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The Mechanism of Degradation of Polyphosphate in Yeast Cells by Freezing and Thawing*

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Abstract

Following the freezing and thawing of yeast cells, it was shown that a considerable amount of phosphorus compounds escaped from the cells. It was also demonstrated that the level of the phosphate in the cell had a close connection to the cell growth.

The phosphates, obtained from the cells by extraction and purification, were determined to be composed of several kinds of inorganic polyphosphates showing different behaviors of adsorption on Dowex 1 column, and were found to be located outside the protoplasmic membrane of the cell.

Certain phosphatase, which was capable of decomposing the phosphates, was also obtained from the same yeast by extraction and partial purification, and was found to be located inside the protoplasmic membrane.

The freeze-thawing treatment resulted in some degree of damage to the protoplasmic membrane in yeast cells; lactose and polyphosphate, which normally can only penetrate the cell wall, became capable of penetrating the protoplasmic membrane.

From the results obtained, it was assumed that such damage to the protoplasmic membrane caused by freeze-thawing may bring about a contact of phosphatase and phosphate, which are located inside and outside the membrane, respectively. As a result the polyphosphate was decomposed to orthophosphate and released from the cells.

Introduction

There have been many morphological and physiological investigations on the freezing injury of yeast cells (Nei, 1960; Hansen and Nossal, 1955; Mazur, 1961). It may also be important to approach the cellular injury biochemically. Thus we attempted a study on cellular injury by elucidating the mechanism of liberation of certain phosphate compounds from the cell, which is brought about by freeze-thawing. This was based on our assumption that the phosphate was playing an important role on cell growth and that the loss of the phosphate might have a considerable effect on cell metabolism. In this experiment the nature of the phosphorus compound and that of the phosphatase which decomposes this phosphorus compound and the location of these substances in the cell were investigated. The damage to protoplasmic membrane of the cell due to freeze-thawing was also examined on the assumption that the membrane is a barrier which ordinarily prevents the contact of the phosphorus compound and phosphatase within a cell but allows them come in contact only when such a barrier is damaged by freeze-thawing.

* Contribution No. 777 from the Institute of Low Temperature Science.

I. Experimental Results

1. THE PHOSPHORUS COMPOUNDS

a) Decomposition of phosphate by freeze-thawing the cell. Normal yeast cells kept in malt extract accumulated a large amount of unknown phosphates in the cell. The

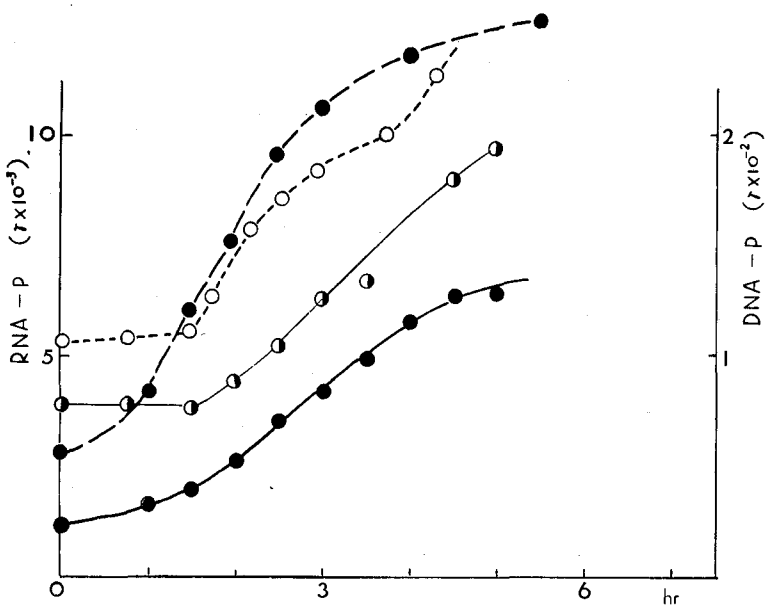

Fig. 1. Phosphorus contents in normal cell. --●--, total phosphorus in 1 N $HClO_4$ extract; —●—, RNA-phosphorus; —◐—, DNA-phosphorus; ···○···, cell number

