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THE RELATIONSHIP OF THE CELL SURFACE TO METABOLISM

IV. THE ROLE OF CELL SURFACE PHOSPHATASES OF YEAST

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Abstract

Previous studies have shown that a number of phosphatases are located on the cell surface of yeast. The present paper is concerned with the role of these enzymes in the over-all economy of the cell.

By using molybdate or tungstate to inhibit the surface phosphatases, it was possible to show that these enzymes play no direct role in carbohydrate metabolism or in phosphate transport into the cell. However, experiments with phosphorylated sugars indicate that the cell surface phosphatases are able to split organic phosphates which the cell cannot utilize directly, into non-phosphorylated organic residues which the cells can utilize. The surface phosphatases thus play an indirect role in metabolism by enabling the cells to utilize phosphorylated compounds.
THE RELATIONSHIP OF THE CELL SURFACE TO METABOLISM

IV. THE ROLE OF CELL SURFACE PHOSPHATASES OF YEAST

Introduction

It has been shown by isotope techniques (Rothstein and Meier, 1948) that a number of acid phosphatases are located on the cell surface of yeast. These phosphatases can hydrolyze such substrates as adenosine-triphosphate, adenosine diphosphate, inorganic triphosphate and pyrophosphate, phenyl phosphate and glycerophosphate, with the production of inorganic phosphate. Although some of the properties of the enzymic reactions, such as the time course of hydrolysis, the effect of substrate concentration and the effect of pH have been investigated in some detail, no information concerning the role of these enzymes in the over-all economy of the cell has been available. In fact, very little information is available concerning specific functions of any of the phosphatases. The properties of the yeast cell surface phosphatases can be readily studied in the living cell. For this reason, it seemed possible that the relationship of these particular phosphatases to other cell functions could be investigated experimentally.

Three possible relationships seemed worthy of consideration. Firstly, the surface phosphatases may play a direct role in carbohydrate metabolism. In previous papers (Rothstein and Larrabee, 1948; Rothstein, Frenkel and Larrabee, 1948), it was shown that uranium forms a complex with certain groups on the surface of the cell. These cell surface groups are involved in some manner in the initial steps in sugar metabolism. There is some evidence that these groups are polyphosphates (unpublished data). If the uranium-inhibited reactions should involve the formation of phosphorylated intermediates from the sugars, then the cell-surface phosphatases might regulate the rates of these reactions by diminishing the concentrations of
the phosphorylated intermediates. An example of such a control was described by Meyerhoff (1945) who has found that ATP-ase can play a regulating role in metabolism of hexose diphosphate by brain extracts. With no ATP-ase present, all adenylic acid is converted to ATP by the metabolic reactions and none of the former compound is available as a phosphate acceptor. With too much ATP-ase, nearly all ATP is broken down to adenylic acid and no ATP is available for phosphorylation reactions. With the correct concentration of ATP-ase, a proper balance of ATP synthesis and hydrolysis is maintained and metabolism proceeds at a rapid rate.

Secondly, the surface phosphatases may play a role in the transport of phosphate into the cell. In yeast, phosphate passes into the cell in appreciable quantities only during active metabolism of external substrate (Lawrence et al., 1941; Mullins, 1942) and this process seems to involve surface groups of some kind (Nickerson and Mullins, 1948). In fact the uptake of phosphate by all micro-organisms and possibly by all tissues seems to be an "active" process rather than a simple diffusion (Kamen and Spiegelman, 1949; Sacks, 1949). In any case, it seemed possible that the yeast surface phosphatases might be a part of the "phosphate-transport mechanism".

Thirdly, the surface phosphatases may serve the function of splitting organic phosphates that the cell cannot utilize into products that can be utilized. For example, it has been generally accepted that living yeast cells are impermeable to phosphorylated sugars, and it has been stated that these substances cannot therefore be utilized by the intact cell (Nord, 1926), even though they are rapidly metabolized by cell free extracts. If the surface phosphatases can hydrolyze sugar phosphates to sugar plus inorganic phosphate and then if one or both products can be utilized, the phosphatases will have served a useful function.
In order to determine which, if any, of the three postulated functions was correct, a search was made for a specific phosphatase inhibitor. A number of substances were tested for inhibitor activity. Molybdate, which has been shown to inhibit almond-paste, alkaline phosphatases (Courtois and Bossard, 1944) was found also to inhibit the yeast cell surface phosphatases. By using molybdate as an experimental tool and by making a number of subsidiary studies on the metabolism of phosphorylated sugars by intact cells, it has been possible to elucidate in part the role of cell surface phosphatases in metabolism.

