JOURNAL OF BACTERIOLOGY
Vol. 87, No. 6, pp. 1429-1435 June, 1964

QUANTITATIVE STUDIES ON GLYCOLYTIC ENZYMES IN *LACTOBACILLUS PLANTARUM*

II. INTRACELLULAR CONCENTRATIONS OF GLYCOLYTIC INTERMEDIATES IN GLUCOSE-METABOLIZING WASHED CELLS

SHÔJI MIZUSHIMA AND KAKUO KITAHARA

Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan

Received for publication 5 February 1964

ABSTRACT

MIZUSHIMA, SHÔJI (University of Tokyo, Tokyo, Japan), AND KAKUO KITAHARA. Quantitative studies on glycolytic enzymes in *Lactobacillus plantarum*. II. Intracellular concentrations of glycolytic intermediates in glucose-metabolizing washed cells. J. Bacteriol. **87**:1429–1435. 1964.—The intracellular concentrations of glycolytic intermediates in glucose-metabolizing cells could be estimated by using C^{14}-glucose of high specific activity. Separation of each intermediate was accomplished by column chromatography with Dowex 1-X8 (formate) ion-exchange resin. If the intermediates were inseparable, one was converted into another substance separable by chromatography. Almost all the glycolytic intermediates were found to be radioactive, and all of the radioactive substances appearing on the chromatograms were limited to glycolytic intermediates or their related substances. The results clearly show the role of the glycolytic system for lactic acid fermentation in the organism. Among the intermediates, fructose-1,6-diphosphate and 3-phosphoglycerate were found to be present in relatively high concentrations. The reason for the high concentrations of these compounds is discussed.

In the previous paper (Mizushima, Machida, and Kitahara, 1963), the intracellular concentrations of inorganic ions and coenzymes in glucose-metabolizing washed cells were determined. To estimate the intracellular activities of glycolytic enzymes, it is also essential to determine the intracellular concentrations of the related intermediates in glucose-metabolizing cells. Recently, Bartlett (1959*a*, *b*) presented a method for the isolation of glycolytic intermediates by ion-exchange column chromatography and determined the intracellular contents of some of these intermediates in washed human red cells. However, the intracellular concentrations of these intermediates in cells actively engaged in glycolysis have never been reported.

By modifying Bartlett's technique by the use of C^{14}-glucose of high specific activity, the intracellular concentrations of glycolytic intermediates in glucose-metabolizing cells could be estimated. The present paper deals with the method for the determination of these intermediates and their concentrations in the cytoplasmic solution.

MATERIALS AND METHODS

Materials. Uniformly labeled glucose (C^{14}-glucose) was a sample prepared in the authors' Institute (Maruo, Takahashi, and Hattori, 1960). Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, 3-phosphoglycerate, phosphoenolpyruvate, 6-phosphogluconate, nicotinamide adenine dinucleotide (NAD), and pyruvate kinase were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Reduced NAD ($NADH_2$) was prepared from NAD by use of alcohol and the alcohol dehydrogenase system (Rafter and Colowick, 1957). D-Lactate dehydrogenase was prepared from *Lactobacillus plantarum* 11 (Mizushima, Hiyama, and Kitahara, 1964); glucose-6-phosphate dehydrogenase was from *Leuconostoc mesenteroides* B07 (Takebe, Shirakawa, and Kitahara, *personal communication*).

Preparation of chromatography sample. L. plantarum 11 was cultivated as described previously (Mizushima et al., 1963). An amount of 25 mg (dry weight) of the cells thus obtained was suspended in 4 ml of 0.2 M phosphate buffer (pH 7.0, 30 C) containing 26 μmoles of uniformly carbon-labeled glucose (144 counts per min per mμmole). After 10 min of incubation, the suspension was poured into 40 ml of boiling water to stop the fermentation instantaneously. After being maintained at 100 C for 1 min, the suspen-

sion was removed and allowed to cool to room temperature, and was then centrifuged at 10,000 $\times$ g for 10 min to remove the cell debris. The clear supernatant solution thus obtained was used as sample A.