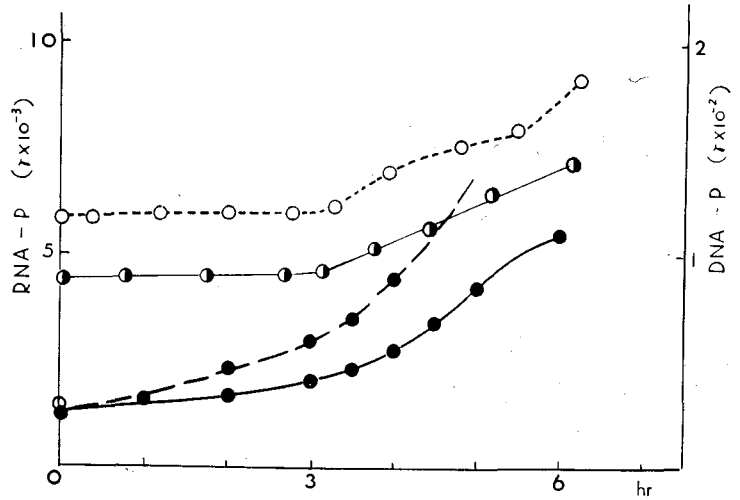

Fig. 2. Phosphorus contents in frozen-thawed cell. --●--, total phosphorus in 1 N $HClO_4$ extract; —●—, RNA-phosphorus; —◐—, DNA-phosphorus; ···○···, cell number

relationship between the level of the phosphate and cell growth is shown in Fig. 1. When the cells were frozen-thawed, however, this phosphate disappeared. The reduction of cell viability and the loss of phosphorus compound were much greater in rapid freezing than in slow freezing. Cells surviving the rapid freezing grew after a considerably prolonged lag phase. In such cells, reaccumulation of the phosphorus compound was always found; moreover, it seemed to begin at almost the same time as the renewal of cell growth (Fig. 2). These facts suggested that there is a close relationship between cellular injury and the loss of phosphorus compound in frozen-thawed cells.

The changes in the content of various phosphorus compounds in baker's yeast cells due to freeze-thawing are shown in Table 1. In the comparison of 7 fractions of phosphorus component (Ogur and Rosen, 1950) in intact and frozen-thawed cells, it was shown that the phosphorus compound was mainly liberated, by freeze-thawing, from the 1 N perchloric acid extractable fraction, and nearly the same amount of the phosphate lost in that fraction was discovered as Pi in the suspending medium and in the 70% alcohol wash of the frozen-thawed sample. However, there was no change in other phosphorus components, including RNA.

Table 1. Distribution of phosphorus compounds in intact and frozen-thawed yeast

Phosphorus component	Phosphorus μg/100mg dry cell	
	Intact	Frozen-thawed
Suspending medium	5	505
70% Alcohol wash	380	650
Hot alcohol-ether extract	85	135
0.2 N Cold $HClO_4$ extract	320	100
1 N Cold $HClO_4$ extract	1,250	750
0.5 N Hot $HClO_4$ extract	200	190
2% Hot NaOH extract	85	90
Total	2,325	2,420

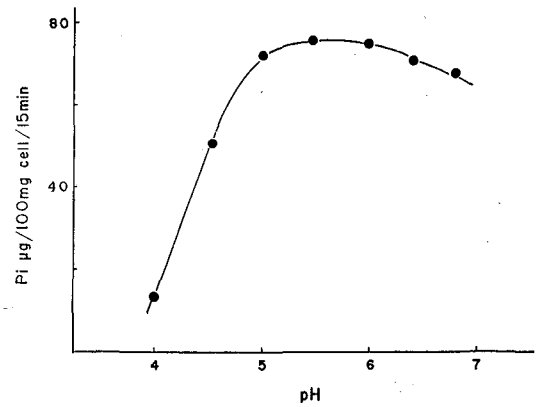

Fig. 3. The pH dependency of Pi liberation from yeast cells by freeze-thawing

The reaction of decomposition of the phosphate shows a pH dependency (Fig. 3); it was active in a range of pH 4 to 7, where the optimum activity was at about pH 6. The decomposition of the phosphate was inhibited by fluoride ion; inhibition started at 1×10^{-3} M of NaF and reached 55% in 3.5×10^{-2} M (Fig. 4). The reaction of decomposition of the phosphate was heat unstable; the cells, which were previously treated at temperatures ranging from 45 to 60°C for 60 seconds, were frozen-thawed, and the liberation

