

# Some Observations on Cell Membrane Transport in Yeasts\*

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The function of the surface structure of yeast cells was investigated from the viewpoint of sugar transport.

It was found that glucose and sugar phosphates could not penetrate into the cell without the action of hexokinase and phosphatase, which located on the surface structure of the cell. Namely, glucose incorporation was performed under the active state of hexokinase, and sugar phosphates incorporation was carried out after the substrates had been splitted into two components, sugar and inorganic phosphate, by the action of phosphatase outside of the cell. Sugar moiety was incorporated by the action of hexokinase, while inorganic phosphate remained outside of the cell.

Enzymes locating on the surface of the cell, seem to play a significant role for yeast metabolism as a kind of "Permease".

## INTRODUCTION

The surface structure such as cell wall and cell membrane in a micro-organism has a function to form the shape and to retain the intracellular materials of cell and also plays a role as the barrier to control permeation of materials.

Since the metabolizable substrates or the metabolic products can penetrate into or out of the cell against the gradient of its concentration of the surroundings, it is evident that the mechanism of the transference of materials through the surface structure into or out of the cell is not performed by diffusion alone.

During the metabolism, microbial cell surface shows often some specific semi-permeability and is known to have a specific transport system. With respect to the specific transport system in microbial cell, some hypotheses have been proposed. However, none of them can explain the mechanisms completely in detail.

It is suggested that at the begining of metabolism the trans-location of materials would takes place through the surface structure. The metabolizable materials may be attracted to the site of the metabolic enzymes or the other specific translocase to form the enzyme-material or the translocase-material complex on the cell surface and may be trans-located into the cell.

Cohn and Monod<sup>1)</sup> explained the specific systems concerning the material transport in terms of "Permease", which is located on the cell surface. But "Permease" has not been recognized as a single enzyme.

Several enzymes are detected on the surface structure of microbial cell<sup>2)</sup>, but the role of these enzymes for the transport of materials has not been studied.

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The object of this paper is to describe the experimental results about the function of enzymes (hexokinase and phosphatase) which are distributed on the surface structure of yeast in the transport of materials.

It is mentioned that these enzymes have an important role for the transport of substrate, and that without these enzymes no metabolizable material can penetrate into the cell freely.

## EXPERIMENTAL

### Materials and Methods

**Yeast.** *Saccharomyces cerevisiae* Y-21 (brewer's yeast) was grown in liquid malt extract medium. Medium (100 ml.) was shaken in a 500 ml. shaking flask for 36 to 48 hrs. at 30°C and the harvested and washed cells were employed. Baker's yeast (Toyobo) was also used.

**Chemicals.** Commercial glucose and fructose-1, 6-diphosphate dipotassium salt were used. Glucose-1-phosphate and glucose-6-phosphate were prepared in this laboratory.  $^{14}\text{C}$ -glucose was obtained from Daiichi Pure Chemicals Co., Glucose-6-phosphate dehydrogenase was prepared from fresh beer yeast according to the method of Kornberg<sup>4</sup>.

Snail's digestive juice was obtained from the gute of *Euhadra hickonis* (Kobelt) and *Euhadra awaensis vortex*. It was shown to have the following contents: Water 84 to 89 %, total nitrogen 0.19 to 0.30 %, (pH 5.6 to 6.2)

Protoplast, cell membrane, cytoplasm and cell wall fraction of yeast were prepared as follows: Yeast cells were suspended in Michaelis phosphate buffer (pH 5.8) containing 10 % (*w/v*) of carbowax-1500 to protect from the osmotic lysis of protoplast. To this suspension, snail's digestive juice was added. The mixture was incubated at 30°C for 2 to 3 hrs. with gentle agitation. Intact cells, remaining in the reaction mixture, were separated by centrifuging at 1,500 r.p.m. for 5 min. and resulting sediments were discarded. Supernatant, which contained protoplast and dissolved cell wall, was centrifuged at 3,000 r.p.m. for 10 min. then protoplast was sedimented. Protoplast was harvested in yield of 80 to 90 % from intact cells. A portion of protoplast was employed to prepare the cell membrane (ghost) and cytoplasm. When the protoplast was poured into about 8 to 10-fold of water, it was bursted to form ghost. From the resulting solution cytoplasm and ghost were separated by centrifuging at 3,000 r.p.m. for 10 min..