To determine the extracellular amounts of glycolytic intermediates or end products which were released during the fermentation, 6.5 μmoles of uniformly carbon-labeled glucose were fermented for 10 min in 1 ml of 0.2 M phosphate buffer (pH 7.0, 30 C) containing 6.3 mg (dry weight) of washed cells. The reaction was stopped by pouring the suspension into 10 ml of ice-cold water. The clear supernatant solution obtained after centrifugation (10,000 $\times$ g, 10 min) was used as sample B.

The volumes of cytoplasmic solution in both cases are calculated to be about 22.5 and 5.7 μliters, respectively (Mizushima et al., 1963).

Chromatographic separation of glycolytic intermediates on column. In general, the chromatographic method applied in the present study was that described by Bartlett (1959*a*). A resin bed of Dowex 1-X8 formate (200- to 400-mesh commercial grade, 1 by 14 or 1 by 10 cm) was used. Chromatographic separation was performed by linear gradient elution (0 to 0.6 N formic acid or 0 to 4 N ammonium formate, pH 3.0) at room temperature. Eluates were collected in test tubes (10 ml per tube), and 1 ml from each tube was dried for the determination of radioactivity. The radioactivity was determined with a radiation counter (model 32; Scientific Research Institute Ltd., Tokyo, Japan).

General analytical methods. Glucose was determined by the anthrone method, and lactate was assayed by the method of Barker and Summerson (1941). Inorganic phosphate was determined by the method of Allen as modified by Nakamura (1950). Glycerol-phosphate, 6-phosphogluconate, and phosphoglycerate in chromatographic eluates were detected as follows; 1 drop of a 2% solution of β-naphthol and 5 ml of H_2SO_4 were added to a 0.5-ml sample and heated at 100 C for a few minutes. The 6-phosphogluconate gave a red color, whereas glycerol-phosphate and phosphoglycerate gave a blue color. Hexose-phosphate and pyruvate in the chromatographic eluates were detected by the phenol-H_2SO_4 method (Hodge and Hofreiter, 1962).

Quantitative conversion of radioactive intermediates into others. To determine the amounts of intermediates which were not separable by chromatography, it is necessary to convert the glycolytic intermediate into another substance quantitatively. Before the conversion, formic acid or ammonium formate in eluates was removed as described by Bartlett (1959*a*). Glucose-6-phosphate was converted into 6-phosphogluconate in 10 ml of 0.2 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 7.5) containing 3×10^{-4} moles of $MgSO_4$, 3×10^{-6} moles of NAD phosphate (NADP), and a glucose-6-phosphate dehydrogenase preparation. Hexose-1-phosphate and hexose-diphosphate were converted into hexose and hexose-6-phosphate, respectively, by hydrolysis in 0.1 N HCl at 100 C for 130 min. Glyceraldehyde-3-phosphate was converted into lactate by treatment with 1 N NaOH at 30 C for 30 min. Conversion of 2,3-diphosphoglycerate to 3-phosphoglycerate was made at 100 C for 1 hr in 1 N acetic acid in which the pH was adjusted to 2.0 with HCl. In our preliminary experiment, 2,3-diphosphoglycerate was completely converted into 3-phosphoglycerate under the condition described above. Conversion of phosphoenolpyruvate into lactate was made as follows. To the dried sample were added 0.5 ml of 0.2 M phosphate buffer (pH 7.0) and 2 ml of distilled water; the solution was neutralized to pH 7.0 with 4 N NaOH (total volume, 2.6 ml). To the solution were added 0.2 ml of the pyruvate kinase preparation, 0.05 ml of the D-lactate dehydrogenase preparation, 0.1 ml of 10^{-2} M adenosine diphosphate (ADP), 0.1 ml of 5×10^{-2} M $MgCl_2$, and 0.1 ml of 2.2×10^{-3} M phosphoenolpyruvate. The conversion of phosphoenolpyruvate to lactate was started by the addition of 0.2 ml of 2×10^{-3} M $NADH_2$.

Paper partition chromatography. Paper partition chromatography of phosphate esters was performed with Toyo Roshi no. 50 filter paper with solvent A (ethyl acetate-acetic acid-water, 3:3:1, at 4 C) or solvent B (methyl Cellosolve-methylethylketone-3 N NH_4OH, 7:2:3, at 26 C). Radioactivity on the paper was scanned with a counting rate meter (model PR-A3; Scientific Research Institute Ltd., Tokyo, Japan). Color development of phosphate esters was carried out according to the method of Bandurski and Axelrod (1951). For organic acids, the solvent used was butanol-formic acid-water (4:1.5:1, 25 C); the spots were developed with bromocresol purple.