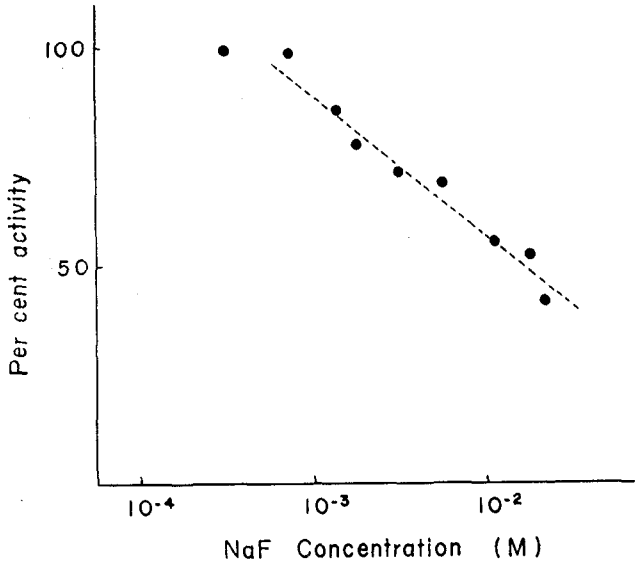

Fig. 4. Effect of the concentration of NaF upon the inhibition of the liberation of Pi from yeast cells by freeze-thawing

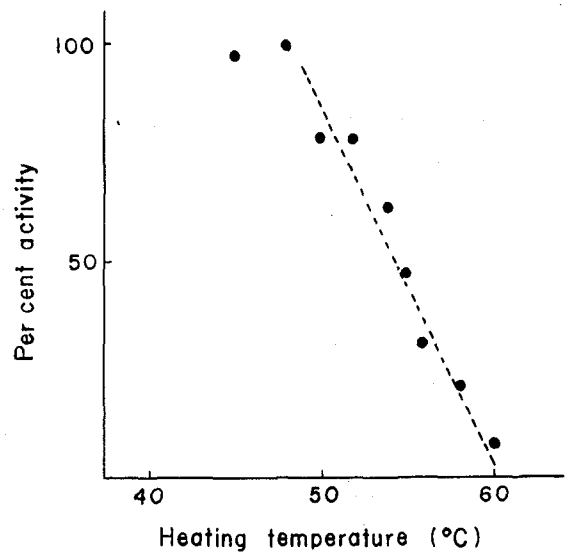

Fig. 5. Inhibitory effect of heating on the liberation of Pi from yeast by freeze-thawing

of Pi was measured in aliquots of supernatant fluid. The rate of liberation was reduced by about 22% in the cells treated at 50°C, and almost 100% in those treated at 60°C (Fig. 5).

b) The nature of the phosphorus compounds in 1 N perchloric acid extractable fraction. The phosphorus compound which accumulated in the yeast after 15 hours of culture was extracted following the method of Ogur and Rosen (1950). The cells washed and boiled with ethyl-ether were suspended in a 10% cold trichloroacetic acid and stirred for 1 hour. The extract was obtained by centrifugation. The extracted phosphorus compound can be divided into four components, chromatographically on Dowex 1 column (Kornberg, 1956), according to the KCl concentration in the eluant. All of the adsorbed phosphorus compounds can be released from the column by changing the KCl concentrations stepwise from 0.1 to 0.7 M. The eluate of the 0.1 M KCl solution which was mainly composed of Pi and nucleotide comprised approximately 13% of the total phosphorus compounds. Including the small fraction eluted by the 0.3 M KCl solution, the 0.1 M KCl eluate was followed by two large components, eluted by the 0.5 and 0.7 M KCl solutions, respectively. The component of these two zones was identified as poly-

