The Role of Polyphosphates in the Transport Mechanism of Glucose in Yeast Cells

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ABSTRACT Several cations inhibit anaerobic fermentation of glucose by intact yeast cells. Some ions (*e.g.* Hg^{++}) penetrate into the cytoplasm and cause an irreversible inhibition of fermentation. Other ions (*e.g.* UO_2^{++}, Ni^{++}, and Co^{++}) are reversibly bound to a substance at the outside of the yeast cell identified as polyphosphate. Although the cations are bound to exactly the same extent, their influences on fermentation differ greatly. Thorium ions are bound not only to the polyphosphates, but in addition, to phosphatides in the cell membrane. Under circumstances in which glucose is transported into the cell, the amount of polyphosphate in the outer face of the membrane decreases considerably. If yeast is poisoned with monoiodoacetate, the number of glucose molecules that can still be taken up equals the original number of cation-binding sites at the outer surface of the membrane. These data suggest that one molecule of glucose is taken up in connection with the disappearance of one polyphosphate monomer. The hypothesis is framed that the uptake of glucose into the yeast cell is associated with an enzymic phosphorylation (possibly of the carrier), with polyphosphate as phosphate donor. The inhibition of glucose uptake caused by certain metal ions may be the consequence of induced changes in the spatial arrangement of polyphosphate chains; the greater the change in configuration, the larger is the inhibition.

INTRODUCTION

Many years ago one of us (Booij, 1940) found that certain metal ions have a pronounced influence on anaerobic fermentation of glucose by *Saccharomyces cerevisiae*. Some ions inhibit fermentation completely at low concentration (Ag^{+}, Hg^{++}, Cu^{++}, UO_2^{++}, and Th^{4+}). Others (Zn^{++}, Cd^{++}, and Ni^{++}) decrease fermentation at low concentration, but the inhibition is only partial, even at high concentrations (except at very high concentrations, where all salts decrease fermentation by a non-specific osmotic effect). The hypothesis was framed that the cations are bound somewhere in the protoplasmic membrane, resulting in a decrease of glucose uptake.

The aim of this study is to investigate the nature of the binding of certain cations to the membrane of the yeast cell, the identity of the cation-binding substances, the relationship of cation-binding to the rate of glucose uptake, and finally, the role of the metal-binding substance in glucose transport.

METHODS

Baker's yeast, strain Delft I, was cultured aerobically at 27°C on a liquid medium of the following composition (see Schultz and Atkin, 1947): glucose, 90 gm; KH_2PO_4, 6 gm; citric acid, 12 gm; tert. K citrate, 60 gm; $(NH_4)_2SO_4$, 15 gm; $MgSO_4 \cdot 7\ H_2O$, 3 gm; KCl, 5 gm; $CaCl_2 \cdot 2\ H_2O$, 1.5 gm; $FeCl_3 \cdot 6\ H_2O$, 90 mg; $MnSO_4$, 30 mg; inositol, 30 mg; biotin, 15 mg; Ca pantothenate, 30 mg; thiamine, 30 mg; pyridoxine, 30 mg; nicotinic acid, 30 mg; riboflavin, 30 mg; NaCl, 250 mg; $ZnSO_4$, 250 mg; water to 5 liters. After a culturing time of 48 hours the medium was sucked off through a G-4 glass filter. The yeast cells were washed several times on the filter with distilled water. The yeast was then starved overnight in distilled water with aeration and subsequently washed again twice, with 20 volumes of distilled water. By this method the endogenous respiration is reduced to a very low level and the chance that interfering metabolites will be excreted into the medium during the experiments is minimal.

Yeast weight is expressed as wet, packed cell weight.

The binding of metal ions to yeast cells was determined by measuring the disappearance of the ions from the medium. In calculating the total volume of medium it was taken into account that 20 per cent of the wet, packed yeast volume consists of intercellular liquid (Conway and Downey, 1950). The pH was always maintained at a value in between 3.4 and 3.7, because at higher values some salts would hydrolyze, while lower values may be detrimental to the yeast cells. The pH was regulated by means of dilute hydrochloric acid.

Metal ions were determined chemically by utilizing the following methods of analysis: nickel, dimethylglyoxime (Makepeace and Craft, 1944); cobalt, according to Wise and Brandt (1954); uranyl, according to Francois (1958); mercury, dithizone, see Sandell (1959 *a*); copper, according to Hoar (1937); silver, dithizone, see Sandell (1959 *b*); iron, according to Dean and Lady (1953); thorium, according to Grimaldi and Fletcher (1956).

Ponceau Red was determined with the aid of a colorimeter at a wave length of 510 mμ. Analysis of phosphorus-containing substances was done according to the scheme of Juni *et al.* (1948). When only polyphosphates and nucleic acids were determined, polyphosphates were extracted and analyzed according to Lohmann and Langen (1957), whereas nucleic acids were extracted and assayed by the method of Ceriotti (1955). Glucose was determined according to Nelson's method (1944); when iodoacetic acid (IAA) was present in the medium, glucose was determined by the method of Hagedorn and Jensen (see Gorter and de Graaff, 1956), as IAA interferes with Nelson's method.

RESULTS

A. *The Binding of Cations to Baker's Yeast Cells*

The uptake of uranyl ions by yeast cells shows the following characteristics:

1. Within 2 minutes a certain quantity of UO_2^{++} is taken up, but in the next 60 minutes (which is the longest time we studied) no further binding occurs.
2. At very low uranyl concentrations virtually all uranyl ions are taken up by the cells; consequently the equilibrium concentration in the medium is very low. When the uranyl concentration in the medium is increased to only about 1.1 times the maximum amount which the yeast cells can bind, the binding shows a rather abrupt saturation (Fig. 1). This means that uranyl is bound in a complex that is only slightly dissociated.