RESULTS

Dowex 1-formate separation of sample A. The results obtained by chromatography of sample A are presented in Fig. 1. Before the chromatography, 10 μmoles each of glucose-6-phosphate, pyruvate, and fructose-diphosphate were added to the sample. Recovery of the radioactivity was about 90%. The experimental column separation described herein was performed only once. The results presented are, therefore, those obtained in a single experiment. However, quite similar results to those in Fig. 1 were obtained in the preliminary experiment where 18 μmoles of C^{14}-glucose were used as a substrate.

Fractions 2 to 9. The amount of glucose in fractions 2 to 9 was determined. All the radioactivity in these fractions was present in glucose (glucose not utilized, 11 μmoles).

Fractions 15 to 20. Portions (30 ml) of fractions 15 to 20 were dried in vacuo, redissolved in a small amount of distilled water, and then applied on paper for partition chromatography. Examination of the radioautogram showed that almost all of the activity in these fractions was due to gluconate (gluconate, 0.15 μmoles).

Fractions 21 to 27. Determination of the amount of lactate in fractions 21 to 27 revealed that all the radioactivity was present in lactate. From this, the washed cells were found to form 5.7 μmoles of lactate per mg of dry cells per hr. This is a normal rate of lactate formation for this strain (lactate formed, 20 μmoles).

Fractions 67 to 75. From the results shown in Fig. 1 and those of Bartlett (1959*a*), fractions 67 to 75 apparently contained hexosephosphates. The separation of one hexosephosphate was carried out with one-third of the combined fractions. First the sample was hydrolyzed with HCl to convert hexose-1-phosphate into hexose, and then the remaining glucose-6-phosphate was enzymatically converted into 6-phosphogluconate. The resulting solution, which contained hexose, fructose-6-phosphate, and 6-phosphogluconate, was rechromatographed on a Dowex 1-formate column and eluted with ammonium formate (Fig. 2). Fructose-6-phosphate was identified by paper chromatography with solvents A and B. From these results, the amounts of hexose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were determined as follows: hexose-1-phosphate, 7 mμmoles; glucose-6-phosphate, 33 mμmoles; and fructose-6-phosphate, 15 mμmoles.

Fractions 125 to 131. The compound contained in fractions 125 to 131 was considered to be phosphoglycerate because of the acid-stability of the phosphate ester bond and the position of the ester on the chromatogram. Almost all of the radioactivity in these fractions was found in 3-phosphoglycerate. Possible contamination by hexose-diphosphate was measured by treatment with 1 N HCl followed by chromatography on a Dowex column. No appreciable amount of hexose-diphosphate was found in these fractions (3-phosphoglycerate, 0.8 μmoles; 2-phosphoglycerate, <14 mμmoles).

Fractions 132 to 137. As shown in Fig. 1, these fractions contained fructose-1,6-diphosphate. The amounts of fructose-diphosphate and glucose-diphosphate were estimated as follows. A dried sample obtained from 5 ml of the eluate was hydrolyzed in 2 ml of 1 N HCl at 100 C for 30 min. The solution was then rechromatographed on a Dowex 1-formate column with the addition of 20 μmoles each of fructose-diphosphate and fructose-6-phosphate. The result (Fig. 3) showed the quantitative conversion of the hexose-diphosphate into hexose-6-phosphate. Fractions 15 to 18 of the second chromatogram were treated with

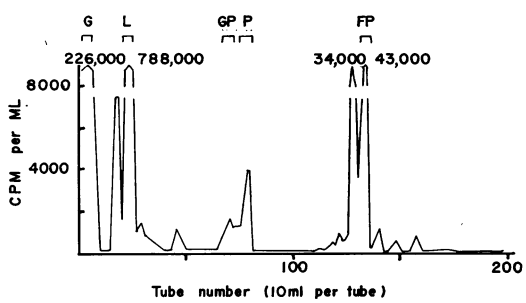

FIG. 1. *Dowex 1-formate separation of glycolytic intermediates from a cell extract. Sample A, which contained 10 μmoles each of glucose-6-phosphate (GP), pyruvate (P), and fructose-1,6-diphosphate (FP), was run through a column (1 by 14 cm) of Dowex 1-X8 formate, and the absorbed compounds were successively eluted at 1 ml per min with 1 liter of linear gradient 0 to 0.6 N formic acid and 0 to 4 N ammonium formate (pH 3.0). The brackets at the top of the figure indicate the eluting range of each added substance. G, glucose; L, lactate. Numerical values indicate the maximal radioactivity (counts/min) of these peaks.*

