REGULATION AND ENERGIZATION OF CATION TRANSPORT IN YEAST

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Aan mijn ouders

Voor Elja, Olaf en Sanne

. Some characteristics of
phenylphosphonium uptake

CHAPTER I

GENERAL INTRODUCTION

In general, microorganisms such as yeasts, are completely dependent upon their environment for growth and survival. Solutes are transported into the cell from the environment and waste products are excreted into it. In this way cells maintain a nearly constant intracellular composition. For uptake and excretion solutes have to pass both the cell wall and the cell membrane. The cell wall is composed in such a way that most solutes have a free passage. The cell or plasma membrane, on the other hand, is most impermeable for most solutes. Therefore the cells must contain highly specialized transport systems for uptake and excretion. In general, the cytoplasmic composition differs appreciably from the environment. It is clear that energy, derived from the cell metabolism, must be supplied to maintain the cytoplasmic composition. Cellular energy, mostly stored in chemical bonds of for example ATP, is supplied directly or indirectly to the transport system.

Though little is known about transport processes at the molecular level one can still obtain information about the translocation mechanism via indirect means. Many studies have appeared in which theoretically derived rate equations either based upon a simple model in which the translocation process is considered formally to be analogous to an enzymic process or being based upon more complex transport models are used (1-4). Thus a kinetical description of transport processes is used to characterize uptake or excretion processes. Fitting these kinetical data into theoretical models may reveal information about the translocation mechanism. The simplest form of a rate equation for an enzymic process is the Michaelis-Menten equation (5),

$$v = \frac{V_m \cdot s}{K_m + s} \qquad (1)$$

where V_m is the maximal rate of substrate translocation at infinite substrate concentration s. The Michaelis-Menten constant,

the K_m, is the concentration of s at which $v = \frac{1}{2} V_m$. This equation relates the way in which a substrate is converted into the products mediated by an enzyme with one substrate binding site to the substrate concentration in the medium. As stated by Borst-Pauwels (6) the Michaelis-Menten equation may formally describe the translocation of a solute across the membrane, mediated by a mechanism with one binding site, only under restricted conditions.

Frequently, deviations from Michaelis-Menten kinetics are observed. For example, deviations have been attributed to the simultaneous operation of two or more transport systems each having one binding site (7-10), to the operation of a transport system with two or more binding sites (11-15), to the operation of allosteric transport systems (16-20) or to the operation of a transport system with one binding site, of which K_m and V_m may discontinuously change on increasing the concentration of the solute being transported (21-23). In addition, Theuvenet and Borst-Pauwels (24) have shown, that in case of solute transport across a charged membrane, deviations from Michaelis-Menten kinetics can be observed, even if solute transport is mediated by a single-site mechanism. Since most biological membranes bear a net negative surface charge, e.g. due to the negative groups of the phospholipids, the negative surface potential resulting from these groups will "attract" cations and "repell" anions towards and away from the membrane respectively. This results in a change in the apparent K_m in eqn. (1). It is even possible that the ion, of which the uptake is studied, influences the magnitude of the surface potential on increasing the substrate ion concentration resulting in deviations from Michaelis-Menten kinetics. From the effects of other ions on the kinetical parameters of the uptake of a certain ion and on applying theoretical discrimination criteria, it is possible to draw conclusions about the mechanism of solute translocation (25). Furthermore, Barts (26) has shown that in the case of ion translocation via a mobile carrier the V_m and K_m in eqn. (1) may depend upon the membrane potential. Also under these conditions deviations from Michaelis-Menten kinetics may be found.

Although the transport systems for many solutes have been described in detail, still little is known about the energization of solute transport. Depending on the way solute transport is energized, one generally discerns primary active and secondary active transport (27). In primary active transport the energy derived from the cell metabolism is directly supplied to the transport system. The transport system contains an enzyme, which is capable to catalyse the hydrolysis of the energy-rich compound ATP. Such an enzyme is called an ATPase. During ATP hydrolysis the enzyme may undergo conformational changes by which transport of solutes across the membrane can take place. Examples of primary active transport systems are the animal ($Na^+ + K^+$) ATPase (28) and the gastric ($K^+ + H^+$) ATPase (29 and references therein). The yeast plasma membrane also contains an ATPase (30) of which its biological function at the present time is still under examination. This ATPase regulates the primary active transport of protons from the cell to the medium (31). During extrusion of protons the cells build up not only a concentration gradient for protons but also an electrical potential difference across the membrane. When there is a net translocation of charge across the membrane, the translocation is said to be electrogenic. Thus, in yeast, the membrane potential is believed to originate, at least to a considerable extent from the electrogenic extrusion of protons. The energy stored originally in the chemical bonds of ATP is converted into an electrochemical potential difference for protons (proton motive force or pmf) across the membrane. The membrane potential (ΔE) and the proton gradient (ΔpH) are related to the pmf according to

$$pmf = \Delta E - 2.3\ RT/_F\ \Delta pH\ , \qquad (2)$$

where R,T and F have their usual meaning and where ΔE and ΔpH are defined in the same direction, namely cell interior minus cell exterior. Recently it was proposed by Goffeau et al. (32) that the yeast plasma membrane ATPase not only catalyzes proton efflux but also simultaneously K^+ uptake. This exchange should

still be electrogenic which means that more protons are excreted than K^+ is accumulated. Until now, however, no one has shown that the membrane ATPase is a (K^++H^+) ATPase. Hauer et al. (33) proposed recently that there is a neutral K^+/H^+ antiport system operative in the yeast cell. Also for this system there is no direct evidence until now.

In secondary active transport uptake of solutes is not energized directly by hydrolysis of ATP but coupled to the energy stored in the electrochemical potential difference for protons, build up by primary active processes. There are several ways in which the coupling of solute uptake with the pmf may occur. In the case of cation transport this coupling may be quite simple via the electrical part of the pmf. In fact, divalent cation uptake is believed to be energized via the membrane potential (31). Because the membrane potential is generally negative inside the cell with respect to the medium, cations can be accumulated inside the cells to equilibrium concentrations that are higher than those in the medium. Uptake of neutral solutes may be coupled to the total pmf. This may be achieved via a mechanism which transports one or more protons along with the neutral solute. In an analogous way anion uptake can be energized via a cotransport with two or more protons. In fact, phosphate and sulphate uptake in yeast are believed to be energized via a cotransport mechanism (31). It might be hypothesized that also K^+ uptake in yeast is energized by the proton motive force (31). It has been argued both by Goffeau et al. (32) and Borst-Pauwels (31) that the membrane potential is in fact too low in order to account for the huge accumulation of K^+ into metabolizing yeast cells. However, when the K^+ uptake proceeds via a cotransport with protons, the energy available from the pmf may be sufficient to account for the K^+ accumulation. It is obvious that the membrane potential plays an important role in solute translocation.

Attemps to neasure the membrane potential of yeast cells by microelectrodes have not been very succesfull yet, due to the small size of these cells. In larger cells of the yeast *Endomyces magnusii* the results may be perhaps more promising (34). Another

frequently used method for measuring membrane potentials is to measure the equilibrium distribution of lipophilic cations (35). It is assumed that these cations distribute themselves across the cell membrane according to the membrane potential. Then from the steady-state distribution ratio the membrane potential can be calculated.

Although much studies have dealt with a kinetical description of solute transport, much less attention has been paid to the regulation of the cellular solute content. The most abundant cellular cation is K^+. K^+ probably plays an important role in cellular growth (36), regulation of the osmotic value of the cell (37) and is one of the factors that contributes to the maintenance of the cell pH (31,38). Furthermore various cellular enzymic processes are activated specifically by K^+ (39).

In this study three aspects of ion translocation in the yeast *Saccharomyces cerevisiae* have been investigated. In the first place we have looked for a further characterization of the Rb^+ uptake mechanism by kinetical studies, since there are indications that the Rb^+ uptake is mediated by a apparent three-site translocation mechanism (11-14, Chapter II).

In the second place we studied the energization of cation transport in yeast. Primarily we looked for an appropriate method to measure the membrane potential of S.cerevisiae. It will be shown in Chapter III that the steady-state distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) can be used to calculate relative values of the membrane potential. In Chapter V the effect of inhibitors of the yeast plasma membrane ATPase upon the membrane potential in relation to Rb^+ and Sr^{2+} uptake is discussed. In Chapter VI a possible coupling between the pmf and K^+ fluxes is discussed. In Chapter VII the energization of the Na^+ efflux from Na^+-rich cells is examined.

The third aspect we have considered concerns the regulation of cation transport during Rb^+ uptake, which is dealt with in Chapter IV.

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CHAPTER II

IS THE Rb^+ UPTAKE IN YEAST MEDIATED BY A THREE-SITE TRANSLOCATION MECHANISM?

IS THE Rb^+ UPTAKE IN YEAST MEDIATED BY A THREE-SITE TRANSLOCATION MECHANISM?

Summary

The uptake of Rb^+ at high Rb^+ concentrations has been examined more closely in order to investigate whether three sites are involved simultaneously in Rb^+ transport. The concentration dependence of initial uptake rates obtained via linear regression is described by a quadratic rate equation pointing to the involvement of a two-site transport mechanism and the concentration dependence found on applying non-linear regression is described by a cubic rate equation indicating that an apparent three-site transport mechanism is involved. With linear regression a high intercept with the y-axis in the time course of Rb^+ uptake is obtained. With non-linear regression this intercept is much lower and is of the same magnitude as that calculated from the Rb^+ uptake in non-metabolizing cells. It is concluded that, though tentatively, the Rb^+ uptake in yeast is mediated by an apparent three-site translocation mechanism. Finally the meaning of the third site is discussed.

Introduction

Since the work of Armstrong and Rothstein (1964,1967) it became clear that in the uptake of monovalent cations in the yeast *Saccharomyces cerevisiae* at least two sites were involved. According to these authors the transport system contains a so-called substrate site which is actively involved in ion transport and a so-called modifier site, which has only binding capacity to the various ions present in the medium and which does not participate directly in the translocation of the cations through the cell membrane. Occupation of this site by e.g. protons or other monovalent cations decreases the rate constant of substrate translocation through the membrane whereas at higher substrate concentrations the inhibitor cations at the modifier site are replaced by the substrate cation giving rise to a relief of the inhibition. It is obvious that uptake via

a so-called two-site mechanism will give rise to deviations from Michaelis-Menten kinetics (see Borst-Pauwels (1981) and references therein).

Results obtained by Borst-Pauwels et al. (1981) pointed to the existence of a third site involved in monovalent cation transport. This site, called activation site, has affinity to K^+ and Rb^+ and their affinities for this site are greater than for the substrate site. It was found by Borst-Pauwels et al. (1971) that convex deviations in the Hofstee plot (Hofstee,1952) came to the fore instead of a concave curve as was found by Armstrong and Rothstein (1964). This convex curve corresponds with a sigmoidal relation between the rate of Rb^+ uptake and the Rb^+ concentration.

One might now question whether there are three sites involved in Rb^+ (or K^+) uptake, or that only two sites are involved simultaneously. In the latter case one has to assume that the modifier site discovered by Armstrong and Rothstein (1964, 1967) and the activation site discovered by Borst-Pauwels et al. (1971) are identical. The differences in the form of the Hofstee plot found, should in that case be attributed to differences in the affinities of the ions, both substrate ions and competing ions for the two sites, in the strains of yeast used by the two groups. At this stage of knowledge we cannot distinguish between these two possibilities. Still there are some indications for the simultaneous involvement of three sites in Rb^+ uptake. From the data of Borst-Pauwels et al. (1971)it could not be completely excluded that at high Rb^+ concentrations also concave deviations in the Hofstee plot came to the fore. On the other hand, Armstrong and Rothstein (1964,1967) found no convex deviations in the Hofstee plot. This, however, can be readily explained by the fact that at the concentrations they applied the activation site was already saturated.

We have now examined the Rb^+ uptake at high Rb^+ concentrations more closely in order to find out if the three sites are involved simultaneously in Rb^+ translocation. In fact, it has been shown by Derks and Borst-Pauwels (1979) that the kinetics

of Cs^+ uptake at low medium pH were described by a three-site translocation mechanism, giving rise to a so-called cubic rate equation. At low Cs^+ concentrations a convex curve was found in the Hofstee plot, whereas at high Cs^+ concentrations a concave curve was found.

Materials and Methods

Yeast cells (2%,w/v), strain Delft II were exhausted of endogeneous substrate by aeration overnight at room temperature in distilled water. Then the cells were washed twice with distilled water and transferred into 45 mM Tris buffer, adjusted to pH 4.0 or 4.5 with succinic acid. Metabolizing cells were obtained by preincubating the yeast cells (2.2%, w/v) with glucose (3%, w/v) under anaerobic conditions, i.e. by bubbling N_2 through the suspension, for one hour at 25°C. The uptake of Rb^+ was determined as described by Theuvenet and Borst-Pauwels (1976a). Initial uptake rates were determined by linear regression or by means of a first-order curve fitting program (Universitair Rekencentrum, Nijmegen).

Results

The uptake of Rb^+, at two extreme Rb^+ concentrations at pH 4.0 is shown in fig. (1). The uptake of 1 mM Rb^+ (fig. 1A) was a typical example for the uptake of Rb^+ at concentrations lower than 25 mM. The uptake was within the incubation time completely linear (correlation coefficient r = 0.998) and the uptake rate could be simply determined by linear regression analysis. The uptake of 100 mM Rb^+ (fig. 1B) was a typical example for the Rb^+ uptake at concentrations higher than 25 mM. Still linear regression analysis was applicable (r = 0.95) but then an intercept with the y-axis was found and this intercept amounted to 5.6 $mmol.kg^{-1}$ (dry yeast). An intercept with the y-axis may be ascribed to the fraction of $^{86}Rb^+$ which was not removable by the washing procedure applied. On the other hand, the question may be raised, whether such an intercept may be real or not. It must be remembered that on increasing the Rb^+

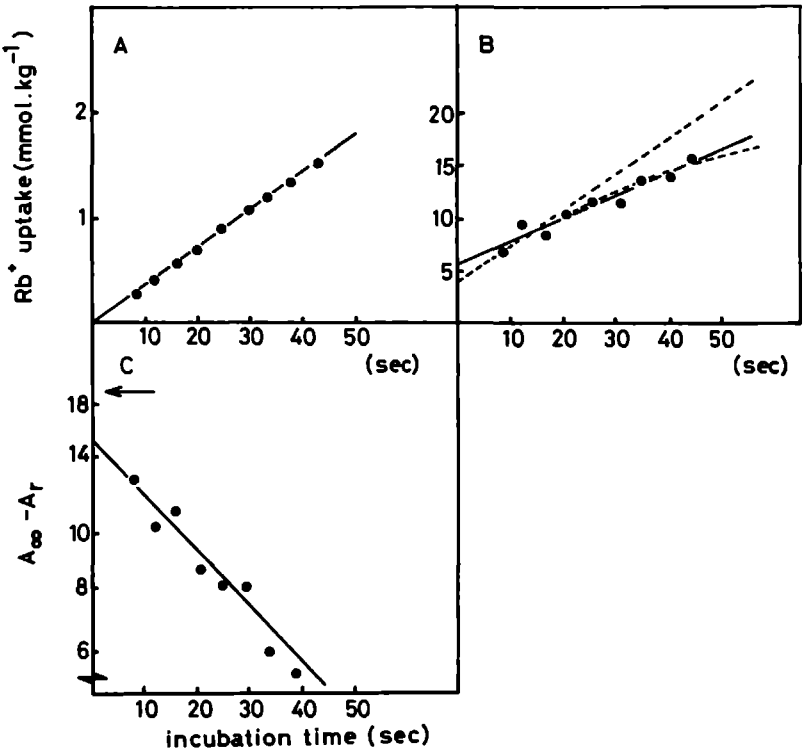

Fig. (1): Uptake curves of Rb^+ at low (1A, 1 mM) and high (1B, 100 mM) concentrations at pH 4.0.
(1C): rearrangement of the data of fig. (1B) according to first-order curve fitting with a free value of the intercept. A_r is the radioactivity in the cells. A_∞ is the radioactivity in the steady-state. A_∞ was estimated by trial and error, in such way that a straight line was obtained. The arrow indicates the value of A_∞.

concentration in the medium the specific activity of $^{86}Rb^+$ in the medium is decreased. Therefore the binding of $^{86}Rb^+$ to the cells at high Rb^+ concentrations should at least be equal or lower than the binding of $^{86}Rb^+$ at low Rb^+ concentrations. This means that the intercept, found at high Rb^+ concentrations, on applying linear regression may be too high. Consequently the calculated initial rate of uptake may be underestimated. We therefore also analysed the uptake data according to a non-

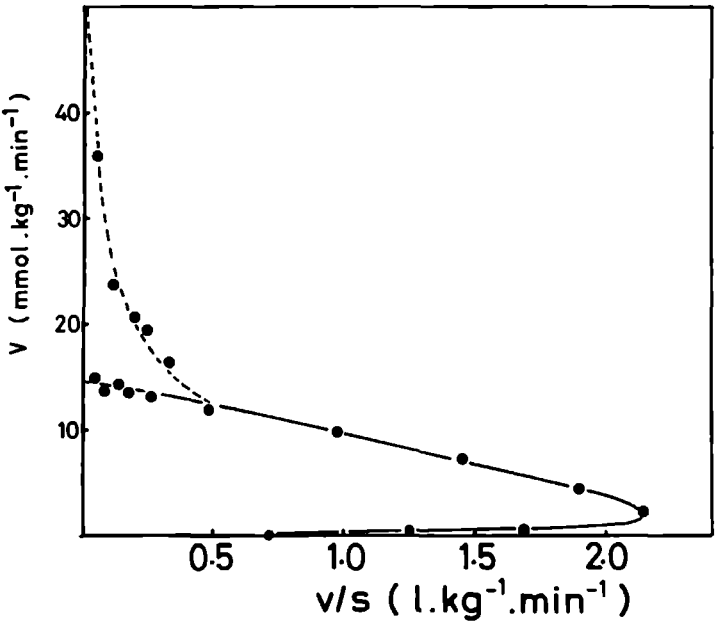

Fig. (2): Hofstee plot of Rb^+ uptake at pH 4.0. The full-drawn line refers to uptake rates computed via linear regression. This curve is calculated according to a quadratic rate equation. The dotted line refers to uptake rates computed via first-order curve fitting with a free value of the intercept. This curve is calculated according to a cubic rate equation.
Mean of duplicate experiments.

linear regression method, using a first order kinetic curve fitting program with a free intercept. The initial influx rate could be calculated from the slope of the tangent to the curve. Fig. (1C) shows the data of fig. (1B) according to a first-order fit. The intercept, calculated according to this method was reduced to 3.5 mmol.kg^{-1} (dry yeast). The initial uptake rates, calculated with both methods differed a factor 1.6.

Fig. (2) shows the concentration dependence of the Rb^+ influx-rate, calculated with both methods, according a Hofstee plot. We have analysed these data according a quadratic and cubic rate equation, respectively, see also Eqn. (1) and (2)

$$v = \frac{AS + BS^2}{C + DS + S^2} \quad (1) \qquad v = \frac{A^1S + B^1S^2 + C^1S^3}{D^1 + E^1S + F^1S^2 + S^3} \quad (2)$$

When a transport model with independent binding sites is involved, the dissociation constants of the Rb^+ carrier complexes can be calculated according to $C = K_1K_2$, $D = K_1 + K_2$, $D^1 = K_1{}^1K_2{}^1K_3{}^1$, $E^1 = K_1{}^1K_2{}^1 + K_1{}^1K_3{}^1 + K_2{}^1K_3{}^1$ and $F^1 = K_1{}^1 + K_2{}^1 + K_3{}^1$. The K-values with proper index are the dissociation constants of the Rb^+-carrier site complexes. Table I shows the values of the kinetical constants. It is seen that $K_3{}^1$, the dissociation constant for the third site was of the order of magnitude of 0.5 M at pH 4.0. Similar experiments were carried out at pH 4.5. Also in that case the initial rates of uptake at high Rb^+ concentrations depended strongly upon the method of evaluation, see fig. (3). The kinetical constants calculated via both the quadratic and the cubic rate equation are given in Table I, too.

Table I

Kinetical coefficients of Rb^+ uptake by yeast.

	Quadratic (1)			Cubic (2)	
pH	4.0	4.5		4.0	4.5
A	1.1	0.8	A^1	472	46
B	14.5	14.8	B^1	6737	755
C	1.5	0.2	C^1	50	40
D	4.8	1.5	D^1	667	15
			E^1	2312	98
K_1	0.34	0.15	F^1	462	53
K_2	4.5	1.36			
			$K_1{}^1$	0.29	0.15
			$K_2{}^1$	5.0	1.9
			$K_3{}^1$	457	51

Data of fig. (2) and (3) respectively. (1) and (2) refer to equation (1) and (2) respectively. A and B^1 are expressed in mmol $^2.1^{-1}.kg^{-1}$, B and C^1 in mmol.$kg^{-1}.min^{-1}$, C and E^1 in mM^2, D,F^1,K,K^1 in mM, A^1 in mmol^3. $^{1-2}.kg^{-1}$ and D^1 in mM^3.

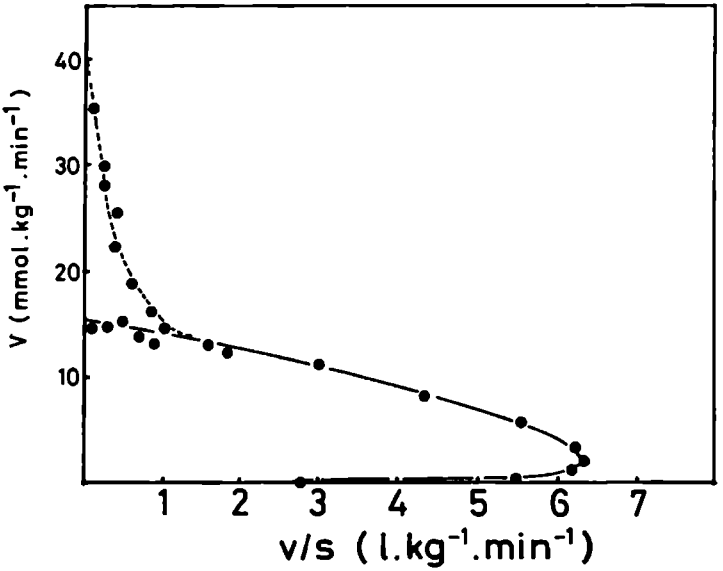

Fig. (3): Hofstee plot of Rb^+ uptake at pH 4.5. See legend to fig. (2). Mean of duplicate experiments.