Inulin was measured quantitatively and inulin space was examined by using 4 % solution of inulin, and intercellular space of cell mass was calculated as follows: Ten ml. of yeast mass (centrifuged at 3,000 r.p.m. for 15 min) is composed of 7 ml. of cell and 3 ml. of water.

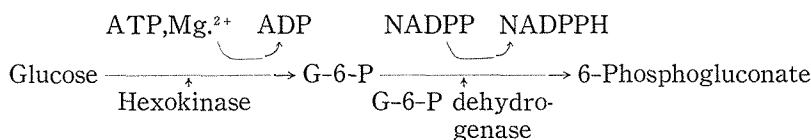
Volume of cell wall was calculated as follows<sup>5</sup>: Assuming the diameter of cell is  $5\mu$ , so the volume of cell comes to be  $2.5^3 \times 4/3 \times \pi = 65.41 \mu^3$ , the thickness of cell wall is  $0.32\mu$ , so that volume of protoplast is  $2.18^3 \times 4/3 \times \pi = 42.59 \mu^3$ . The volume of cell wall is  $65.41 - 42.59 = 22.89 \mu^3$  and whole volume of cell wall comes to be  $7 \text{ (ml.)} \times 22.89/65.41 = 2.44 \text{ (ml.)}$ . The intercellular space in the 10 ml. of yeast mass comes to be  $3 + 2.44 = 5.44 \text{ (ml.)}$ .

Glucose and glucose-6-phosphate were determined colorimetrically with Hitachi

photo-electric colorimeter according to the Somogyi-Nelson method<sup>6)</sup>.

Phosphatase was determined colorimetrically according to the Fiske-Subbarow method<sup>7)</sup>.

Hexokinase activity<sup>8)</sup> was determined by NADPP (Nicotine Adenine Dinucleotide Phosphate) reduction coupled with glucose-6-phosphate dehydrogenase which was assayed by measuring optical density at 340 m $\mu$  with Shimadzu spectrophotometer as shown in the following scheme.



Phosphatase was assayed by determining labile inorganic phosphate and sugar. CO<sub>2</sub> evolution was measured with the Warburg manometer at 30°C under the phase of air.

Radio activity was measured by GM counter.

## RESULTS AND DISCUSSIONS

**Free diffusion of materials and inulin space in yeast mass.** The free diffusion of materials into the cell and the intercellular spaces in yeast mass were examined.

The cells of baker's yeast were washed three times with distilled water and centrifuged at 3,000 r.p.m. for 15 min. Ten ml. of yeast mass was suspended in 40 ml. of inulin solution and the mixture stood at room temperature for 3 hrs. After the suspension was centrifuged at 3,000 r.p.m. for 15 min., 10 ml. of supernatant solution was evaporated. at 105°C and the resulting dry inulin was weighed.

Original inulin solution might be diluted with the intercellular water and by diffusion into the cells. From the results shown in Table 1, 40 ml. inulin solution was diluted with 4.2 ml. of intercellular water. In the same way, the dilution ratio of the other sugars was examined.

As presented in Tables 1 and 2, penetration into the cell was not observed at all.

If those materials could penetrate into the cell freely, conspicuous dilution of solutes should be observed. However, the dilution ratio obtained from the experiments in which xylose, galactose and glucose were employed, was less than that

Table 1. Inulin and the other sugar space.

| Sugars                                  | Xylose | *Galactose | *Glucose | Inulin |
|---|--------|------------|----------|--------|
| Increase of water by adding cell mass** | 43.1   | 43.1       | 44.5     | 44.2   |
| Increased volume                        | 3.1    | 3.1        | 4.5      | 4.2    |
| Dilution ratio (%)                      | 0.75   | 0.75       | 1.13     | 1.05   |

\* Monoiodo acetic acid 1/63 M added.

\*\* Calculated as follows: 40x sugar mg./ml. before incubation/sugar mg./ml. after incubation.

Reaction mixture contents: Yeast 10 ml., 4% sugar solution 40 ml., Stand for 3 hrs. at room temp..

