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Inhibition of Rb^+ uptake in yeast by Ca^{2+} is caused by a reduction in the surface potential and not in the Donnan potential of the cell wall

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Enzymic removal of the yeast cell wall does not affect the kinetics of Rb^+ uptake by the cells, nor the inhibition of this uptake by Ca^{2+}. The maximum rate of Rb^+ uptake is not affected, whereas the apparent K_m is increased by Ca^{2+}, in protoplasts as effectively as in intact cells. Comparison of this effect of Ca^{2+} with that exerted on the interaction of the surface potential fluorescent probe 9-aminoacridine with the cells reveals that the inhibition of Rb^+ uptake is caused by a decrease in the interfacial Rb^+ concentration near the membrane surface rather than by competition of Rb^+ and Ca^{2+} for binding to the translocator. Moreover, the present results show that the decrease in interfacial Rb^+ concentration is the consequence of a reduction in the negative surface potential of the membrane by Ca^{2+} and is not due to the concomitant reduction in the Donnan potential of the cell wall.

Introduction

The negative electrostatic potential (ψ_0) being a property of most biological membranes is generated by fixed charges, attracting cations to and repelling anions from the membrane surface (see, e.g., Ref. 1). In a theoretical study, we showed that due to this negative surface potential the apparent K_m of cation transporters is increased [2]. This has been confirmed experimentally by us for Rb^+ uptake in the yeast *Saccharomyces cerevisiae*, strain Delft II. The negative surface potential was reduced by adding polyvalent cations at varying concentrations. It was concluded that the increase in apparent K_m was due to competitive inhibition [3–5]. Since however, the yeast cell is surrounded by a cell wall bearing also negative charges [6–7], part of the effects found might have been caused by a reduction in the negative Donnan potential of the cell wall.

In order to examine this possibility, we compared the effect of Ca^{2+} upon Rb^+ uptake both in intact cells and in cells of which the cell wall has been removed enzymatically. In addition, the effect of Ca^{2+} upon the interaction of the fluorescent dye 9-aminoacridine which probes the surface potential of yeast [8] has been determined in both types of cells.

The results of this study provide evidence for the notion that Ca^{2+} inhibits the Rb^+ uptake in yeast via a reduction in the surface potential.

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Materials and Methods

Preparation of cells. Cells of the non-flocculent brewing yeast *Saccharomyces cerevisiae* A 294 (obtained from Whitbread and Co., Ltd., Luton, U.K.) were grown as described in Ref. 9. After harvesting in the late exponential phase of growth by centrifugation, the cells were washed three times with distilled water and resuspended in distilled water to a density of 10% (w/v).

Protoplasts were prepared as described by Theuvenet and Bindels [9]. The protoplasts were suspended in 0.82 M sorbitol supplemented with 1 mM $CaCl_2$ at a cell density of $0.5 \cdot 10^9$ protoplasts/ml, which was equivalent to 39 mg dry wt. intact cells/ml.

Electron microscopy. Thin section and freeze-fracture electron microscopy were carried out using standard procedures. For thin sections, the glutaraldehyde and osmium tetraoxide fixation method was used and samples were stained en bloc with uranyl acetate. Freeze fracturing and shadowing were performed in a Balzers BAF freeze-etch unit.

Rb^+ uptake. The washed cells or protoplasts were preincubated anaerobically in 45 mM Tris-succinate buffer (pH 4.5) supplemented with 3% (w/v) glucose at a cell density of $1 \cdot 10^8$ cells/ml and at 21°C. The suspending medium for the protoplasts in addition contained 0.82 M sorbitol and 1 mM $CaCl_2$. After a 6 min preincubation, $^{86}RbCl$ supplemented with nonradioactive RbCl and $CaCl_2$ of the desired concentration was added to the suspensions. Care was taken to keep the osmolarity of the suspending medium for the protoplasts at 865 mOsM by adjusting the sorbitol concentration. Nine successive 0.5-ml samples of the suspension were taken over a period of 2 min, the uptake of Rb^+ being stopped by the dilution of the 0.5 ml in 0.5 ml ice-cold buffered solution (pH 4.5) containing 50 mM $MgCl_2$. This stop-buffer also contained 0.82 M sorbitol and 1 mM $CaCl_2$ in case protoplasts were sampled. Underneath the stop-buffer was a 200 μl layer of silicone oil, Alcatel 214.