The dissociation constant for the third site, calculated by means of the cubic rate equation amounted to approximately 50 mM. Which of the two methods applied gave the most reliable value for the initial uptake rates depends upon the actual value of the Rb^+ uptake at zero time. In order to get information about this value we conducted some experiments in which we compared the uptake of Rb^+ in metabolizing and non-metabolizing cells. The latter are cells that were pretreated in the same way as metabolic cells, except that glucose was omitted. Fig. (4) shows the uptake of 100 mM Rb^+ in both type of cells at pH 4.5. It is seen that the uptake in non-metabolizing cells was low. From this uptake the intercept with the y-axis was determined with linear regression analysis and amounted to 4.1 mmol.kg^{-1} (dry yeast). This intercept was fixed in the calculation of the initial uptake rate in the metabolizing cells, using first order kinetics, see fig. (5). When the initial uptake rate was

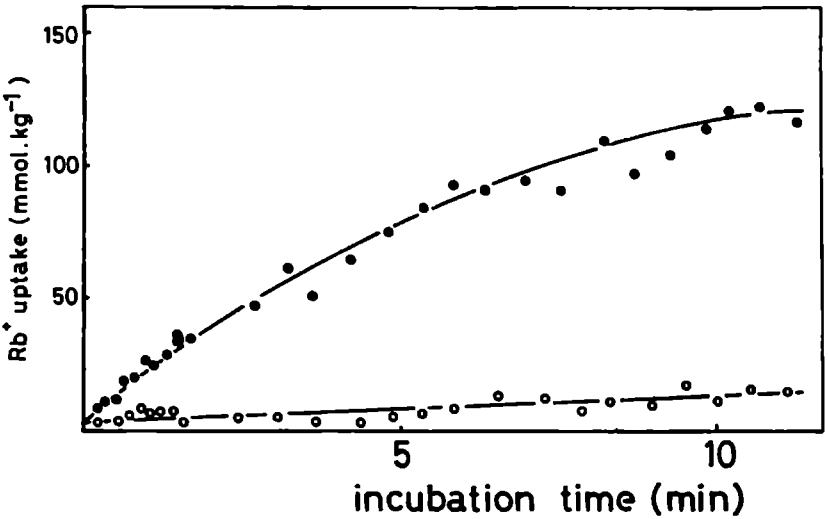

Fig. (4): Uptake of 100 mM Rb^+ at pH 4.5. o uptake in non-metabolizing cells (intercept 4.1 mmol.kg^{-1}) ● uptake in metabolizing cells. Mean of triplicate experiments.

calculated according to first-order curve fitting using a free value of the intercept, this intercept amounted to 2.9 mmol. kg^{-1} (dry yeast). When the initial uptake rate was calculated according to linear regression the value of the intercept was calculated to be 11.6 mmol.kg^{-1} (dry yeast).

Discussion

In this study we have examined if there are indications for the simultaneous involvement of more than two sites in Rb^+ translocation.

Figs. (2) and (3) show that at low medium pH concave deviations in the Rb^+ Hofstee plot can be found at high Rb^+ concentrations. This, however, may be only apparent. Depending on the method used to evaluate the initial uptake rate, either a concave deviation or no deviation can be found. The value of the intercept with the y-axis plays a crucial role in the evaluation of the initial uptake rates at high Rb^+ concentrat-

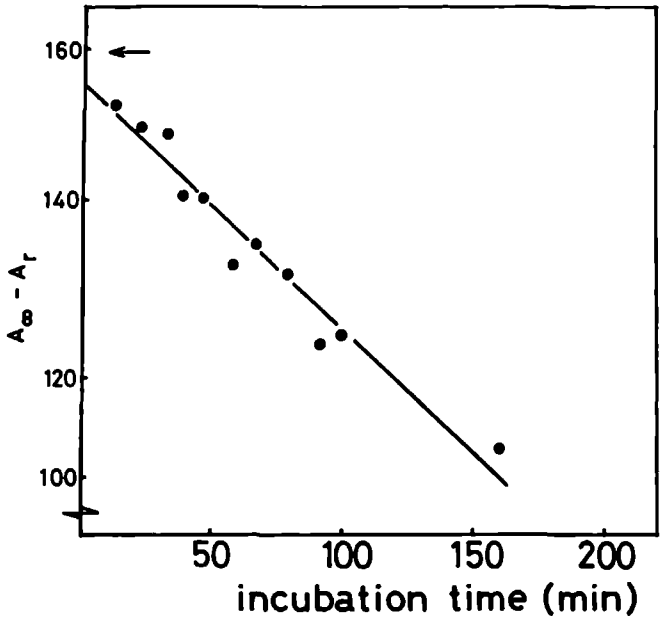

Fig. (5): Semi-log plot of the data of fig. (4). See legend to fig. (1C). The computations were carried out with a fixed intercept on the y-axis (4.1 mmol.kg^{-1}).

ions, see fig. (1). This value remains uncertain, unless we are able to take yeast samples far more rapidly after the addition of the radioactive Rb^+ to the yeast suspension. Unfortunately, an automatic sampling device developed for this purpose was not available in due time. In order to overcome this technical limitation we have tried to get informed about the value of the intercept by studying the Rb^+ uptake in non-metabolizing cells as in these cells the Rb^+ uptake is greatly reduced. Fig. (4) shows that the intercept calculated from the Rb^+ uptake in non-metabolizing cells is much lower than the intercept calculated from the Rb^+ uptake in metabolizing cells via linear regression. When the intercept is calculated from the Rb^+ uptake in metabolizing cells using first-order curve fitting with a free value of the intercept with the y-axis, this intercept is somewhat lower than the intercept calculated from the Rb^+ uptake in non-

metabolizing cells,but the differences are only small.Our results indicate therefore that the concentration dependence of the Rb^+ uptake based upon initial uptake rates calculated via non-linear regression gives a more realistic view than that based upon linear regression.

We conclude therefore, though tentatively, that the Rb^+ uptake in yeast is mediated by a three-site translocation mechanism. In fact, Derks and Borst-Pauwels (1979) found also a concave deviation for Cs^+ uptake at high Cs^+ concentrations, whereas at low Cs^+ concentrations convex deviations from the Hofstee plot were found, pointing to the simultaneous involvement of three sites in Cs^+ uptake.

Finally we will remark that a possible third site may be only apparent. High substrate cation concentrations may reduce the surface potential by screening the negative charges on the membrane. This results in a lower effective concentration of the substrate cation near the membrane. Theuvenet and Borst-Pauwels (1976b) have shown that due to screening concave deviations may appear in the Hofstee plot. This notion is supported by the fact that the "third" site according to Armstrong and Rothstein (1964) is not directly involved in the translocation of ions through the membrane.

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CHAPTER III

SOME CHARACTERISTICS OF TETRAPHENYLPHOSPHONIUM UPTAKE INTO *SACCHAROMYCES CEREVISIAE*

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SOME CHARACTERISTICS OF TETRAPHENYLPHOSPHONIUM UPTAKE INTO *SACCHAROMYCES CEREVISIAE*

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The characteristics of the uptake of the lipophilic cation tetraphenylphosphonium (TPP^+) into *Saccharomyces cerevisiae* have been investigated in order to establish whether this compound can be used to monitor the membrane potential of this organism. Unlike dibenzyldimethylammonium, TPP^+ is not translocated via the thiamine transport system, nor via another inducible translocation mechanism. On changing the experimental conditions the equilibrium potential of TPP^+ varies according to expected changes of the membrane potential. TPP^+ accumulation is higher in metabolizing cells than in non-metabolizing cells. In addition, decreasing the medium pH, addition of the proton conductor 2,4-dinitrophenol and addition of K^+ all cause an apparent depolarization, whereas Ca^{2+} apparently hyperpolarizes the cell membrane. It is concluded that TPP^+, if applied at low concentrations, can be used to measure the membrane potential of *S. cerevisiae*.

Introduction

It has not been possible as yet to measure membrane potentials in yeast cells directly with microelectrodes. A possible exception is *Endomyces magnusii*. Vacata et al. [1] have punctured the rather large cells of this yeast with microelectrodes, but they did not give experimental evidence that the potential difference that they measure in this way is really the membrane potential. Therefore a number of indirect methods have been applied to obtain information about the membrane potential in yeast. Thus changes in the membrane potential occurring during galactoside transport in *Saccharomyces fragilis* have been monitored with a fluorescent carbocyanine dye [2] but this method yields only qualitative information about the membrane potential. The distribution of the lipophilic cations dibenzyldimethylammonium (DDA^+) and triphenylmethylphosphonium ($TPMP^+$) can neither be used to obtain quantitative information about the membrane potential in *Saccharomyces cerevisiae* since these compounds are translocated across the cell membrane of this yeast via the inducible thiamine transport system [3].

Another lipophilic cation, which is also frequently used for measuring the membrane potential in microorganisms is tetraphenylphosphonium (TPP^+) [4–8]. Though Serrano et al. [9] reported that attempts to use TPP^+ as a probe for the membrane potential in *S. cerevisiae* failed under a wide range of experimental conditions, Vacata et al. [1] found that TPP^+ accumulates into *S. cerevisiae* at high medium pH, but they stated that this result should be interpreted with caution since it was not excluded that the cation might be trans-

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Abbreviations used: TPP^+, tetraphenylphosphonium; DDA^+, dibenzyldimethylammonium; $TPMP^+$, triphenylmethylphosphonium.

located via the inducible thiamine transport system of this yeast like DDA^+ and $TPMP^+$ TPP has also been shown to accumulate into other yeast species, namely *Endomyces magnusii* [1], *Saccharomyces bayanus* [1] and *Rhodotorula gracilis* [1,10,11], but not in *Candida parapsilosis* [12]

We have studied the characteristics of TPP^+ uptake into *S cerevisiae* in order to establish whether or not this cation is accumulated via the thiamine translocator In addition TPP^+ is studied under conditions in which the membrane may be expected to become depolarized or hyperpolarized, in order to investigate whether this compound can be used as a probe for the membrane potential in *S cerevisiae*

Materials and Methods

The yeast *S cerevisiae* strain Delft II, was aerated overnight in distilled water at room temperature in order to exhaust endogeneous substrates Before the experiments started the yeast was washed twice by centrifuging and resuspending the pellet in distilled water The cells were finally resuspended in buffer (45 mM Tris, brought to the desired pH with succinic acid) Unless otherwise stated, the yeast was pre-incubated during 5 min in the presence of glucose (5% w/v)

Uptake of radioactively labelled TPP^+ was measured at 25°C as described in Ref 3 The initial concentration of added labelled TPP^+ was 0 18 nM

Comparison of DDA^+ and TPP^+ uptake was done in experiments by means of electrodes that are sensitive to these cations Experimental set-up and construction of the electrodes was as described in Ref 13 It appeared that for the TPP^+-sensitive electrodes the same membranes could be used as for the DDA^+-electrodes The electrode responses yielded straight lines down to a concentration of 0 5 μM and had slopes of 50–62 mV per decade Before each uptake experiment, the electrode was calibrated in 20 ml buffer with 5% glucose, by successive additions of TPP^+ or DDA^+ to a final concentration of 13 6 μM Uptake was started by addition of 5 ml 25% yeast in buffer with glucose to this solution containing the lipophilic cations

The pH of the suspension was measured regularly At pH 4 5 and in the presence of glucose the pH did not fall more than 0 1 unit, at higher pH the decrease was somewhat greater The values of the medium pH reported correspond to the values measured at the end of the incubation period In the absence of added glucose the pH remained constant

^{14}C-labelled tetraphenylphosphonium bromide was purchased from the Radiochemical Centre, Amersham, England ^{14}C-labelled dibenzyldimethylammonium chloride was synthesized according to the method described in Ref 14 The yeast was kindly provided by Gist-Brocades, Delft

Results

The lipophilic cation TPP^+ is accumulated into the yeast, *S cerevisiae* strain Delft II, at a very low rate In Fig 1 the uptake of the cation is shown under different conditions Both metabolizing cells and starved non-metabolizing cells accumulate TPP^+, but the uptake rate and the final accumulation level are higher for the metabolizing cells The medium pH has a pronounced effect On decreasing the external pH, the uptake becomes smaller and slower, both in metabolizing and in starved cells On addition of 2,4-dinitrophenol (100 μM) at low pH, the TPP^+ which was taken up into the cells, is partially released (Fig 1b) Also KCl (50 mM) causes an efflux of previously accumulated TPP^+ (Figs 1a and 1b) On the other hand $CaCl_2$ (1 mM), added together with TPP^+, increases both the uptake rate and the final cellular TPP^+ concentration (Fig 2)

If TPP^+ partitions passively between the medium and the cell water according to its electrochemical gradient, the membrane potential equals the equilibrium potential of TPP^+ (E_{TPP}) provided that the activity coefficients of TPP^+ in medium and cell water do not differ

$$E_{TPP} = -59 \log[C_{cell}/C_{medium}] \text{ (mV)} \qquad (1)$$

C_{cell} and C_{medium} are the concentrations of TPP^+ in the cell water and the medium, respectively

Fig 3 shows that E_{TPP} increases with the medium pH and that the time needed to reach the half-maximal level ($t_{1/2}$) decreases with the medium pH The equilibrium levels of the cellular

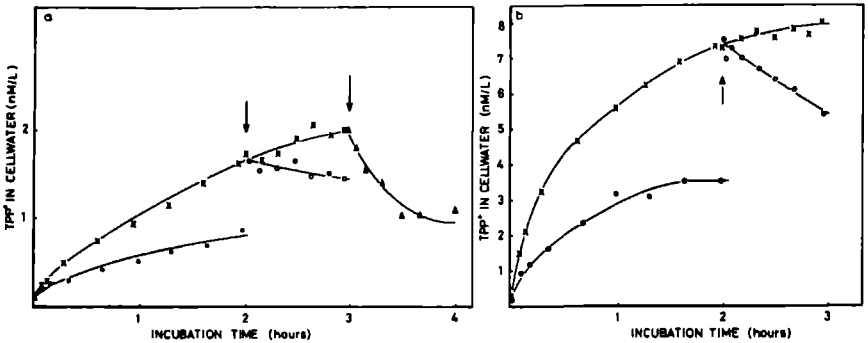


Fig 1 TPP^+ uptake by resting cells and metabolizing cells and the effect of 2,4-dinitrophenol (100 μM) and KCl (50 mM) Metabolizing cells are starved cells which are pre-incubated (during 5 min) and incubated in the presence of 5% glucose (w/v) Resting cells are cells which are pre-incubated and incubated in the absence of glucose Means of duplicate experiments (a) Uptake at pH 4 5 ×, metabolizing cells ●, resting cells ○, accumulation after the addition of KCl at $t = 2$ h At $t = 3$ h dinitrophenol is added to the metabolizing cells (b) Uptake at pH 7 0 ×, metabolizing cells, ●, resting cells, ○, accumulation after the addition of KCl The final pH was 6 2 for metabolizing cells and 7 0 for resting cells

concentrations and $t_{1/2}$ were estimated by means of first order fits.

Addition of iodoacetic acid at a 3 mM concentration, at which glycolysis is inhibited almost completely [15], to cells metabolizing on glucose under anaerobic conditions causes a release of previously accumulated TPP^+ (Fig. 4). Also, blocking the metabolism of cells respiring on ethanol, by simultaneous addition of antimycine and deoxyglucose [16] and bubbling nitrogen through the suspension instead of air results in an efflux of accumulated TPP^+ (data not shown).

Since the lipophilic cation DDA^+ is taken up by *S. cerevisiae* via the transport system for thia-

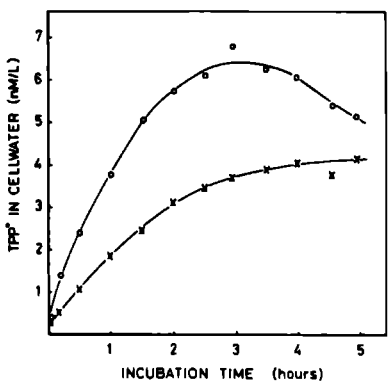


Fig 2 Effect of $CaCl_2$ (1 mM) on TPP^+ uptake at pH 5 6 by metabolizing cells, which were pre incubated during 5 min and incubated in the presence of 5% glucose (w/v) × without added $CaCl_2$ ○ with $CaCl_2$ added at $t = 0$ Mean of duplicate experiments

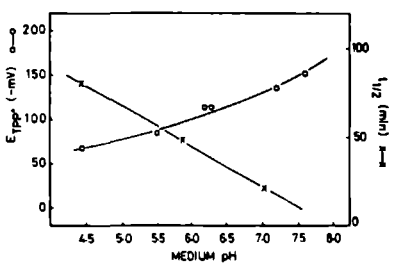
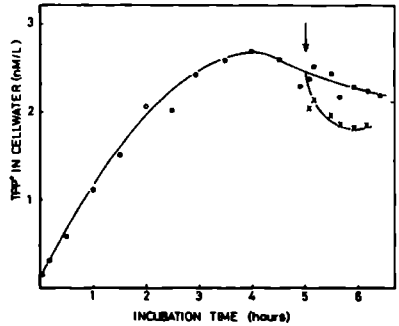


Fig 3 Effect of the medium pH on the equilibrium potential of TPP^+ (E_{TPP^+}) (see Eqn 1) and the half-maximal time constant ($t_{1/2}$) Metabolic cells (see legend to Fig 1) Mean of triplicate experiments



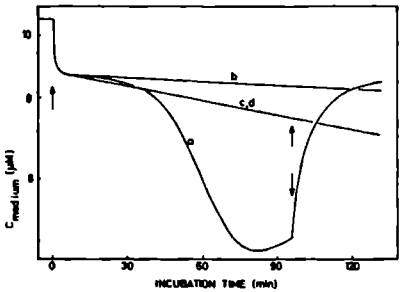


Fig 4 Effect of 3 mM iodoacetic acid on TPP^+ accumulation by metabolizing cells at pH 4 5 Pre-incubation (5 min) and incubation were carried out in the presence of 5% glucose (w/v) In order to prevent exhaustion of the glucose each 1 5 h extra glucose was added to a final concentration of 2% (w/v) O control × TPP^+ accumulation after the addition of the iodoacetic acid at $t = 5$ h Mean of duplicate experiments

Fig. 5 Effect of thiamine disulfide on DDA^+ and TPP^+ uptake as measured with ion-selective electrodes Pre-incubation during 5 min and incubation with 5% glucose Yeast cells were added to the buffer at $t = 0$ DDA^+ uptake with thiamine disulfide added at (a) $t = 95$ min and (b) $t = 0$ and TPP^+ uptake with thiamine disulfide added at (c) $t = 0$ and (d) $t = 95$ min

mine [3], we have investigated whether the same is true for TPP^+ The transport system, by which thiamine and DDA^+ are translocated is inducible [3] This means that the uptake rate of these compounds increases according as the cells are pre-incubated in the presence of a suitable substrate during a longer period The uptake rate of TPP^+, however, was independent of the pre-incubation period no differences were measured after pre-incubation of the cells during 5, 60 and 120 min (data not shown)

In Fig. 5 the difference between the inducible uptake of DDA^+ and the non-inducible uptake of TPP^+ into cells, that were pre-incubated during 5 min in the presence of glucose, is clearly demonstrated A large increase of the uptake rate of DDA^+ is observed after approx 40 min whereas such a phenomenon is not observed during TPP^+ uptake Thiamine disulfide (10 μM), a potent inhibitor of thiamine transport [17], had no effect on TPP^+ uptake, when added together with TPP^+, nor upon the level of TPP^+ accumulated into the cells after 120 min DDA^+ uptake, however, was decreased greatly and previously accumulated DDA^+ was released again on adding this inhibitor In addition non-radioactive DDA^+ had even

TABLE I

CONCENTRATION DEPENDENCE OF TPP^+ UPTAKE BY METABOLIZING CELLS AT PH 4 5

Pre-incubation during 5 min and incubation were carried out in the presence of 5% glucose C_{TPP} is the initial concentration of TPP^+ in the medium v is the initial uptake rate $t_{1/2}$ is the half maximal response time and E_{TPP} is the equilibrium potential of TPP^+

C_{TPP} (μM)	v (μmol h^{-1})	v/C_{TPP} (h^{-1})	E_{TPP} (mV)	$t_{1/2}$ (min)
0 18 10^{-3}	6 8 10^{-4}	3 79	−66	110
300	609	2 03	−49	124
1000	1554	1 55	−42	120

at a concentration of 1 mM at which concentration the thiamine carrier is already saturated to an appreciable extent [3] no effect upon the initial rate of uptake of radioactive TPP^+ (data not shown)

Finally we have examined how TPP^+ uptake depends on the concentration of TPP^+ As shown in Table 1 E_{TPP} becomes smaller as the TPP^+ concentration (C_{TPP}) increases The initial uptake rate (v) increases less than proportionally with C_{TPP} since the quotient v/C_{TPP} becomes smaller on increasing C_{TPP}

Discussion

The yeast strain used by us accumulates TPP at a very low rate The time needed to reach the half-maximal level becomes smaller with increasing medium pH This decrease is possibly a consequence of changes in the membrane potential and the surface potential In an other strain of *S cerevisiae* we found a more rapid uptake (data not shown) with half-maximal response times of the order of magnitude as found in *R gracilis* [10] This also applies to the strain of *S cerevisiae* used in Ref 1 Thus the different accumulation rates found between the two yeast species seem to be due not so much to interspecific differences as to differences in membrane properties such as surface charges which may exist between strains of the same species

The decrease in the final equilibrium distribution of TPP^+ on lowering the medium pH can of course not be ascribed to differences in surface potential but is probably due to depolarisation of the yeast plasma membrane by protons

We have found no indications that TPP^+ like DDA^+ and TPMP is translocated via the thiamine transport system The inhibition of thiamine transport by TPP^+ found previously [3] might be attributed to binding of TPP to the thiamine transport site without being translocated via this system As prolonged pre incubation of the yeast cells in the presence of glucose does not affect the initial uptake rate of the cation TPP is not translocated by an inducible transport system

The extent of TPP^+ accumulation in the cells seems to reflect changes in the membrane potential under different conditions in a correct way Both addition of the proton conductor 2,4-dinitrophenol and impairment of metabolism lead to an apparent depolarization In addition, TPP^+ uptake into starved cells is lower than in metabolizing cells This is in accordance with current views on the operation of an electrogenic proton pump or possibly electrogenic H^+/K^+ pump which is dependent on cell metabolism, see literature referred to in Ref 18 Moreover, in the related ascomycete, *Neurospora crassa*, in which the membrane potential can be measured directly with microelectrodes depolarization has been measured also on blocking metabolism [19] Also the effects of medium pH or added cations are in accordance with the expectations On decreasing the pH or on adding K^+ to the medium the membrane potential, estimated from the TPP^+ accumulation, becomes less negative, whereas calcium ions hyperpolarize the membrane These effects of pH, K^+ and Ca^{2+} are also observed in *N crassa* [20] In Ref 1 a decrease of TPP^+ accumulation in *S cerevisiae* was also observed after the addition of a proton conductor or K^+ Comparable changes in TPP^+ accumulation as we have found for *S cerevisiae* have been reported for *R gracilis* [10] An exception is the effect of Ca^{2+}, in Ref 10 it was found that 100 mM Ca^{2+} depolarizes the membrane of *R gracilis* whereas we measure with 1 mM Ca^{2+} a hyperpolarization in *S cerevisiae* This however, may be due to the difference in the concentrations applied

The value for the membrane potential that we calculate for the equilibrium distribution of TPP^+ at pH 7 5 is higher than found in Ref 1, where a value of -75 mV is reported for *S cerevisiae* at the same pH Probably the composition of the medium (300 mM Tris in Ref 1 instead of 45 mM Tris used by us) the metabolic state of the cells and the TPP^+ concentration applied (160 μM TPP^+ in Ref 1 instead of 0 18 nM TPP^+ used by us) are responsible for this difference

The non-linear relation between the initial uptake rate and the concentration of TPP^+ is probably due to the diffusion potential of the cation which will exist as long as TPP^+ has not yet equilibrated across the membrane The higher the concentration of TPP^+ added to the medium the greater the initial depolarization will be This depolarization will reduce the uptake rate of TPP^+

The decrease of the equilibrium potential of TPP^+ (E_{TPP}) found at high TPP^+ concentrations may point to a depolarization, for example caused by the toxic properties of this compound [21] These effects, however, are negligibly small if the cation is applied at low concentrations (0.18 nM in the uptake experiments with radioactively labelled TPP^+) Hence, it appears that TPP^+ if applied at sufficiently low concentrations, can be used as a quantitative probe for the membrane potential in *S cerevisiae*.