Table 2. Diffusion of  $^{14}\text{C}$ -glucose in yeast cell under the condition of MIA existed.

|                             | Supernatant | Wash water | Cell                                    |
|-----------------------------|-------------|------------|---|
| Suspended for 2 hrs. (cpm.) | 88,800      | 5,800      | 5,300 { Wall 5,300<br>Proto-<br>plast 0 |
| Distribution ratio (%)      | 88.8        | 5.8        | 5.3 (6.9*)                              |

\* Cell wall volume in which diffusion may occur by calculation.

Reaction mixture contents: Yeast cell 4ml. was suspended in 11.5 ml. of 4% glucose which contains 20  $\mu\text{C}$   $^{14}\text{C}$ -glucose (100,000 cpm.).

of the volume calculated, and free diffusion of the materials into the cell could not be observed. From the results obtained with  $^{14}\text{C}$ -glucose it was obvious that the distribution of radio activity was limited outside of the cell (Table 2).

**Relationship between hexokinase and hexose transport.** Hexokinase distribution: Three fractions of cell particles were examined about their hexokinase activity. Content of reaction mixture used for measurement of the activity is shown in Table 3. As shown in Fig. 1, hexokinase activity of the intact cell, the protoplast and the ghost were almost the same. Therefore, it would be supposed that the activity measured in the intact cell and the protoplast was derived from that of the ghost, namely, the activity had been existed neither in the cell wall nor the cytoplasm. With respect to the fact mentioned above, as shown in Fig. 2 b, experiments were undertaken about the activity of the cytoplasm to obtain an actual proof for this assumption, but the cytoplasm had considerably high activity.

This misconception may be attributed to the obstructive action of osmotic barrier in hexokinase assay. When the osmotic barrier was broken, the high activity of hexokinase was observed, because the hexokinase in the cytoplasm and in the cell membrane acted on substrate (Fig. 3a). The dried cell, in which osmotic barrier was broken, showed higher rate of NADPP reduction than those of the intact cell and the protoplast.

The reason for similarity of air dried yeast and the cytoplasm, prepared from protoplast, in NADPP reduction curve may be attributed to the difference in destruction method of osmotic barrier. Fig. 3 b shows the lack of hexokinase act-

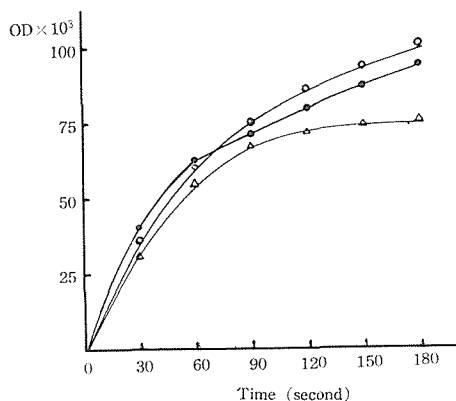


Fig. 1. Hexokinase activity of intact cell, protoplast and ghost. -●-, intact cell; -○-, protoplast; -△-, ghost.

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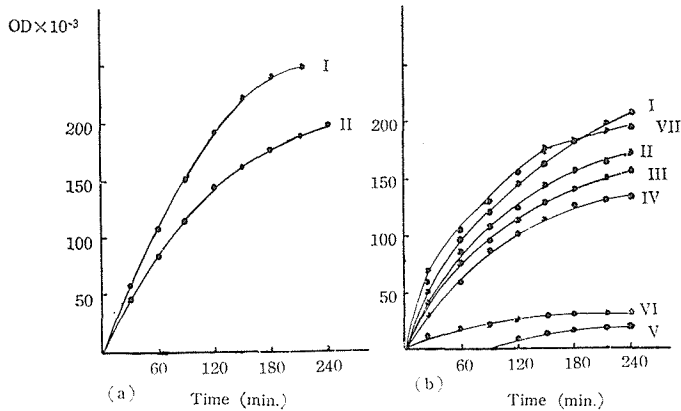


Fig. 2. Hexokinase distribution. In fig. (a) I, sonicate; II, intact cell; and in fig. (b) I, air dried cell; II, protoplast; III, intact cell; IV, ghost; V, snail juice; VI, wall plus snail juice; VII, cytoplasm.