Table 2. Chemical analysis of polyphosphates obtained from yeast

Fraction	Phosphorus μg/ml			Optical density		Nitrogen
	Total P	Acid-labile P	Pi	260 mμ	280 mμ	μg/ml
0.1 M KCl	55	1	40	4.50	1.15	24
0.5 M KCl	110	106	1	0.10	0.05	0
0.7 M KCl	100	101	1	0.30	0.15	0

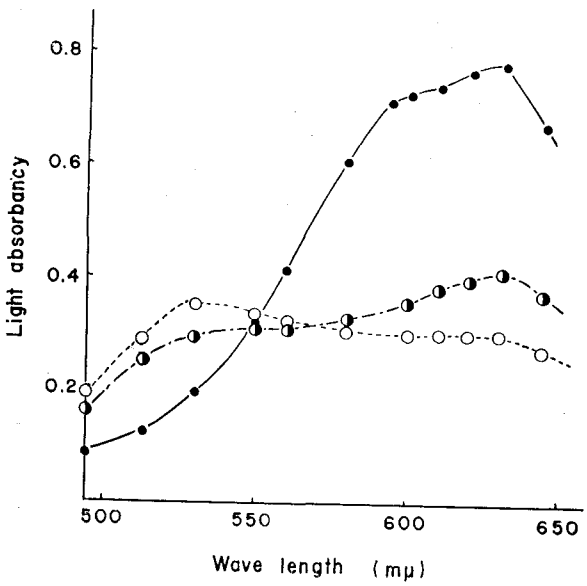

Fig. 6. Metachromatic property of polyphosphate. 3.3×10^{-5} M toluidine blue. ●, in water; ◐, in 0.5M KCl eluate; ○, in 0.7M KCl eluate

Table 3. Effect of freeze-thawing on chromatographical separation of polyphosphates

M KCl fraction		0.1	0.3	0.5	0.7
Phosphorus (Per cent)	Control	13	11	51	25
	Frozen-thawed	12	6	51	31

phosphate in the following manner. All of the phosphate was found to be acid labile. There was virtually no ultraviolet absorption, and also no evidence of nitrogen, indicating an extremely slight contamination by nucleotide (Table 2). The phosphate induced metachromasy by mixing with toluidine blue after the method of Wiame (1947); as shown in Fig. 6, the 0.5 and 0.7 M KCl eluate, mixed with toluidine blue solution, made a shift of the absorption maximum from 630 to 530 mμ, when measured spectrophotometrically. Wiame suggested that the metachromatic reaction is specific to polyphosphate in the phosphorus compounds of a biological systems.

In comparison to these fractions which were obtained by the chromatographical separation of intact and frozen-thawed samples, it was found that quantitatively the percentage of phosphorus was not affected by freeze-thawing, as shown in Table 3.

2. POLYPHOSPHATASE

a) Extraction and characterization of polyphosphatase. It can be assumed that the decomposition of polyphosphate by freeze-thawing is attributable to the function of the enzyme polyphosphatase, as baker's yeast possesses this enzyme activity, and because the decomposition did not occur when frozen-thawed in the extracted solution. The pH dependency, NaF sensitivity and heat stability in this reaction will support this assumption.

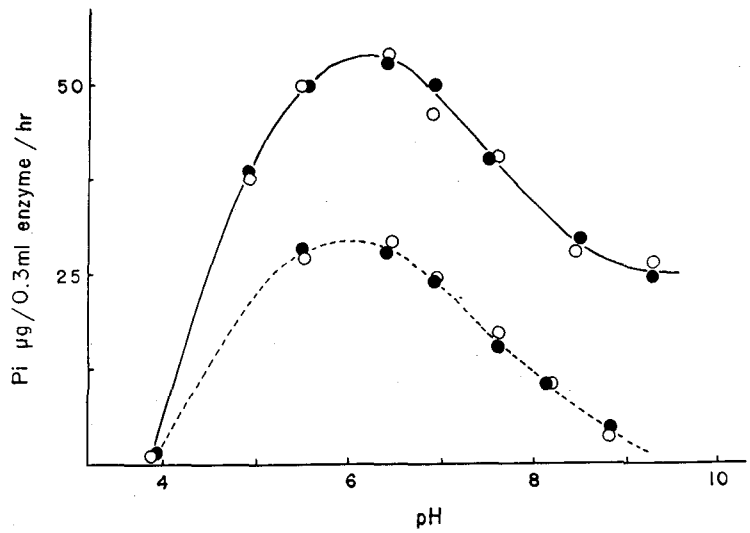

Fig. 7. The pH-activity curves of polyphosphatase obtained from yeast. ●, control; ○, frozen-thawed; ——, Mg salt added; ·····, no Mg salt added