Immediately after dilution of the sample, the Eppendorf vial was centrifuged for 15 s in a microfuge at maximum speed ($10000 \times g$). The buffer was then removed by aspiration. Subsequently, 1 ml ice-cold stop-buffer was carefully added and removed by aspiration thus washing away any remaining radioactivity. Thereupon, the oil layer was removed by aspiration taking care not to disturb the cell pellet. After resuspension of the pellet in 600 μl distilled water, 500 μl was dissolved in 4.5 ml Packard scintillation fluid. Radioactivity was measured in a Philips PW 4540 scintillation analyser.

The concentrations of Na^+ and K^+ in the suspending media were determined by flame spectrophotometry.

9-Aminoacridine binding. The binding of the dye to yeast cells was determined according to Theuvenet et al. [8] at 25°C by measuring the dye concentration in supernatants of the cell suspension fluorimetrically. In order to prevent uptake of 9-aminoacridine into the yeast cell, which can take place via the derepressed thiamine transport system, 25 μM thiamine disulfide, a potent inhibitor of this transport system [10], was added to the cell suspensions. The amount of acridine 'bound' by the cells (B) is divided by the remaining concentrations in the supernatants (F). The value of B/F represents the relative amount of acridine bound.

Chemicals. Zymolyase-5000 was purchased from Kirin Brewery Co., Ltd. and Cellulase-Onozuka R-10 from Kinki Yakult Co., Ltd. 9-Aminoacridine was from Sigma and ^{86}Rb from Amersham International, U.K. Silicone oil of the type Alcatel 214 was purchased from VG Instruments B.V., Nieuw Vennep, The Netherlands. All other reagents were A.R. grade and were obtained from commercial sources.

Theory

Effect of Ca^{2+} on the apparent K_m of Rb^+ uptake

The dependence of the Rb^+ uptake on the Rb^+ concentration can be accounted for by a two-site immobile single carrier transport model [11], the two sites being called the activation and the substrate sites [12]. The relation between the Rb^+ uptake rate (v_i) and the concentration of Rb^+ in the medium (s_i) has the mathematical form:

$$v_i = \frac{A_i s_i + B_i s_i^2}{C_i + D_i s_i + s_i^2} \qquad (1)$$

The kinetic coefficients A_i, C_i and D_i although being independent of s_i do depend upon the concentration of other ions in the suspending medium. B_i represent the maximum rate of uptake and is independent of s_i and other ions, except protons [13]. C_i and D_i are dependent on the surface potential [3–4]. D_i is the sum and C_i is the product of the dissociation constants of Rb^+ at the activation and substrate sites. Since the apparent dissociation constant of Rb^+ for the substrate site-Rb^+ complex is much larger than that for the Rb^+-activation site complex [14], the coefficient D_i equals approximately the former dissociation constant and represents the apparent K_m of Rb^+ uptake under conditions that the activation site is saturated with Rb^+. In the present study, D_i is the relevant coefficient that will be considered.

The dependence of D_i upon other monovalent cations s_j, also present in the medium, and the surface potential is given by:

$$D_i = \frac{d_{i,0}}{y} + \sum_{j \neq i} d_{i,j} s_j \tag{2}$$

$$\text{in which } y = \exp(-F\psi_0/RT) \tag{3}$$

where ψ_0 is the surface potential, $d_{i,0}$ is the value of D_i for the case that the surface potential is zero and no competitive inhibitors are present. $d_{i,j}$ is a proportionality constant the value of which depends on the type of competitive inhibitor (see for example, Ref. 5). F, R and T have their usual meaning [3–4].

From the effect of divalent cations of concentration s_d on the kinetic coefficients of Rb^+ uptake in yeast found earlier [3–4], it was derived that

$$1/y = 1/y_0 + \beta\sqrt{s_d} \tag{4}$$

where y_0 is the value of y in the absence of added divalent cations and β is a proportionality constant which depends on the type of divalent cation added [15].