Methods

Fresh Baker's yeast (Standard Brands, Inc.) was washed several times with 10 times its volume of distilled water. During the first washing, centrifuge speeds were kept low so that only about 85 to 90% of the cells were carried down. By this procedure light cells, cell fragments and colloidal material were discarded leaving heavy, easily centrifuged, viable cells. The yeast was then starved in distilled water with aeration for 2 to 3 hours, then adjusted to the required pH with NaOH or HCl. The cell suspension was then flushed with nitrogen, and substrates and inhibitors added. Stirring was maintained by bubbling nitrogen through the suspension through a sintered glass disc. At various times samples were withdrawn and centrifuged down rapidly at high speeds (10,000 times gravity) with an angle head centrifuge.

The glucose phosphates incorporated with radioactive phosphate (P32) were prepared from muscle extract by a modification of the technique of Anderson and Fantl (1941). The purity of the Ba salts of glucose-1 and glucose-6-phosphate was at least 97% in terms of barium, phosphate and glucose content. Inorganic phosphate was less than 1%. Adenosine triphosphate was prepared by the method of Dounce, et al. (1948). All other chemicals were commercial C.P. grade.
The beta activity of the $P_{32}$ samples were determined with a thin mica window, G.M. tube and a commercial scaling circuit. Two ml of an unknown solution was placed in a stainless steel cup at a fixed distance from the counting tube. The concentration of $P_{32}$ in the sugar phosphates was sufficiently high that 2 ml of unknown solution gave counts more than 20 times that of background. The counting period of 10 minutes was sufficiently long so that the standard error of counting was less than $1.5\%$.

Chemical methods used in various experiments included:

- glucose - Folin and Malmnos (1929)
- inorganic phosphate - method of Fiske and Subbarrow (1929) as modified by Meyerhoff and Osper (1947)
- labile phosphate - the increase in inorganic phosphate after 7 minutes of hydrolysis at $100^\circ$ C with 1 N HCl
- total phosphate - the inorganic phosphate after wet washing with concentrated $H_2SO_4$ and superoxal
- pH measurements were made with the glass electrodes.

**Results**

**Inhibition of Cell Surface Phosphatases.** A number of substances were tested for phosphatase-inhibiting properties. These substances were selected because they had been reported to inhibit other phosphatases or because they were available in the laboratory and were known to inhibit other enzymes. The inhibition was tested using $10^{-3}$ M adenosinetriphosphate (ATP) as a substrate. The substrate was added to a suspension of yeast containing 10 milligrams of cell per milliliter of suspension (10 mg/ml), plus inhibitor, both adjusted to pH 3.5, the pH optimum for the hydrolysis of ATP by yeast (Rothstein and Meier, 1948). Each poison was added 15 minutes prior to the addition of substrate. The data are summarized in Table 1.
Table 1. The Effect of Various Substances on the Hydrolysis of ATP by Cell Surface Phosphatases

The inhibitions are based on the appearance of inorganic phosphate during the ten minute period following the addition of substrate. The control values with no inhibitor were 20 micrograms of phosphate liberated per milligram of yeast (wet weight).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>Substance</th>
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Digitoxin I and Digitoxin II were samples obtained from two different pharmaceutical companies.
A number of substances tested showed no inhibitions at concentrations of 1 to 5 x 10^{-4} M. These included sodium azide, dinitrophenol, iodoacetate, sodium citrate and calcium chloride. Other substances such as magnesium chloride, beryllium sulfate and o-iodoso-benzoic acid (the sulfhydryl reagent of Hellerman et al. (1941), showed a little inhibiting action in this range of concentrations. Sodium citrate was found to inhibit to some extent at relatively high concentrations.

The only substances which gave high inhibitions at low concentrations were digitoxin and molybdate. Digitoxin has been shown to inhibit ATP-ase of frog heart (Kimiura and Dubois, 1947). In experiments with yeast, it was found that a sample of digitoxin from one pharmaceutical manufacturer gave a 22% inhibition at 1 x 10^{-5} M and 50% inhibition at 1 x 10^{-4} M. However, a sample of digitoxin from another source gave no inhibition at 10 times this concentration. For this reason and because of the limited solubility of digitoxin, this substance did not seem to be a useful inhibitor. The only inhibitor found which gave almost complete inhibition at low concentrations was sodium molybdate. This substance has been shown by Courtois and Bossard (1944) to inhibit almond paste alkaline phosphatase. It was decided to investigate in some detail the effects of molybdate on the cell surface phosphatases in the hope that this inhibitor might be a useful tool in evaluating phosphatase function.