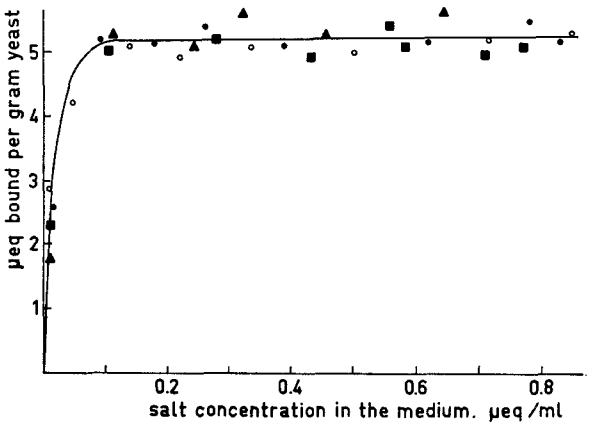

FIGURE 1. Binding of UO_2^{++} (○), Ni^{++} (■), Co^{++} (●), and Fe^{++} (▲) by yeast cells. The yeast concentration was 2.5 per cent. The final ion concentration in the medium (measured after 15 minutes) is plotted along the abscissa. The maximal binding for each of these ions (mean value of 20 determinations ±SD) is: UO_2^{++}, 4.9 ± 0.2; Ni^{++}, 5.0 ± 0.2; Fe^{++}, 5.3 ± 0.3; Co^{++}, 5.1 ± 0.1 µeq/gm yeast.

The uptake of uranyl is constant in one batch of yeast, provided that all experiments are performed in a short time (1.5 hours). Storage of yeast for several days leads to a gradual decrease of binding capacity. The uranyl-binding capacity of several batches of yeast varied from 4.5 to 6.2 µeq per gm.

Ni^{++}, Co^{++}, and Fe^{++} show exactly the same binding characteristics as uranyl ions. The amount bound is independent of time, concentration, and temperature, and varies between 4.5 and 6.2 µeq per gm for different batches of yeast. For any given batch the amounts of Ni^{++}, Co^{++}, and Fe^{++} bound are exactly equal to the quantity of uranyl that can be bound (Fig. 1). In view of

these data it may be suggested that UO_2^{++}, Ni^{++}, Co^{++}, and Fe^{++} are bound to the same cation-binding substance (provisionally called "X"). To prove this, combinations of two cations were added to different yeast suspensions. In the combinations of Ni^{++}/Co^{++} and Fe^{++}/Ni^{++} it was observed that the sum of the quantities of the two ions bound equals the binding of each ion measured separately. This additive effect indicates that these ions compete for the same binding sites. Furthermore, Co^{++}, Ni^{++}, and Fe^{++} may be displaced from the yeast cells by uranyl ions. It may be concluded that UO_2^{++}, Ni^{++}, Co^{++}, and Fe^{++} (we will call this the "uranyl group") are bound to the same substance of the yeast cells. Moreover, the affinities for the binding groups are more or less equal, a finding of importance in determining the chemical nature of the cation-binding groups.

The binding of uranyl ions to yeast cells had already been studied by Rothstein (1954). Our experiments regarding the influence of time and uranyl concentration are in complete agreement with those of Rothstein except for the difference that Rothstein, Frenkel, and Larrabee (1948) found a considerably lower level of binding (about 2.2 μeq/gm yeast). Later a possible explanation for this difference will be given. The authors mentioned proved that the uranyl ions do not penetrate into the cell, but are bound to the outside of the cell. They give a number of arguments, which are also supported by our work. For example, ethylenediaminetetraacetate (EDTA) frees these cations within 2 minutes from their binding to yeast cells. Also, variation in temperature (from 3 to 25°) has no appreciable influence on the level of binding or on the rate of binding. Apparently, the binding is not the consequence of a diffusion of ions into the cytoplasm. If a diffusion phenomenon were involved one would expect a pronounced influence of temperature (Danielli and Davson, 1935; Booij, 1956). As nickel, cobalt, and ferrous ions are bound to the same substance as uranyl ions, it can be concluded that these ions are also bound to the outside of the cell.

The binding of thorium ions by yeast shows the same characteristics as the binding of uranyl ions. Here too a saturation effect is observed: maximal thorium binding is reached within 2 minutes and is independent of the thorium concentration in the medium (above a certain minimum concentration, of course); and the temperature has no influence between 3 and 25°C. The only difference between thorium and the ions of the uranyl group is that the thorium ions are bound to a larger extent, the difference always amounting to 4.0 to 4.2 μeq/gm. Thus, the total amount of thorium bound to yeast cells varies between 8.5 and 10.4 μeq per gm yeast, compared to the uranyl binding of 4.5 to 6.2 μeq per gm yeast.

The fact that variation in the thorium-binding capacity of different batches of yeast parallels the variation in the binding capacity for ions of the uranyl group suggests that thorium ions are also bound to "substance X," but that

in addition thorium ions are bound to other sites (provisionally called "Y"), which do not bind ions of the uranyl group. The quantity of "substance Y" is rather constant, *viz.* 4.0 to 4.2 μeq per gm. The characteristics of thorium binding and the fact that EDTA rapidly releases the thorium from the cells strongly suggest that substance Y, like substance X, is located at the outside surface of the cells.

The binding of thorium ions to substance X can be proved by demonstrating competition between thorium and uranyl ions. If a mixture of thorium nitrate and uranyl nitrate is added to a yeast suspension, the total amount of ions bound (expressed as microequivalents) is equal to the maximal quantity of thorium ions that can be bound. The affinity of thorium ions for substance X about equals that of uranyl ions. This is suggested by the following experiment. A certain batch of yeast cells showed a thorium-binding capacity of 9.8 μeq and a uranyl-binding capacity of 5.8 μeq per gm. The difference (4 μeq) is presumably the quantity of thorium bound to substance Y. To 1 gm of yeast, 16 μeq thorium nitrate and 12 μeq uranyl nitrate were added. The binding amounted to 7.1 μeq of thorium and 2.7 μeq of uranyl ion. The excess thorium was added to "saturate" substance Y, which binds thorium but not uranyl. The binding to substance X must therefore be 3.1 μeq of thorium and 2.7 μeq uranyl. It may be concluded that thorium ions have only a slightly stronger affinity for substance X than uranyl ions.