The dependence of D_i on the concentrations of s_d (Ca^{2+} in this study) is found by eliminating the factor y in Eqn. 2 by means of Eqn. 4.

$$D_i = d_{i,0}\left(\frac{1}{y_0} + \beta\sqrt{s_d}\right) + \sum_{j \neq i} d_{i,j} s_j \tag{5}$$

For convenience, we define corrected values of D_i by subtracting the contribution due to competitive inhibitors always present in the suspension (Na^+ and K^+ according to Ref. 16 and H^+ according to Ref. 5 with respective $d_{i,j}$ values of 0.21, 5.5 and 31.3 mM^{-1}). This corrected D_i value will be called the apparent K_m of the Rb^+ uptake and is denoted K'_m. Thus,

$$K'_m = D_i - \sum_{j \neq i} d_{i,j} s_j = \frac{d_{i,0}}{y_0} + d_{i,0}\beta\sqrt{s_d} \tag{6}$$

Eqn. 6 predicts that although Ca^{2+} has no affinity for the Rb^+ transport system, the apparent K_m will be increased on raising the Ca^{2+} concentration, giving rise to an apparent competitive inhibition of the Rb^+ uptake. Typically, the apparent K_m will increase linearly with $\sqrt{s_d}$, instead of directly with the Ca^{2+} concentration as is expected for a real competitive inhibition.

Effect of Ca^{2+} on 9-aminoacridine binding

In a recent publication, we showed that 9-aminoacridine may be applied as a probe of the surface potential of yeast cells [8]. The distribution ratio of the dye concentration 'bound' to the plasma membrane of yeast (B) and the concentration of free dye in the medium (F) depends on the surface potential according to Eqn. 7.

$$B/F = \alpha y \tag{7}$$

where α is the value of B/F under the condition that the surface potential is zero ($y = 1$). Eliminating y from Eqn. 7 by means of Eqn. 4 leads after rearranging the equation to:

$$F/B = \frac{1}{\alpha}\left(\frac{1}{y_0} + \beta\sqrt{s_d}\right) \tag{8}$$

According to this equation, F/B increases linearly with $\sqrt{s_d}$, just as is predicted for the apparent K_m of the Rb^+ uptake.

Results

Electron microscopy

Fig. 1 shows thin sections and freeze-fracture micrographs of yeast cells before and after enzymic digestion of the cell wall. Thin-section mi-