The enzyme solution, obtained by extraction and partial purification from the mechanically degraded yeast, showed a strong activity to disintegrate polyphosphate. As shown in the pH-activity curves of this enzyme in Fig. 7, the enzyme activity was manifested in a wide pH range between 4 and 9, where the optimum activity was at about pH 6, and it was not affected by freeze-thawing.

The enzyme activity was inhibited by an addition of NaF. The inhibition began at a concentration of about 1×10^{-3} M and increased with the concentration of NaF until reaching the highest concentration, as shown in Fig. 8. The enzyme activity was also

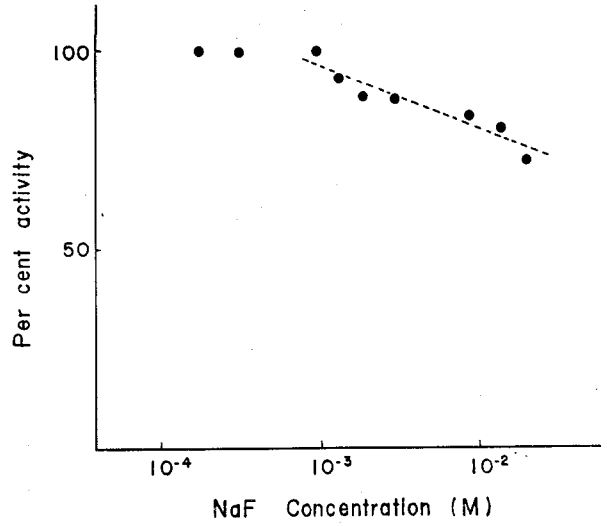

Fig. 8. Inhibitory effect of NaF concentration on the activity of polyphosphatase

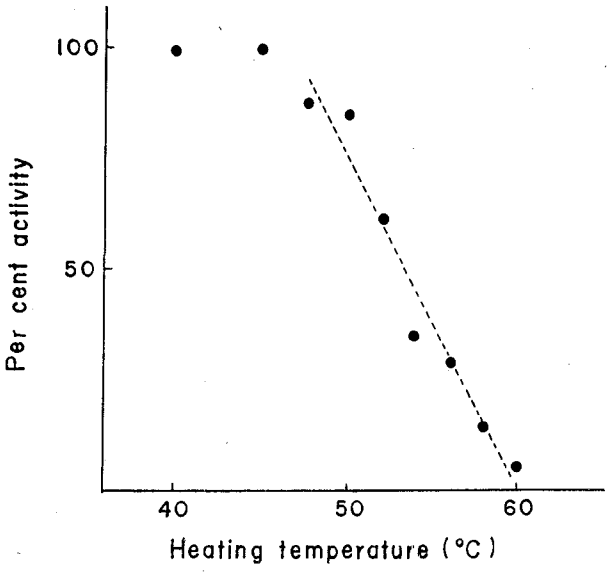

Fig. 9. Heat inactivation of polyphosphatase

decreased by heating. The effect of heating was examined by treating specimens for 60 seconds at various temperatures ranging from 40 to 60°C. As shown in Fig. 9, the enzyme activity was not affected by heating at 45°C, but it was remarkably reduced with a rise of temperature; 25% of the activity was lost at 50°C, and it was almost entirely lost at 60°C. The similarity of the behavior of polyphosphate decomposition in isolated enzyme and in frozen-thawed yeast in pH activity curve, NaF inhibition and heat inactivation brought about the conclusion that the decomposition of polyphosphate in the cells due to freeze-thawing is caused by the action of polyphosphatase.

b) Location of polyphosphatase in the cell. The experiment to determine whether or not polyphosphatase originally contained in the cells can decompose the polyphosphate