It has been shown previously (Rothstein and Meier, 1948) and additional evidence will be shown in the present paper, that a large number of phosphate compounds can be split by the cell-surface phosphatases. It has been found that molybdate inhibits the hydrolysis of all of the substrates so far tested. However, the concentration of molybdate required was not the same in each case. Typical inhibition curves are presented in Figure 1 for some of the substrates that have been studied in some detail. The curves are nearly parallel and roughly S-shaped on a semi-log plot. The concentration
Figure 1. Inhibition by Molybdate of the Hydrolysis of Various Phosphate Compounds by Yeast Cells

The inhibition was calculated from the inorganic phosphate liberated in 10 minutes by a yeast suspension containing 10 mg wet weight per ml of suspension. Substrate concentrations were $10^{-3}$ M; the pH was 3.5.
of molybdate necessary for 50% inhibition varied from $10^{-5}$ M for inorganic pyrophosphate to $2 \times 10^{-7}$ for glycerophosphate. The enzymes were very sensitive to molybdate with essentially 100% inhibition of all substrates at $10^{-4}$ M. Some inhibition is found at concentrations as low as $1 \times 10^{-8}$ M. Each inhibition curve encompasses a wide range of molybdate concentrations. For example, in passing from 10 to 90% inhibition for ATP, the range of molybdate concentrations is 1000-fold, from about $10^{-8}$ M to $10^{-5}$ M.

The inhibition of phosphatase activity by molybdate can be characterized as a competitive inhibition on the basis of kinetic studies. In Figure 2, the reciprocal of the rate of hydrolysis is plotted against the reciprocal of the substrate concentration with pyrophosphate as the substrate. With no molybdate present, there is a straight-line relationship indicating that the Michaelis-Menten (1913) equation can be used to characterize the reaction. The hydrolysis of ATP by yeast surface phosphatases has also been shown to fit the Michaelis-Menten equation (Rothstein and Meier, 1948). With no molybdate present, a straight-line relationship is also found (Figure 2). The intercept on the rate axis is the same, but the slope is larger than in the control curve (no molybdate). This is typical of a competitive inhibition (Lineweaver and Burk, 1934).

A competitive inhibition implies a reversible inhibition. Experiments were set up to test the reversibility of the molybdate inhibition. Yeast was poisoned with $3 \times 10^{-5}$ M molybdate, and the ability to hydrolyze $10^{-2}$ M pyrophosphate at pH 3.0 was tested. The inhibition was 74% based on the activity of the same yeast with no molybdate. The cells were then centrifuged down and resuspended in 100 volumes of water at pH 3.0, stirred for 5 minutes, then recentrifuged. The washing procedure was carried out four times. The cells were then tested using pyrophosphate as a substrate and compared with control cells (no molybdate) washed in the same manner. The inhibition was thereby reduced to 13%. If the cells were washed with $10^{-2}$ M pyrophosphate instead of water, and tested by the same procedure, the in-
Figure 2. The Kinetics of Molybdate Inhibition of Pyrophosphate Hydrolysis by Surface Phosphatase

The yeast concentration was 10 mg/ml and the pH, 3.0
Because tungstate is similar chemically to molybdate, the former substance was also tested as a possible phosphatase inhibitor. Figure 3 shows that tungstate is even a little more effective than molybdate, as an inhibitor of pyrophosphate hydrolysis. An inhibition of 50% was achieved by $1.1 \times 10^{-5}$ M molybdate compared with $5.6 \times 10^{-6}$ M for tungstate. Roughly 1/2 as much tungstate was required to achieve 50% inhibition.

**Effect of Molybdate on Sugar Metabolism.** Molybdate in concentrations that completely inhibit phosphatase activity for all substrates has very little effect on the fermentation of glucose as measured either by CO$_2$ production or by glucose consumption. Figure 4 for example shows the effect of $10^{-5}$ M molybdate on CO$_2$ production from glucose. At the most, the inhibition was about 10%. This concentration of molybdate, however, was sufficient to inhibit markedly the phosphatase activity (Figure 1). Inhibition measured in terms of glucose consumption under the same conditions was less than 10% with $10^{-4}$ M molybdate which is sufficient to completely inhibit the phosphatases. Molybdate in $10^{-5}$ M concentration had no measurable effect on glycogen synthesis from glucose nor did it affect glycogen degradation during starvation.

In short, the inhibition of phosphatase activity by molybdate was not accompanied by changes in metabolism.