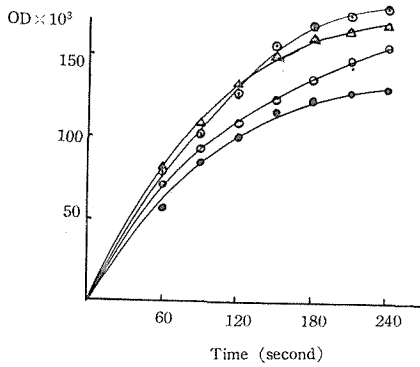


Fig. 3(a). The effect of destruction of osmotic barrier in the hexokinase assay. -●-, intact cell; -⊙-, air-dried cell; -△-, cytoplasm (shockate of protoplast); -○-, sonicate. (Yeast: 25mg./wet wt).

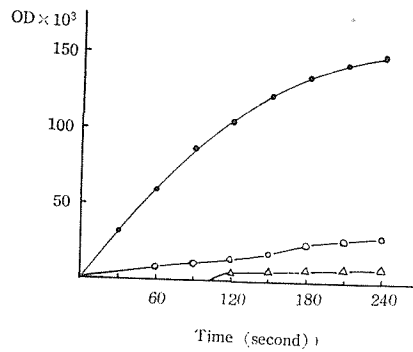


Fig. 3(b). Hexokinase activity of cell wall, Yeast employed as enzymes 25mg. of wet cell. -●-, intact cell; -○-, cell wall plus snail juice (0.2ml.); -△-, snail juice.

Table 3. Reaction mixtures of hexokinase assay.

|  |         |
|--|---------|
| 0.1 M Potassium phosphate buffer pH 6.5  | 1.0 ml. |
| Glucose (5 μM)   | 0.1     |
| ATP (0.2 mg.)  | 0.1     |
| NADPP (0.2 mg.)  | 0.1     |
| MgSO <sub>4</sub> ·7.H <sub>2</sub> O (1 mg.)                                  | 1.0     |
| G-6-P dehydrogenase  | 0.5     |
| KCN (10 <sup>-3</sup> M)   | 0.1     |
| Enzymes: Intact cell, protoplast, ghost,<br>cytoplasm, cell wall and sonicate. | 0.5     |
| Total  | 3.4     |

Enzyme was prepared from 25 mg. of raw brewer's yeast and reaction was carried out in a 5 ml. cuvette.

ivity in the cell wall preparation. According to the results obtained, it is probable that the hexokinase activity of the intact cell was represented by the activity of the cell membrane, and the activity of cytoplasm could not be measured in an intact state.

**The role of hexokinase for transport.** Glucose metabolism was inhibited by the addition of M/63 MIA (Monoiodo acetic acid) in the medium. The hexokinase activity in the cell membrane may possibly has the most significant part for glucose metabolism in yeast. Glucose could not penetrate into the cell without the action of hexokinase (Tables 1 and 2).

As shown in Figs. 1 and 2, the cell membrane was abundant in hexokinase, and glucose could penetrate into the cell by the action of hexokinase. This fact suggests that the hexokinase on the cell membrane has a correlation with glucose permeation and moreover has some specific actions like permease for glucose transport.

**Effect of insulin on glucose permeation.** Glucose metabolism of yeast was not stimulated by insulin unlike that of the mammalian blood vessel,<sup>9)</sup> and insulin also showed no effect on the recovery of the hexokinase activity of yeast cell, which had been inhibited by MIA (Table 4 and Fig. 4).

**Relationship between phosphatase and sugar phosphate transport.** It has been suggested that sugar phosphates can hardly be used as the substrate for metabolism in the intact cell.

Table 4. Effect of insulin to the recovery of hexokinase activity inhibited by MIA.

| Time (min.)                | Glucose mg./ml. |    |      |      |
|----------------------------|-----------------|----|------|------|
|                            | 0               | 30 | 120  | 180  |
| Control (1/63 M) MIA added | 38              | 34 | 34.3 | 34   |
| 1/63 M MIA + insulin       | 38              | 34 | 34   | 34.7 |

Reaction mixture contents: Yeast (3,000 rpm. ppt.) 10 ml., glucose 1,600 mg. (40 mg./ml.), MIA 1/63 M, insulin  $5 \times 10^{-2}$  unit.