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CHAPTER IV

THE REGULATION OF THE Rb^+ INFLUX RATE DURING Rb^+ ACCUMULATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

THE REGULATION OF THE Rb^+ INFLUX RATE DURING Rb^+ ACCUMULATION IN THE YEAST *SACCHAROMYCES CEREVISIAE*

Summary

In the yeast *Saccharomyces cerevisiae*, the net uptake of 10 mM Rb^+ reaches a steady-state in approximately 20 min. This is due to a decrease in the Rb^+ influx rate. The increase in the Rb^+ efflux rate is small and is of only minor importance in the regulation of the net Rb^+ uptake. Thus a so-called "constant-pump and leak" system is not operating. It has been excluded that the Rb^+ influx rate is due to a decrease in the rate of glycolysis, changes in cellular osmotic value, depolarisation of the cell membrane or to a decrease in the p.m.f. During Rb^+ uptake the cell pH increases and the cell ATP content decreases. These changes are, however, not large enough to account quantitatively for the decrease in the Rb^+ influx rate. However, the decrease in the Rb^+ influx rate can be explained by assuming that the Rb^+ carrier is an allosteric carrier of which the conformational state depends upon the concentration of K^+ and Rb^+ inside the cell. According to this hypothesis, during Rb^+ uptake the state of the carrier changes, which results in a decreased influx rate. In conclusion it may be hypothesized that the Rb^+ uptake is regulated by some feedback mechanism of which the exact nature should still be clarified.

Introduction

K^+ is the most abundant cellular cation of yeast cells cultivated in a low Na^+ medium (1-3). K^+ probably plays an important role in the regulation of yeast cell growth (4). A great percentage of the osmotic value of the yeast cell is determined by the cellular K^+ concentration (5). In addition K^+ influx is one of the factors contributing to the maintenance of the physiological cell pH. During metabolism, K^+ uptake prevents acidification of the cell cytoplasm by an exchange of

abbreviations DDA^+ = dimethyldibenzylammonium
TPP^+ = tetraphenylphosphonium

cellular protons with K^+ from the medium (6,7). Furthermore various cellular enzymatic processes, as the protein synthesis, are activated specifically by K^+ (8).

Despite these important physiological functions, the regulation of the cellular K^+ content is not very intensively studied. Most of the studies with yeast have dealt with the ion transport system itselve, rather than the factors which determine the K^+ content of the cells.

In *Saccharomyces cerevisiae* with propanol-2 as substrate Ryan and Ryan (9) found under aerobic conditions that the rate of net K^+ accumulation decreases during K^+ uptake due to an increased K^+ efflux, which compensates finally the influx. Rothstein and Bruce (10) found the same in *S.cerevisiae*, with glucose/O_2 as substrate at low K^+ concentrations in the medium. However, at relatively high K^+ concentrations the decrease in net K^+ accumulation rate was mainly due to a decrease in the rate of K^+ influx, whereas the K^+ efflux rate remained unaltered during K^+ accumulation.

We have now studied the Rb^+ influx rate at relatively high Rb^+ concentrations (10 mM) at which the monovalent cation transport mechanism is almost saturated (7). We have examined whether the Rb^+ influx rate is also decreased during Rb^+ accumulation and how the influx rate is regulated.

One of the factors which may regulate the influx rate is the cell pH (9,11). An increase in the cell pH leads to a decrease in the rate of either K^+ or Rb^+ uptake. This decrease is ascribed to a decrease in the maximal rate of uptake. The affinity of Rb^+ to the carrier is unaltered (11). Another factor which may regulate the Rb^+ influx rate is the energy state of the cells. We therefore examined changes in cell ATP content and the membrane potential and also the proton motive force during Rb^+ uptake.

In plant root cells the K^+ influx rate is also decreased during K^+ uptake. It is hypothesized that the K^+ influx rate is regulated by the cellular K^+ content via a conformational change of the K^+ translocator and an increase in cell K^+ will lead to

a decreased turnover of this translocator (12-16).

Also in some bacteria the rate of K^+ influx is decreased during K^+ uptake (17,18). This decrease however, is ascribed to an increase in the cellular osmotic value (18).

Materials and Methods

Yeast cells (2%, w/v), *S.cerevisiae*, strain Delft II, were exhausted of endogeneous substrate by aeration overnight at room temperature in distilled water. Then the cells were washed twice with distilled water and transferred into 45 mM Tris buffer, adjusted with succinic acid to pH 4.5. Metabolism was started by adding glucose (3%, w/v) to a yeast cell suspension (2%, w/v) under anaerobic conditions i.e. by bubling N_2 through the suspension. For precise experimental conditions see legends to the figures.

The uptakes of Rb^+, DDA^+, Sr^{2+} and TPP^+ were determined as described in Ref. (19-21).

The ATP content of the cells was determined according to Ref. (22). The bioluminescence was measured using the luciferin method on a Lumac M 1030 celltester (Lumac N.V./S.A.; Belgium).

The cell pH was determined as described in Ref. (23).

The K^+ content of the medium was determined by centrifuging the cell suspension (5 min , 3000 rpm) and measuring the K^+ concentration of the supernatant. For the determination of the K^+ content of the cells 1 ml cell suspension was filtered off on preweighted filters (Schleicher and Schüll; 602 H). The filters were washed subsequently with 2 ml ice-cold 50 mM $MgCl_2$ solution, 2 ml ice-cold distilled water, dried with acetone and weighted again. The filters were extracted with 2 ml 35% (v/v) nitric acid for one hour and after suitable dilution the K^+ content was measured. K^+ concentrations were measured by flamephotometry. When Rb^+ was present appropriate corrections were made for the contribution of Rb^+ to the light emission.

The glycolytic rate was determined by measuring the amount of CO_2 liberated under anaerobic conditions with glucose as substrate using a CO_2 electrode (Radiometer; Denmark).

All experiments were carried out at 25°C. Labelled compounds were purchased from the Radiochemical Centre, Amersham; England. All other chemicals were purchased from commercial sources.

Results

Fig. (1) shows that the net uptake rate of 10 mM Rb^+ gradually decreased during Rb^+ uptake. The intracellular Rb^+ concentration reached a steady-state level in approximately 20 min. The main factor contributing to the decrease in the net Rb^+ uptake rate appeared to be a decrease in the Rb^+ influx rate. A second factor which contributed to the regulation of the Rb^+ net uptake was the Rb^+ efflux rate. The Rb^+ efflux rate increased during Rb^+ uptake (see inset fig. (1)). The increase in the efflux rate, however, was of minor importance for the regulation of net Rb^+ uptake during the first 10 min of Rb^+ uptake. After prolonged incubation, the relative contribution of the Rb^+ efflux rate to the regulation of net Rb^+ uptake became of the same order of magnitude as the Rb^+ influx rate, due to a greatly decreased Rb^+ influx rate.

We have examined whether the decrease in Rb^+ influx rate could be attributed to a decreased rate of glycolysis. However, incubation of metabolising yeast cells with 10 mM Rb^+ did not lead to an inhibition of the glycolysis. On the contrary, a slight stimulation of the rate of glycolysis was found (data not shown).

Fig. (2) shows that the cell pH increased during Rb^+ uptake, which is in accordance with Ref. (9,11). The rate of Rb^+ influx could be markedly increased by decreasing the cell pH (see Table I). Addition of 4 mM Tris-butyrate or 1 mM Tris-phosphate led to a transient increase in the rate of Rb^+ influx and to a decrease in the cell pH. Within 40 min the rate of Rb^+ influx was decreased again to the same value as found in the control experiments, i.e. in the absence of butyrate or phosphate. The acidification of the cells, caused by butyrate or phosphate, however, appeared to be not transient.

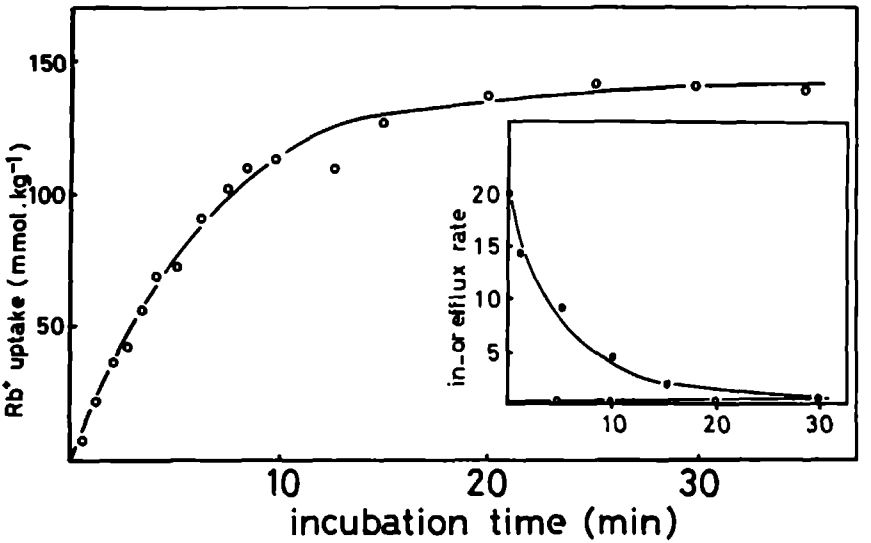

Fig. (1): Uptake of 10 mM Rb^+ by metabolizing yeast cells. Starved cells [2%; w/v] were preincubated for one hour in the presence of glucose [3%; w/v] and N_2, before adding 10 mM Rb^+, labelled with c.f. $^{86}Rb^+$.
Inset fig. (1): Rb^+ influx and efflux rates determined at appropriate times during the uptake of 10 mM non-radioactive Rb^+ into yeast cells. (●) influx-rate : starved cells [2%, w/v] were preincubated for 45 min with glucose [3%; w/v] and N_2. The cells were concentrated to 20% (w/v) and preincubated further for 10 min with glucose and N_2. Then 10 mM Rb^+ was added. At the times indicated the cells were diluted to 2% (w/v) into media containing 10 mM Rb^+ (final concentration), labelled with c.f. $^{86}Rb^+$, whereafter the initial rate of Rb^+ uptake was determined. (o) efflux rate : the efflux rate was calculated from $V_{net} = V_{influx} - V_{efflux}$, where V_{net} is the net uptake rate (slopes to the curve of fig. (1)) V_{influx} and V_{efflux} are the influx and efflux rate respectively. Means of duplicate experiments.

Fig. (2) further shows that during Rb^+ uptake the cellular ATP content decreased, whereas no such a decrease was found in the absence of added Rb^+. Similarly the rate of Rb^+ influx decreased only when the cells were incubated in the presence of 10 mM Rb^+, but not when the cells were incubated in the absence of Rb^+. It is apparent from this figure that the relative decrease in the Rb^+ influx rate was much greater than the relative decrease in cell ATP content.

The cellular ATP content could also be varied by adding

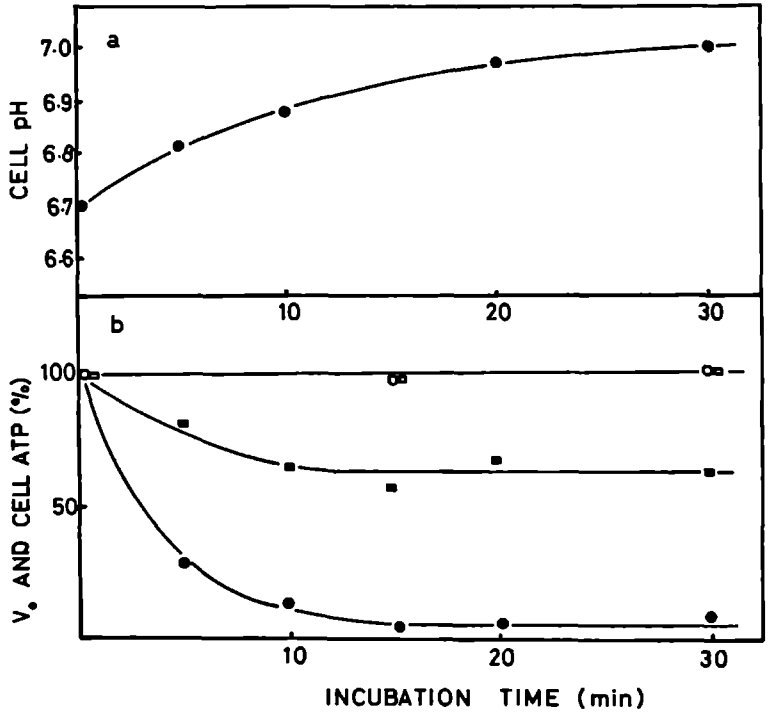

Fig. (2): a. Change in cell pH during uptake of 10 mM Rb^+ (see also subcript to fig. (1)). The yeast concentration was 2% w/v. Means of triplicate experiments.

b. Change in cellular ATP content and Rb^+ influx rate during Rb^+ accumulation, both expressed as % of the control.

(o,●) influx rate : see subscript to inset of fig. (1). In the control experiment no addition of Rb^+ to the 20% cells (w/v) was made.

(o) control, with 100% = 23.67 mmol.kg^{-1} (dry yeast).min^{-1}. (●) during Rb^+ accumulation.

(□,■) cellular ATP content : see subscript to inset of fig. (1). At the times indicated the cells were diluted to 2% (w/v) into media containing 10 mM Rb^+. Immediately after the dilution the ATP content was determined. In the control experiment no addition of Rb^+ to the 20% cells (w/v) was made.

(□) control, with 100% = 0.70 mmol ATP.kg^{-1} (dry yeast) (■) during Rb^+ accumulation.

Mean of duplicate experiments.

Table I

Effect of phosphate and butyrate on the initial rate of Rb^+ uptake and cell pH at pH 4.5.

time	control		1 mM phosphate		4 mM butyrate	
min.	v	pH_i	v	pH_i	v	pH_i
0	13.11	6.72	-	-	-	-
60	0.40	7.00	0.92	6.73	1.52	6.52
80	0.38	6.99	0.48	6.72	0.48	6.50
100	0.41	6.97	0.40	6.73	0.42	6.52

Yeast cells (20%;w/v) were incubated with 10 mM Rb^+. See legend to fig. (1). At t = 50 min the cells were diluted to 2% (w/v) into media containing 10 mM Rb^+ and 3% glucose. Butyric acid and phosphoric acid were brought to pH 4.5 with Tris and were added respectively at t = 54 and t = 59.8 min, to the final concentrations indicated. At t = 60, 80 and 100 min labelled c.f. $^{86}Rb^+$ was added, whereafter the 10 mM Rb^+ influx rate (v, $mmol.kg^{-1}$ (dry yeast).min^{-1}) and cell pH (pH_i) were determined. Mean of duplicate experiments.

different amounts of iodoacetic acid, an inhibitor of glycolysis (24), to the yeast cell suspension. Fig. (3) shows that on increasing the iodoacetic acid concentration, the initial rate of Rb^+ uptake as well as the cellular ATP content decreased. In the presence of iodoacetic acid, the relative decrease in Rb^+ influx rate was approximately equal to the relative decrease in cellular ATP content.

The membrane potential of the yeast cell may be one of the driving forces for monovalent cation transport ((7) and references therein). We have examined therefore whether the membrane potential decreased during Rb^+ uptake. The apparent membrane potential can be calculated from the equilibrium distribution of the lipophilic cation TPP^+, on applying the Nernst equation (25). The value of the membrane potential, calculated in this way, may be overestimated due to probe binding to cellular constituents (26). This matter will be dealt with in Chapter VI. No corrections for possible TPP^+ binding were made. The uptake of TPP^+ is very slow at pH 4.5 and measurements of TPP^+

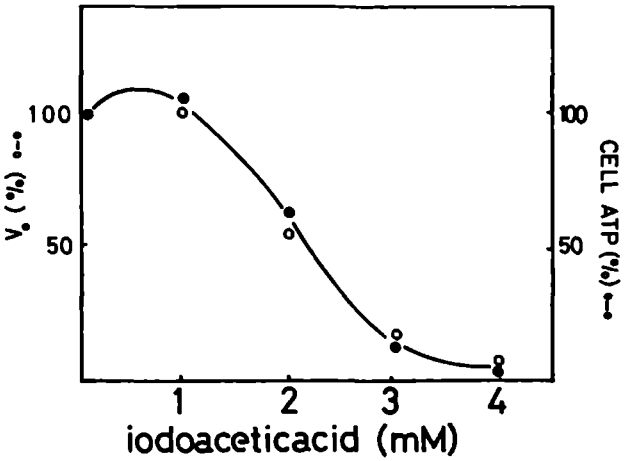

Fig. (3): Effect of iodoacetic acid on the influx rate of 10 mM Rb^+ and cell ATP content. Cells (2%;w/v) were preincubated for one hour with glucose (3%;w/v) and N_2. The iodoacetic acid was added at time 59.5 min. The Rb^+ influx rate and ATP content were determined at t = 60 min.
(o) Rb^+ influx rate (V_o); 100% = 13.27 mmol $Rb^+.kg^{-1}$ (dry yeast).min^{-1}.
(•) cell ATP; 100% = 0.72 mmol $ATP.kg^{-1}$ (dry yeast).
Mean of duplicate experiments.

equilibrium distribution ratios cannot be obtained within the interval during which the rate of Rb^+ influx decreased. Therefore we examined whether the initial rate of TPP^+ uptake could be used as a relative measure of the membrane potential. Fig. (4) shows that a correlation existed between the initial rate of TPP^+ uptake and the membrane potential, calculated from the steady-state distribution of TPP^+ under various experimental conditions (see legend to fig. (4)). Therefore we can take the initial rate of TPP^+ uptake as a measure of the relative membrane potential, provided that possible adsorption of TPP^+ to yeast cell constituents is not altered appreciably during uptake of Rb^+. Table II shows that addition of 10 mM Rb^+ led to an almost immediate depolarisation of the yeast cell membrane of approximately 10 mV. This depolarisation remained stable over at least 30 min. This means that no change in membrane potential occurred under conditions that the Rb^+ influx rate decreased considerably.

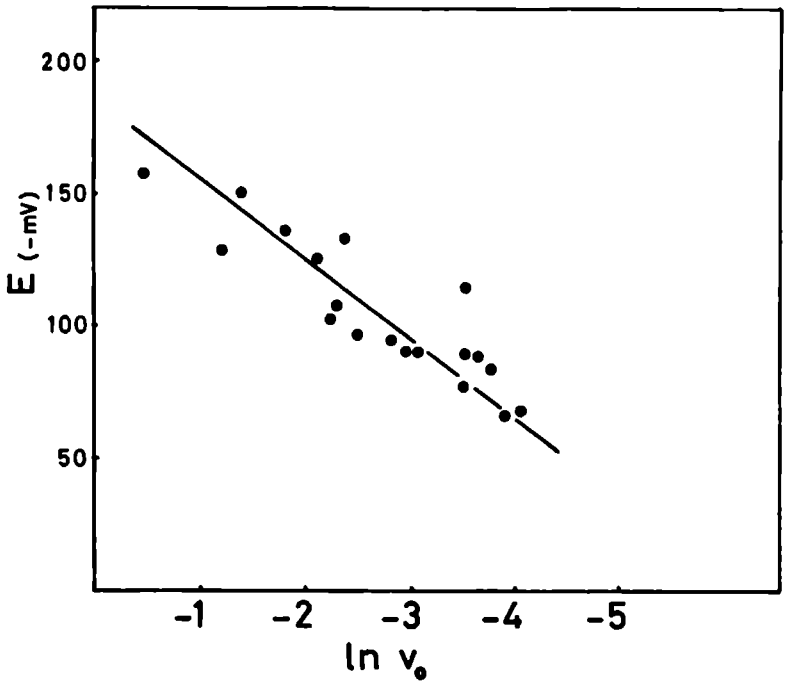

Fig. (4): Correlation between the initial rate of TPP^+ uptake and the equilibrium membrane potential, calculated from the steady-state distribution of TPP^+ between cells and medium. The cells (2%;w/v) were preincubated with glucose (3%;w/v) and N_2 for 5 min, where after the TPP^+ uptake was determined. The membrane potential was varied by variations in the medium pH, in the concentrations of added $CaCl_2$ (0-10 mM) or KCl (0-100 mM) and Trisphosphate (0-1 mM). Mean of duplicate experiments.

We have also examined whether the decrease in the rate of Rb^+ influx, was accompanied by a decrease in the rate of influx of other compounds, which are translocated via energy dependent transport processes. Table III shows that the initial uptake rate of DDA^+, which cation, in the strain of yeast used, is translocated via the thiamine carrier (21), and the initial rate of Sr^{2+} uptake were not decreased by preincubating the cells for 30 min with 10 mM Rb^+ as compared with a preincubation of only 1 min.

Table II

Effect of 10 mM Rb^+ on the apparent membrane potential of yeast cells at pH 4.5.

	E (mV)	
time (min)	control	+ 10 mM Rb^+
1	-63±1	-52±1
30	-62±1	-53±1

Yeast cells were preincubated as described in the legend to fig. (1). At t = 0 10 mM Rb^+ and 5% glucose or in the control only 5% glucose were added to the cell suspension (20%;w/v). At the times indicated the cells were diluted to 2% (w/v) into a medium containing 10 mM Rb^+ and labelled TPP^+, where after the initial rate of TPP^+ uptake was determined. From the initial rate of TPP^+ uptake we calculated the apparent membrane potential by means of fig. (4). The data are not corrected for probe binding to cell components.
Mean of duplicate experiments.

Table III

Initial rate of DDA^+ and Sr^{2+} uptake during Rb^+ accumulation at pH 4.5.

	1 µM DDA^+		1 µM Sr^{2+}	
	t = 0	t = 30	t = 0	t = 30
control	100%	105%	100%	115%
10 mM Rb^+	76%	80%	100%	103%

Yeast cells were preincubated as described in the legend to Table II. At the times indicated (min) the cells were diluted to 2% (w/v) into media containing 10 mM Rb^+ and labelled DDA^+ or Sr^{2+} whereafter the initial uptake rates of these compounds were determined.
(expressed as % of the control value)
Means of duplicate experiments.