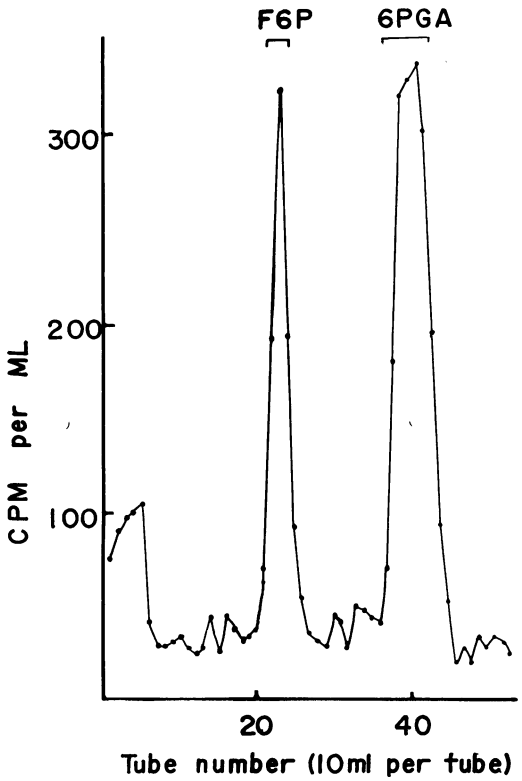

FIG. 2. *Dowex 1-formate separation of glucose, fructose-6-phosphate, and 6-phosphogluconate. A solution derived from fractions 67 to 75 containing 10 μmoles each of added fructose-6-phosphate (F6P) and 6-phosphogluconate (6PGA) was run through a column (1 by 10 cm) of Dowex 1-X8 formate, and the absorbed compounds were eluted at 2 ml per min with 1 liter of linear gradient 0 to 2 N ammonium formate (pH 3.0). The brackets at the top of the figure indicate the eluting range of each added substance.*

glucose-6-phosphate dehydrogenase to convert glucose-6-phosphate into 6-phosphogluconate and further rechromatographed to separate fructose-6-phosphate from 6-phosphogluconate (Fig. 4). From these results, the amounts of fructose-diphosphate and glucose-diphosphate in sample A were determined: fructose-1,6-diphosphate, 0.37 μmoles; glucose-1,6-diphosphate, 85 mμmoles.

Fractions 138 to 145. Phosphoenolpyruvate in a 30-ml sample of fractions 138 to 145 was converted into lactate by using a pyruvate kinase-lactate dehydrogenase system and was diluted to 40 ml with distilled water. The resultant solution was rechromatographed with the addition of 10 μmoles of lactate. The results (Fig. 5) show that the radioactive compound in these fractions was exclusively phosphoenolpyruvate (phosphoenolpyruvate, 11 mμmoles).

Fractions 154 to 163. 2,3-Diphosphoglycerate in fractions 154 to 163 was converted into 3-phosphoglycerate as indicated in Materials and Methods. After dilution to 40 ml, the hydrolysate was rechromatographed with the addition of 20 μmoles of 3-phosphoglycerate. As shown in Fig. 6, the radioactive substance was fully converted into 3-phosphoglycerate (2,3-diphosphoglycerate, 2.6 mμmoles).