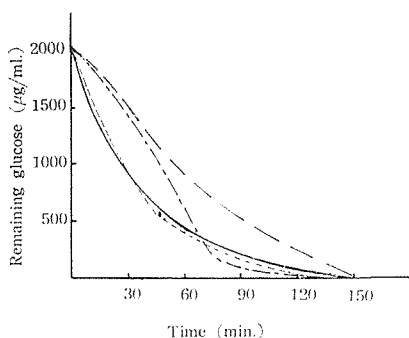


Fig. 4. Effect of insulin in glucose metabolism. Insulin concentration: ———  $10^{-2}$  unit/ml., .....  $10^{-3}$ , ———  $10^{-4}$ , - - - -  $10^{-5}$ .

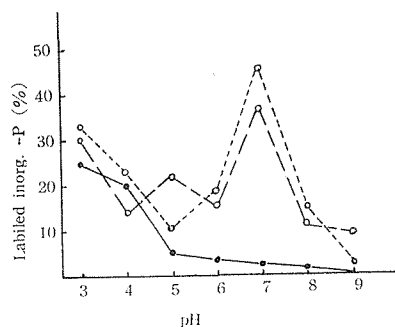


Fig. 5. Optimum pH of phosphatase in yeast. Reaction mixture: Yeast 100 mg., substrate  $10 \mu\text{M}$ , Total volume 10ml. Glycine buffer pH 1.2-3.3, acetate buffer pH 3.2-6.2, veronal buffer pH 6.8-9.6. -●-, G-1-P; .....○, G-6-P; - - ○ - -, F-1, 6-P.

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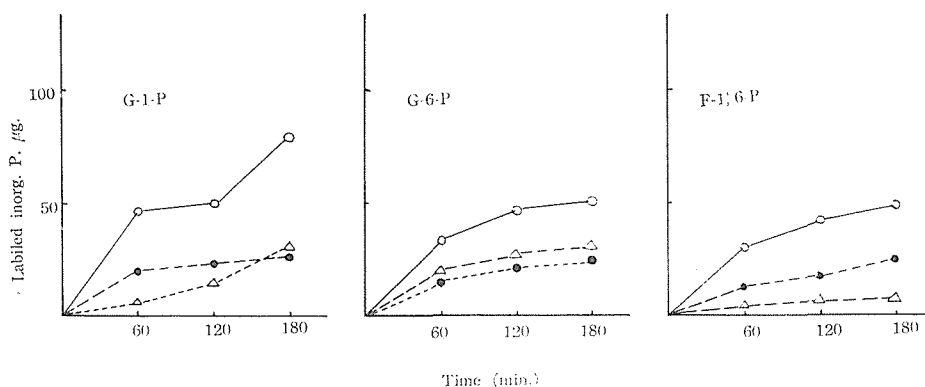


Fig. 6. Distribution of phosphatase in yeast cell.  
 --●--, cell wall; —○—, cell membrane; ---△---, cytoplasm

Rothstein<sup>10</sup> detected the activity of phosphatase in yeast cell and showed the function of it for the utilization of ATP (Adenosine triphosphate).

In this experiment the phosphatase distribution in cell and its role for sugar phosphate metabolism were examined.

**Phosphatase distribution.** It is evident that yeast phosphatase has high specificity for substrate showing two different optimal pH ranges for glucose-6-phosphate and fructose-1,6-phosphate (Fig. 5).

According to the investigation of three fractions of cell, the activity was located mainly in cell membrane about 1.5 to 2-fold more than in the other two fractions (Fig. 6).

**Mechanism of sugar phosphates utilization.** As shown in Fig. 7, glucose-6-phosphate was hydrolyzed to glucose and inorganic phosphate by intact cell and they were accumulated out of the cell in the presence of MIA. (4 and 5 in Fig. 7).

The phosphatase activity of yeast was inhibited by sodium molybdate at the concentration of  $2 \times 10^{-4}$  M. So accumulation of glucose and labile phosphate, and

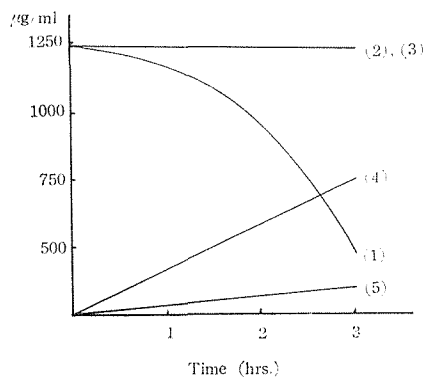


Fig. 7. G-6-P utilization by yeast.  
 (1) G-6-P decrease at the absence of inhibitor.  
 (2),(3) Presence of inhibitor.  
 (4) Glucose accumulation at the presence of MIA.  
 (5) Increase of labeled phosphate.