We also have examined whether the increase in the osmotic value of the cells had affected the Rb^+ influx rate. When, during Rb^+ uptake, the cellular Rb^+ content increases, the osmotic value of the cells will increase, too. By this, the

cells will swell though in a restricted way because of the presence of the cell wall. If the swelling of the cells is the cause of the decrease in the rate of Rb^+ influx, an increase in the osmotic value of the medium should lead to a restoration of the Rb^+ uptake capacity of the cells. That this is true in some bacterial cells is shown in Ref. (18), where an osmotic upshock led to an increased rate of K^+ influx. Table IV shows, however, that in yeast cells neither addition of 300 mM sucrose nor concentrations of sorbitol upto 800 mM significantly increased the initial rate of Rb^+ influx.

In plant root cells, the decrease in monovalent cation influx rate is ascribed to inhibition of a hypothetical allosteric cation carrier, occurring on increasing the cellular cation content (12-16). The decrease in cation influx rate is related to the cellular cation content by an equation of the form (1), which is only an approximation of the rate equation for an allosteric process (27,28).

$$v_{in} = \frac{V_{max} \cdot s^n}{K_m + s^n} \qquad (1)$$

where v_{in} = cation influx rate

V_{max} = maximal cation influx rate, expected when $s = 0$ and $n < 0$

K_m = Michaelis-Menten constant for cation uptake

s = intracellular cation content

$|n|$ = Hill parameter, representing the minimal number of allosteric sites.

Equation (1) can be linearized according the Hill equation:

$$\log \frac{v_{in}}{V_{max} - v_{in}} = \log K + n \log s \qquad (2)$$

We have examined whether our experimental data can also be described by eqn. (2). For the intracellular cation content S we took the sum of the Rb^+ and K^+ content. This may be justified,

Table IV

Effect of sorbitol and sucrose on the initial rate of Rb^+ uptake in Rb^+ loaded yeast cells at pH 4.5.

	Initial rate of Rb^+ uptake (mmol.kg^{-1} (dry yeast).min^{-1})			
time min.	control	sorbitol 300 mM	sucrose 300 mM	sorbitol 800 mM
0	13.81±0.33	12.11±0.64	13.75±0.53	13.56±1.09
1	8.17±0.57	8.98±0.51	8.09±0.43	n.d.
5	3.81±0.73	3.85±0.67	3.65±0.97	n.d.
10	2.28±0.10	2.55±0.81	2.17±0.42	n.d.
15	1.28±0.26	1.31±0.67	1.65±0.40	1.38±0.89

Yeast cells (20%;w/v) were incubated with 10 mM Rb^+. See legend to fig. (1). At the times indicated the cells were diluted to 2% (w/v) into media containing 10 mM Rb^+, labelled with $^{86}Rb^+$, sorbitol or sucrose, whereafter the initial rates of Rb^+ uptake were determined. Mean of duplicate experiments. n.d. = not determined.

since Rb^+ uptake in yeast cells is only slightly affected by replacing the greater part of the cell K^+ by Rb^+ (29). We also conducted some experiments, wherein the uptake of c.f. $^{86}Rb^+$ in the presence of either 10 mM non-radioactive Rb^+ or 10 mM non-radioactive K^+ was determined. Under these conditions the same steady-state distribution ratios for $^{86}Rb^+$ between cells and medium were obtained (data not shown). This also indicates that in yeast cells, Rb^+ and K^+ behave very similar.

The K^+ content of yeast cells was approximately 300 mmoles kg^{-1} (dry yeast) and did not change much within 30 min during 10 mM Rb^+ uptake. In fact a slight loss of cell K^+ was observed, but this amounted to less than 5% of the total cell K^+ (data not shown). We also examined whether loading of the cells with 10 mM K^+ affected the influx rate of c.f. $^{86}Rb^+$ into metabolizing cells. Fig. (5) shows both sets of data plotted according eqn. (2). The value of V_{max} was obtained by an itterative fitting procedure. From the linear parts of the curves, the Hill para-

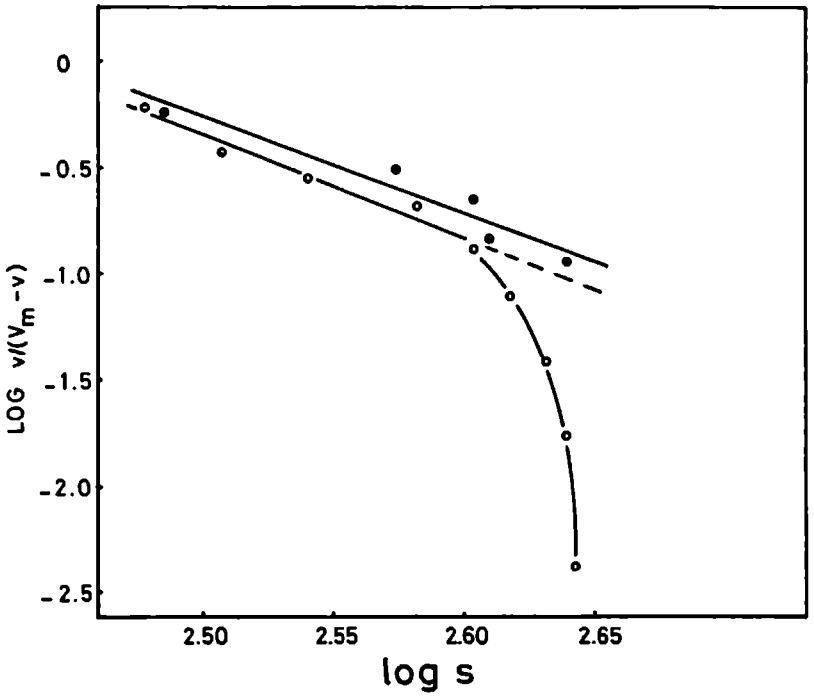

Fig. (5): Dependence of the Rb^+ influx rate on the cellular cation content. Metabolizing yeast cells (20%;w/v) (see also subscript to inset of fig. (1)) were incubated with 10 mM non-radioactive Rb^+ or K^+. At appropriate times during the incubation the cells were diluted into media containing 10 mM Rb^+ (final concentration), labelled with c.f. $^{86}Rb^+$ (o) or 10 mM K^+ (final concentration, labelled with c.f. $^{86}Rb^+$ (•), whereafter the initial rates of $^{86}Rb^+$ uptake and cell cation content were determined. With Rb^+ incubation $s = Rb^+_{cell} + K^+_{cell}$ (mmol.kg^{-1} dry yeast). With K^+ incubation $s = K^+_{cell}$ (mmol.kg^{-1} dry yeast). Data are plotted according eqn. (2), where n is the slope of the linear part of the curve.
Mean of duplicate experiments.

meters were obtained and these agreed very closely for Rb^+ uptake in cells preincubated with either Rb^+ or K^+ (-4.9 and -4.5 respectively).

Discussion

As shown in fig. (1), the net Rb^+ uptake from 10 mM Rb^+ stops when a steady-state level of approximately 140 mmoles.kg^{-1} (dry yeast) is reached. The Rb^+ concentration used by us is relatively high, so that the carrier is almost saturated and the Rb^+ uptake rate is only slightly lower than the maximal uptake rate (30). Under the conditions applied the net accumulation of Rb^+ appears to be primarily regulated via the Rb^+ influx, whereas the Rb^+ efflux only contributes markedly to the total net Rb^+ flux after prolonged incubation with Rb^+. Similar results are found for K^+ uptake (at relatively high K^+ concentrations) in yeast cells, with glucose/O_2 as substrate, where the net K^+ flux is predominantly regulated by the rate of K^+ influx (10). Apparently, with glucose as substrate, a so called "constant pump and leak system" is not operating.

A factor which contributes to the regulation of the net monovalent cation uptake is the cell pH. An increase in cell pH leads to a decrease in the maximal rate of Rb^+ influx (11). Therefore part of the decrease in the Rb^+ influx rate may be ascribed to the increase in cell pH, which occurs during Rb^+ uptake (fig. (2)). The increase in cell pH, however, cannot account quantitatively for the decrease in the Rb^+ influx rate. Table I shows that on restoring the cell pH or even acidifying the cells more than the control by preincubating the cells with phosphate or butyrate only leads to a slight increase in Rb^+ influx rate. In addition the Rb^+ uptake rate decreases again, whereas the cell pH remains constant.

A factor which may also be involved in the regulation of Rb^+ influx during Rb^+ uptake is the energy transduction to the Rb^+ transport system. The way in which monovalent cation transport in yeast is energized is still unknown. It has been suggested that monovalent cation transport is driven by the membrane potential (7 and references therein), but this is rather unlikely since the K^+ accumulation ratio is much greater than the value of the membrane potential may ever account for (7 and chapter VI). Therefore two other possible way of energiz-

ation have been considered. Either the proton motive force (pmf) may be the driving force for monovalent cation uptake (7 and chapter VI) or the carrier is directly energized by ATP. In the latter case the cation carrier may be identical to the plasma-membrane ATPase of the yeast (32).

It is seen in Table II that on addition of 10 mM Rb^+ the membrane depolarises approximately 10 mV, whereafter the membrane potential remains constant. The Rb^+ influx rate, however, is decreased to less than 5% of the control value in the same period that no change occurred in the membrane potential. This means that the membrane potential itself does not contribute to the regulation of the Rb^+ influx rate during accumulation of Rb^+ at high medium concentrations.

The pmf is related to the membrane potential (ΔE) and the difference in pH between cells and medium according to:

$$pmf = \Delta E - 2.3\ RT/F\ \Delta pH\ (mV) \qquad (3)$$

where R, T and F have their usual meaning and where ΔE and ΔpH are defined in the same direction as cell interior minus cell exterior. It is seen in fig. (2) that the cell pH increases during Rb^+ uptake. Thus ΔpH increases, while the membrane potential remains constant. Therefore the pmf increases, too and it may be concluded that the pmf does not contribute to the regulation of the Rb^+ influx rate during Rb^+ uptake.

According to the hypothesis that the monovalent cation influx is directly energized by ATP, the decrease in Rb^+ influx rate may be due to a decrease in cellular ATP content. Although the influx rate of 10 mM Rb^+ decreases concomitantly with the cellular ATP content (see fig. (2)), the relative decrease in Rb^+ influx rate (more than 95%) is far more greater than the relative decrease in ATP content. On the other hand, when the glycolysis is inhibited by increasing concentrations of iodoacetic acid (see fig. (3)), the relative decrease in the Rb^+ influx rate is almost equal to the relative decrease in cellular ATP content. It can be concluded therefore that the decrease in cell ATP during Rb^+ accumulation is not large enough in order

to account quantitatively for the decrease in Rb^+ influx rate, unless part of the cell ATP is not directly available for energization of the Rb^+ influx during progressive accumulation of Rb^+ into the cells.

The results presented in Table III support the view that the energization of the cells is not altered by preincubating the cells with Rb^+, since the uptakes of Sr^{2+} or DDA^+, which cations are also translocated via energy-dependent processes (7,21) are not affected by preincubating the cells with Rb^+

On considering that neither the change in cell pH nor the change in energization of the yeast cells can account for the decrease in the Rb^+ influx rate during Rb^+ uptake we think, that it is more likely that the turnover rate of the monovalent cation carrier is decreased during Rb^+ accumulation into the cells and that this decrease is due to either a direct or indirect effect of the increased cation content of the cells, rather than to a change in the energy state of the cells.

Also in other organisms it is found that the influx rate of monovalent cations decreases during cation uptake. This is found in bacteria (17,18) as well as in plant roots (12-16). In the latter type of organism the regulation of the cation influx rate is attributed to changes in the conformational state of the carrier with increasing cellular K^+. This is based upon the fact that the rate of K^+ influx is related to the cellular K^+ concentration by an experimental function of the form of Eqn. (2). When we fit our data to Eqn. (2) we obtain Hill parameters of a negative sign, just as found for K^+ uptake in plant roots (12-16), indicating a negative cooperativety in the regulation of the carrier by cell K^+. Such a system may protect the cells from obtaining too high levels of intracellular cation and consequently may protect the cells from swelling too much.

Remarkably, at a cellular cation content of approximately 400 $mmol.kg^{-1}$ (dry yeast), a strong increase in the Hill parameter is observed in the Rb^+ uptake experiments. To explain this phenomenon a more sophisticated model should be developed.

It may be of physiological importance for a cell to maintain a constant intracellular cation content via regulation of

the influx, without affecting the energy state of the cells much, rather than a regulation via deenergization of the cells. This allows the cells to accumulate different kinds of solutes more or less in an independent way. It is also a better way than regulation via a "constant pump and leak" system, since no waste of energy occurs for pumping K^+ inwards against an increased K^+ leak.

It is concluded that the Rb^+ uptake in yeast cells is regulated by some feedback mechanism, although the exact nature of the mechanism is still unknown. Also changes in cell pH and cellular ATP content play a role in the regulation of Rb^+ uptake. This role, however, is probably only of minor importance.

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CHAPTER V

SOME ASPECTS OF THE ACTION OF INHIBITORS OF THE YEAST PLASMA MEMBRANE ATPase UPON THE MEMBRANE POTENTIAL, K^+ MOVEMENTS, CELL pH AND ALSO UPON Rb^+ AND Sr^{2+} UPTAKE

SOME ASPECTS OF THE ACTION OF INHIBITORS OF THE YEAST PLASMA MEMBRANE ATPase UPON THE MEMBRANE POTENTIAL, K^+ MOVEMENTS, CELL pH AND ALSO UPON Rb^+ AND Sr^{2+} UPTAKE

Summary

1. Inhibitors of the yeast plasma membrane induce K^+ efflux. The effects of low concentrations of inhibitors upon K^+ movements point to all-or-none effects, which complicate the interpretation of the results.
2. Concentrations of the inhibitors DCCD, EDAC and D10-9, which clearly induce K^+ efflux do not decrease the Rb^+ uptake. TPTCl, DES and F^- inhibit the Rb^+ uptake. The effect of TPTCl is probably for a great part due to all-or-none damage of the yeast cells. The effect of DES is probably only for a minor part due to all-or-none cell lysis.
3. DCCD and EDAC have no effect upon the membrane potential. TPTCl, F^-, D10-9 and EB depolarise. DES hyperpolarises.
4. Upon addition of the inhibitors no changes in cell pH are detected. This means that changes in the influx rates are only a reflection of changes in the membrane potential, irrespectively whether the energization is due to the pmf or to the membrane potential alone.
5. DCCD, EDAC and TPTCl have no effect upon the Sr^{2+} influx rate and accumulation level. EB, D10-9 and DES increase both. The effect of EB, D10-9 and DES can be explained on assuming a dual effect on the cells. On addition of inhibitor part of the cells is hyperpolarised and the remainder of the cells is depolarised. It is shown that conditions are considerable where the overall membrane potential will decrease and the overall Sr^{2+} accumulation will increase.

abbreviations used
- $I_{0.5}$ = the concentration that gives 50% inhibition
- TPP^+ = tetraphenylphosphonium
- DES = diethylstilbestrol
- EDAC = N-ethyl-N^1-(3-dimethylaminopropyl)-carbodiimide
- DCCD = N,N^1-dicyclohexylcarbodiimide
- D10-9 = antibiotic of unknown structure
- TPTCl = triphenyltinchloride
- EB = ethidiumbromide

Introduction

In 1962 Eddy and Indge (1) predicted the existence of a plasmamembrane ATPase in the yeast *Saccharomyces cerevisiae*. Experimental evidence for the existence of such an ATPase has been obtained, not only for *S.cerevisiae* but also for *Schizosaccharomyces pombe*, *Candida albicans*, *Candida tropicalis* and *Neurospora crassa* (2-8). Still little is known about its structure and biological function. It has been suggested (9-11) that this ATPase expels protons from the cells, thereby generating a membrane potential (ΔE), inside negative and a pH gradient (ΔpH), inside alkaline. Thus the cells can generate an electrochemical potential difference for protons, the so-called proton motive force (pmf). ΔE and ΔpH are related to the pmf according eqn. (1).

$$pmf = \Delta E - 2.3 \frac{RT}{F} \Delta pH \ , \qquad (1)$$

where R, T and F have their usual meaning and where ΔE and ΔpH are defined in the same direction, namely cell interior minus cell exterior.

Two possible roles of the plasmamembrane ATPase in solute transport may be considered. This role may be indirect i.e. that the uptake of solutes is energized by the pmf or components of the pmf or this role may be direct i.e. that the ATPase is a transport ATPase, like the animal Na^+/K^+ ATPase or the gastric H^+/K^+ ATPase (12,13). Goffeau et al. (14) have considered the possibility that the yeast membrane ATPase is directly involved in an electrogenic H^+/K^+ antiport. There is, however, until now no experimental evidence that the yeast plasma membrane ATPase is a transport ATPase.

In this study we examined the effects of inhibitors of the plasma membrane ATPase upon the components of the pmf and on the ATP content of the cells. We further studied the effect of the inhibitors on the Rb^+ and Sr^{2+} uptake. When Rb^+ and Sr^{2+} uptake are directly coupled to ATP hydrolysis mediated by the membrane ATPase, impairment of the ATPase will always lead to inhibition

of the Rb^+ and Sr^{2+} uptake. It has been shown, however, by Nieuwenhuis et al. (15) that divalent cation uptake is not mediated by the membrane ATPase. When both Rb^+ and/or Sr^{2+} uptake are driven by the membrane potential or by the pmf (11,16-21,see also Chapter VI) inhibition of the membrane ATPase will not obligatory lead to an impairment of cation transport. In such a case a correlation would be expected between the membrane potential or the pmf and the uptake rate of the ion involved.

Materials and methods

Yeast cells, *S.cerevisiae*, strain Delft II were exhausted of endogeneous substrate by aeration overnight. Then the cells were washed twice with distilled water and transferred into 45 mM Tris brought to pH 4.5 with succinic acid. Metabolism was started by adding glucose (3%, w/v) to the cell suspension (2%, w/v). Anaerobic conditions were maintained by bubbling nitrogen through the suspension for one hour. The uptake of Rb^+ (applied as chloride salt), using $^{86}Rb^+$ as a tracer, was studied according to Ref. (23). The uptake of Sr^{2+} (applied as chloride salt) using $^{89}Sr^{2+}$ as a tracer was studied according to Ref. (24). The uptake of TPP^+, using c.f. tetra $\left[U - {}^{14}C\right]$ phenylphosphonium bromide (final concentration 0.18 nM) was studied according to Ref. (25).

The yeast plasma membrane ATPase was isolated according to Ref. (26). ATPase activity was determined by measuring orthophosphate liberation from 2 mM Na_2H_2ATP (vanadate-free, Boehringer Mannheim) during 60 min in a 25 mM Tris - 25 mM MES buffer of pH 6.8 containing further 2 mM $MgSO_4$ and 250 mM KCl as described in Ref. (26). The K^+ was added in order to simulate the *in vivo* conditions as much as possible. This also applies to the pH, which is the mean pH of metabolizing cells (27). When the time course of P_i liberation was not linear, the ATPase activity was calculated from the slope of the tangent to the curve at t = 0. The K^+ concentration in the medium was determined after centrifuging 5 ml of the cell suspension (5 min, 3000 rpm). The supernatant was used for K^+ analysis using a

Zeiss flame spectrofotometer. The cell pH was determined according to Ref. (28). The ATP content of the cells was determined according to Ref. (29). The bioluminescence was measured using the luciferin method on a Lumac M1030 celltester (Lumac N.V./S.A., Belgium).

Acetone-treated cells were made by suspending 100 g yeast (wet weight) into 500 ml acetone. The suspension was mixed thoroughly and centrifuged (5 min, 3000 rpm). The pellet was resuspended in acetone and centrifuged. The cells were dried in the air and stored at 4°C until further use. Before use the cells were once washed with distilled water and once with buffer.

All experiments were carried out at 25°C. DES, D10-9 and DCCD were dissolved in ethanol. The final ethanol concentration was less than 1% (v/v). All other chemicals were dissolved in buffer.

Labelled compounds were purchased from the Radiochemical Centre, Amersham England. D10-9 was kindly donated by Gist-Brocades, Delft, The Netherlands. All other chemicals were of analytical grade and purchased from commercial sources.

Results

Inhibition of the yeast plasma membrane ATPase

The following inhibitors of the yeast plasma membrane ATPase were used: DES (11), which also inhibited the plasma membrane of *N.crassa* (30), EDAC (4), D10-9 (18,22,31-34), DCCD (11,31,33-35), TPTCl (36) and NaF (31,33,34). We also used the organic cation EB, which is known to inhibit the K^+ and Rb^+ uptake (37) and to stimulate Ca^{2+} uptake (38,39), just like D10-9 (18). In addition EB inhibits the yeast membrane ATPase as well (P.Peters, unpublished data).

Fig. (1) shows the ATPase activity measured with different amounts of inhibitors. The $I_{0.5}$, i.e. the concentration that gave 50% inhibition, for DES, TPTCl and NaF was 10 µM, 5 µM and 10 mM respectively. D10-9 hardly inhibited the ATPase activity. However, addition of the detergent Triton X-100 (0.1%, w/v) increased the sensitivity of the enzyme for D10-9 greatly

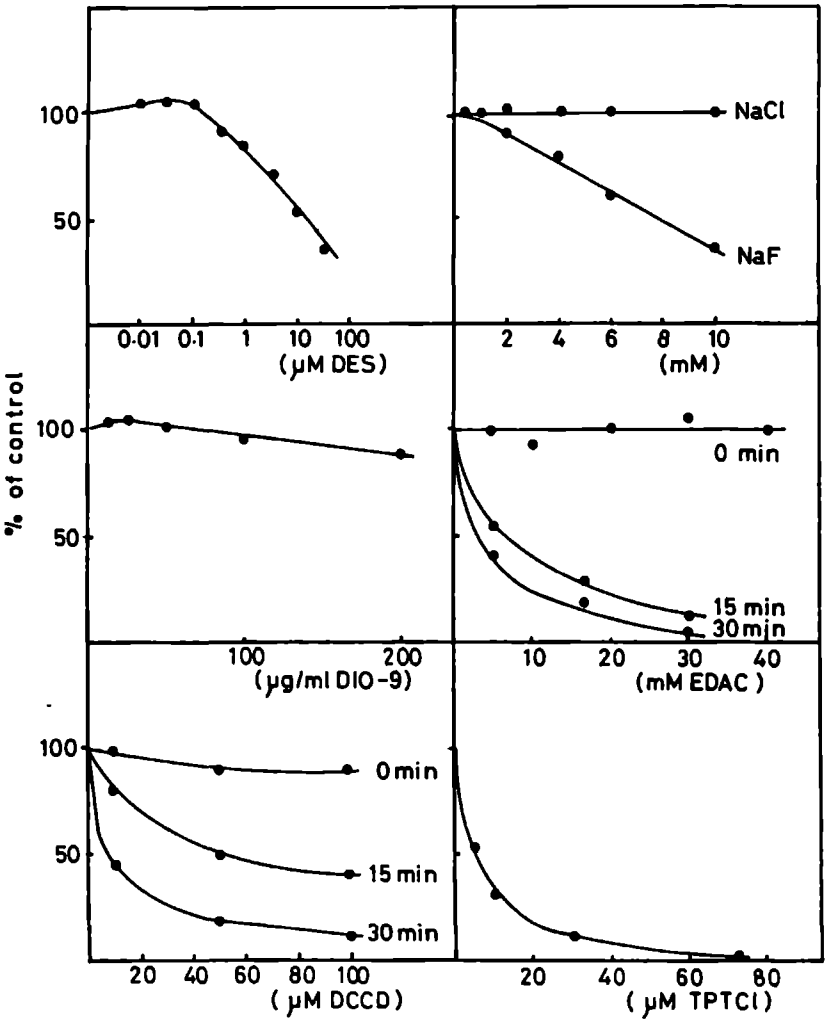

Fig. (1): Effect of the various compounds on the ATPase activity. See materials and methods. Mean of duplicate experiments.