The uptake of Hg^{++}, Ag^{+}, and Cu^{++} by yeast cells follows a totally different pattern from that of the uranyl group. The amounts taken up increase considerably in the course of time, and may exceed the maximal uranyl binding by many times. Thus it is apparent that these ions penetrate into the yeast cell. A further argument is that EDTA releases only a small proportion of these ions from the cells, even though their affinity for EDTA is certainly not less than that of Ni^{++} (Flaschka, 1955).

The physiological actions of Hg^{++}, Ag^{+}, and Cu^{++} are also different. They have a disintegrating influence on the structure of the yeast cells (Passow and Rothstein, 1960; Demmink, 1963; Van Steveninck, 1962). As a result, the cells lose intracellular substances to the medium.

The influence of several cations on anaerobic fermentation is summarized schematically in Fig. 2. From these data and the cation-binding studies it can be concluded that the inhibition of fermentation caused by some metal ions (*e.g.* Hg^{++}, Ag^{+}, and Cu^{++}) can be explained on the basis of their disintegrating influence on the cell structure. Therefore these ions did not seem to be of value for the study of glucose uptake. Uranyl ions, on the other hand, are bound at the cell surface to substance X, this binding being associated with a complete inhibition of fermentation. This suggests that substance X is involved in the mechanism of glucose transport into the cell. UO_2^{++}, Ni^{++}, Co^{++}, and Fe^{++} are bound to the same sites at the cell surface with approxi-

mately equal affinities. Thus the different influences of these ions on fermentation (Fig. 2) cannot be explained on the basis of different modes of binding.

B. *Identification of the Cation-binding Sites at the Cell Surface*

From the colloid chemical point of view it may be expected that the ions of the uranyl group might be bound to macromolecules (or association colloids, *viz.* phosphatides etc.) present at the protoplasmic membrane or in the cell wall. In natural colloids three negative groups may possibly bind cations: sulfate, carboxyl, and phosphate groups. *A priori* one would not expect a large

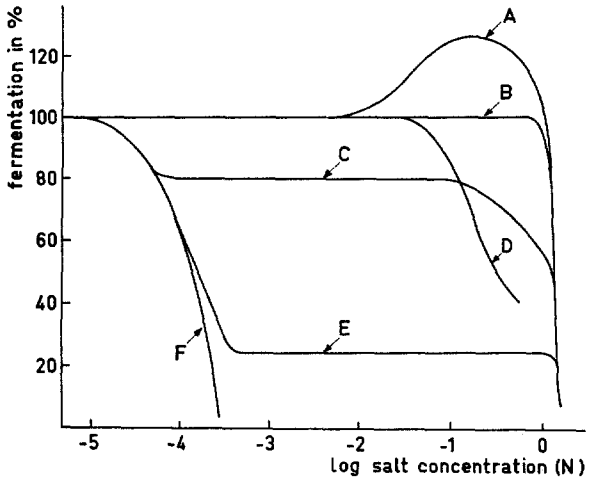

FIGURE 2. Influence of metal ions on anaerobic fermentation (schematic, Booij, 1940). Fermentation is given in percentage of the control, salt concentrations in normal units. A = K^+, Na^+, NH_4^+; B = Ca^{++}; C = Zn^{++}; D = Co^{++}; E = Ni^{++}, Cd^{++}; F = Ag^+, UO_2^{++}, Hg^{++}, Cu^{++}, Th^{4+}.

number of colloids containing sulfate groups at the outside of yeast cells. From the remaining groups the phosphate group has, in general, a stronger cation-binding capacity, especially at low pH.

To test the working hypothesis that the cations of the uranyl group are bound to phosphate groups, a number of experiments on phosphorus-deficient yeast were performed. It is possible to cultivate yeast containing only about one-quarter of the normal amount of phosphorus. Ten gm of freshly cultivated yeast is grown in a modified culturing liquid (see Methods), which contains no KH_2PO_4. After 24 hours the amount of yeast has increased to about 50 gm. The phosphorus content is only 1.5 mg per gm yeast, compared to the normal value of 5 to 6 mg per gm.

The phosphorus-deficient yeast ferments glucose with a velocity that is only 30 per cent of that of normal yeast. If, however, KH_2PO_4 (0.02 M) is added to the medium, the velocity increases to the normal level within an hour. If the

phosphorus-deficient yeast is grown in a normal culture medium, the velocity of reproduction is equal to that of normal yeast. Thus this method of treatment causes no irreversible damage.

The phosphorus-deficient yeast binds much less UO_2^{++}, Co^{++}, Ni^{++}, and Fe^{++}. The binding capacity is only 1.1 to 1.5 μeq/gm compared to the normal level of 4.5 to 6.2 μeq per gm, a decrease of about 80 per cent. This large difference clearly suggests that the cation-binding substance X contains phosphate groups. It would be unlikely that a phosphorus deficiency would lead to a large decrease of carboxyl or sulfate groups at the outside of the yeast.

It has been pointed out that Rothstein *et al.* (1948) found a lower cation-binding capacity. It may be suggested that the yeast that was used in their experiments was somewhat deficient in phosphate and, therefore, in cation-binding capacity. Rothstein *et al.* did not give the total amount of phosphorus; they reported the amount of orthophosphate in the cells (0.3 mg P/gm). In our phosphorus-rich yeast this value is 0.96 mg P per gm as orthophosphate As Rothstein used the same method for the extraction of orthophosphate, these values are comparable.

The conclusion that thorium is also bound to substance X leads to the supposition that the thorium-binding capacity of phosphorus-deficient yeast should be less than that of normal yeast. In fact, a decrease is found that is always exactly equal to the decrease in uranyl-binding capacity, suggesting that the quantity of substance Y, which binds thorium ions only, is not decreased in phosphorus-deficient yeast.