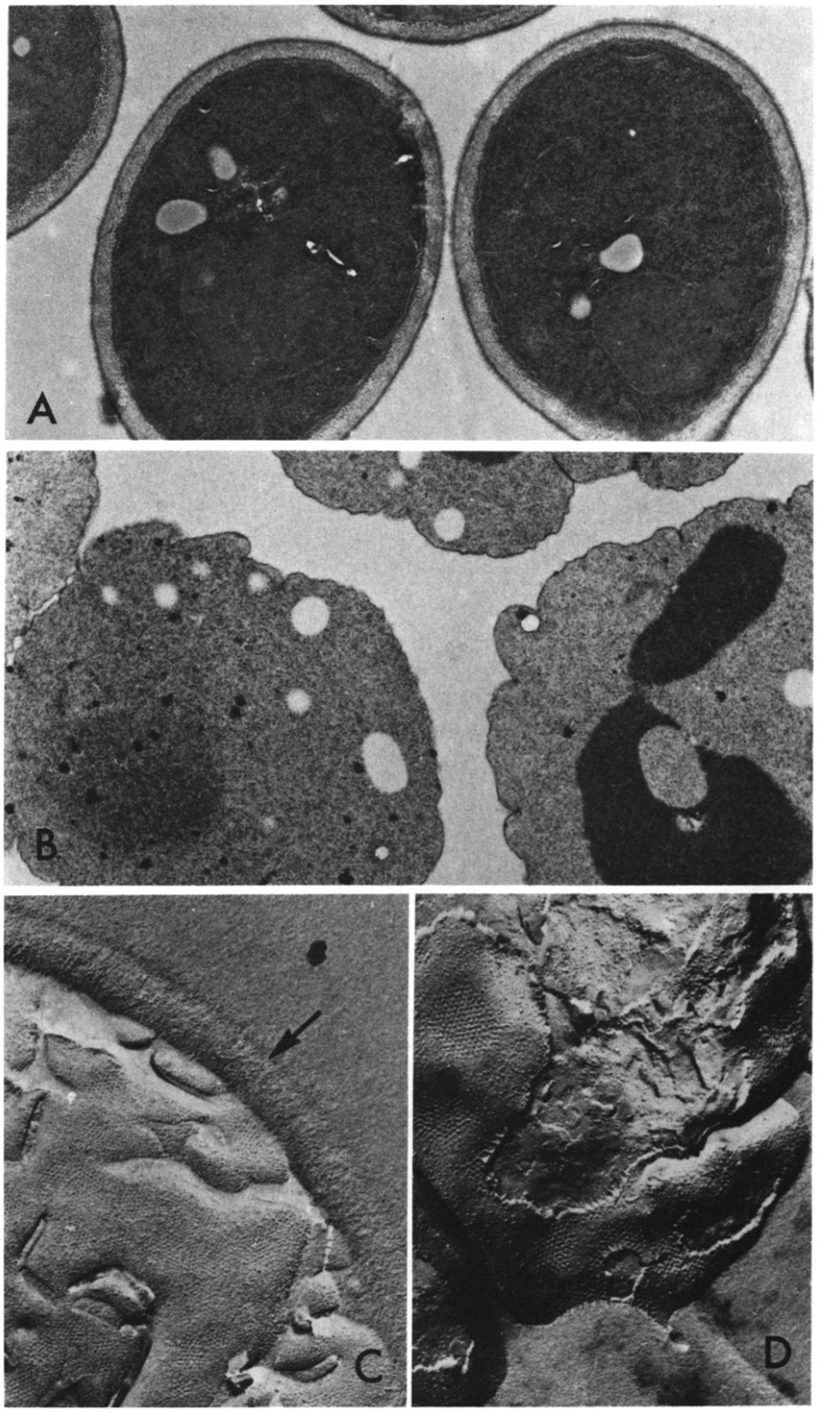

Fig. 1. Thin-section and freeze fracture micrographs of intact yeast cells and protoplasts. (A) Thin-section micrograph of intact cells. (B) Thin-section micrograph of cells after 30 min enzymic digestion of the cell wall. (C) Freeze-fracture micrograph of intact cells. Arrow marks the cell wall. (D) Freeze-fracture micrograph of cells after 30 min enzymic digestion of the cell wall. Magnification in (A–B) 20000×; in (C–D) 32000×.

crographs of intact cells (Fig. 1A) show clearly the presence of the cell wall. Thin-section micrographs of cells taken after the 30 min enzymic digestion period routinely applied by us (Fig. 1B), however, show a complete absence of the cell wall. The cell organelles in the latter case show more contrast, due to the fact that in the former case the cell wall prevents intracellular penetration of the fixative to a great extent, the cell wall itself being heavily stained. This result is confirmed by the freeze-fracture micrographs wich show clearly the presence of the cell wall (see arrow) approx. 200 nm in width (Fig. 1C), whilst the membrane fracture borders on ice (Fig. 1D). Moreover, freeze-fracture micrographs exhibit ultrastructural details of the fractured plasma membrane. A close examination reveals that the particle distribution and specific intramembraneous particles are not altered.

This electron miroscopic characterization of the cells demonstrates the removal of the cell wall by the enzymic digestion method applied. This is supported further by our previous findings that after enzymic digestion of the cell wall no phosphomannan is detectable in the ^{31}P-NMR spectrum of the cells [17]. Apparently, this typical intrinsic compound of the yeast cell wall is completely absent in preparation of protoplasts.

Rb^+ uptake

Removal of the cell wall renders the yeast cell osmofragile [9]. Consequently, for carrying out ion-uptake studies, sampling of the protoplasts using the filtration method previously applied with intact cells [14] is not applicable. Also, sampling by centrifuging the protoplasts as applied earlier by us [9] appeared unsuitable for processing great numbers of yeast samples required in the kinetical studies aimed. We therefore applied the more suitable method described under Materials and Methods. This method is a modification of the one previously applied for collecting organelles from a radioactive medium [18–19]. Following this procedure, the cells are spun down through a layer of silicone oil thus separating the cells from the radioactive medium. In order to stop uptake of Rb^+ and to wash off adsorbed radioactivity, the samples were diluted in ice-cold stop buffer prior to spinning the cells down through the silicone oil. Fig. 2 shows the time-course of the Rb^+ uptake in

