A, in the case of intact cells, glutathione was accumulated in side the cells and was not excreted out of the cells. Thus, industrial production of glutathione has been mainly carried out by extracting it from cells, although the glutathione content of the cells is low and extraction procedures are tedeuous and intricate. Fortunately, however, immobilization of yeast cells with polyacrylamide gel removes the barrier of glutathione transport across the cell membrane. Although the modification caused by immobilization is unknown, this excretion of glutathione is very promising for efficient glutathione production, since extraction from the cells can be eliminated and feed back inhibition of γ-glutamylcysteine synthetase by intracellular glutathione may be lowered.

To produce glutathione, a high glucose concentration was necessary (Fig. 2). If 1 mol of glucose generates 2 mol of ATP in the conversion of glucose to ethanol and carbon dioxide, the utilization efficiency of generated ATP for glutathione production is only 0.5%, as calculated from the data in Fig. 2. To explain the low efficiency of ATP utilization, the following three reasons were considered: (1) ATP generated is degraded by the action of ATPase or phosphatase; (2) the affinity of glu-

tathione synthetic enzymes for ATP is low in the "immobilized state"; or (3) ATP generated is not fully available to the site of glutathione synthetic reactions. We are now investigating how the efficiency of ATP utilization is influenced by the conditions under which the glutathione synthetic reactions are carried out.

Phosphate buffer inhibits glutathione production. However, glutathione production was markedly reduced in the absence of buffer, although glucose consumption gradually proceeded. This indicates that the rapid consumption of glucose, that is, a highly potent ATP supply, was necessary for glutathione production. As mentioned above, to enhance ATP generation over ATP degradation and to compensate for the low efficiency of ATP utilization, rapid glucose consumption may be indispensable. Therefore, suppression of undesirable side reactions such as ATPase reaction may enhance glutathione production.

Glutathione once produced was degraded after prolonged incubation (Fig. 5). So, to produce glutathione efficiently, the reaction time should be controlled, and if possible, the glutathione-degrading activities should be suppressed.

Lastly, as shown in our previous paper (Murata et al. 1978), NAD, the essential cofactor, of the glycolytic pathway, was specifically released from the gel during repeated use. Therefore, to produce glutathione continuously, NAD, supply is indispensable. The reason why ATP does not leak out from the gel is unknown.

Based on these considerations, continuous glutathione production was carried out using an immobilized *S. cerevisiae* cell column. In addition to examinins the glycolytic pathway as an ATP regeneration system, we investigated the utilization of the acetate kinase reaction in *Escherichia coli* cells as an ATP regeneration system for the production of glutathione (Murata et al. 1979 , 1980a and b). In this case, glutathione was also found to be efficiently produced by immobilized *E. coli* cells in a polyacrylamide gel (Murata et al. 1980b). Among these ATP regeneration systems, the glycolytic pathway seems most promising for the useful lifetime for glutathione production. However, the productivity of this system is low compared to the conventional fermentation method or chemical synthesis. By elevating the glutathione-producing activity of immobilized cells, this glutathione production system may be a more promising one. We are now investigating a highly efficient ATP regeneration system for the production of glutathione.

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