These results made it *a priori* improbable that the ions of the uranyl group would be bound to phosphatides. Indeed the decrease of the cation-binding capacity in phosphorus-deficient yeast to about 25 per cent of the normal value would then mean a drastic change in the lipid layers at the outside of the cell, without a manifest change in vitality. This seems rather unlikely.

To elucidate this problem the following experiments were done. Pieces of filter paper were impregnated with a solution of lecithin. These pieces of filter paper—after drying—were put into solutions of an uranyl, a nickel, or a cobalt salt in water. The salt concentrations were the highest used in the experiments with yeast (*viz.* 1.5 mM). The assay of the ions remaining in solution showed that no cations were bound to lecithin in filter paper even after 24 hours. From this experiment it may be concluded that it is highly unlikely that ions of the uranyl group are bound to phosphatides in the yeast cell membrane.

The same kind of experiments showed that thorium ions are, on the contrary, bound to lecithin, in a ratio of one thorium ion to four lecithin molecules. Thus it can be suggested that substance Y, which binds only thorium ions, may be a phosphatide in the lipid cell membrane. To test this hypo-

thesis the binding of uranyl ions to yeast cells was measured in the presence of Ponceau Red (a negative dye with three sulfonic acid groups). Neutral phosphatides (*e.g.* lecithin) will react with uranyl and Ponceau Red, to form a so-called tricomplex with the formula:

$$Lec_6(UO_2)_3Ponc_2 \text{ (Lec = lecithin and Ponc = Ponceau Red).}$$

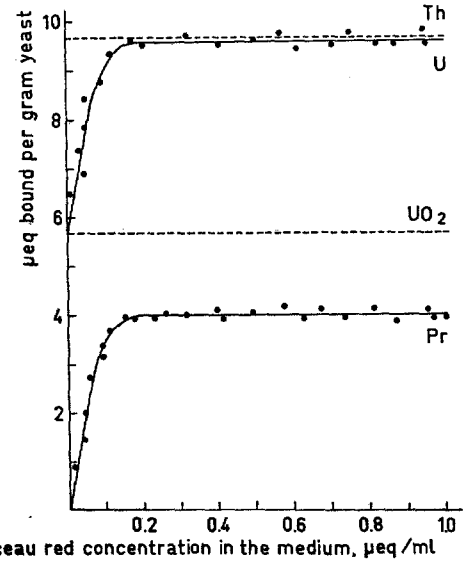

FIGURE 3. Binding of uranyl ions to yeast in the presence of increasing concentrations of Ponceau Red, compared to the level of binding of uranyl and thorium ions when present alone. Broken lines: *Th* = level of the Th^{4+} binding; UO_2 = level of UO_2^{++} binding when present alone. Full lines: *U* = binding of UO_2^{++} and *Pr* = binding of Ponceau Red, with increasing Ponceau Red concentrations and a fixed (excess) uranyl concentration in the medium.

This method may be used for the quantitative determination of phosphatides, for instance on a paper chromatogram (Hooghwinkel and Van Niekerk, 1960). It would be supposed that yeast cells, too, would bind uranyl ions and Ponceau Red, if the phosphatides in the membrane are accessible to the reagents. Yeast suspensions were incubated at pH 4.5 with varying concentrations of uranyl and Ponceau Red. At different times the cells were centrifuged and the concentrations of uranyl and Ponceau Red in the supernatant were determined. After 2 minutes the concentrations of uranyl and Ponceau Red in the supernatant remain constant. As in the preceding experiments the binding takes place in a very short time, which again suggests that the binding occurs on the outside of the cell. The experiments showed (time of contact always about 15 minutes, Fig. 3):

1. If only uranyl nitrate is added, a binding to substance X is found (5.5 μeq per gm yeast).
2. If only Ponceau Red is added, no dye is bound to the yeast cells.
3. If an excess uranyl nitrate (25 μeq/gm yeast) is added and then Ponceau Red in increasing concentrations, at low concentrations practically all of the dye is bound to the yeast cells. The binding increases to a maximum of 4 μeq dye per gm of yeast.
4. Parallel to the binding of Ponceau Red ions an extra binding of uranyl ions is observed in exactly equivalent amounts. From the moment the maximal uptake of Ponceau Red is reached, no more uranyl ions will be bound.
5. The total amount of uranyl ions bound in the presence of an excess of Ponceau Red equals the amount of thorium ions which the same yeast will bind.

Thus the difference between the thorium-binding capacity and the uranyl-binding capacity of yeast is equal to the extra binding of uranyl ions in the presence of Ponceau Red. This experiment—in combination with the model experiments on thorium binding of lecithin *in vitro*—strongly suggests that substance Y consists of phosphatides. The experiments showed that the amount of substance Y remains constant, even in phosphorus-deficient yeast.

To ascertain which phosphorus-containing substance would show the properties of X, mentioned before, the phosphorus-containing substances from yeast were isolated according to the scheme of Juni *et al.* A quantitative analysis showed that the total phosphorus content of the yeast was 6 mg per gm yeast. It was distributed in the following way:

	per cent
1. Orthophosphate	16
2. Organic P (metabolites, ATP, ADP, etc.)	10
3. Pyrophosphate	4
4. Phospholipids	6
5. Polyphosphates	20
6. Phosphoproteins	2
7. Nucleic acids	31
8. Unknown substances	11

The first three groups contain substances of low molecular weight. It is difficult to regard these compounds as components of a membrane structure. It has been pointed out that phospholipids cannot be responsible for the binding of ions of the uranyl group. Phosphoproteins are found in yeast only to a small extent. Under the most favorable circumstances the number of phos-

phate groups in these macromolecules would be barely sufficient to bind the ions of the uranyl group. This would necessitate the presence of all phosphoproteins at the outside of the cell and it would be imperative that all phosphate groups in these proteins be present in the form of monoesters. It seems unlikely that both requirements are fulfilled. It seems that most of the phosphorus in phosphoproteins is present as diester (Perlmann, 1954). This would mean a much lower cation-binding capacity.