Fractions 76 to 82. Almost all pyruvate in fractions 76 to 82 was discarded by mistake in the procedure for removing formic acid by ether extraction. Among glycolytic intermediates, however, only pyruvate is easily extracted by

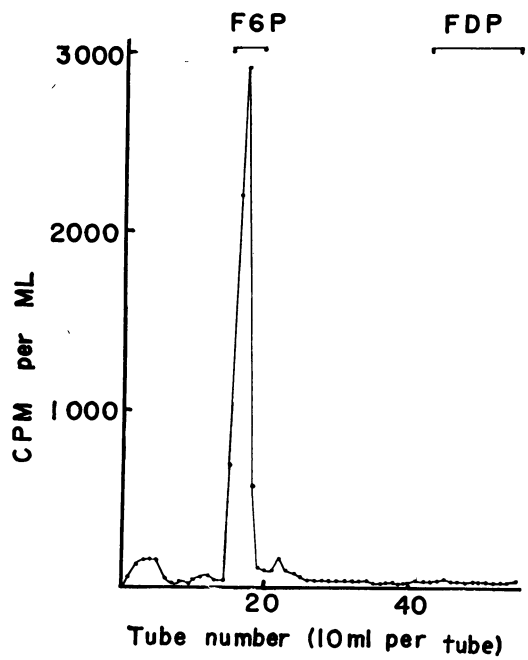

FIG. 3. *Dowex 1-formate separation of fructose-6-phosphate and fructose-1,6-diphosphate. A solution derived from fractions 132 to 137 containing 10 μmoles each of added fructose-6-phosphate (F6P) and fructose-1,6-diphosphate (FDP) was run through a column (1 by 5 cm) of Dowex 1-X8 formate, and the absorbed compounds were eluted at 2 ml per min with 1 liter of linear gradient 0 to 2 N ammonium formate (pH 3.0). The brackets at the top of the figure indicate the eluting range of each added substance.*

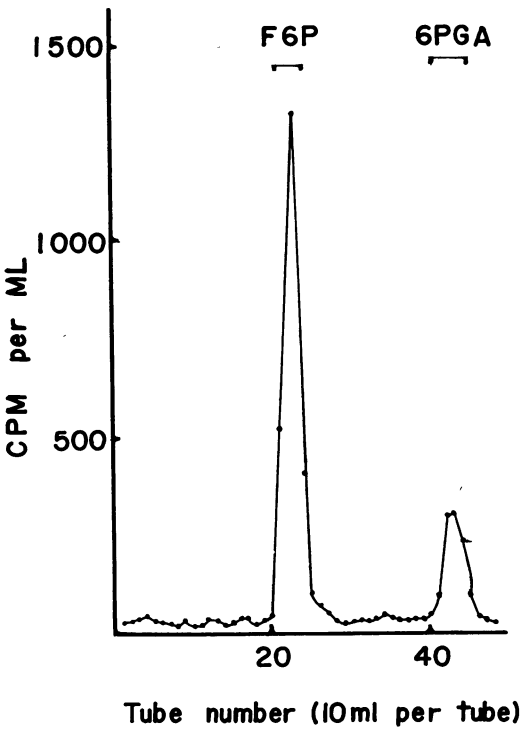

FIG. 4. *Dowex 1-formate separation of fructose-6-phosphate and 6-phosphogluconate. A solution derived from formate eluate of Fig. 3 containing 10 μmoles each of added fructose-6-phosphate (F6P) and 6-phosphogluconate (6PGA) was run through a column (1 by 10 cm) of Dowex 1-X8 formate, and the absorbed compounds were eluted at 2 ml per min with 1 liter of linear gradient 0 to 2 N ammonium formate (pH 3.0). The brackets at the top of the figure indicate the eluting range of each added substance.*

ether. Therefore, the amount was estimated from the loss of radioactivity through the ether extraction. Glyceraldehyde-3-phosphate in these fractions was dephosphorylated by mild NaOH hydrolysis. Dephosphorylated substance was separated by Dowex 1-formate, but we could not detect radioactive lactate (pyruvate, 14 mμmoles).

Amount of glycolytic intermediates in extracellular fermenting medium. Figure 7 presents the results which were obtained by chromatography of sample B on a Dowex 1 column. The recovery of radioactivity was about 90%. The substances corresponding to the peaks of radioactivity were identified according to the method described above. In this case, the glycolytic activity of intact cells was found to be about 5.7 μmoles (lactate formation) per mg of cells per hr. From the figure, the amounts of intermediates released during the fermentation were determined as follows: pyruvate, 10 mμmoles; hexose-diphosphate, 7.7 mμmoles; monophosphoglycerate, 11 mμmoles; and gluconate, 74 mμmoles.

The results show that all the glycolytic intermediates were almost completely located in cells.