(P. Peters, unpublished data). Also DCCD and EDAC did not inhibit the ATPase when added together with ATP at time zero to the enzyme. However, when preincubating the enzyme for 15 min

these two compounds inhibit the enzyme, too. The $I_{0.5}$ was 44 μM and 5 mM for DCCD and EDAC, respectively. After 30 min of pre-incubation the $I_{0.5}$ was 8 μM and 3.5 mM, respectively.

Effect of inhibitors of the membrane ATPase upon the membrane potential

Fig. (2) shows the uptake of TPP^+, which uptake is driven by the membrane potential (40). The uptake of TPP^+ was almost immediately decreased or stopped on adding D10-9, EB, TPTCl or F^- and was increased by DES. The value of the membrane potential could only be calculated from the steady-state distribution, that means after 1-2 hours after addition of the inhibitor. Recently it was reported that TPP^+ might bind to cellular components (41). This point will be dealt with in Chapter VI. No corrections for possible TPP^+ binding were made. Table I shows the TPP^+ accumulation ratio and the calculated membrane potential. DCCD and EDAC had no effect on the membrane potential.

Effect of inhibitors of the membrane ATPase upon the Sr^{2+} uptake

Foury et al. (22) described that D10-9, an inhibitor of the yeast *S.pombe* membrane ATPase stimulated Ca^{2+} uptake whereas Peña (38) found that EB also stimulated Ca^{2+} uptake in *S.cerevisiae* and Nieuwenhuis (42) found that EB stimulated Sr^{2+} uptake in *S.cerevisiae*. We have examined whether this was also true for the other ATPase inhibitors. We studied their effects on the initial rate of 1 μM Sr^{2+} uptake. It is seen in Table II that EDAC, DCCD and TPTCl had no effect whereas D10-9, EB and DES stimulated the Sr^{2+} uptake. F^- could not be tested because of complex formation between F^- and Sr^{2+}.

Fig. (3) shows the effects of the inhibitors on the Sr^{2+} steady-state accumulation ratio. These steady-state accumulations appeared to be, at least qualitatively, correlated with the initial rates of Sr^{2+} uptake.

Effect of inhibitors of the membrane ATPase upon the Rb^+ uptake

All inhibitors tested were added 5 min prior to the start of the Rb^+ uptake, except DCCD and EDAC that were added at

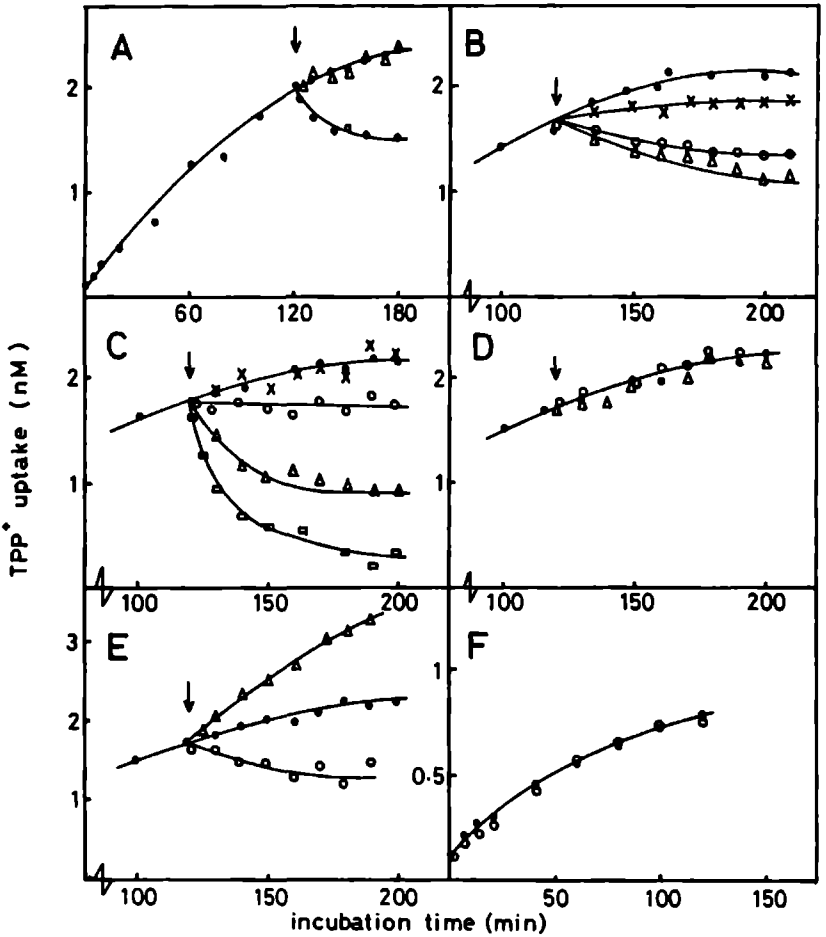

Fig. (2): Effect of inhibitors of the plasmamembrane ATPase upon the TPP^+ distribution at pH 4.5. A-E metabolizing cells, F non-metabolizing cells 2A ● control, Δ 10 µM TPTCl, o 100 µM TPTCl . 2B ● control, x 50 µg/ml D10-9, o 100 µg/ml D10-9, Δ 200 µg/ml D10-9 . 2C ● control, x 0.3 mM EB, o 0.5 mM EB, Δ 1 mM EB, □ 3 mM EB . 2D ● control, Δ 1 mM DCCD, o 30 mM EDAC . 2E ● control, o 10 mM NaF, Δ 100 µM DES. 2F ● control, o 100 µM DES.
Mean of duplicate experiments.

t = -20 min.

Fig. (4) shows that D10-9, EB, EDAC and DCCD did not affect

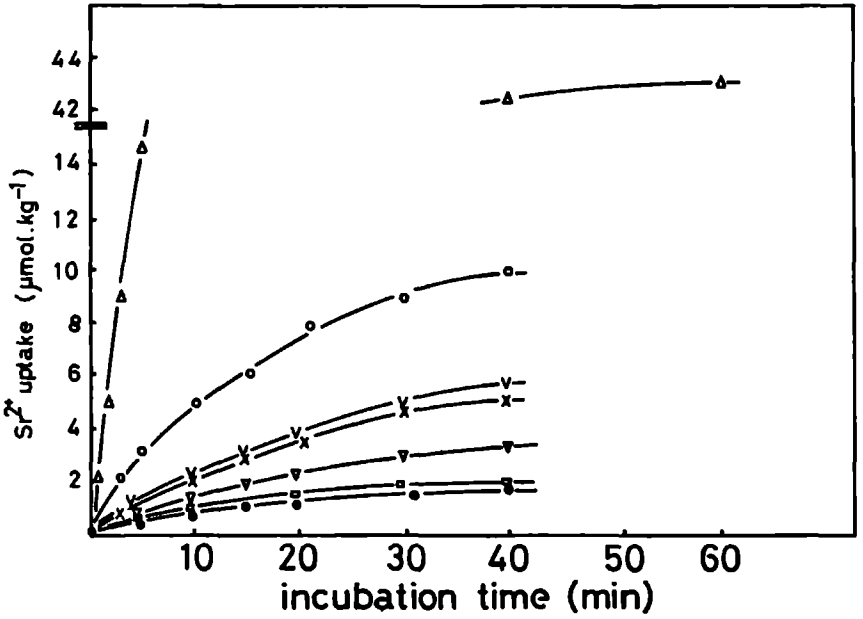

Fig. (3): Enhancement of the Sr^{2+} uptake at pH 4.5. Metabolizing cells. ● control, □ 100 µM TPTCl, ▽ 50 µg/ml D10-9, v 100 µg/ml D10-9, o 200 µg/ml D10-9, Δ 3 mM EB. Mean of duplicate experiments.

the initial rate of 10 mM Rb^+ uptake at pH 4.5. Higher concentrations of DCCD were not tested, due to precipitation of DCCD after diluting the alcoholic solution with the aqueous medium. DES, TPTCl and F^- were potent inhibitors of the initial Rb^+ influx. The $I_{0.5}$ of DES, TPTCl and F^- were 56 µM, 100 µM and 8 mM, respectively.

In Fig. (5) it is shown that 100 µM DES decreased the initial rate of Rb^+ uptake at all Rb^+ concentrations applied to the same relative extent, that means in a non-competitive way. The data are represented according to a Hofstee plot (43). In case of a concentration dependence described by a Michaelis-Menten equation a straight line would be found. The convex deviation found can be ascribed to the operation of a two-site

Table I

Effect of inhibitors of the membrane ATPase upon the membrane potential at pH 4.5. ΔE was calculated from the steady-state TPP^+ distribution.

	TPP^+ in/TPP^+ out	ΔE(-mV)
control	13.7	67
1 mM DCCD	14.2	68
30 mM EDAC	12.6	65
10 μM TPTCl	14.2	68
100 μM TPTCl	9.6	58
10 mM NaCl	13.7	67
10 mM NaF	9.2	57
50 μg/ml D10-9	10.0	59
100 μg/ml D10-9	5.8	45
200 μg/ml D10-9	4.8	40
0.3 mM EB	13.7	67
0.5 mM EB	10.0	59
1 mM EB	5.6	44
3 mM EB	1.5	10
100 μM DES	40.8	95

The membrane potential (ΔE) was calculated from the data of fig. (2).

transport mechanism (20). We further examined the effect of D10-9 and TPTCl on the uptake of 1 mM Rb^+. Fig. (6) shows that D10-9 added at concentrations up to 200 μg/ml did not decrease the initial rate of Rb^+ uptake as was also found at 10 mM Rb^+. In fact, even a slight enhancement of the initial rate of uptake was found, see also Table III. On the other hand TPTCl decreased the initial rate of uptake to about the same relative extent as was found for the uptake at 10 mM Rb^+. At prolonged incubation with inhibitor, however, the uptake of Rb^+ also decreased in the presence of D10-9. This indicates that D10-9 also decreased the steady-state accumulation levels of Rb^+. We have not examined whether this decrease in steady-state level was due to a decrease in the influx rate during incubation with D10-9 or to an enhancement of Rb^+ efflux by D10-9. We have

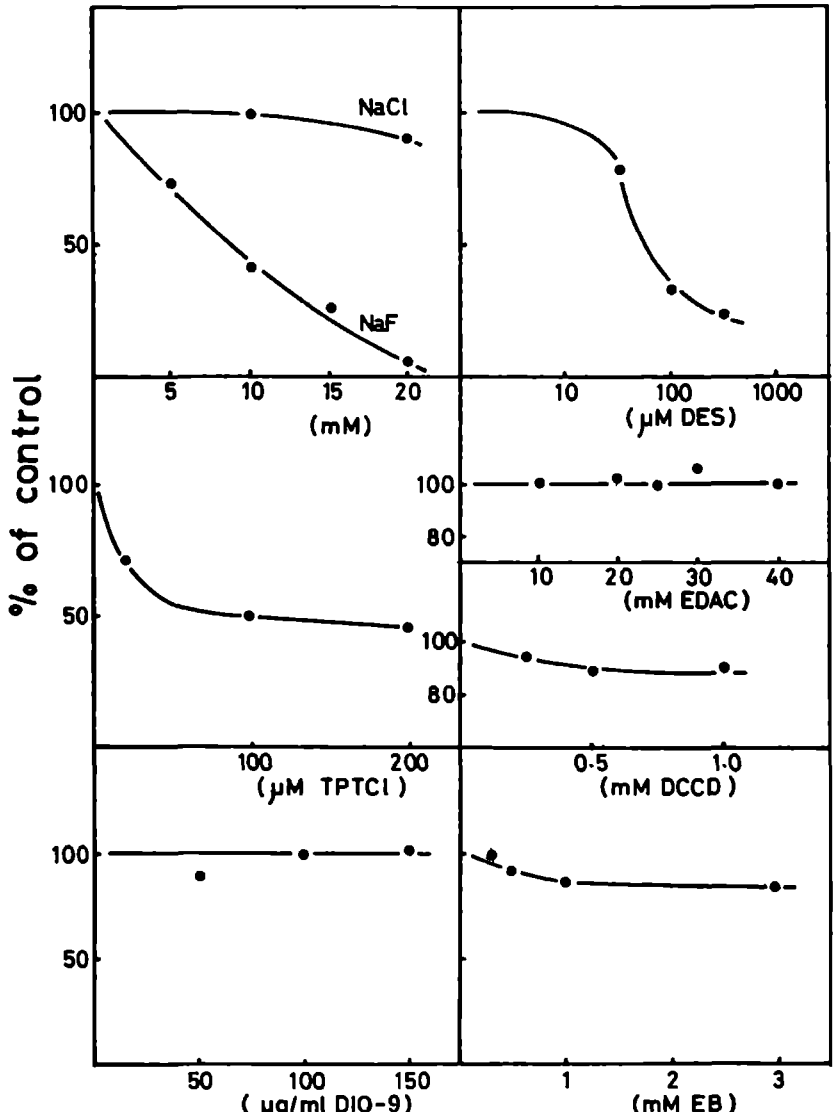

Fig. (4): Decrease of the initial rate of 10 mM Rb^+ uptake at pH 4.5. DCCD and EDAC were added at t = -20 min. The other compounds were added at t = -5 min. Metabolizing cells.
Mean of duplicate experiments.

Table II

Effect of inhibitors of the plasma membrane ATPase upon the initial rate of Sr^{2+} uptake at pH 4.5.

	$[Sr^{2+}]_{\mu M}$	$V_{control}$	$V_{inhibitor}$	$\frac{V_{inhibitor}}{V_{control}}$
100 µM DES	1	$8.50\ 10^{-2}$	$2.15\ 10^{-1}$	2.53
100 µM DES	300	22.5	50.0	2.22
100 µM DES	1000	54.0	128.2	2.37
50 µg/ml D10-9	1	$7.92\ 10^{-2}$	$1.29\ 10^{-1}$	1.63
100 µg/ml D10-9	1	$7.92\ 10^{-2}$	$2.97\ 10^{-1}$	3.75
200 µg/ml D10-9	1	$7.92\ 10^{-2}$	$9.75\ 10^{-1}$	12.31
0.3 mM EB	1	$8.43\ 10^{-2}$	$1.31\ 10^{-1}$	1.56
0.5 mM EB	1	$8.43\ 10^{-2}$	$2.83\ 10^{-1}$	3.36
1 mM EB	1	$8.43\ 10^{-2}$	$5.36\ 10^{-1}$	6.36
3 mM EB	1	$8.43\ 10^{-2}$	2.21	26.20
100 µM TPTCl	1	$8.43\ 10^{-2}$	$8.67\ 10^{-2}$	1.03
1 mM DCCD	1	$8.14\ 10^{-2}$	$8.21\ 10^{-2}$	1.01
30 mM EDAC	1	$8.14\ 10^{-2}$	$8.02\ 10^{-2}$	0.99

Metabolizing cells. All inhibitors were added at t = 0.
Mean of duplicate experiments.
V is the initial influx rate in $\mu mol.kg^{-1}$ (dry yeast).min^{-1}.

compared the Rb^+ accumulation ratios with the TPP^+ accumulation ratios found under comparable conditions. Both values were expressed in percentages of the control, see Table III. For TPTCl these percentages were almost equal, whereas for D10-9 the percentual decrease in TPP^+ accumulation ratio was, at all concentrations examined, greater than the percentual decrease in the Rb^+ accumulation ratios.

Effect of inhibitors of the membrane ATPase upon the ATP content of the cells

All inhibitors were added 10 min prior to the determination of cell ATP. It was found that this content was not affected appreciably by 100 µM DES (105% of the control value), 30 mM EDAC (97%), 1 mM EB (92%), 1 mM DCCD (99%) and 200 µg/ml D10-9

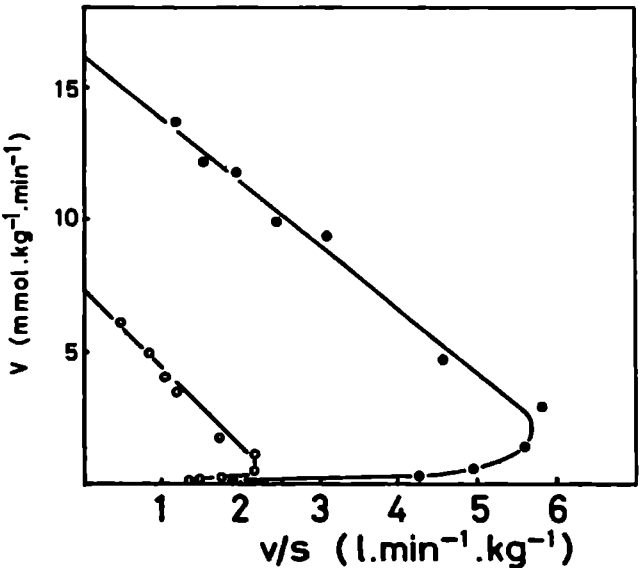

Fig. (5): Effect of DES on the Rb^+ Hofstee plot at pH 4.5. Metabolizing cells. DES was added at t = -5 min. The initial rate of Rb^+ uptake is plotted against the quotient of this rate and the Rb^+ concentration (mM). • control K_m = 2.14 mM, o 100 µM DES K_m = 2.01 (corrected for K^+ in the medium).
Mean of duplicate experiments.

(102%). Similar results have been described already earlier (11,22,36). Fig. (7) shows the effect of increasing F^- concentrations on the ATP content of the cells. At concentrations up to 15 mM the cell ATP content remained almost constant, whereas at higher concentrations the ATP content decreased to zero, probably by interference with the cellular metabolism (44).

Proton and potassium movements induced by inhibitors of the membrane ATPase

As shown earlier for *S.pombe* (33), several inhibitors of the plasma membrane ATPase e.g. D10-9 and DCCD elicited a rapid efflux of K^+ into the medium with concomitant influx of protons. Fig. (8) shows that not only D10-9 but also the other inhibitors tested, caused an efflux of K^+ from metabolizing cells. Moderate

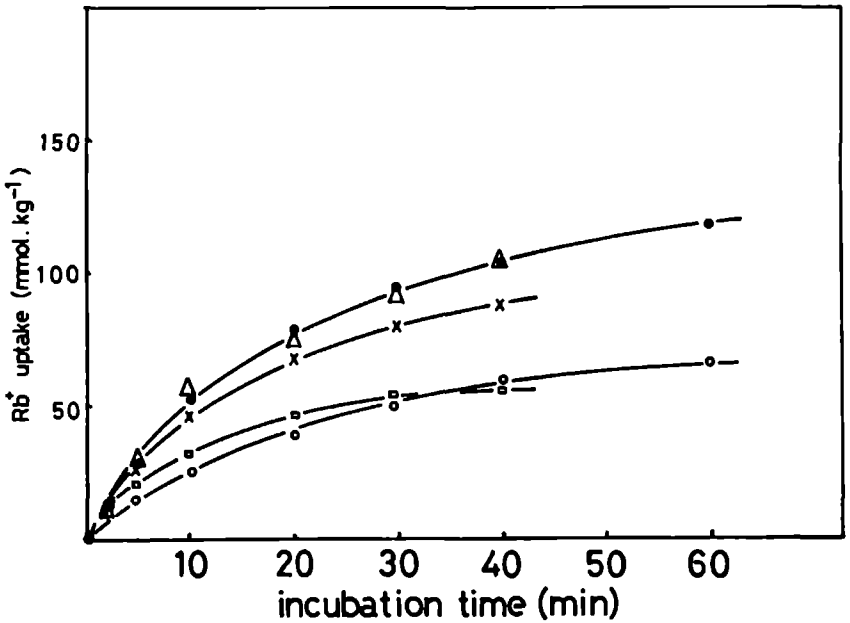

Fig. (6): Effect of D10-9 and TPTCl on the 1 mM Rb^+ uptake at pH 4.5. Metabolizing cells. • control, Δ50 µg/ml D10-9, x 100 µg/ml D10-9, □ 200 µg/ml D10-9, o 100 µM TPTCl.
Mean of duplicate experiments.

concentrations of the inhibitors induced only a transient efflux of K^+, whereas at high inhibitor concentrations the K^+ loss was irreversible. No changes in cell pH could be detected upon addition of the inhibitors. When additions of inhibitors were made to unbuffered suspensions (pH 4.5) also no changes in medium pH could be detected (data not shown).

Can the stimulatory effect of EB and D10-9 on the Sr^{2+} uptake be ascribed to a permeabilizing effect on the plasma membrane?

Roon et al. (32) suggested that the effect of D10-9 upon translocation processes was due to an increase in membrane permeability rather than to an interaction with the energy transduction system. Also EB may give rise to an increase in membrane permeability (45). If D10-9 and EB acted by a

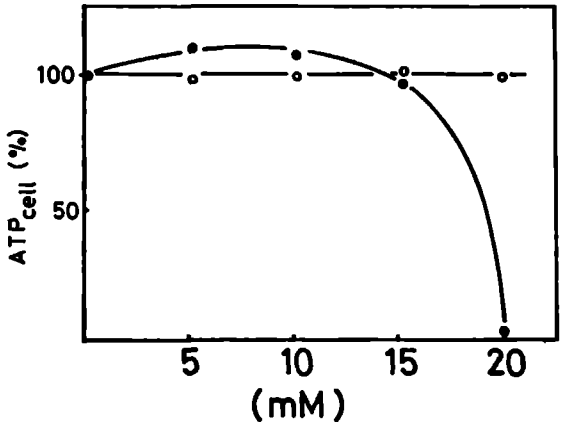

Fig. (7): Effect of NaF and NaCl on the cellular ATP content at pH 4.5 after 10 min of preincubation. Metabolizing cells. ● NaF, o NaCl, 100% = 0.71 mmol.kg^{-1} (dry yeast).
Mean of triplicate experiments.

Table III

Effect of D10-9 and TPTCl on the initial rate of 1 mM Rb^+ uptake and the steady-state accumulation ratio of Rb^+ at pH 4.5.