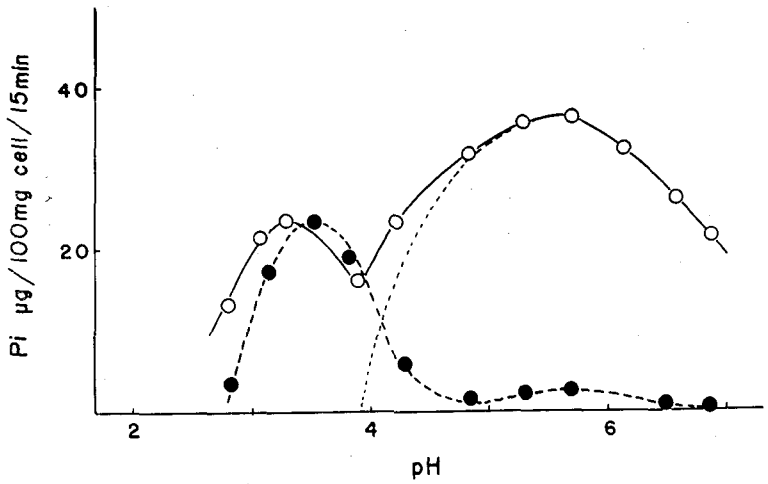

Fig. 10. The pH-activity curves of the decomposition of externally added polyphosphate in intact and frozen-thawed yeast cells. ----●----, intact cells; ——○——, frozen-thawed cells; ·····, activity manifested by freeze-thawing

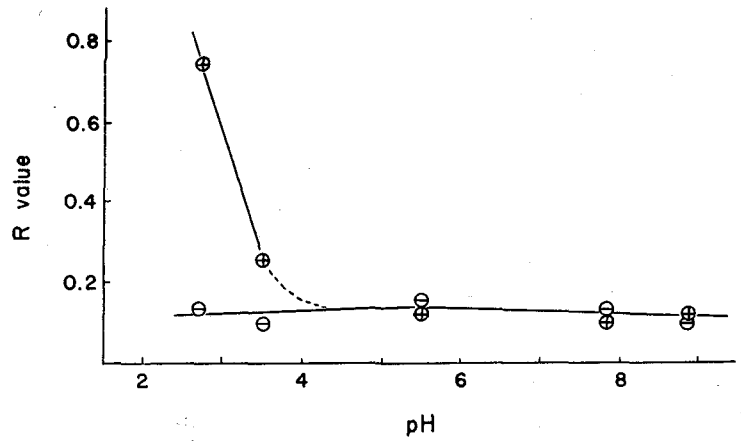

Fig. 11. The "R values" for phosphate compounds in intact yeast cells at various pH. ⊕, polyphosphate; ⊖, tripolyphosphate

assumed to enter from the outside was done using starved cells lacking polyphosphate. In the case of intact cells (dotted line in Fig. 10) a very slight activity in decomposing polyphosphate at pH 4 to 8 was seen, whereas, the activity of the acid phosphatase was extremely high. Such results suggest that the polyphosphate can penetrate the cell wall. This was confirmed by measuring a particular space in the cell, where polyphosphate can readily permeate, by the method of Conway and Downey (1950). The value, estimated on polyphosphate and authentic tripolyphosphate in a range of pH 3 to 9, is shown as "R value" in Fig. 11. The average "R value" of these substances was approximately 0.15 throughout the entire pH range. Conway and Downey identified the space, which corresponds to approximately 15% of the total cell quantity with the cell wall. It will be assumed that polyphosphate externally added can penetrate the cell wall but not the protoplasmic membrane in normal cells.

The "R values" for lactose and polyphosphate in frozen-thawed cells, however, were more than twofold as compared to those in intact cells (Table 4). This result suggests

Table 4. "R values" for lactose and polyphosphate in yeast cells

Materials		"R value"	
		Intact cell	Frozen-thawed cell
Lactose		0.14	0.35
Polyphosphate	pH 3.5	0.25	7.00
	pH 8.8	0.11	0.32

that some damage to the protoplasmic membrane takes place in frozen-thawed cells and polyphosphate can freely permeate the cells. The pH-activity curve of decomposing an externally added polyphosphate in the frozen-thawed cells is also shown in Fig. 10 (solid line). In spite of an increased permeation of polyphosphate into the cell, the maximum activity of acid phosphatase was not augmented. This suggests that the enzyme in the yeast is located exclusively in the cell wall.