Obviously, it is difficult to discuss group 8 (the unknown substances). For the moment it is assumed that the cation-binding substance X is not identical with one of the unknown substances. Thus we are left with two possible candidates: nucleic acids and polyphosphates.

In order to make a choice between these two substances, the following experiment was performed. Freshly cultured yeast was treated in a variety of ways. At different times the amount of polyphosphate, the binding of ions of the uranyl group, and the amount of nucleic acids were measured. The amount of polyphosphate fluctuates considerably under the influence of several factors. Under anaerobic conditions and without a fermentable substrate in the medium, the quantity of polyphosphate decreases (more quickly at higher temperatures). Perhaps this fact may be regarded as a consequence of the role of polyphosphates as an energy pool, because under these circumstances the only source of energy is high energy phosphate. When the suspension is aerated, the amount of polyphosphate increases slightly, presumably as a result of endogenous respiration. If cultured in a phosphate-deficient medium, the yeast will lose the greater part of its polyphosphates. Then, after addition of KH_2PO_4 (1/60 M) and glucose, polyphosphate is synthesized at a very high rate. After 2 hours the quantity is about two times the original value. Under anaerobic conditions the amount of polyphosphate again decreases rather sharply.

The results of this experiment are plotted in Fig. 4. (The abscissa is not a normal time axis. Every interval represents a period in which the yeast was kept under a certain specified condition. To make a quick survey possible, the various determined values are connected by straight lines. The original values were given the arbitrary value of 100 per cent. The other values are expressed as percentages of the original values.) From Fig. 4 the following conclusions may be deduced:

1. A close resemblance between the values for binding of ions of the uranyl group and the amount of polyphosphate in the cells is observed. It cannot be expected that these values will always be exactly the same, as only part of the polyphosphate will be available for ion binding. This fraction—present at the outer surface of the yeast cell—will vary under different circumstances, not only absolutely, but also relatively. At the start of the experiment about 15 to 20 per cent of the polyphosphate will be present at

the outside of the cell. In phosphorus-deficient yeast the amount of polyphosphate decreases very considerably but the fraction situated at the outside seems to be relatively larger (approximately 90 per cent). In a phosphorus-rich yeast the reverse takes place.

2. No relation between the amount of nucleic acids and the ion-binding capacity of yeast can be demonstrated. The quantity of nucleic acids varies only under drastically altered conditions (culturing in phosphorus-deficient medium). Resynthesis of nucleic acids seems to take place only slowly, when glucose and orthophosphate are added to the medium.

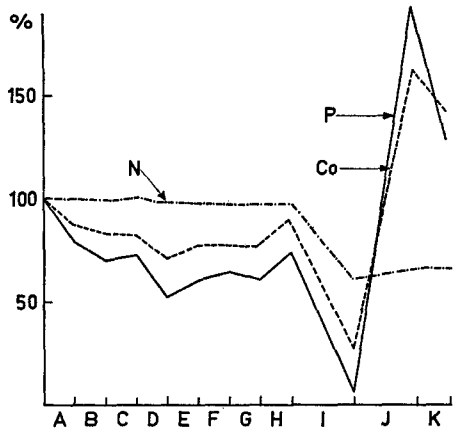

FIGURE 4. Polyphosphate concentration (*P*), nucleic acid concentration (*N*), and Co^{++}-binding capacity of yeast (*Co*) in different circumstances. *A*, 12 hours 4°C, anaerobic; *B*, 2 hours 18°C, anaerobic; *C*, 2 hours 18°C, aerobic; *D*, 10 hours 18°C, anaerobic; *E*, 2 hours 18°C, aerobic; *F*, 3 hours 18°C, anaerobic; *G*, 10 hours 18°C, anaerobic; *H*, 2 hours 18°C, aerobic; *I*, cultured for 18 hours in phosphorus-deficient medium; *J*, 2 hours 25°C, aerobic, glucose and KH_2PO_4 added; *K*, 4 hours 18°C, anaerobic.

From this experiment it may be concluded that the cation-binding capacity of yeast cells must be ascribed to polyphosphate. No indications were found for a binding of cations in relatively large amounts to other substances, but, of course, this possibility is not completely excluded. In view of the definite correlation between the amount of polyphosphate and the ion-binding capacity at the outside of yeast cells, a possible binding of cations to other negative groups (if present) seems to be quantitatively of less importance. In agreement with this, mass law plots of UO_2^{++}, Ni^{++}, and Co^{++} binding to yeast cells show only one component.

C. *The Role of Polyphosphate in Glucose Uptake*

It is not unlikely that polyphosphates play a role in glucose uptake. Many authors defend the thesis that glucose is taken up in a phosphorylated state

(see Picken, 1960). If this is true an energy-rich phosphate is needed, as in the hexokinase reaction.

The binding of uranyl and thorium ions to yeast cells is not influenced by addition of glucose. On the other hand, the amount of bound Ni^{++} or Co^{++} decreased rapidly after the addition of glucose to about 25 per cent of the original value. The binding capacity thereafter remains constant until all glucose is consumed, but returns to the original value (Fig. 5). The result is the same if glucose is added first, followed by the salt. Moreover, the concentration of glucose is not of primary importance (it was varied from 0.25 to 5 per cent).

As uranyl and thorium binding—in contrast to Ni^{++} and Co^{++} binding—is

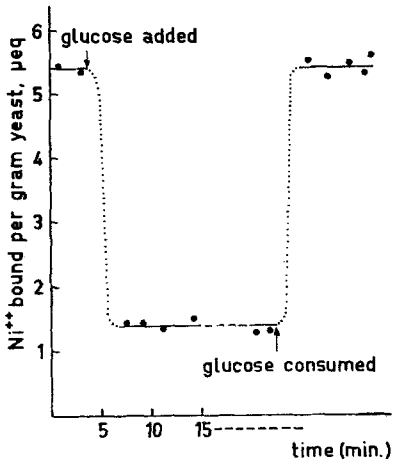

FIGURE 5. Influence of added glucose on the binding of Ni^{++} ions by yeast cells. The binding of Co^{++} ions is influenced in a similar way.

not influenced by the addition of glucose to the medium, it seems that not the presence, but the uptake of glucose into the cell is the condition necessary for the decrease in cation-binding capacity. This uptake occurs in the presence of Ni^{++} and Co^{++} ions, but not when uranyl or thorium ions are present (see Fig. 2). During glucose uptake polyphosphate (the cation-binding substance) disappears from the outside of the cells or becomes inaccessible to cations in the medium. As uranyl and thorium ions inhibit glucose uptake completely, these ions stay bound to the cells, notwithstanding the addition of glucose. This seems to point to a direct relation between the cation-binding substance and glucose transport into the cell.