	V	%	Rb^+ in/Rb^+ out	%	TPP^+ in/TPP^+ out (%)*
control	5.45	100	158	100	100
100 µM TPTCl	2.52	46	120	76	70
control	5.64	100	161	100	100
50 µg/ml D10-9	6.50	115	164	102	73
100 µg/ml D10-9	6.52	116	98	61	42
200 µg/ml D10-9	6.24	111	94	58	35

Metabolizing cells. Inhibitors were added at t = -5 min. Rb^+ in/Rb^+ out was calculated from the steady-state distribution of Rb^+. V is the initial influx rate in mmol.kg^{-1} (dry yeast).min^{-1}. Initial rates were corrected for the increase in the K^+ content in the medium caused by the inhibitors involved.
Mean of duplicate experiments.
* Data from Table I, expressed in % of the control.

disruption of the membrane, not only K^+ leakage can be accounted for readily, but maybe also the increase in divalent cation uptake.

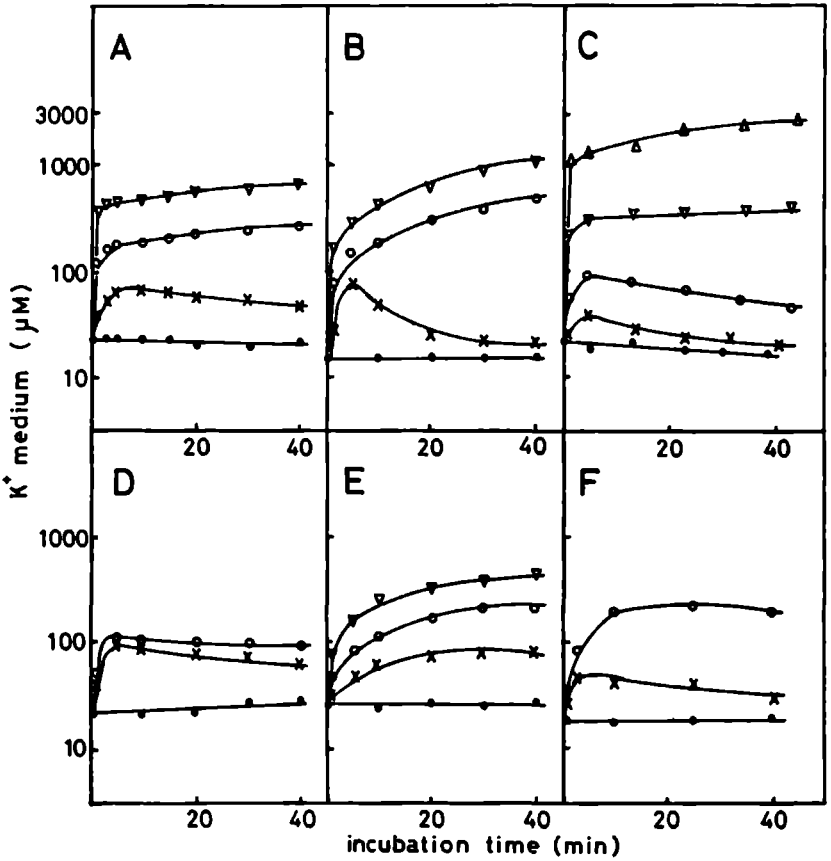

Fig. (8): Increase in K^+ movements at pH 4.5. Metabolizing cells. ● control in all experiments. 8A: D10-9, x 50 µg/ml, o 100 µg/ml, ∇ 150 µg/ml, 8B: EDAC, x 20 mM, o 30 mM, ∇ 45 mM, 8C: EB, x 0.3 mM, o 0.5 mM, ∇ 1 mM, Δ 3 mM, 8D: DES, x 50 µM, o 100 µM, 8E: NaF, x 5 mM, o 10 mM, ∇ 15 mM, 8F: TPTCl, x 10 µM, o 100 µM.
Mean of duplicate experiments.

We measured therefore Sr^{2+} uptake in yeast cells that were made leaky by treatment with acetone and compared it with the uptake of Sr^{2+} into cells that were treated with EB. It is seen in fig. (9) that Sr^{2+} accumulated slightly more in acetone-treated cells than in the control cells. The Sr^{2+} uptake was very rapid which could be ascribed to the increased accessibility

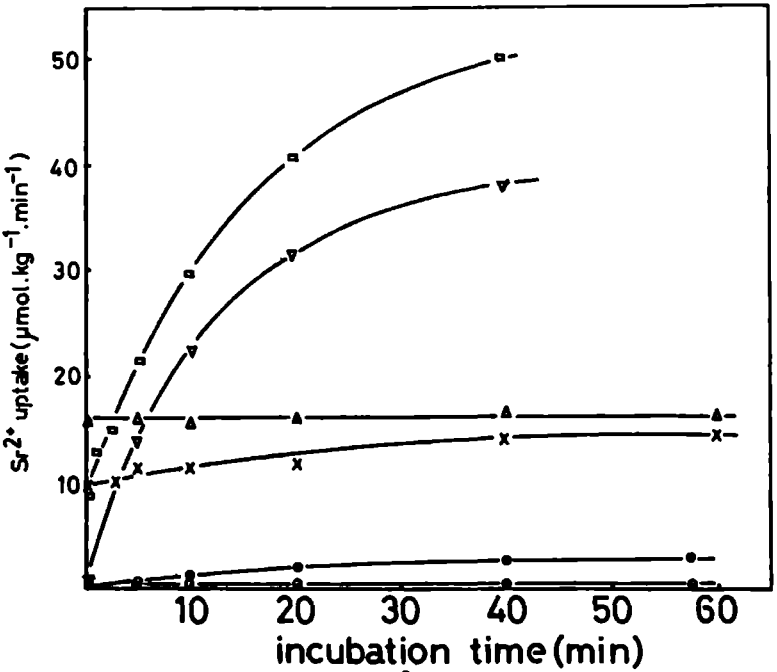

Fig. (9): Effect of 3 mM EB upon the Sr^{2+} uptake in either normal or acetone treated cells. The cells were washed with either EDTA solution or distilled water (DW). ● control/EDTA, x control/DW, Δ acetone/DW, o acetone/+ EB/EDTA, □ control/EB/DW, ▽ control/EB/EDTA. Mean of duplicate experiments.

of the binding groups located intracellularly. The Sr^{2+} accumulated, however, could be washed out completely, by washing the cells with the EDTA solution, used for removing extracellular Sr^{2+} bound to intact cells. Therefore the huge Sr^{2+} accumulation observed after addition of EB, cannot be explained by an increase in binding capacity for Sr^{2+}, caused by an increase in membrane permeability.

Discussion

It is shown by us that the plasma membrane ATPase inhibitors reported in the literature (4,11,22,30-36) exert also an inhibitory action on the isolated plasma membrane ATPase of

S.cerevisiae at K^+ and H^+ concentrations equal to the cellular concentrations. D10-9 does not inhibit the ATPase unless in the presence of a detergent. DCCD and its water soluble analogue EDAC exert only an inhibitory action after preincubation of the enzyme. The value of $I_{0.5}$ of F^- for the ATPase activity is in good agreement with the value of $I_{0.5}$ found in the yeast *S.pombe* (31) but higher than the value found in Ref. (34) with *S.cerevisiae* but this may be due to differences in the composition of the medium and the time of incubation. The inhibitory action of NaF is only due to the presence of F^-, since equimolar concentrations of NaCl do not inhibit the ATPase activity (see fig. (1)). The value of $I_{0.5}$ of DES for the ATPase activity is only slightly higher than the value found for its inhibitory action on the plasma membrane ATPase activity of *N.crassa* (30).

When the inhibitors of the ATPase are added at moderate concentrations to a yeast cell suspension these compounds induce a rapid efflux of K^+, which is only transient. Apparently, the K^+ released initially, is taken up again. Efflux of cellular K^+, provoked at higher concentrations of the inhibitors, is not reversed. Similar results have been obtained for the effect of EB upon K^+ movements (37-39). The effect of EB can be explained by an all-or-none effect of EB on the yeast cells (45). Part of the cells lose almost all their K^+, whereas the remainder of the cells remain virtually intact. Probably the still intact cells are capable to accumulate the K^+ lost by the other cells. Apparently higher inhibitor concentrations increase the K^+ permeability of the greater part of the cells, so that re-accumulation of K^+ is not detectable anymore. Probably the inhibitors tested do not give rise to a complete breakdown of the membrane. For in acetone-treated cells, which are completely permeable to small molecules, the uptake of Sr^{2+} is still rather low, whereas several of the inhibitors examined give rise to a stimulation of the Sr^{2+} uptake in metabolising cells, to an extent far more greater than in acetone-treated cells (see fig. (9)). The absence of an inhibitory effect of D10-9 and EB on the initial rate of Rb^+

uptake indicates also that the cells remain relatively intact, at least during short periods of incubation (see fig. (4)). On addition of these compounds the cell pH did not change indicating, that in contrast to the observations with *S.pombe* (33) the K^+ efflux was not compensated by a proton influx.

It has been suggested that D10-9 and EB (31,37) might hyperpolarise the yeast cell membrane by increasing the permeability of the cell membrane for K^+. Due to the steep K^+ gradient from cytoplasm to medium this increased K^+ permeability will cause hyperpolarisation. This hyperpolarisation may give rise to the enhancement of Ca^{2+} uptake found with D10-9 (22) and with EB (38) and may also cause the increased Sr^{2+} influx which we have found with these inhibitors. Table I shows that DCCD and EDAC, which did not enhance Sr^{2+} uptake also did not hyperpolarise the membrane. On the other hand DES hyperpolarises the cell membrane and enhances Sr^{2+} uptake. We have tested whether the hyperpolarisation induced by DES was an artefact due to complex formation between DES and TPP^+ after accumulation of these compounds into the cell, which may give rise to an increased TPP^+ accumulation. However, in non-metabolizing yeast cells, in the presence of 5 mM deoxyglucose and 15 µM antimycin A, which compounds completely block metabolism, an enhanced TPP^+ accumulation was not found on adding DES (see fig. 2F). The notion that DES hyperpolarises metabolizing yeast cells was supported, although indirectly, by Barts (unpublished data) who found that DES hyperpolarises the fungus *N.crassa* on applying microelectrodes in this organism. On the other hand with D10-9 and EB, which compounds stimulate Sr^{2+} uptake, a decrease in the accumulation ratio of TPP^+ is found indicating that these two inhibitors depolarise the yeast cell membrane. This depolarisation, however, may be apparent. It has been shown that both compounds (45,46) give rise to an all-or-none K^+ efflux from the yeast cells besides a graded K^+ efflux. Part of the cells become K^+-poor cells whereas the remainder of the cells are still relatively rich in K^+. It might well be possible that the K^+-poor cells are depolarised and the K^+-rich cells are

hyperpolarised. Such hyperpolarised cells may be capable to accumulate Sr^{2+} to such an extent, that the net Sr^{2+} accumulation found in the total yeast suspension is still greater than that found before addition of the inhibitors, despite the fact that part of the cells are depolarised. For the monovalent cation TPP^{+} the chance that this ion also shows an increase in net accumulation is much less, than for the divalent cation Sr^{2+} as is illustrated in the Appendix.

We like to present here an alternative explanation, with respect to the hyperpolarisation theory, for the observed increase in Sr^{2+} accumulation level. Recently it was proposed that the steady-state Sr^{2+} accumulation in yeast may be regulated by a specific Sr^{2+} efflux system (15). When D10-9, EB and possibly also DES exert an inhibitory action on the efflux of Sr^{2+}, this may also lead to an increase in Sr^{2+} accumulation.

Fig. (4) shows that only F^{-}, DES and TPTCl decrease the initial rate of 10 mM Rb^{+} uptake, whereas the other compounds have no effect upon the Rb^{+} influx. However, at this concentration the substrate site of the monovalent cation carrier is almost saturated and possible effects upon the "K_m" of Rb^{+} uptake will not be detected. As a matter of fact at low Rb^{+} concentrations Peña (37) found an inhibition of the Rb^{+} uptake by EB, whereas at high saturating Rb^{+} concentrations EB did not inhibit the Rb^{+} uptake appreciably. This results in an increase in K_m caused by EB. In fig. (4) it is seen that D10-9 at concentrations up to 200 µg/ml does not inhibit 10 mM Rb^{+} uptake. Table III shows that uptake of 1 mM Rb^{+} is inhibited neither. There are even indications that the Rb^{+} uptake is slightly enhanced by D10-9. This enhancement may be due to hyperpolarisation as discussed above. Fig. (5) shows that DES inhibits Rb^{+} uptake via a non-competitive type of inhibition. This is also true for the effect of TPTCl (see Table III and fig. (4)). This non-competitive inhibition may be partially due to the all-or-none effect which these two compounds exert upon the yeast cells. Part of the cells are permeabilized showing an increased accessibility to large molecules like bromophenolblue (Borst-Pauwels,

Theuvenet and Stols, unpublished data). As far as DES is concerned the contribution of this all-or-none effect is probably not large enough in order to account quantitatively for the observed decrease in Rb^+ uptake. Then a far more greater increase in K^+ efflux would be expected than was found, see fig. (8). Only 3% of the K^+ of the cells was released in the presence of 100 μM DES, whereas the inhibition of the Rb^+ uptake amounted to 60%.

Hauer et al. (36) have found that TPTCl inhibits K^+/H^+ exchange without affecting the value of the membrane potential. This is not supported by our findings. Rb^+ uptake is inhibited by TPTCl, whereas also a decrease in membrane potential is found. Because of the all-or-none effects, which TPTCl exerts upon the yeast cell membrane, both the depolarisation of the yeast cell and the inhibition of the Rb^+ uptake may at least partially be traced to permeabilization of part of the cells.

The absence of any effect upon the ATP content of the cells is surprising. One would expect that the cell ATP content will increase, when the membrane ATPase is impaired, see for example (11). However, since part of the cells may be damaged by the inhibitors applied, the cellular ATP content of the still intact cells may be underestimated.

It is also surprising that the cell pH is not affected by the various inhibitors. According to Ref. (33) D10-9 induces K^+/H^+ exchange by which the cells will be acidified. Possibly the all-or-none effects exerted by the inhibitors also mask cellular pH changes in single cells.

At this moment we can only speculate about the way DES interacts with the yeast cell. It might be possible that DES does not inhibit the plasma membrane ATPase under *in vivo* conditions. DES is highly lipophilic and therefore a great part of the DES may be absorbed in apolar parts of the cells, by which the effective concentration is greatly reduced. If DES interferes with the Rb^+ translocation at concentrations at which the yeast membrane ATPase is still not affected some possible models accounting for the results found may be con-

sidered. A possible explanation for the observed hyperpolarisation found, is, that due to inhibition of Rb^+ uptake, the cell is acidified. This will lead to an increase in membrane ATPase activity, since the pH optimum of the membrane ATPase is far below the normal cell pH value (26). Though we do not find any changes in cell pH, these changes may be masked, see above. When the Rb^+ uptake proceeds via an electrogenic Rb^+/H^+ exchange (14) inhibition of the Rb^+ uptake will lead to depolarisation. If, on the other hand, DES uncouples Rb^+ and H^+ movements the cell may be hyperpolarised. There are, however, no indications that low concentrations of DES stimulate the ATPase activity, see fig. (1). Also in *N.crassa* a stimulatory effect of DES on the ATPase activity is not found (30).

Appendix

It might be hypothesized that the enhancement of Sr^{2+} uptake by EB and D10-9 is due to an increase in the negative membrane potential. We will now show that this hypothesis is not neccessarily in contradiction with the finding of a decrease in the TPP^+ accumulation ratio caused by D10-9 and EB. When in intact cells the membrane potential is the driving force for Sr^{2+} uptake, the steady-state accumulation ratios of TPP^+ and Sr^{2+} are given by:

$$\frac{TPP^+ \text{ in}}{TPP^+ \text{ out}} = e^{-F\Delta E/RT} = y$$

and

$$\frac{Sr^{2+} \text{ in}}{Sr^{2+} \text{ out}} = e^{-2F\Delta E/RT} = y^2$$

rearranging leads to:

$$TPP^+ \text{ in} = y \; TPP^+ \text{ out}$$
$$Sr^{2+} \text{ in} = y^2 \; Sr^{2+} \text{ out}$$

When upon addition of inhibitors of the ATPase part of the cells remain intact, say fraction x, than fraction 1-x must be leaky. Suppose that in leaky cells no uptake of cations occurs than,

taking y^* as the value of y expected for the intact cells after addition of the inhibitor, we get:

$$TPP^+ \text{ in} = xy^* \; TPP^+ \text{ out}$$

$$Sr^{2+} \text{ in} = xy^{*2} \; Sr^{2+} \text{ out}$$

When hyperpolarisation of the membrane occurs then $y^* > y$ and when

$$\frac{y^2}{y^{*2}} < x < \frac{y}{y^*} \qquad (o < x < 1)$$

then $\quad y^{*2} x > y^2 ,$

which means that the experimentally found Sr^{2+} accumulation ratio, calculated from the Sr^{2+} uptake will increase on accounting all cells present in the cell suspension.

and $\quad y^* x < y$

which means that the experimentally found TPP^+ accumulation ratio will decrease.

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CHAPTER VI

ENERGIZATION OF K^+ UPTAKE IN YEAST

Summary

It is examined whether the TPP^+ equilibrium distribution between yeast cell water and medium is a good measure for the membrane potential or that membrane potentials calculated from this ratio are too high due to probe binding to cellular components. It appeared,though tentatively,that the value of the membrane potential needed to be corrected by adding 43 mV. We further examined whether K^+ uptake may be due to secondary active transport or not. We conclude that K^+ uptake can be either due to secondary active transport consisting of a co-transport of one K^+ ion with two proton or to primary active transport consisting either of an electrogenic antiport of K^+ and protons or of a neutral antiport of K^+ and protons. The conclusions are reached with the provision that the binding of TPP^+ in permeabilized cells is of the same magnitude as the binding of TPP^+ in intact cells.

Introduction

According to Mitchell (1) energy derived from ATP hydrolysis can be used directly for solute transport (primary active transport, see Chapter I) or can be transformed into a transmembrane electrochemical potential difference for protons, called the proton motive force (pmf). Both the electrical potential difference (ΔE) and the pH gradient (ΔpH) across the membrane contribute to the pmf. These quantities are related to the pmf according to:

$$pmf = \Delta E - 2.3\ RT/F.\Delta pH \qquad (1)$$

where R, T and F have their usual meaning and where ΔE and ΔpH are defined in the same direction namely cell interior minus

abbreviations used TPP^+ = tetraphenylphosphonium
CCCP = carbonyl cyanide m-chlorophenylhydrazone
DNP = 2,4-dinitrophenol

cell exterior.

Solute transport may be driven by the pmf by coupling with H^+ transport. This type of transport is called secondary transport, see Chapter I. In Appendix I it is shown how monovalent cation accumulation may depend upon the pmf or its components.

For studying the dependence of monovalent cation accumulation upon the pmf knowledge about the value of the membrane potential is needed. Since *Saccharomyces cerevisiae* cells are too small for direct maesurements of the membrane potential with microelectrodes, information about the membrane potential can only be obtained by indirect means. A frequently applied method is determining the equilibrium distribution of lipophilic cations. It is assumed that these cations distribute themselves across the membrane according to ΔE. In Ref. (2) or Chapter III evidence is presented that the lipophilic cation TPP^+ may fulfill this requirement. Also evidence with two independent techniques, namely directly with microelectrodes and indirectly with TPP^+, agreed very closely (3). Recently, however, it has been shown that TPP^+ may bind to a considerable extent to cellular components (4,5), so that caution must be taken when accumulation ratios of TPP^+ are "translated" into values of the membrane potential.

Yeast cells contain a plasma membrane ATPase which functions as a proton extruding system (6,7), but the proper role of this ATPase in solute transport is still under investigation. Suggestions have been made (6) that monovalent cation uptake is indirectly energized by ATP via the action of the plasma membrane ATPase. Due to electrogenic H^+ efflux, mediated by this enzyme, an electrogenic potential is generated, which may drive monovalent cation uptake. It has been pointed out, however, that the energy available from ΔE is not large enough to account for the observed high K^+ accumulation ratios (6,8). It has therefore been suggested that the K^+ uptake is driven by the pmf (6) or that K^+ uptake is driven directly by ATP via an electrogenic K^+/H^+ antiport (8), which system may be identical to the plasma-membrane ATPase. Another model proposed for K^+ uptake is a neutral K^+/H^+ antiport (9) which may also consist of a membrane

ATPase and thus may be driven by ATP, too.

In this study we first examined whether the equilibrium TPP^+ distribution is a good measure for the membrane potential or that membrane potentials calculated from this ratio are too high due to binding of TPP^+ to cellular components. Appendix II shows the way in which we have corrected the TPP^+ distribution ratios for binding of TPP^+ to cellular components. Secondly, the K^+ steady-state distribution is determined and it is examined whether this distribution can be accounted for by one of the models presented in Appendix I.

Materials and Methods

Yeast cells (2%, w/v), strain DELFT II, were aerated overnight in distilled water at room temperature in order to remove endogeneous substrate. Before the experiments started the cells were washed twice with distilled water and finally suspended in buffer (45 mM Tris, brought to the desired pH with succinic acid or Hepes). The cell suspension (2%, w/v) was preincubated during 10 min in the presence of glucose (5%, w/v) and a continuous flow of nitrogen was bubbled through the suspension. After the preincubation the cells received labelled TPP^+ (carrier-free, 0.18 nM). The cell pH and the K^+ content of the cell and medium were determined in a parallel experiment, where the label was omitted.

The uptake of [U - ^{14}C]TPP^+ was measured as described in (2).

The cell pH was determined after boiling the washed cells as described in (10) or was calculated from the distribution of the weak ^{14}C labelled butyric acid as described in (10).

The K^+ content of the medium was determined in the supernatant after centrifugation of the cell suspension (5 min, 3000 rpm).

For the determination of the K^+ content of the cells, 1 ml cell suspension was filtered off on weighted filters (Schleicher and Schüll, 602 H). The filters were once washed with 2 ml ice-cold 50 mM $MgCl_2$ solution and 2 ml ice-cold distilled water,

dried with acetone and weighted again. The filters were extracted with 2 ml 35% nitric acid for one hour and after suitable dilution the K^+ content was measured by means of a Zeiss flame spectrofotometer. The K^+ concentration in the cell water was calculated according to Ref. (10). The pH of the cell suspension was measured regularly and did not fall more than 0.3 pH unit.

All experiments were carried out at 25°C.

CCCP was dissolved in ethanol in such a way that the final ethanol concentration in the cell suspension was less than 1% (v/v). All other chemicals were dissolved in buffer.

Labelled compounds were purchased from the Radiochemical Centre, Amersham, England. All other chemicals were purchased from commercial sources.

Results

Since TPP^+ may bind considerably to cellular components (4,5) we examined the binding of TPP^+ in permeabilized yeast cells, obtained by destructing the cell membrane by repeatedly freezing and thawing. The binding was measured as described in Appendix II. It is shown in Table I that the membrane potential calculated from the equilibrium TPP^+ distribution needed to be corrected by adding 43 mV to the values calculated and that this correction was hardly influenced by changes in the pH applied. The pH was varied from 6.0 to 7.4 since that pH range covered the mean cellular pH values found (11). All values of the membrane potential presented in this Chapter were corrected accordingly.