In a region between pH 4 and 8, the reaction of decomposing the polyphosphate showed a sudden increase by freeze-thawing. The pH-activity curve, revealed on frozen-thawed cells in this pH region, was similar to that of isolated polyphosphatase. This indicates that the decomposition of the externally added polyphosphate due to freeze-thawing of the cells is attributable to the action of polyphosphatase in the cells. From these results it is concluded that in yeast cells polyphosphatase is located inside the protoplasmic membrane and can not come in contact with an externally given substrate, but upon freeze-thawing it becomes capable.

3. LOCATION OF POLYPHOSPHATE IN THE CELL

On the pH-activity curve of the decomposition of intracellular polyphosphate by incubation of 15 hour cultured yeast in a wide pH range, a peak at about pH 3.5 and two distinct peaks at about pH 3.2 and 5.5 were noted in intact and frozen-thawed cells, respectively (Fig. 12). The amount of Pi liberated from intact or frozen-thawed cells by the action of acid phosphatase is almost the same. In a pH range of around 5.5, however,

Pi liberation remarkably increased on freeze-thawing, in contrast to intact cells. The pH-activity curves illustrated in Fig. 12 are closely similar to those of Fig. 10. Liberation of Pi did not occur in the cells in which polyphosphate was exhausted previously. This result suggests that the polyphosphate accumulated in the cells was decomposed by acid phosphatase and polyphosphatase in a similar manner to the externally added substrate. Consequently, it was concluded that polyphosphate was located outside the protoplasmic membrane in the cells.

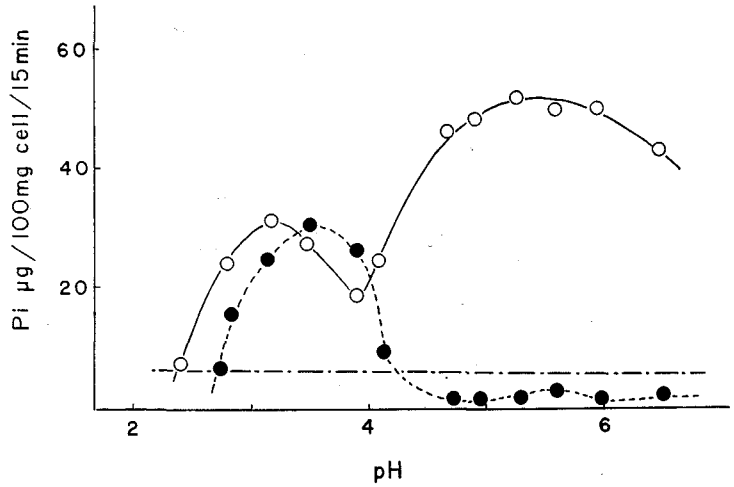

Fig. 12. The pH-activity curves of the decomposition of intracellularly accumulated polyphosphate by intact and frozen-thawed yeast. ---●---, intact cells; ——○——, frozen-thawed cells; ——– –——, polyphosphate lacking cells

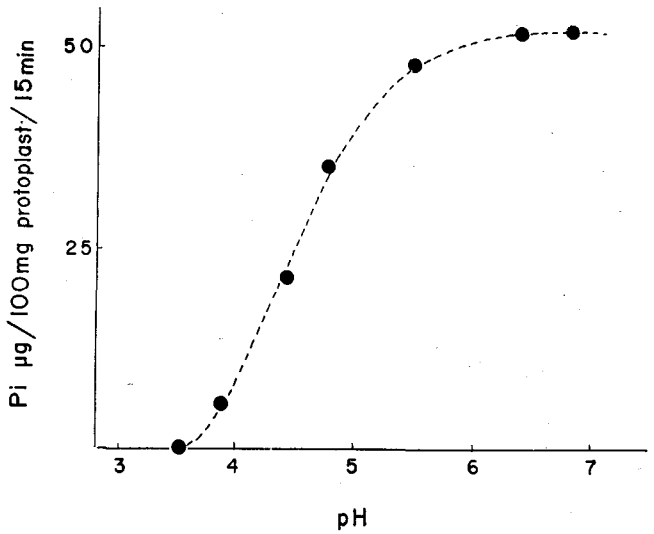

Fig. 13. The liberation of Pi from incomplete protoplasts by the action of polyphosphatase