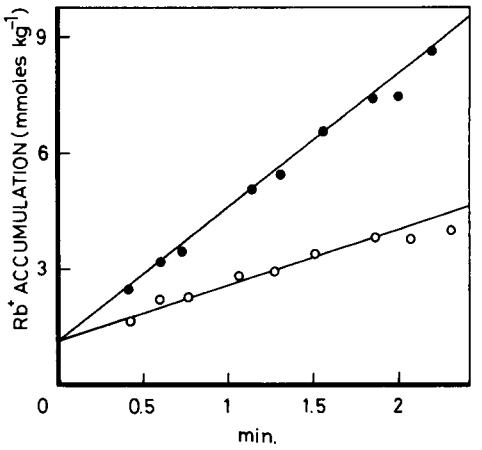

Fig. 2. Time-course of the Rb^+ uptake in intact cells and protoplasts. The Rb^+ accumulation was studied at an initial Rb^+ concentration of 1 mM in 45 mM Tris-succinate (pH 4.5) containing 3% (w/v) glucose, 0.82 M sorbitol and 1 mM $CaCl_2$ under anaerobic conditions and at a cell density of $1 \cdot 10^8$ cells per ml. (○) Uptake in the intact cells; (●) uptake in protoplasts.

metabolizing cells and protoplasts. The accumulation of Rb^+ increases by approximation linearly with time during the first minute after which a leveling-off can be seen. Initial rates of uptake are calculated from the slopes of the linear traject of the uptake curves. Fig. 2 illustrates that the washing procedure applied by us is an effective one, since the intercepts of the uptake curves with the ordinates for intact cells are not significantly different to that for the protoplasts. If the washing was incomplete, a higher intercept for intact cells than for protoplasts would be expected. This would be due to accumulation of Rb^+ into the cell wall [9]. The non-zero intercept found with both intact cells and protoplasts may at least partly be due to the fact that the time indicated refer to the time at which the centrifuge is started, whereas separation of the cells and the medium will be always a little later. Secondly, withdrawal of some medium by the cells through the oil layer may also contribute to the intercept.

The initial rate of Rb^+ uptake in protoplasts is significantly higher than in intact cells. This is partly due to competitive inhibition by K^+ as the concentration of K^+ in the suspension of intact cells is appreciably higher than in the protoplast suspension (218 and 20 μM, respectively), and