This relation between polyphosphate and glucose transport is further demonstrated by the following experiment. Anaerobic fermentation is inhibited completely with iodoacetic acid (IAA), 7.5×10^{-4} M. If a small amount of glucose is added to these poisoned cells, part of it will still be taken up. The rate of uptake is rather low, but after 15 minutes no more glucose is taken up. The amount of glucose taken up is independent of the final glucose

concentration in the medium in the range of 0.3×10^{-4} to 6×10^{-4} mmole glucose per ml. Here the same saturation phenomenon is observed that was already met with when dealing with the binding of cations to yeast cells. At glucose concentrations smaller than 0.3×10^{-4} mmole/ml the maximal uptake is not reached; at concentrations higher than 6×10^{-4} mmole/ml the experimental error grows too high. In all cases the amount of glucose taken up (expressed in micromoles) appears to be the same as the amount of UO_2^{++}, Co^{++}, Ni^{++}, or Fe^{++} (expressed in microequivalents) which the yeast may bind before IAA poisoning. This observation was made with several yeast cultures (Fig. 6); also, in phosphorus-deficient yeast the cation-binding

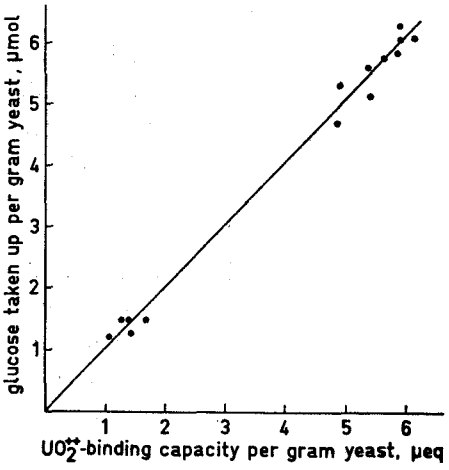

FIGURE 6. Relationship between UO_2^{++}-binding capacity (untreated yeast) and glucose uptake (yeast poisoned with monoiodoaceticacid). Points at the left are from phosphorus-deficient yeast.

capacity and the glucose uptake after poisoning with IAA were always decreased to the same low levels.

The decrease of the Ni^{++} and Co^{++} binding capacity after addition of glucose to the medium suggests that during the uptake of glucose polyphosphate groups disappear from the outside of the cells. However, as the uptake of glucose by non-poisoned yeast goes on as long as this sugar is present in the medium, polyphosphate must be resynthesized. This also tallies with the facts that the cation-binding capacity does not drop to zero and that it regains its original value when all glucose has been consumed. The diminished cation-binding capacity points to a dynamic equilibrium between disappearance and recovery of polyphosphate during glucose uptake.

After poisoning with IAA, exactly as many glucose molecules are taken up as there were cation-binding sites originally. This indicates that for each glucose molecule taken up, one polyphosphate monomer disappears from the outside of the cell and that no recovery of polyphosphate will take place under these circumstances. This is not surprising as the synthesis of polyphosphate

presumably depends on ATP formation (compare Hoffmann-Ostenhof *et al.*, 1954; Kornberg, Kornberg, and Simms, 1956; Kornberg, 1957).

Theoretically it must be expected that yeast, poisoned with IAA and then provided with glucose, will no longer bind ions of the uranyl group. All cation-binding sites will have disappeared through the uptake of glucose and no polyphosphate will be reformed at the outside of the cells. Experiments did not prove this with certainty, as the presence of IAA disturbs these experiments. Under the circumstances mentioned the binding capacity for ions of the uranyl group in fact disappears completely, but the addition of IAA alone results in a 50 per cent decrease. Washing of the cells with water leads to a recovery of the original cation-binding capacity. The manner in which IAA interferes with the binding of cations could not be elucidated.

Experiments on glucose uptake by yeast at 2°C and by yeast poisoned with HCN (0.1 M) showed a small but significant decrease in the total cellular polyphosphate content. This is in agreement with the hypothesis that polyphosphate is utilized in the process of glucose transport into yeast cells.

DISCUSSION

Whereas some metal ions (*e.g.* Cu^{++}, Hg^{++}, and Ag^{+} ions) are capable of penetrating into the intact yeast cell, many bivalent metal ions (*e.g.* UO_2^{++}, Ni^{++}, and Co^{++} ions) do not penetrate, but are bound at the cell surface to a common binding site. Experimental evidence indicates that these cation-binding sites are polyphosphates. Comparison of the properties of polyphosphates with the properties of the yeast cell–binding sites gives strong additional support to this conclusion:

1. Both contain phosphorus.
2. Both have a very strong affinity to various bivalent metal ions. As was shown, uranyl, nickel, ferrous and cobalt ions have about equal affinities to the cation-binding groups of yeast cells. This is an exception to the rule that uranyl ions have in most cases a much stronger affinity to phosphate groups than most of the other metal ions (Booij, 1956). It is, however, in good agreement with studies on polyphosphate–metal ion complexes showing that the affinities of various bivalent metal ions to polyphosphate are all of the same order of magnitude (Van Wazer and Campanella, 1950; Van Wazer and Callis, 1958).
3. In experiments on the competition of metal ions for the yeast-binding sites a rather uncommon phenomenon was found whenever a mixture of uranyl and either cobalt or nickel ions was added to a yeast suspension. There was clearly a competition of uranyl and the other metal ions for the binding sites, but the total number of ion equivalents bound exceeded the number of binding sites as measured with any of the metal ions alone.