**The Effect of Molybdate on Phosphate Uptake.** It has been shown (Lawerence, et al., 1941) that yeast cells take up phosphate only during active metabolism of sugars. This uptake is not stoichiometrically related to the amount of glucose consumed, but is only 1/10 to 1/20 as high. Figure 5 indicates that $2.6 \times 10^{-5}$ M molybdate had no effect whatsoever on the uptake of phosphate during glucose metabolism, although this concentration of molybdate almost completely inhibits phosphatase activity (Figure 1).

**The Metabolism of Phosphorylated Sugars.** It has been suggested that yeast cells are impermeable to phosphorylated sugars and that intact cells
Figure 3. Tungstate Compared with Molybdate as an Inhibitor of Phosphatase Activity

Substrate was $10^{-3}$ M pyrophosphate; the yeast concentration was 10 mg/ml; the time of hydrolysis 10 minutes; and the pH, 3.0.
Figure 4. The Effect of Molybdate on Fermentation of Glucose as Measured by CO₂ Production

Substrate concentration was 0.02 M and pH 4.5
Figure 5. The Effect of Molybdate on the Uptake of Phosphate by Yeast Cells During Glucose Metabolism

The yeast concentration was 25 mg/ml; 0.1 M; and the initial phosphate 0.0002 M.
cannot therefore utilize these substances (Nord, 1926). The data presented below indicate that although yeast cells cannot directly utilize sugar phosphates they can split these compounds into free sugar plus phosphate and can then metabolize the free sugar. Thus Figure 6 indicates that when glucose-1-phosphate or glucose-6-phosphate was added to a yeast suspension at pH's between 2.5 and 7.0, there was a decrease in the total amount of sugar remaining in the medium, and an equivalent increase in the concentration of inorganic phosphate in the medium. This mole to mole correspondence indicates that the glucose portion of the molecule of the sugar phosphate is utilized by the cell only when accompanied by a simultaneous or prior splitting off of the phosphate group, which then remains behind in the medium.

The maximum rate of glucose disappearance from glucose phosphate at the pH optimum is less than 1/20 of the rate of utilization of pure glucose. The pH-activity curve for pure glucose is almost a plateau between pH 7 and 3 and falls to about 60% of maximum at pH 2.0 (unpublished data). On the other hand, the pH activity curves of Figure 6 resemble those for the splitting of other phosphate compounds (Rothstein and Meier, 1948). It seems, therefore, that the limiting factor in the utilization of glucose from glucose phosphate at various pH's is the rate at which cell phosphatases can split off the phosphate group, thereby liberating free glucose. In addition, it was found in some experiments that within the first few minutes after the addition of glucose phosphate to a yeast suspension, a small amount of free glucose appeared in the medium, apparently due to an initial lag period in the utilization of glucose. After about 10 minutes, free glucose was no longer found. The initial appearance of free glucose supports the contention that the sugar phosphate is first split into glucose plus inorganic phosphate, followed by utilization of the liberated glucose.

It was pointed out previously that during the active metabolism of glucose, there is an uptake of a small amount of inorganic phosphate from
Figure 6. The Effect of pH on the Production of Inorganic Phosphate and on the Changes in Total Sugar When Sugar Phosphates Added to a Yeast Suspension.

The yeast concentration was 40 mg/ml and the substrate concentration $3 \times 10^{-3}$ M.

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- glucose disappearance
- PO$_4$ produced
the medium (Figure 5). In the case of the phosphorylated sugars, the sugar residue is consumed, but the phosphate remains in the medium. The failure of the cells to take up phosphate under these conditions is due to the slow rate of metabolism. Glucose from glucose phosphate is consumed at a rate only 1/10 to 1/20 of that when free glucose is the substrate. This slow rate of glucose metabolism is not accompanied by any appreciable phosphate uptake. Phosphate uptake only occurs when there is a rapid metabolism obtained with high concentrations of free glucose.