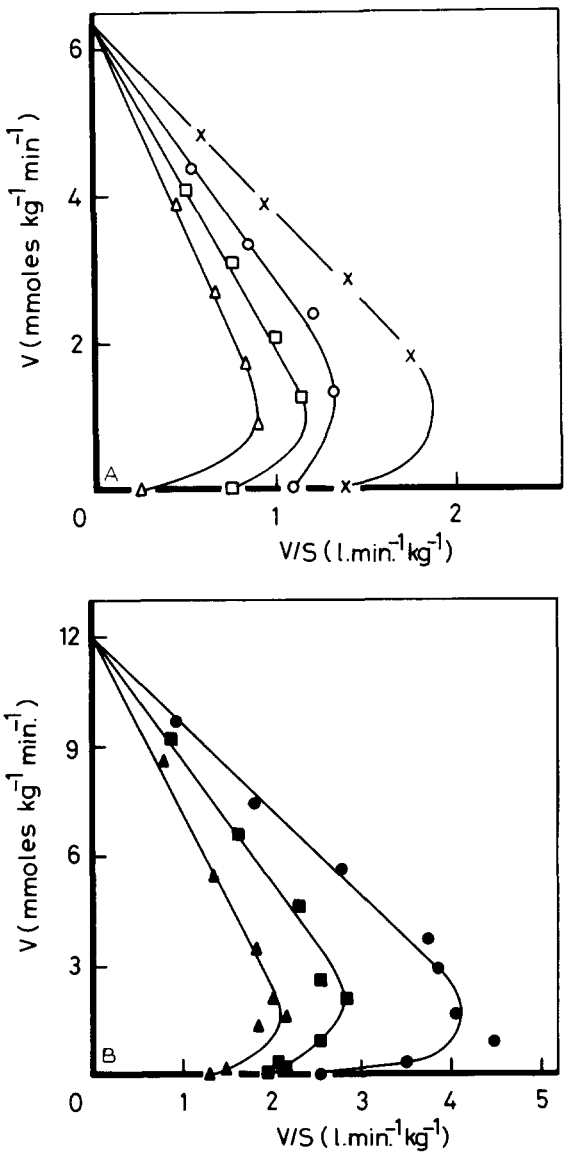

Fig. 3. Effect of Ca^{2+} on the concentration dependence of the Rb^+ uptake rates at pH 4.5. The initial rate of the Rb^+ uptake (v) is plotted against the quotient of this rate and the Rb^+ concentration (s). (A) Uptake in intact cells and at $CaCl_2$ concentrations of (×) 0 mM, (○) 0.85 mM, (□) 8 mM, (△) 32 mM. (B) Uptake in protoplasts at $CaCl_2$ concentrations of (●) 1 mM, (■) 9 mM and (▲) 36 mM.

partly to a reduced V_{max} in intact cells as compared to the V_{max} shown by protoplasts (see Fig. 3).

The effect of Ca^{2+} on the concentration dependence of Rb^+ uptake is graphically represented in

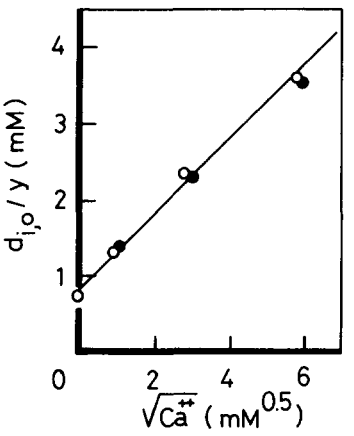

Fig. 4. Dependence of the apparent K_m of the Rb^+ uptake ($K'_m = d_{i,0}/y_0$, see Eqn. 6) on the Ca^{2+} concentration at pH 4.5. (○) Intact cells; (●) protoplasts.

Fig. 3 according to Hofstee [20]. Ca^{2+} inhibits the Rb^+ uptake in intact cells in an apparently competitive way, in accordance with previous findings with the *S. cerevisiae*, Delft II [3]. This is also true for Rb^+ uptake in protoplasts. Apparently, the Rb^+ uptake in protoplasts is more effectively inhibited by Ca^{2+} than in intact cells. However, after correcting the D_i values estimated from the slopes of the linear parts of the Hofstee plots at the higher Rb^+ concentrations for competitive inhibition by H^+, Na^+ and K^+, the apparent K_m of Rb^+ uptake in protoplasts does not differ significantly from that of intact cells.

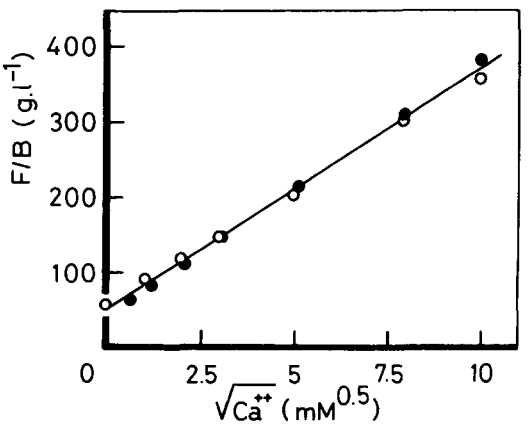

Fig. 5. Dependence of the ratio of free 9-aminoacridine and bound dye (F/B) on the Ca^{2+} concentration in the cell suspensions at pH 4.5. (○) Intact cells; (●) protoplasts.

Fig. 4 shows that the apparent K_m for both intact cells and protoplasts is increased by Ca^{2+} to the same extent, and that its dependence on the Ca^{2+} concentration can be described by Eqn. 6, under Theory.

9-Aminoacridine binding

Ca^{2+} reduces the binding of 9-aminoacridine to both intact cells and protoplasts which results in an increase in the ratio of the acridine concentration in the suspending medium (F) and that bound to the plasma membrane (B).

Fig. 5 shows that, at least to a good approximation, F/B increases linearly with the square root of the Ca^{2+} concentration, as was predicted under Theory. In addition, Fig. 5 shows that the binding of 9-aminoacridine to protoplasts differs very little from that to intact cells. This is in accordance with previous findings [8].