Fig. (1) shows the pH dependence of the membrane potential, the pH gradient and the K^+ accumulation ratio. We have expressed all data as "membrane potential equivalents", that means that the K^+ accumulation ratio was expressed as $E_K = - RT/F \ln K_i^+/K_o^+$ and the pH difference as $E_H = - RT/F \ln H_i^+/H_o^+ = 2.3 RT/F.\Delta pH$. The membrane potential became more negative on increasing the pH (- 26 mV at pH 4.5 to - 71 mV at pH 7.0). ΔpH decreased from 2.2 units (E_H = 130 mV) at pH 4.5 to 0.1 unit (E_H = 5 mV) at pH 7.0. The decrease in E_H was linear above pH

Table I

Parameters used in the calculation of the correction of the membrane potential at pH 7.0. Effect of changes in the pH upon the correction term.

	intact cells	permeabilized cells	pH	correction
NSW	1.364 ± 0.097	-	6.0	39 ± 7
R_D	1.976 ± 0.220	-	6.6	44 ± 11
$f_{ads.}$	2.308 ± 0.321	-	7.0	43 ± 9
			7.4	48 ± 9
NSW*	-	0.060 ± 0.023		
R_D*	-	1.13 ± 0.08		
$f^*_{ads.}$	-	4.54 ± 1.94		

The correction term of the membrane potential was determined as described in Appendix II.
Mean of triplicate experiments.

5.5 and the slope of this straight line amounted to -56 mV per pH unit. E_K remained almost constant in the pH range tested and was approximately -250 mV, which corresponded to a K^+ accumulation ratio of 18,000. Fig. (1) further shows the pH dependence of the pmf calculated according to eqn. (1). We also calculated the energy needed in order to account for the K^+ accumulation found when either a 1:1 or a 2:1 cotransport of proton(s) with one K^+ was involved. Fig. (2) shows that for a 1:1 cotransport the energy available for K^+ accumulation, see Appendix I, Eqn. (6), was not high enough. When, however, no corrections for TPP^+ binding to cellular components were made, the energy for a 1:1 cotransport is just sufficient to account for the observed K^+ accumulation. The energy available for a cotransport of two protons, see Appendix I, Eqn. (7), with one K^+ was up to pH 6.25 more than sufficient and above this pH the energy was approximately large enough.

The dependence of ΔE and E_H on the external K^+ concentration at pH 7.0 is shown in fig. (3). The membrane depolarised for only 20 mV, when the K^+ concentration in the medium was increased up to 100 mM. E_K on the other hand was reduced

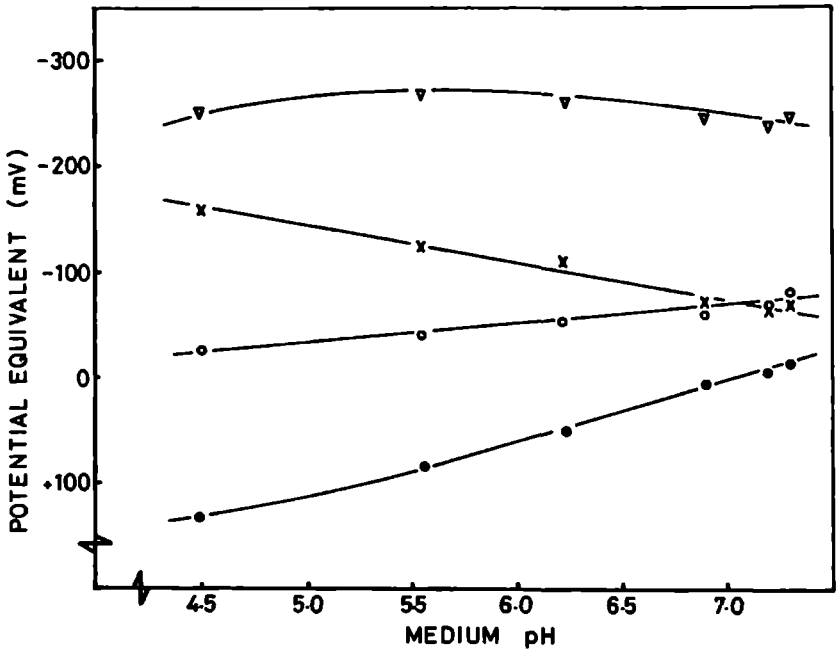

Fig. (1): Effect of varying the medium pH upon ΔE, E_H, pmf and E_K. Metabolizing cells. ΔE is the membrane potential (o), $E_H = 2.3\ RT/F$. ΔpH (●), pmf = $\Delta E - E_H$ (x), $E_K = -\frac{RT}{F} \ln K_i^+/K_o^+$ (▽). Mean of duplicate experiments.

strongly on increasing the external K^+ concentration. A linear relation was found between E_K and log K_o^+ and the slope of this straight line amounted to 56 mV per decade. Furthermore a slight increase in ΔpH was found by which E_H became more positive. The value of E_K became less negative than the value of ΔE when the external K^+ concentration exceeded 100 mM. The pmf remained constant in the K^+ concentration range tested.

The effect of the uncouplers CCCP and DNP is shown in Table II. These uncouplers added at the concentrations applied did not abolish the pH gradient, whereas ΔE was completely abolished or was even reversed of sign. The cell pH was determined in two independent ways, namely by a glass electrode after boiling the

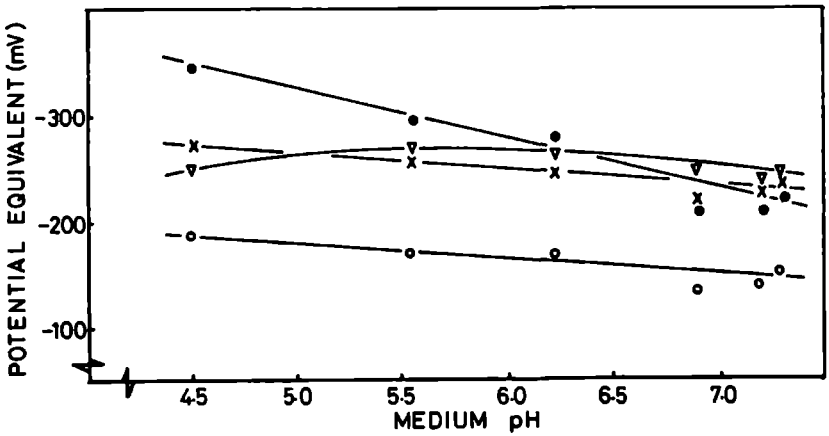

Fig. (2): Energy available for a cotransport of one K^+ ion with one respectively two protons. Metabolizing cells. o $2\Delta E - E_H$: energy available for a 1:1 cotransport, see model (3), Appendix I. • $3\Delta E - 2E_H$: energy available for a 2:1 cotransport, see model (4). x $2\Delta E - E_H$: energy available a a 1:1 cotransport, but in this case no corrections for TPP^+ binding to cell components were made. ∇ E_K: data of fig. (1). Mean of duplicate experiments.

Table II

Effect of uncouplers on E_K and the components of the pmf at pH 4.5.

	pH_o	pH_i	E_H (mV)	ΔE (mV)	E_K (mV)
control	4.45	6.67	131 ± 1	-24 ± 1	-246 ± 1
200 µM DNP	4.45	6.64	129 ± 1	0	-216 ± 1
control	4.51	6.61	124 ± 1	-19 ± 1	-241 ± 1
25 µM CCCP	4.51	6.61	124 ± 1	0	-209 ± 1

Metabolizing cells. ΔE was calculated from the TPP^+ equilibrium distribution. In a parallel experiment, without added TPP^+, E_H and E_K were determined. For meaning of the symbols see legend to fig. (1). Mean of duplicate experiments.

cells or from the distribution of butyric acid. Both methods gave essentially the same results. Addition of uncouplers led to K^+ efflux. E_K became less negative by approximately 30 mV.

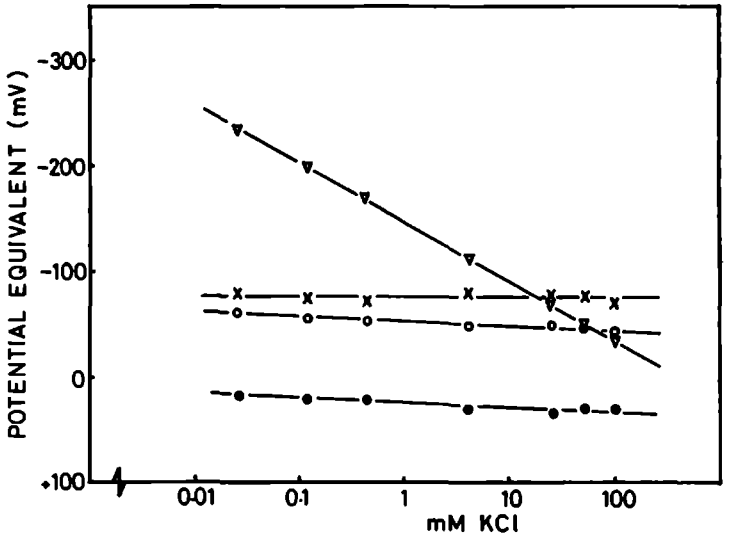

Fig. (3): Effect of varying the external K^+ concentration upon ΔE, E_H, pmf and E_K at pH 7.0. Metabolizing cells. For meaning of the symbols see legend to fig. (1).
Mean of duplicate experiments.

The cells lost 5% of their K^+.

Since addition of salts of weak permeant acids may cause net K^+ uptake (11) we examined the effect of butyrate on the parameters of the pmf. Table III shows that butyrate caused a slight, but significant, net K^+ uptake i.e. E_K became more

Table III

Effect of butyrate on E_K and the components of the pmf at pH $\pm$ 4.5.

	pH_o	pH_i	E_H (mV)	ΔE (mV)	K_i^+/K_o^+	E_K (mV)
control	4.52	6.63	124 $\pm$ 1	-21 $\pm$ 1	15,973	-248 $\pm$ 1
4 mM butyrate	4.52	6.40	111 $\pm$ 1	-19 $\pm$ 1	19,415	-253 $\pm$ 1

Metabolizing cells. See further legend to Table II.

negative, whereas E_H was slightly reduced by 13 mV. The membrane potential did not change significantly upon addition of butyrate.

Discussion

In order to examine secondary active transport knowledge of the value of the membrane potential is important. In Chapter III it is stated that TPP^+ can be used as a probe for the membrane potential but as pointed out in a still increasing number of reports about the binding of TPP^+ to cell components, the value of the membrane potential may be overestimated. This study clearly shows that the negative membrane potentials calculated from the TPP^+ equilibrium are too high and need to be corrected. This correction amounts to 43 $\pm$ 5 mV. When the medium pH is increased the membrane is hyperpolarised. In the whole pH range tested the values of the membrane potential are more positive than E_K. Only at high K^+ concentrations, see fig. (3), a membrane potential is observed which equals or is more negative than E_K. It can therefore be concluded that at low external K^+ the negative membrane potential is not large enough in order to account for the K^+ accumulations observed. This rules out model (1), see Appendix I. The hyperpolarisation of the membrane found on increasing the medium pH may be attributed to a reduction in the contribution of protons to that part of the potential which consists of the diffusion potential. This hyperpolarisation, however, has almost no effect on the K^+ distribution as is expressed by the constant value of E_K over the wide pH range (fig. (1)). If K^+ is taken up according to model (1) the effect of butyrate on the net K^+ uptake should be accompanied by an increase in the negative membrane potential. Since no such effect is found, see Table III, this is an additional argument against K^+ uptake via model (1).

It is also ruled out that K^+ uptake is due to an electroneutral K^+/H^+ exchange driven by the pH gradient (model (2)) since $-E_K$ in the entire pH range is larger than E_H.

It is suggested that K^+ uptake in *Neurospora crassa* (12) and *Streptococcus feacalis* (13) is accompanied with one proton. We

examined whether this is also true for yeast, see model (3). On calculating the energy available for such a cotransport it appeared that the energy content is not large enough to account for the observed K^+ accumulations. We have also tried to account for the observed K^+ accumulations via a cotransport of one K^+ ion with two protons, see model (4). According to this model the energy available for such a cotransport is by approximation large enough. It is generally accepted that uncouplers abolish membrane potentials by making membranes permeable to protons. Our observations made with *S.cerevisiae* do not fit into this scheme. Table II clearly shows that CCCP and DNP do not abolish the pH gradient. CCCP and DNP, however, abolish the membrane potential completely or may even reverse this potential. The same phenomena have been found earlier for yeast (14) and it is suggested that uncouplers act directly upon the membrane ATPase. On the other hand, quite opposite effects of uncouplers on the components of the pmf in *S.cerevisiae* have been described. De La Peña et al. (15) found that uncouplers only transiently influenced ΔE, whereas ΔpH is drastically decreased. Uncouplers reduce E_K for only 30 mV, whereas the membrane potential is completely abolished. This is again an additional argument against K^+ uptake according to model (1).

In conclusion secondary active transport via most of the models presented in Appendix I can be ruled out. Only a cotransport of one K^+ with two protons may account at least by approximation for the K^+ accumulation found. A cotransport of K^+ with only one proton would be only possible if the binding of TPP^+ to permeabilized cells found is an artefact due to unmasking of negative groups and that these groups are not available for TPP^+ binding in the intact cells. This possibility is still under investigation

Besides the possibility of cotransport of K^+ with two protons primary active transport of K^+ should be considered. This transport may be energized directly by ATP hydrolysis. Such a system may consist of the membrane ATPase and may account for the large K^+ accumulations found. Goffeau et al. (8) have suggested that this pump is an electrogenic cation pump. In that

case more protons should leave the cell than K^+ is taken up. About the stoichiometry we can only speculate. This may be either a $K^+/2H^+$ antiport or a $2K^+/3H^+$ antiport in analogy with the animal (Na^++K^+) ATPase. Since two sites are involved in K^+ influx (6), see however Ref (16) for a single-site transport mechanism for Rb^+ (K^+) uptake also accounting for the apparent two-site kinetics experimentally found, three sites may be involved in proton efflux in order to account for the generation of an electrogenic potential, negative inside. Hauer et al. (9) suggested a neutral K^+/H^+ antiport mediated directly by ATP besides a proton pump for the generation of the membrane potential. More studies are needed in order to elucidate the K^+ uptake mechanism in more detail.

Appendix I

When K^+ transport is mediated by the pmf or its components, it can be deduced (17) that the steady-state distribution is related to the electrochemical gradient of protons in the following way:

$$E_K = - RT/F \ln K_i^+/K_o^+ = (n+1)\ \Delta E - 2.3\ RT/F.n.\Delta pH \qquad (2)$$

where K_i^+ and K_o^+ are the concentration (or rather activities) of K^+ inside and outside the cell respectively and $|n|$ is the number of protons cotransported (n is positive) or antiported (n is negative) with K^+ and E_K is the K^+ Nernst-potential.

Energization of K^+ uptake may occur in various ways.

Model (1). The uptake of carrier-mediated K^+ is driven by the membrane potential difference.

thus $n = 0$ and therefore $E_K = \Delta E$ (3)

Model (2). The uptake of K^+ proceeds via an 1:1 K^+/H^+ antiport.

thus $n = -1$ and therefore $E_K = 2.3\ RT/F.\Delta pH$ (4)

Model (3). The uptake of K^+ is an 1:1 cotransport with a proton.

thus $n = 1$ and therefore $E_K = 2\ \Delta E - 2.3\ RT/F.\Delta pH$ (5)

or $E_K = \Delta E + pmf$ (6)

Model (4). The uptake of K^+ is a 1:2 cotransport with two protons.

thus $n = 2$ and therefore $E_K = 3\ \Delta E - 4.6\ RT/F.\Delta pH$ (7)

Appendix II

For correcting the membrane potential for TPP^+ binding to intracellular components four parameters must be known, namely the amount of cell water, the Donnan potentials of both the cell wall and the cell inner of permeabilized cells and the adsorbtion coefficient of TPP^+ for binding to the cell wall. The amount of cell water can be calculated from the mannitol-inaccessible space and the Donnan potentials can be calculated from the Na^+ distribution between medium and cells.

Method:

2% Starved yeast cells were used. Part of the cells were permeabilized by 10 times freezing and thawing. Either the intact cells or permeabilized cells were suspended in 50 mM Hepes buffer brought to the desired pH with Tris. After thoroughly mixing labelled carrier-free mannitol, Na^+ or TPP^+ were added whereafter 5 ml cell suspension was centrifuged after 30 seconds (3000 rpm, 5 min). The activity in the supernatant was determined (A_s, activity per 0.5 ml). The activity of the pellet was also determined (A_r, activity per pellet). We further determined the dry-weight (DW) and the wet-weight (WW) of the pellet of 5 ml suspension. The difference between WW and DW gave the total water content (TW).

The calculation of the adsorption of TPP^+ to the intracellular components consisted of the following steps.

a) amount of cell water (non-solvent water, NSW) in intact cells determined with ^{14}C-mannitol,

$$NSW = \frac{TW}{DW} - \frac{A_r}{2A_s DW} \qquad [ml.g^{-1}\ DW] \tag{8}$$

b) the Donnan ratio, R_D, of the cell wall determined with $^{22}Na^+$ in intact non-metabolizing cells

$$R_D = \frac{\frac{A_r}{2A_s DW} - \frac{TW}{DW} + NSW + \frac{V_{CW}}{DW}}{\frac{V_{CW}}{DW}} \tag{9}$$

where V_{CW}/DW is the volume of the cell wall per g DW. This value amounts to approximately 0.27, thus

$$R_D = \frac{\frac{A_r}{2A_s DW} - \frac{TW}{DW} + NSW + 0.27}{0.27} \tag{10}$$

c) adsorbtion coefficient of TPP^+ binding to the cell wall of intact cells ($f_{ads.}$) determined with ^{14}C - TPP^+.

$$f_{ads.} = \frac{\frac{A_r}{2A_s DW} - 0.27\ (R_D - 1) - \frac{TW}{DW} + NSW}{0.27\ R_D} \tag{11}$$

d) amount of cell water in permeabilized cells (NSW*) determined with ^{14}C-mannitol

$$NSW^* = \frac{TW}{1.5DW} - \frac{A_r}{3A_s DW} \tag{12}$$

where the factor 1.5 denoted a correction term for the difference in DW of intact cells and permeabilized cells.

e) the Donnan ratio, R_D^*, of permeabilized cells determined with $^{22}Na^+$

$$R_D^* = \frac{\frac{A_r}{3A_s DW} - 0.27\ (R_D - 1) - \frac{TW}{1.5DW} + NSW}{NSW - NSW^*} \tag{13}$$

f) adsorbtion coefficient for TPP^+ binding to intracellular components ($f^*_{ads.}$) determined with ^{14}C-TPP^+

$$f^*_{ads.} = \frac{\frac{A_r}{3A_sDW} - \frac{TW}{1.5DW} + NSW + 0.27(R_D + f_{ads.}R_D - 1) - R_D^*\,(NSW-NSW^*)}{R_D^*\,(NSW-NSW^*)} \quad (14)$$

The values of A_r and A_s refer in each calculation to the labelled compound added.

The amount of TPP^+, which is free in the cells was calculated from the total TPP^+ accumulated into the cells via

$$TPP^+_{total} = TPP^+_{bound} + TPP^+_{free} \quad (15)$$

$$TPP^+_{total} = f^*_{ads.} \cdot TPP^+_{free} + TPP^+_{free} \quad (16)$$

$$TPP^+_{free} = \frac{TPP^+_{total}}{1 + f^*_{ads.}} \quad (17)$$

The membrane potential can be calculated according to:

$$\Delta E = -2.3\ RT/F\ \log \frac{TPP^+_{free,in}}{TPP^+_{out}} \quad (18)$$

or

$$\Delta E = -2.3\ RT/F\ \log \frac{TPP^+_{total}}{TPP^+_{out}} + 2.3\ RT/F\ \log\ (1 + f^*_{ads.}), \quad (19)$$

where 2.3 RT/F log $(1 + f^*_{ads.})$ is the correction for the apparent membrane potential calculated from the total TPP^+ uptake.

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CHAPTER VII

Na^+ EFFLUX FROM YEAST

Summary

Na^+ efflux from Na^+-loaded yeast cells is dependent upon metabolism. It is ruled out that this dependence is an artefact caused by the increase in the osmotic value of the medium on adding glucose. Na^+ efflux from yeast may be described by several models. At this moment it is difficult to differentiate between the various models. As concerned Na^+ efflux into media to which no extra K^+ is added we conclude that Na^+ efflux is either due to an electrogenic Na^+-pump or to a Na^+-anion symport mechanism. When K^+ is present in the medium the Na^+ efflux is greatly enhanced, while no change in membrane potential is found. The latter points to the possibility that Na^+ and K^+ fluxes are tightly coupled according to an electroneutral exchange mechanism. The exchange of Na^+ for K^+ is an active process proceeding against the electrochemical gradient of Na^+

Introduction

Under normal conditions yeast cells contain much less Na^+ than K^+. When yeast cells are grown in media containing K^+ as well as Na^+, the cells preferentially take up K^+ due to the high affinity of K^+ for the monovalent cation carrier. At not too high K^+ concentrations yeast cells can also take up Na^+ via the monovalent cation carrier. Inaddition Na^+ can be taken up via an inducible Na^+-phosphate cotransport system (1). Apart from the Na^+ uptake systems also one or more Na^+ efflux systems are operating in the yeast (2,3).

The Na^+ efflux system is suggested by Conway et al. (2,4) to consist of a redox pump, but this idea has neven been confirmed. Several other authors have studied the Na^+ efflux from yeast but still there is no clear insight into the mechanism(s)

Abbreviations used: TPP^+ = tetraphenylphosphonium
INT = 2p-Iodophenyl-3p-Nitrophenyl-5-phenyltetrazolium
DES = diethylstilbestrol
DCCD = N,N'-dicyclohexylcarbodiimide

involved (5-10). Fig. (1) shows the possible models for Na^+ efflux from yeast.

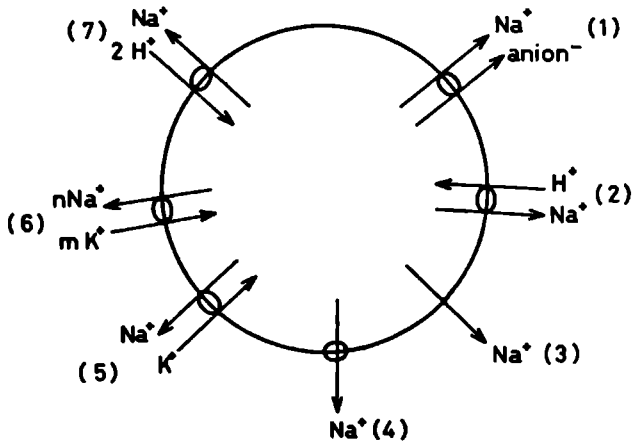

Fig. (1): Models for Na^+ efflux. See text for further explanation.