This assumption was examined by use of incomplete protoplasts, in which the cell wall was partially destroyed by incubation with snail enzyme preparation (Eddy and Williamson, 1957) but which still retained polyphosphate. Polyphosphate in these incomplete protoplasts was decomposed by the enzyme added externally (Fig. 13). Since both enzyme and substrate can not pass through the protoplasmic membrane as mentioned before, polyphosphate must be located outside the protoplasmic membrane. When the transformation of the protoplasts proceeded, however, the Pi liberation from the protoplasts by freeze-thawing decreased markedly, suggesting that the polyphosphate had already been liberated from the cells (Table 5).

Table 4. Pi liberation from cells or protoplasts due to freeze-thawing

State of specimens	Pi liberated μg/100 mg specimen/15 min
Intact cells	69
Incomplete protoplasts	51
Protoplasts	11

4. EFFECT OF FREEZE-THAWING ON THE CELL

The activity of decomposition of externally given polyphosphate by intact or frozen-thawed protoplasts is shown in Table 6. Intact protoplasts showed a slight activity in suspension, but, the phosphatase activity in frozen-thawed protoplasts was 10 times as much as that in intact protoplasts and almost three fourths of the total activity was liberated from protoplasts into the suspending media. In freeze-thawing of intact cells, however, neither acid phosphatase nor polyphosphatase was released from the cells (Table 7). This indicates that the cell wall was not damaged to such an extent as to enable these large molecular substances to pass.

It seems likely that, owing to some degree of damage caused by freeze-thawing, to

Table 6. Release of polyphosphatase into supernatant by freeze-thawing of protoplasts

State of protoplasts		Pi liberated μg/15 min/100 mg protoplasts	
Intact protoplasts	Supernatant fluid	1	
	Whole suspension	5	
Frozen-thawed protoplasts	Supernatant fluid	38	55
	Precipitate	17	
	Whole suspension	62	

Table 7. Polyphosphatase activity in supernatant of cell suspensions

pH	Enzyme activity/100 mg of specimen (μg Pi splitted/15 min)	
	Intact cells	Frozen-thawed cells
3.15	0	0
3.50	0	1
5.60	0	1
6.14	0	0

the protoplasmic membrane, polyphosphatase and/or polyphosphate, which are usually separated by the protoplasmic membrane, became capable to penetrate the membrane and come in contact with each other and, bringing about a degradation of polyphosphate. The protoplasmic membrane is extremely fragile and is easily damaged by freeze-thawing. In the protoplasts thus damaged, the dispersion of the enzyme and its contact with substrate in the surrounding media may occur. In the frozen-thawed cells, however, it is assumed that the contact of enzyme and substrate should take place within the cell, as the thick cell wall prohibits the liberation of the enzyme and perhaps also the liberation of the polyphosphate from the cells.

II. Discussion

There are a considerable number of reports stating that the permeability of yeast cells changed in freeze-thawing and certain cellular materials diffused out of the cells (Armstrong, 1961; Mazur, 1963). The mechanism of the damage of cells due to freeze-thawing is not yet made clear in detail. However, large molecular substances, such as acid phosphatase and polyphosphatase, can not penetrate the cell wall, even after the freeze-thawing. This suggests that freeze-thawing injures only the protoplasmic membrane and not the cell wall. The injury of the membrane must be caused by intracellular ice crystal formation or rapid removal of cellular water.

On the role of polyphosphate in cell metabolism a few facts have been established hitherto. For instance, Wiame (1949) reported that polyphosphate is used for nucleic acid synthesis, and this also seems to be supported by our previous work (Souzu and Araki, 1962). As we reported, polyphosphate may play an important role in the yeast cell metabolism. Thus, it can be said that the decomposition of the phosphate has a great influence upon the cells.

Acknowledgments

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