Discussion

Both the electronmicroscopic data shown in this article (Fig. 1) and our earlier findings that the typical intrinsic compound of the cell wall of yeast, phosphomannan, is not present in the ^{31}P-NMR spectrum of protoplasts [17], demonstrate that under the experimental conditions applied, yeast cells are completely converted into protoplasts by the enzymatic treatment. As the protoplasts are devoid of a cell wall and even remnants of this wall are not visible in the electron micrographs, it can be safely concluded that an eventual effect of the Donnan potential of the cell wall on the Rb^+ uptake in intact cells will be completely absent in the Rb^+ uptake by the protoplasts.

The method applied to separate cells and protoplasts from the radioactive medium by centrifuging them through a layer of silicone oil proved very reliable and superior to the method used by Theuvenet and Bindels [9], both by giving better reproducible results and a constant intercept which was alike for protoplasts and cells at all Rb^+ and Ca^{2+} concentrations used.

It was out of the scope of this study to find an explanation for the difference in the maximal rate of uptake observed between intact cells and protoplasts. Possibly, the somewhat lower cell pH of the protoplasts, as determined by means of ^{31}P-NMR of cellular orthophosphate (Gage, R.A., unpublished data), caused the higher maximal rate of uptake [13].

The kinetics of uptake of Rb^+ in yeast is rather complex and can be ascribed to the involvement of a multi-site transport mechanism [14] giving rise to deviations from Michaelis-Menten kinetics.

As shown in Fig. 3, at relatively high Rb^+ uptake concentrations, the transport kinetics approach that of a single-site transport process allowing to evaluate by approximation the dissociation constant of the low-affinity substrate site. In accordance with previous findings, this K_m is linearly related to the square root of the Ca^{2+} concentration. This phenomenon is typical for the interaction of a divalent cation with monovalent cation uptake if this interaction is due to a reduction in the negative surface potential. If due account is made for competitive inhibition of Rb^+ uptake by different amounts of monovalent cations present in the medium of intact cells or protoplasts, the relation between the apparent K_m and the square root of the Ca^{2+} concentration appeared to be quantitatively equal for protoplasts and intact cells. This shows that the increase in the K_m for Rb^+ uptake caused by Ca^{2+} is mainly due to the reduction in the negative surface potential of the yeast and that the expected reduction in the Donnan potential of the cell wall does not contribute much to the inhibition of Rb^+ uptake.

In view of previous theoretical considerations, the inhibition of the Rb^+ uptake by Ca^{2+} can be attributed to a progressive decrease in the ratio of the interfacial Rb^+ concentration near the plasma membrane and its concentration in the bulk aqueous phase on reducing the negative surface potential with increasing amounts of Ca^{2+} [2]. This ratio equals the Boltzmann factor $y = \exp(-F\psi_0/RT)$ and should be equal also for other monovalent cations. Recently, we showed that the factor y, which is a measure for the surface potential, can be conveniently determined with the monovalent cation and fluorescent 9-aminoacridine. This dye appears to be concentrated at the plasma membrane, whereas accumulation of this dye into the cell wall is negligible. The accumulation ratio of this dye between the region near the membranes and the bulk aqueous phase is shown to be proportional to the factor y [8]. Fig. 5 supports our earlier

conclusion that the interaction of this dye with yeast is a membrane phenomenon that depends upon the surface potential of the plasma membrane and that the Donnan potential of the cell wall does not contribute significantly to the attraction of the dye into the near-membrane phase of the cells. Just as has been found for the apparent K_m of Rb^+ uptake, the F/B ratio of the dye is also increased linearly with the square root of the Ca^{2+} concentration in the same way for protoplasts as for intact cells.

The effectiveness of a polyvalent cation in reducing the surface potential depends upon the net negative charge density of the cells [8]. The fact that Ca^{2+} reduces the surface potential to the same extent in protoplasts as with intact cells shows that the enzymic removal of the cell wall has not affected the charge density of the cell membranes.

In conclusion, the negative charges of the cell wall play no quantitatively important role in ion transport across the yeast cell membrane. The apparent competitive inhibition of Rb^+ uptake by Ca^{2+} (and likely also by other polyvalent cations applied previously [3–4]) may indeed be ascribed to a decrease in the interfacial Rb^+ concentration near the membrane caused in turn by a reduction in the negative surface potential of the plasma membrane.

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