Obviously this is only possible when anions (presumably OH^-) are incorporated in the complex. In studies on the metal ion–binding properties of polyphosphates, exactly the same phenomenon was found with combinations of uranyl and either nickel or cobalt ions. The exact reaction mechanism has not yet been studied in detail, but these observations support the conclusion that the binding sites at the outside of yeast cells are polyphosphates.

The observation that thorium ions are bound to yeast cells in higher amounts than other cations, suggested that these ions might be bound to the surface polyphosphates as well as to phosphatides of the cell membrane. The additional binding of uranyl ions, together with an equivalent Ponceau Red binding, to the same ultimate level as the thorium binding and the reported studies on metal ion–binding by lecithin *in vitro* support this hypothesis. According to the membrane theory of permeability (Booij, 1962) every living cell is surrounded by a double layer of phosphatides (in combination with sterols and coatings of macromolecules). The experiments on thorium binding give the opportunity to compute whether this hypothesis is tenable. We start from the following data.

1. The surface of a lecithin molecule in a monomolecular layer is between 40 and 80 A^2 (Van Deenen, Houtsmuller, De Haas, and Mulder, 1962).
2. The mean surface of a yeast cell is 118 μ^2 (Silbereisen, 1960).
3. The number of cells in 1 gm yeast is 10^{10}.

It has been shown that 4 μeq, or 24×10^{17} molecules, of substance Y (phosphatide) per gm is present at the outside of yeast cells. Taking lecithin as a model for all phosphatides with respect to diameter, these molecules are estimated to occupy an area between 9.6×10^{19} and 19.2×10^{19} A^2. (As only the outside of the yeast cells may be reached by the thorium ions or by the combination of uranyl and Ponceau Red ions, we are dealing with only one side of the double layer of lipids.) These figures compare favorably with the total surface of the cells in 1 gm yeast of 11.8×10^{19} A^2.

Though it is obvious that these calculations are an approximation only, the fact that the values of the measured surface of yeast cells and of the surface calculated from the thorium-binding data are of the same order of magnitude supports the hypothesis that thorium ions are bound to membrane phosphatides. This observation may eventually be of more general interest in studies on the cell membrane *in vivo*.

The binding of uranyl ions at the outside of the cell causes a complete inhibition of glucose uptake (see Fig. 2). This indicates that the polyphosphates, by which the uranyl ions are complexed, must be involved in the mechanism of glucose uptake. This conclusion was supported by the decreased cobalt- and nickel-binding capacity of the cells during the process of glucose uptake. That

we are really dealing here with a decrease in the number of cation-binding sites, and not with a complex formation between glucose and Ni^{++} or Co^{++}, is proved by the following observations:

1. Variation of the cation/glucose ratio over a 200-fold range has no influence on the observed phenomena, either qualitatively or quantitatively.
2. The formation of the colored cobalt-2-nitroso-1-naphthol-4-sulfonic acid complex is not influenced by glucose (in concentrations up to 30 per cent).

The decrease in the total amount of cellular polyphosphate during glucose uptake by HCN-poisoned cells and during glucose uptake by unpoisoned cells at 2°C also indicates that polyphosphate is involved in the glucose uptake mechanism. The experiments on glucose uptake by IAA-poisoned yeast cells indicate that the uptake of one molecule of glucose is associated with the disappearance of one polyphosphate monomer from the outside of the cell. The obvious interpretation of these observations is that the uptake of glucose into the cell is associated with a phosphorylating process. This cannot be interpreted, however, as a simple phosphorylation of glucose, as phosphorylated derivatives of glucose are not taken up as such by yeast cells. It might be that the glucose is bound to the carrier by phosphorylation, or that the carrier has to be phosphorylated prior to the binding of glucose. Presumably an enzyme participates in this phosphorylation.

Various cations have completely different influences on glucose uptake by yeast cells (see Fig. 2). Whereas uranyl ions cause complete inhibition of glucose uptake, nickel ions give an inhibition of 75 per cent and cobalt ions do not inhibit at all. Yet the experiments show that these ions are bound to the same extent and with about equal affinities to the polyphosphate-binding sites at the outside of the cells. Probably the solution of this problem can be found in the physicochemical properties of polyphosphates.

Jost (1958), Thilo (1959), and Corbridge (1955, 1956) have shown that the configuration of polyphosphate chains is highly dependent on the metal ions present. This was shown by crystallographic methods, but Thilo proved that the same is true for polyphosphates in solution. These data lead to the following hypothesis. Under physiological circumstances the polyphosphate chains at the outside of yeast cells have a certain configuration (presumably the K^+ shape). They must be considered as the substrate of a phosphorylating enzyme, which operates in glucose uptake. An added (and bound) metal ion may alter the configuration in such a way that the polyphosphate no longer fits the enzyme's active site. The larger the difference between the induced polyphosphate configuration and the physiological shape, the stronger the inhibitory effect on glucose uptake.

This hypothesis implies that one high energy phosphate bond is used during

the uptake of one molecule of glucose. It does not seem likely, however, that this energy would be lost from the cell, as it corresponds to as much as 50 per cent of the total high energy phosphate generated by the fermentative reaction. There are at least two possibilities by which the energy can be conserved:

1. At the inside of the membrane free glucose can be released and polyphosphate resynthesized. It is quite possible that the energy content of the split polyphosphate bond is retained in the carrier complex (either in the form of one other high energy bond or in the addition of two bonds, *e.g.* phosphate carrier plus phosphate glucose) and can be used for the resynthesis of polyphosphate inside the membrane.
2. Inside the membrane, glucose phosphate may be released from the carrier to enter further metabolic pathways, the hexokinase reaction being passed over.

In considering the possibility that the proposed mechanism of glucose uptake by yeast cells might be of more general significance in the uptake of sugars by living cells, certain facts have to be taken into account.