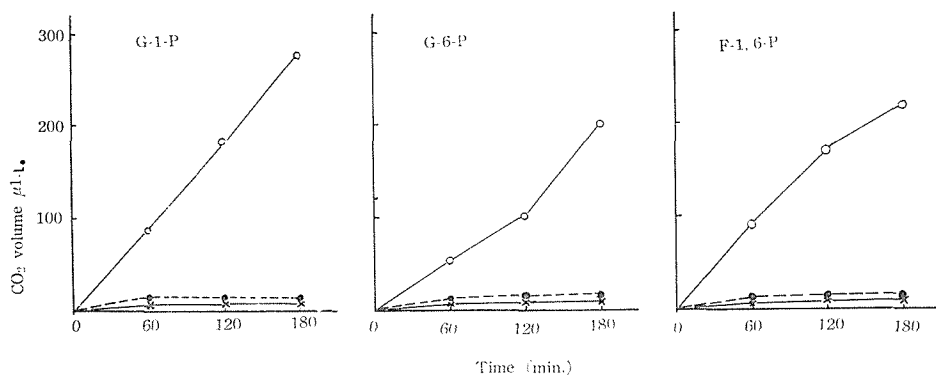


Fig. 8. Inhibition of sugar phosphate metabolism by MIA and Sodium molybdate. Content of reaction mixture: Yeast 100mg., substrate  $10\mu M$  MIA  $1/63M$ , sodium molybdate  $2 \times 10^{-4}M$ ,  
 —○—, no inhibition --○--, MIA; —×—, sodium molybdate.

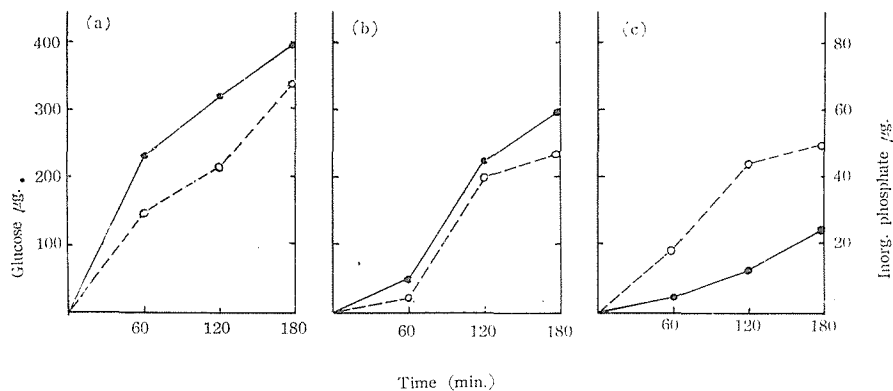


Fig. 9. Accumulation of sugars and inorganic phosphate at the presence of MIA.  
 Reaction mixture contents: Yeast 100mg., substrate  $10\mu M$ , MIA  $1/63M$ , pH3.0.

- (a) Substrate: G-1-P. —●—, glucose; --○--, inorg. phosphate.
- (b) Substrate: G-6-P. —●—, glucose; --○--, inorg. phosphate.
- (c) Substrate: F-1, 6-P. —●—, fructose; --○--, inorg. phosphate.

consumption of glucose-6-phosphate were not observed in the presence of these inhibitors (2 and 3 in Fig. 7). On the other hand, glucose-6-phosphate was metabolized at pH 3.0 (1 in Fig. 7) and inorganic phosphate was accumulated in the medium in absence of the inhibitors. (5 in Fig. 7).

Though the metabolism of sugar phosphates was also inhibited by MIA. (Fig. 8), their hydrolysis was observed (Fig. 9) and sugar and inorganic phosphate were accumulated in the presence of MIA.