Intracellular concentrations of glycolytic intermediates in glucose-metabolizing washed cells. As the volume of cytoplasmic solution corresponding to 1 mg of dry cells had previously been found to be 0.9 μliter (Mizushima et al., 1963), the in-

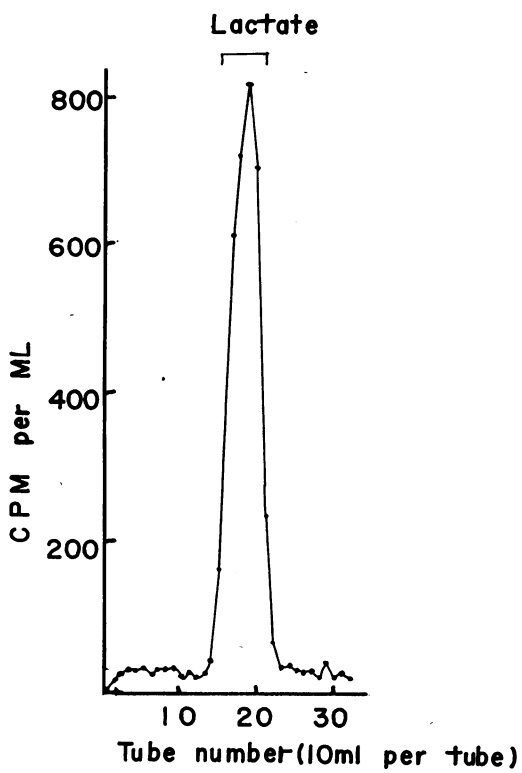

FIG. 5. *Dowex 1-formate separation of lactate and phosphoenolpyruvate. A solution derived from fractions 138 to 145 containing 10 μmoles each of added lactate and phosphoenolpyruvate was run through a column (1 by 10 cm) of Dowex 1-X8 formate, and the absorbed compounds were eluted at 2 ml per min with 1 liter of linear gradient 0 to 0.6 N formic acid. The bracket at the top of the figure indicates the eluting range of the added substance.*

tracellular concentrations of glycolytic intermediates were calculated from the results obtained in the present experiment (Table 1).

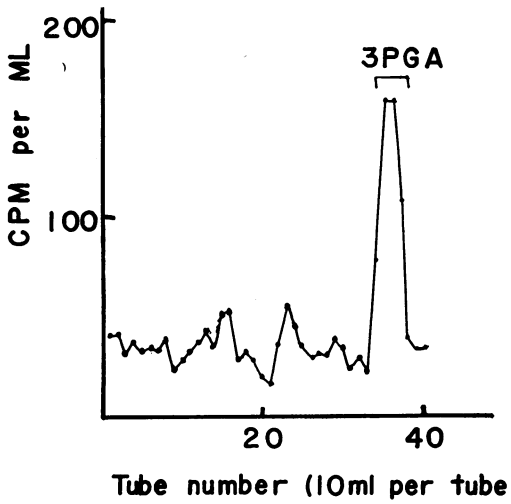

FIG. 6. *Dowex 1-formate separation of 3-phosphoglycerate. A solution derived from fractions 154 to 163 containing 10 μmoles of added 3-phosphoglycerate was run through a column (1 by 10 cm) of Dowex 1-X8 formate, and the absorbed compounds were eluted at 2 ml per min with 1 liter of linear gradient 0 to 4 N ammonium formate (pH 3.0). The bracket at the top of the figure indicates the eluting range of the added 3-phosphoglycerate.*

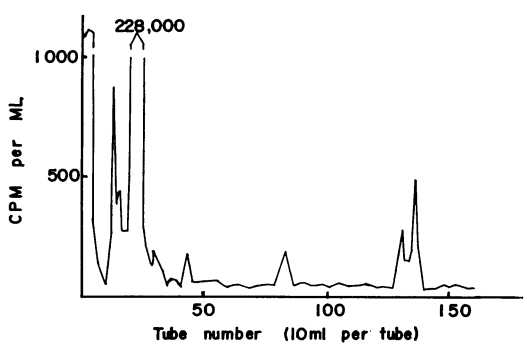

FIG. 7. *Dowex 1-formate separation of glycolytic intermediates released into a reaction medium. Sample B was run through a column (1 by 14 cm) of Dowex 1-X8 formate, and the absorbed compounds were successively eluted at 1 ml per min with 1 liter of linear gradient 0 to 0.6 N formic acid and 0 to 4 N ammonium formate (pH 3.0). Numerical value at the top of the figure indicates the maximal radioactivity (counts per min) of the peak.*