Though the mechanism of glucose transport in erythrocytes shows kinetic resemblance to that of transport of glucose in yeast cells, erythrocytes do not contain polyphosphate. Thus there must exist differences between the glucose transport mechanisms of these two kinds of cells. Further, sugars such as sorbose cannot be phosphorylated by yeast, but are apparently transported by the same system as glucose (Cirillo, 1961 *a*, 1961 *b*, 1962). Here again the hypothesis implies that notwithstanding the apparent resemblance of the uptake mechanisms of glucose and sorbose, major differences must exist between glucose and sorbose transport. Such differences have been found and they will be reported in a forthcoming paper.

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REFERENCES

1. BOOIJ, H. L., 1940, *Rec. trav. bot. néerl.*, **37**, 1.
2. BOOIJ, H. L., 1956, Inleiding tot de Physische Biochemie, Haarlem, F. Bohn N.V., 119.
3. BOOIJ, H. L., 1962, *in* Conference on Permeability held at the Agricultural University of Wageningen, Zwolle, Tjeenk Willink N.V., 5.
4. CERIOTTI, G., 1955, *J. Biol. Chem.*, **214**, 59.
5. CIRILLO, V. P., 1961 *a Tr. New York Acad. Sc.*, **23**, 725.
6. CIRILLO, V. P., 1961 *b*, *Ann. Rev. Microbiol.*, **15**, 197.
7. CIRILLO, V. P., 1962, *J. Bact.*, **84**, 485.
8. CONWAY, E. J., and DOWNEY, M., 1950, *Biochem. J.*, **47**, 347.
9. CORBRIDGE, D. E. C., 1955, *Acta Cryst.*, **8**, 520.
10. CORBRIDGE, D. E. C., 1956, *Acta Crystr.*, **9**, 308.
11. DANIELLI, J. F., and DAVSON, H., 1935, *J. Cell. and Comp. Physiol.*, **5**, 495.
12. DEAN, J. A., and LADY, J. H., 1953, *Anal. Chem.*, **25**, 947.

13. DEMMINK, H. W., 1963, De lekkage en de opname van kaliumionen bij de gistcel, Thesis, Leyden.
14. FLASCHKA, H., 1955, *Fortschr. chem. Forsch.*, **3**, 253.
15. FRANCOIS, C. A., 1958, *Anal. Chem.*, **30**, 50.
16. GORTER, E., and DE GRAAFF, W. C., 1956, Klinische Diagnostiek, Leyden, Stenfert Kroese, 243.
17. GRIMALDI, F. S., and FLETCHER, M. H., 1956, *Anal. Chem.*, **28**, 812.
18. HOAR, T. P., 1937, *Analyst (Amsterdam)*, **62**, 657.
19. HOFFMANN-OSTENHOF, O., KENEDY, J., KECK, K., GABRIEL, O., and SCHÖNFELLINGER, H. W., 1954, *Biochim. et Biophysica Acta*, **14**, 285.
20. HOOGHWINKEL, G. J. M., and VAN NIEKERK, H. P. G. A., 1960, *Koninkl. Ned. Akad. Wetenschap., Proc., Series B*, **63**, 258.
21. JOST, K. H., 1958, *Z. anorg. Chem.*, **296**, 154.
22. JUNI, E., KAMEN, M. D., REINER, J. M., and SPIEGELMAN, S., 1948, *Arch. Biochem. and Biophysics*, **18**, 387.
23. KORNBERG, S. R., 1957, *Biochim. et Biophysica Acta*, **26**, 294.
24. KORNBERG, A., KORNBERG, S. R., and SIMMS, E. S., 1956, *Biochim. et Biophysica Acta*, **20**, 215.
25. LOHMANN, K., and LANGEN, P., 1957, *Biochem. Z.*, **328**, 1.
26. MAKEPEACE, G. R., and CRAFT, C. H., 1944. *Ind. and Eng. Chem., Anal. Ed.*, **16**, 375.
27. NELSON, N., 1944, *J. Biol. Chem.*, **153**, 375.
28. PASSOW, H., and ROTHSTEIN, A., 1960, *J. Gen. Physiol.*, **43**, 621.
29. PERLMANN, G. E., 1954, *Biochim. et Biophysica Acta*, **13**, 452.
30. PICKEN, L., 1960, The Organization of Cells, London, Oxford University Press, **4**, 322.
31. ROTHSTEIN, A., 1954, *Symp. Soc. Exp. Biol.*, **8**, 165.
32. ROTHSTEIN, A., FRENKEL, A., and LARRABEE, C., 1948, *J. Cell and Comp. Physiol.*, **32**, 261.
33. SANDELL, E. B., 1959 *a*. Colorimetric Determination of Traces of Metals, New York, Interscience Publishers, Inc., 3rd edition, 626.
34. SANDELL, E. B., (1959 *b*). Colorimetric Determination of Traces of Metals, New York, Interscience Publishers, Inc., 3rd edition, 812.
35. SCHULTZ, A. S., and ATKIN, L., 1947, *Arch. Biochem. and Biophysics*, **14**, 369.
36. SILBEREISEN, K., 1960, *in* Die Hefen, (F. Reiff, H. Lüers, and M. Lindeman, editors), Nürnberg, Verlag Hans Carl, **1**, 323.
37. THILO, E., 1959, *Naturwissenschaften*, **46**, 367.
38. VAN DEENEN, L. L. M., HOUTSMULLER, U. M. T., DE HAAS, G. H., and MULDER, E., 1962, *J. Pharm. and Pharmacol.*, **14**, 429.
39. VAN STEVENINCK, J., 1962, De invloed van metaalionen op de glucoseopname door gistcellen, Thesis, Leyden.
40. VAN WAZER, J. R., and CALLIS, C. F., 1958, *Chem. Rev.*, **58**, 1011.
41. VAN WAZER, J. R., and CAMPANELLA, D. A., 1950, *J. Am. Chem. Soc.*, **72**, 655.
42. WISE, W. M., and BRANDT, W. W., 1954, *Anal. Chem.*, **26**, 693.