In Figure 7, data are presented which indicate that the splitting of glucose phosphate into glucose and phosphate occurs at the surface of the cell. These data are similar to those presented for ATP in a previous paper (Rothstein and Meier, 1948). Glucose-1-phosphate and glucose-6-phosphate containing radioactive phosphorus (P\textsubscript{32}) were added to yeast suspensions. At various times, the yeast was centrifuged down and the medium and the cells were analyzed. In the case of each sugar phosphate, there was no change in the total phosphate or of the P\textsubscript{32} activity of the medium. No P\textsubscript{32} activity was detected in the cells. There was, however, an appearance of inorganic phosphate in the medium and an equivalent disappearance of total sugar. Despite the fact that over 80% of the glucose-6-phosphate and about 50% of the glucose-1-phosphate were hydrolyzed, all of the inorganic phosphate produced remained in the medium with no exchange occurring between the cell phosphates and the inorganic phosphate (containing P\textsubscript{32}) produced from the sugar phosphate. If the hydrolysis of the glucose phosphates occurred inside the cell, such an interchange would take place and there would probably be a decrease in the phosphate content of the medium. Therefore the splitting of the sugar phosphate must take place on the outside of the cell. Just as in the case of ATP, no hydrolysis of sugar phosphate occurs in cell-free medium. The phosphatase is firmly bound to the cell surface.
Figure 7. The Metabolism of Glucose Phosphates Containing Radioactive Phosphorous (P$_{32}^-$) by Yeast Cells

In the experiment with glucose-1-phosphate, the substrate concentration was 2.6 x 10^-3 M, yeast concentration 20 mg/ml and pH 4.5. In the case of glucose-6-phosphate, the substrate concentration was 1 x 10^-3 M, yeast 40 mg/ml and pH 3.0.
The equivalence between the inorganic phosphate produced and the disappearance of total sugar noted in Figure 6, at different pH's, is also shown in Figure 7, indicating that the glucose portion of the glucose phosphate is metabolized after the latter molecule is hydrolyzed to free glucose plus inorganic phosphate.

The Effect of Molybdate on Metabolism of Sugar Phosphates. It has been shown previously that molybdate in concentrations which inhibit the cell surface phosphatases had little effect on the metabolism of glucose. However, a yeast suspension poisoned with $3 \times 10^{-5}$ M molybdate did not metabolize glucose-1-phosphate or glucose-6-phosphate at all. No inorganic phosphate was liberated and no glucose disappeared. The cells could not utilize the sugar phosphates when the phosphatases were inhibited. These experiments again indicate that sugar phosphates can be utilized only after the cell-surface phosphatases have liberated free glucose into the medium.

Discussion

On the basis of the experiments with molybdate and with the phosphorylated sugars, it can be stated that the cell surface phosphatases play no direct role in carbohydrate metabolism nor in the mechanism of phosphate uptake. However, these phosphatases can make available to the cells substances in the medium which ordinarily cannot be utilized. For example, phosphorylated sugars are apparently unable to penetrate the cell membrane and these substances cannot, therefore, be directly utilized by the cell. However, after the phosphate group is split off by the cell surface phosphatases, the free glucose produced can be utilized. The natural environment of the yeast cell undoubtedly contains phosphorylated compounds which cannot be used directly by the cell. However, the residues produced by the phosphatase reaction may be useful. The process seems to be analogous in some respects to digestion of materials in the gut of higher organisms, except
that the enzymes in this case remain firmly bound on the surface and are not released into the medium by the cell. It seems possible that phosphorylated compounds in the gut of higher animals are also hydrolyzed in a similar manner by surface phosphatases prior to absorption. Cytochemical techniques indicate that the lining of the lumen of the gut is rich in phosphatases (Dempsey and Dean, 1946). Organic phosphates probably constitute a large fraction of the total phosphate of food even after cooking and subjection to acid in the stomach.

**Summary**

1. A number of substances were tested for inhibiting action on yeast cell-surface phosphatases. Molybdate and tungstate in low concentrations were found to inhibit the phosphatases completely.

2. The molybdate inhibition of the surface phosphatases was competitive in nature and completely reversible. The concentration of molybdate required for 50% inhibition varied with each substrate, but in each case an S-shaped inhibition curve was found which covered about a 1000-fold range of inhibitor concentration.

3. Molybdate in concentrations which markedly inhibited the phosphatase activity had little effect on glucose fermentation as measured by CO₂ production and by glucose disappearance, or on glycogen synthesis or degradation, or on phosphate uptake from the medium.

4. Molybdate prevented the utilization of glucose phosphates by the intact cell. However, in the absence of molybdate, the sugar part of the sugar phosphate was utilized after cell-surface phosphatases had split the compounds into free glucose and inorganic phosphate. The inorganic phosphate remained in the medium.

5. The cell-surface phosphatases seem to play no role in carbohydrate metabolism, or in the uptake of inorganic phosphate from the medium. The
phosphatases do play a role in the metabolism of organic phosphates in the medium. These compounds cannot be utilized directly. However, after they are hydrolyzed to inorganic phosphate plus organic residue, the latter may be utilized.
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