From the  $CO_2$  evolution ratio it is obvious that the rate of sugar phosphate metabolism is slower than that of sugar. Addition of cell wall fractions as a source of phosphatase, to the reaction mixture accelerated the reaction rate, and  $CO_2$  evolution from sugar phosphate came nearly equal to that from the sugar (Fig. 11).



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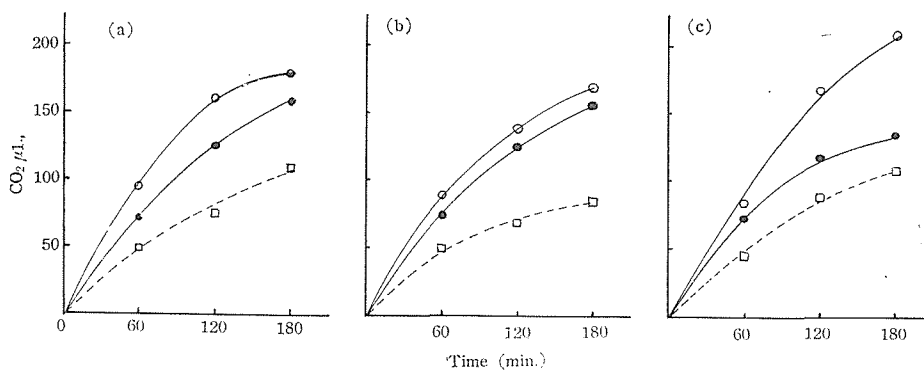


Fig. 10. Effect of the cell wall fraction as phosphatase adding to the sugar phosphate utilizing medium.

Reaction mixture contents: Yeast 100 mg., substrate 10 μM, wall fraction from 2gr. of cell. pH 3.0.

- (a) —○—, glucose; —●—, G-1-P+wall fraction; --□--, G-1-P.
- (b) —○—, glucose; —●—, G-6-P+wall fraction; --□--, G-6-P.
- (c) —○—, fructose; —●—, F-1, 6-P+wall fraction; --□--, F-1, 6-P.

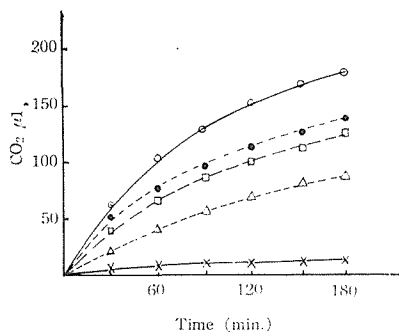


Fig. 11. CO<sub>2</sub> evolution from sugar phosphate.

Reaction mixture contents: Yeast 100mg., substrate 10 μM, pH 3.0, MIA 1/63M, MoNa 2×10<sup>-4</sup>M.

- , glucose; —●—, F-1, 6-P; --□--, G-1-P;
- △---, G-6-P; —×—, inhibitor.

It seemed that substrate specificity of enzyme changed the rate of metabolism.

These facts indicate that sugar phosphates were utilized at the active state of both phosphatase and hexokinase by intact cell. And it is obvious that phosphatase and hexokinase may be essential to complete metabolism of sugar phosphate and may act as "Permease" on the sugar phosphates transport. In any case the cell membrane played a role as a specific "barrier" to the permeation of the materials.

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#### REFERENCES

- (1) G.N. Cohn, and J. Monod, *Bact. Rev.*, 21, 169 (1957).
- (2) P. Mitchell, *Biochem. Soc. Symp.*, No. 16, 74 (1957).
- (3) E. Work, *J. Gen. Microbiol.*, 25, 167 (1961).
- (4) A. Kornberg, *J. Biol. Chem.*, 182, 805 (1957).
- (5) H.D. Agar and H.D. Douglas, *J. Bact.*, 73, 365 (1957).
- (6) M. Somogyi, *J. Biol. Chem.*, 160, 61 (1945).
- (7) C.H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66, 375 (1925).
- (8) R. Siepmann and J. C. MacDonald, *Canadian J. Microbiol.*, 6, 5 (1960).
- (9) T. Kohno, *Prot. Nucleic Acid and Enzyme (Japan)*, 7, 85 (1962).
- (10) A. Rothstein, *J. Biol. Chem.*, 32, 77, 85 (1948); *ibid.*, 33, 261 (1949); *ibid.*, 34, 97 (1956); *ibid.*, 63, 87 (1961).