TABLE 1. *Intracellular concentrations of glycolytic intermediates in a glucose-metabolizing washed cell**

Compound	Concn
	M
Pyruvate	5.2×10^{-3}
Hexose-1-phosphate	0.4×10^{-3}
Fructose-6-phosphate	0.8×10^{-3}
Glucose-6-phosphate	1.7×10^{-3}
Fructose-1,6-diphosphate	17.0×10^{-3}
Glucose-1,6-diphosphate	4.3×10^{-3}
3-Phosphoglycerate	39.0×10^{-3}
Phosphoenolpyruvate	5.5×10^{-3}
2,3-Diphosphoglycerate	1.3×10^{-3}
Gluconate	3.8×10^{-3}

* These values were calculated from the results on sample A by correcting for the results on sample B.

DISCUSSION

The amounts of glycolytic intermediates in the human red cell were systematically determined by Bartlett (1959*b*); however, the determination was carried out on washed resting cells. To determine the intracellular concentrations of glycolytic intermediates in the glucose-metabolizing cells, it is necessary to stop the intracellular enzymatic activity instantly. Since the rate of lactate formation in the glucose-metabolizing washed cell was found to be about 6 μmoles per mg of cells (dry basis) per hr, the change in concentration of these intermediates caused by metabolic turnover was calculated as about 10^{-3} M per sec. In other words, if it takes 1 sec to stop the whole enzymatic reaction, there is a fair chance of changing the intracellular concentrations of certain intermediates by the order of 10^{-3} M. Therefore, in the present study, the reaction was stopped by pouring the reaction mixture into 10 volumes of boiling water. With this method, it takes less than 1 sec to stop the whole reaction. However, errors of the order of 10^{-4} M are unavoidable in the data shown in Table 1.

As shown in Table 1, when C^{14}-glucose was used as a substrate, almost all the glycolytic intermediates found in cells were radioactive, and all radioactive substances appearing in the chromatogram were limited to glycolytic intermediates or their related substances. The

results clearly show that the metabolic pathway of lactic acid formation from glucose in *L. plantarum* is exclusively the glycolytic one. However, radioactive dihydroxyacetonephosphate, glyceraldehyde-3-phosphate, and 2-phosphoglycerate were not detected in the present experiment. Whereas a small part of glucose undoubtedly also serves as the source of pentose phosphate for nucleic acid synthesis, the technique employed was probably insufficiently sensitive to determine the latter intermediates. The high concentrations of fructose-1,6-diphosphate and 3-phosphoglycerate in the cells are noteworthy. As 1,3-diphosphoglycerate is known to be decomposed readily to give 3-phosphoglycerate under physiological conditions (Leloir and Cardini, 1957), the amount of 3-phosphoglycerate listed in Table 1 is considered to be the sum of 3-phosphoglycerate itself and 1,3-diphosphoglycerate. In the cells which ferment glucose without growth, adenosine triphosphate (ATP) generated during glycolysis is not used for biosynthetic reactions. As an ATP-utilizing system in the nongrowing cell, the Halden Young Effect is well known. By this effect, the accumulation of fructose-1,6-diphosphate is stimulated. In fact, the accumulation was observed in the present experiment, but the amount was less than 1% of the amount of ATP theoretically formed during the fermentation. It is possible that the accumulation of fructose-diphosphate causes the accumulation of 1,3-diphosphoglycerate with no change in the concentration of ATP. If most of the 3-phosphoglycerate measured in the present experiment was actually 1,3-diphosphoglycerate, the accumulation can be explained in this way. The increase in the concentration of 1,3-diphosphoglycerate in the cell accelerates the rate of its autodecomposition rather than the enzymatic transformation, and causes the decrease in ATP generation during the glycolytic process. Considering the instability of 1,3-diphosphoglycerate, it may be concluded that the autodecomposing process uncouples a part of the ATP generation in glucose-fermenting washed cells.

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