Part of the Na^+ efflux is energy-dependent (3,9). However, Kotyk and Kleinzeller disputed this (6). They suggested that the increase in Na^+ efflux, found on adding glucose to the medium, is due to an increase in the osmotic value of the medium, rather than to a stimulation of the metabolism. It has been suggested by Conway et al. (2,4) that the Na^+ efflux is linked in a neutral way to the efflux of organic anions such as succinate or bicarbonate via electron transfer between the two carriers involved in both transport processes (model (1)).

The simultaneous efflux of Na^+ and organic anions does not neccessarily point to the existence of a coupling between Na^+ and anion transport systems. Rothstein (9) has suggested that the increase in anion concentration in the medium is due to diffusion of undissociated acids from the cells and that the increase in the proton concentration of the medium gives rise to an enhancement of a neutral H^+/Na^+ exchange (model (2)). An alternative explanation is, that diffusion of undissociated

acids into the medium leads to an increase in the proton influx due to a disturbance in the steady-state proton distribution. The proton influx will lead to depolarisation of the yeast membrane, which in turn may lead to an enhancement of the Na^+ efflux in the direction of its electrochemical gradient (model (3)).

Na^+ efflux may also be due to an electrogenic Na^+-pump analogous to the hypothetical ATP-driven H^+-pump (model (4)). It should be remarked, however, that until now there are no indications that the yeast plasma membrane ATPase is specifically stimulated by Na^+.

When K^+ is present in the medium the anion efflux is partially depressed whereas the Na^+ efflux is stimulated and Na^+ is exchanged for K^+ (2,3,5). According to Conway et al. (2,4) the K^+ and Na^+ movements are also indirectly coupled in a neutral way, via electron transfer between to two carriers involved (model 5). Similarly the K^+/Na^+ exchange is not necessarily due to coupled K^+-Na^+ fluxes. An increased influx of K^+ will lead to depolarisation of the membrane which may give rise to an increase in Na^+ efflux according to its electrochemical gradient (model (3)). Na^+ efflux may also be due to electrogenic K^+/Na^+ exchange, thereby transporting mK^+ against nNa^+ (model (6)). m may be greater than n or n may be greater than m.

Recently it has been suggested (10) that part of the Na^+ efflux occurs via a non-electroneutral H^+/Na^+ antiporter (model (7)), by which two or more protons are exchanged against one Na^+ ion. This model accounts for a metabolism-dependent Na^+ efflux driven by both the proton motive force (pmf) and the pH gradient.

In this study we examined the effect of varying the medium pH upon Na^+ efflux across the yeast cell membrane. Also effects of changes in the osmotic value of the medium were examined. We also investigated the effect of K^+ upon Na^+ efflux. In addition we applied an inhibitor of the membrane ATPase, namely DES, which compound inhibits K^+ uptake despite the fact that the membrane is hyperpolarised, see Chapter V. By determining the effect of K^+/Na^+ exchange upon the membrane potential, it was

examined whether the K^+/Na^+ antiport was electroneutral or electrogenic.

Materials and Methods

The yeast *Saccharomyces cerevisiae*, strain DELFT II was exhausted of endogeneous substrate by aeration overnight in distilled water at room temperature at 4% w/v. The cells were washed twice with distilled water and suspended at 4% w/v into a medium that contained 200 mM Na^+ citrate/citric acid pH 7.0 and glucose (5%, w/v) in order to preload the cells with Na^+. Anaerobic conditions were maintained by bubbling N_2 through the suspension. After two hours the cells were centrifuged (5 min, 3000 rpm) and resuspended in the same medium for another two hours. Non-metabolizing cells were obtained by aeration of the "Na^+-cells" overnight in 200 mM Na^+ citrate/citric acid pH 7.0. Before use the cells were washed twice with distilled water and suspended in the desired medium (see legends to the figures). Metabolizing cells were obtained by preincubating the exhausted cells (2%, w/v) with glucose (3%, w/v) for 10 min under anaerobic conditions. In general Na^+ loaded cells were obtained containing 200 $\pm$ 40 mmol $Na^+.kg^{-1}$ (dry yeast) and 160 $\pm$ 20 mmol $K^+.kg^{-1}$ (dry yeast).

The concentrations of Na^+ and K^+ in the cell and medium were determined by flame spectrophotometry as described for K^+ in Chapter IV.

The concentrations of succinate in the medium was determined from the reduction of INT by succinatedehydrogenase. This enzyme was obtained from crude mitochondrial fractions of yeast. The concentration of succinate was determined spectrophotometrically at 490 nm from a solution containing 1 ml sample, 1 ml reagens and x ml enzyme, depending on the specific activity of the enzyme preparation. The reagens contained phosphate buffer of pH 7.4 (132 mM), sucrose (5 mM), Triton X-100 (0.05 %, w/v) and INT (0.2%, w/v).

The membrane potential was calculated from the steady-state TPP^+ distribution as described in Chapter III and was corrected

for intracellular binding of TPP^+, see Chapter VI.

The cell pH was determined as described in Ref. (11).

All experiments were carried out at 25°C.

Results

Kotyk and Kleinzeller (6) suggested that the increase in the Na^+ efflux rate found on adding glucose to Na^+-loaded yeast might be due to an increase in the osmotic value of the medium rather than to the onset of metabolism. We have examined the effect of some sugars, both fermentable and non-fermentable ones upon Na^+ movements. Fig. (2) shows the time course of Na^+ efflux from Na^+-loaded cells, suspended into a medium that contained no extra added Na^+. From the sugars added, the only one that stimulated this efflux was glucose. The non-fermentable sugars xylose and lactose did not stimulate the Na^+ efflux. We also examined whether lowering the glucose concentration did give rise to a less large increase in Na^+ efflux. However, the increase in Na^+ efflux at 20 mM glucose was not significantly different from that found at 200 mM glucose.

In connection with the findings of H^+/Na^+ antiporters in bacteria (12) and in connection with the suggestion that Na^+ efflux from yeast consists of a non-electroneutral H^+/Na^+ antiport (14), see also model (7), we also examined the effect of varying the medium pH upon the Na^+ efflux from both metabolizing and non-metabolizing Na^+-loaded cells. Table I shows that the Na^+ efflux from both metabolizing cells and non-metabolizing cells increased on increasing the medium pH. The difference in Na^+ efflux rate found with metabolizing cells and with non-metabolizing cells appeared to be almost constant in the pH range tested. The Na^+ concentrations in the medium were at the start of the experiment rather low, Na^+ influx under these conditions did not contribute significantly to the net Na^+ efflux determined. This influx amounted to maximal 0.3 mmol Na^+. kg^{-1} (dry yeast). We also determined the steady-state distribution ratio of Na^+ in metabolizing cells. The Na^+ Nernst potentials (E_{Na}) referring to these distribution ratios are given in

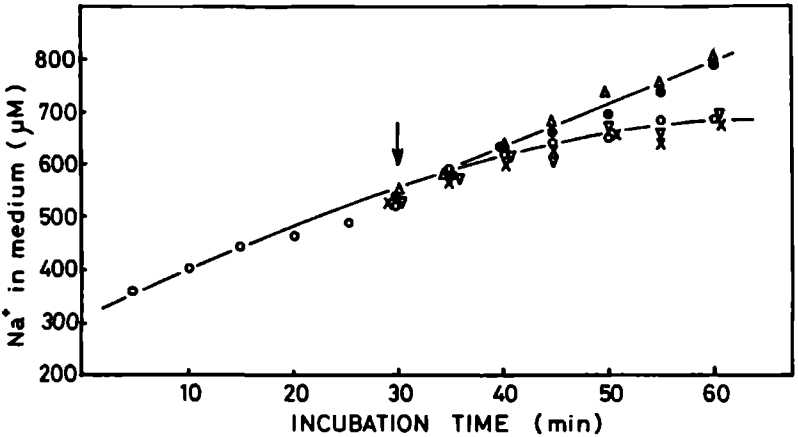

Fig. (2): Effect of glucose, xylose and lactose upon the Na^+ efflux at pH 4.5. Non-metabolizing cells. o control, ∇ 200 mM xylose, x 200 mM lactose, Δ 20 mM glucose, ● 200 mM glucose. Additions were made at t = 30 min.
Mean of duplicate experiments.

Table I

Effect of the medium pH upon the Na^+ efflux rate and cell pH.

pH_o	$pH_i(1)$	Na_i^+	$V_o(1)$	$V_o(2)$	$V_o(1)-V_o(2)$	$pH_i(2)$	E_H	E_{Na}
3.5	5.8	25	6.3	0.4	5.9	4.9	83	-80
4.5	6.5	25	6.2	0.8	5.4	6.4	112	-78
5.0	6.6	35	6.4	1.0	5.4	6.8	106	-88
6.0	6.8	45	6.6	1.4	5.2	7.0	59	-85
7.0	6.9	45	7.5	1.5	6.0	7.2	10	-81

Buffer: 45 mM Tris brought to the desired pH with succinic acid. pH_o is the medium pH. $pH_i(1)$ and $pH_i(2)$ are the cell pH's obtained at t = 1 min and in the steady-state, respectively. $V_o(1)$ and $V_o(2)$ are the initial Na^+ efflux-rates from metabolizing and non-metabolizing cells, respectively. V_o is expressed in mmol $Na^+.kg^{-1}$ (dry yeast).min^{-1}. Na^+ is the Na^+ concentration in the medium at t = 0 (μM). $E_H = 2.3\ RT/F.\Delta pH$ and $E_{Na} = -RT/F.\ln Na_i^+/Na_o^+$ denote the pH difference and Na^+ distribution in the steady-state, expressed in mV.

Table I. These potentials did not depend upon the medium pH and were approximately -80 mV.

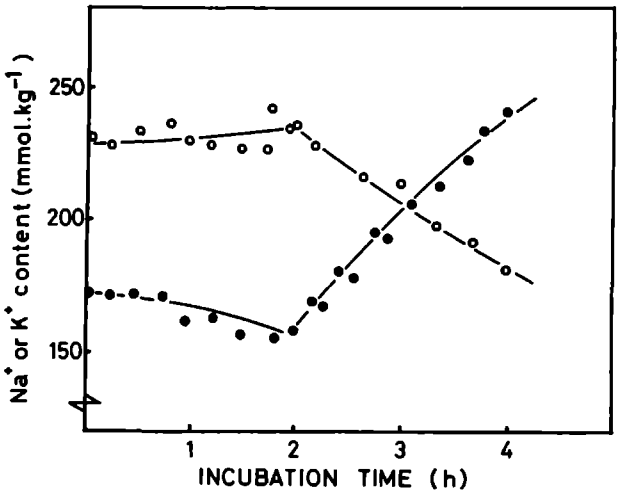

Fig. (3): Effect of K^+ upon the Na^+ efflux at pH 7.0. Metabolizing cells. The medium consisted of 100 mM Hepes/Tris pH 7.0 and 250 mM NaCl. At t = 2 hours 25 mM KCl was added . o Na^+ content, • K^+ content. Mean of triplicate experiments.

When K^+ was added to a suspension of Na^+-loaded cells in a Na^+-poor medium the Na^+ content of the cells fell rapidly (7,9). We examined whether this was also true if K^+ was added to a Na^+-rich medium which contained 250 mM Na^+. Loading the cells with Na^+ yielded cells which contained 160-240 mmol $Na^+.kg^{-1}$ (dry yeast), which corresponded to cellular Na^+ concentrations of 125-180 mM Na^+ referring to the cellwater. If Na^+ efflux occurred into the Na^+-rich medium and the membrane potential was still negative (from inside to outside) this Na^+ efflux would proceed against the electrochemical Na^+-gradient. Fig. (3) shows the effect of addition of 25 mM K^+ on the Na^+ content of the cells at pH 7.0. When no K^+ was present in the medium, the Na^+ and K^+ content of the cells increased and decreased respectively only slightly. After addition of K^+, a great decrease in the Na^+ content was found with a concomitant increase in the K^+ content. The stoichiometry of the Na^+ and K^+ fluxes amounted to $1:1.1\pm0.3$ (mean of triplicate experiments).

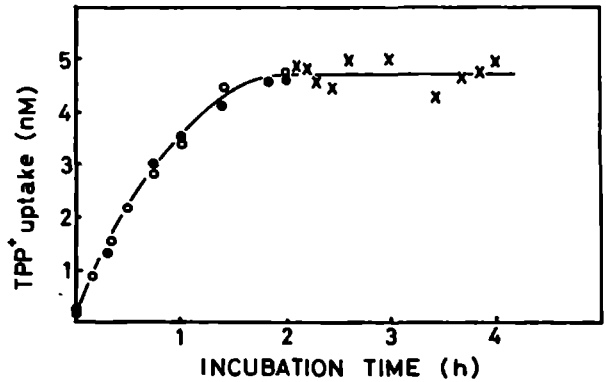

Fig. (4): Effect of K^+ upon the TPP^+ uptake at pH 7.0. For conditions see legend to fig. (3). o control, • 25 mM KCl added at t = 0, x 25 mM KCl added at t = 2 hours.
Mean of duplicate experiments.

Fig. (4) shows the uptake of TPP^+ in Na^+-loaded cells. Addition of 25 mM K^+ did neither affect the initial rate of TPP^+ uptake nor the TPP^+ steady-state distribution. K^+, added after 2 hours, when the TPP^+ uptake had reached its steady-state, neither did effect the TPP^+ distribution. From the TPP^+ distribution an apparent membrane potential could be calculated (13). This membrane potential amounted to -90 mV. However, on accounting for the binding of TPP^+ to cellular components the membrane potential was only -47 $\pm$ 5 mV. See Chapter VI for the correction of TPP^+ binding.

We also examined the amount of succinate liberated under the same conditions. Fig. (5) shows that the amount of succinate liberated from the cells was not significantly affected by K^+. K^+ only increased the lag time for the appearance of succinate. Addition of K^+ after 120 min also led to only a transient decrease in the rate of succinate appearance (data not shown).

Finally we examined the effect of an inhibitor of the yeast plasma membrane ATPase, DES, (see Chapter V)upon the release of Na^+ from Na^+-loaded cells. Table II shows that DES did not inhibit the Na^+ efflux in the presence of glucose. When 1 mM Rb^+

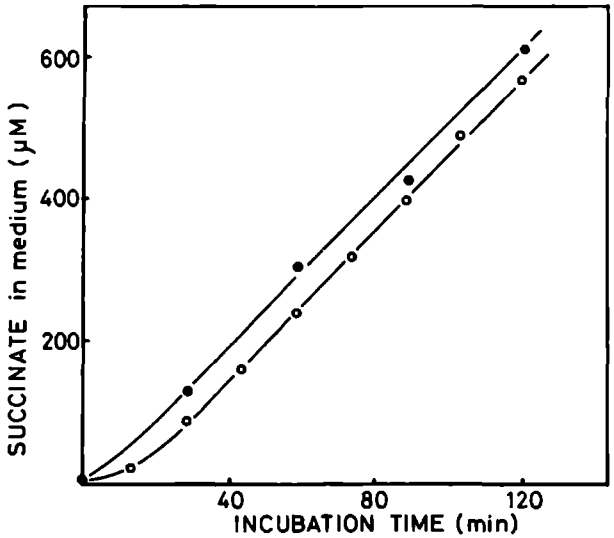

Fig. (5): Effect of K^+ upon the succinate efflux at pH 7.0. For conditions see legend to fig. (3). ● control, o 25 mM KCl added at t = 0. Mean of duplicate experiments.

Table II

Effect of 100 μM DES upon Na^+ efflux-rate and Rb^+ influx-rate at pH 4.5.

	Na^+ efflux-rate	%	Rb^+ influx-rate	%
control	3.90	100		
+ 1 mM Rb^+	6.15	157	15.21	100
+ 100 μM DES	4.18	107		
+ 1 mM Rb^+ + 100 μM DES	3.58	92	9.06	53

Metabolizing cells. The influx and efflux rate are expressed in $mmol.kg^{-1}$ (dry yeast).min^{-1}. Buffer: 45 mM Tris/succinate pH 4.5. Mean of triplicate experiments.

was present, the Na^+ efflux rate was increased approximately 1.6 fold. DES inhibited, however, the Rb^+-stimulated Na^+ efflux completely. We also determined the effect of DES on the initial uptake rate of 1 mM Rb^+ in Na^+-loaded cells. DES decreased this

uptake rate for 53%.

Discussion

Fig.(2) shows that Na^+-loaded cells, when suspended into media containing no added Na^+ of K^+ lose part of their Na^+. Stimulation of the Na^+ efflux on adding a sugar which increases the osmotic value of the medium is only found with glucose. Although xylose and lactose increase the osmotic value of the medium to the same extent as glucose, they do not stimulate Na^+ efflux. Xylose is taken up by the cells but cannot be metabolized under anaerobic conditions and lactose is even not taken up (6). Kotyk and Kleinzeller (6) found that sugars, irrespectively whether they are metabolized or not, increase the rate of Na^+ efflux. Accordingly, these authors attributed the effect of the sugars to the increase in osmotic value of the medium caused by the sugars added. Our results are in contradiction to this notion. In addition lowering the glucose concentration from 200 to 20 mM does not result in a decrease in the Na^+ efflux rate, though at 20 mM glucose the osmotic value is much lower than at 200 mM. This means that the extra Na^+ efflux found on adding glucose is dependent upon the metabolism. In this connection it may be relevant to remark that Rodríguez-Navarro et al. (14) have shown that there exists a good correlation between the efflux rate of the analogous cation Li^+ and the ATP content of the cells.

The values of E_{Na}, see Table I, are much less negative than those found for E_K under comparable conditions in normal cells, see Chapter VI. Since Na^+ can be accumulated into the cell via the same influx mechanism as K^+ (15 and references therein), these Tables clearly show that one or more Na^+ efflux mechanisms are operating in the yeast. We will now discuss which of the possible models summarized in fig. (1) may be involved in the Na^+ extrusion from the cells.

Na^+ efflux according to model (3) should decrease when the membrane potential becomes more negative, which would be expected on starting metabolism, see Chapter III. Na^+ efflux according

to model (1) will be stimulated by adding glucose since the efflux of anions is metabolism-dependent (16). Na^+ efflux according to model (2) will also be stimulated by glucose since glycolysis will in general lead to an increase in cell pH. Na^+ efflux according to models (4) and (7) will also be stimulated and Na^+ efflux according to model (5) must be dependent upon the metabolism since the K^+ gradient is very unfavourable and cannot be the driving force for Na^+ efflux. Since at the onset of metabolism the rate of Na^+ efflux increases, model (3) should be rejected.

The effect of changes in the medium pH upon the Na^+ efflux may be both direct and indirect. An increase in medium pH should directly affect Na^+ efflux according to a neutral H^+/Na^+ antiport (model (2)), unless the affinity of the protons to the transport system is very high. This makes the involvement of Na^+ efflux according to model (2) rather improbable. This may also apply to Na^+ efflux according to model (7). However, in the latter case the reduction in the rate of Na^+ efflux expected may be compensated at least partially by the increase in the negative membrane potential occurring on increasing the medium pH, see Chapter VI. The driving force for Na^+ efflux according to model (7) consists of two components: ΔE the membrane potential between cells and medium and $E_H = -RT/F \ln H_i/H_o$, which depends upon the pH difference between cells and medium. H_i and H_o are the proton activity in cells and medium respectively. When ΔE becomes more negative and H_i/H_o increases, that means E_H decreases, Na^+ ions will be expelled more rapidly. Since two protons are involved in the Na^+ efflux, the driving force will be proportional with $2E_H-\Delta E$. As seen in Table I E_H increases greatly on decreasing the medium pH from pH 7 to pH 4.5. When the contribution of the pH gradient to the driving force for Na^+ efflux will be compensated by a comparable reduction in the negative membrane potential, the yeast cell membrane should be depolarised for 200 mV on decreasing the medium pH from pH 7 to pH 4.5, which is much more than was found in normal K^+-loaded cells, see Chapter VI. Therefore the in-

volvement of model (7) in Na^+ efflux is also not very likely. Neither a pH-independent Na^+ efflux rate nor a pH-independent Na^+ steady-state distribution as are found experimentally, see Table I, would be expected according to this efflux model.

According to Ryan and Ryan (17) Na^+ efflux can also be influenced indirectly by changes in the medium pH namely via changes in the cell pH. This is not found by us. According to Rothstein (9) competition of protons and Na^+ for a single efflux mechanism for both Na^+ and H^+ may underly the effect of the cell pH upon Na^+ efflux. Our results, however, do not confirm this view. On the other hand the absence of an effect of the cell pH upon both Na^+ efflux rate and Na^+ steady-state distribution may be in accordance with the concept of Conway et al. (2,4) of a symport of cellular anions and Na^+ ions, see model (1). It is also possible that an electrogenic Na^+ uniport is involved. This uniport, however, should then be specific for Na^+ as stated above, since there are no indications for a competition between Na^+ ions and protons. In addition there are no indications (until now) that the yeast membrane ATPase, which is supposed to be involved in proton translocation across the yeast cell membrane from cell to medium, is stimulated specifically by Na^+ ions, as would be expected if this system is also involved in Na^+ efflux.

Addition of K^+ should enhance the rate of Na^+ efflux according to models (5) and (6), which has actually been found (2,3,5). According to model (5) addition of K^+ will not influence the membrane potential whereas according to model (6) addition of K^+ will lead to changes in ΔE, depending upon the ratio of n and m. Figs. (3) and (4) show that Na^+ is extruded from cells, which contain approximately 170 mM Na^+ into media containing both 25 mM K^+ and 250 mM Na^+, while the membrane potential is still negative (-47 ± 5 mV). This means that efflux of Na^+ in exchange for K^+ is an active process proceeding against the electrochemical Na^+-gradient. The ratio of Na^+ and K^+ fluxes is approximately 1 and since no changes in membrane potential are observed upon addition of K^+, the fluxes are probably tightly coupled. Therefore a neutral K^+/Na^+ exchange

mechanism may be involved, model (5). It might be hypothesized that this system is energized by ATP. However, until now there are no indications for a plasma membrane bound ATPase, which is synergetically stimulated by Na^+ and K^+. This points to the involvement of more than one Na^+ efflux mechanism, one Na^+ efflux mechanism operating in the absence of added K^+, and one Na^+ efflux mechanism consisting of a K^+/Na^+ antiport. This view is further supported by the finding that DES, an inhibitor of the yeast plasma membrane ATPase and, as shown in Chapter V, also of the Rb^+ uptake, inhibits that part of the Na^+ efflux which is due to Rb^+/Na^+ exchange, but not the Na^+ efflux in the absence of added Rb^+. This is in agreement with the findings of other authors. Uncouplers inhibit only Na^+ efflux in exchange of K^+, but not Na^+ efflux in the absence of added K^+ (3,5,18). In addition DES and an other inhibitor of the membrane ATPase DCCD (see Chapter V) only partially inhibit efflux of the analogous cation Li^+ in a K^+-rich medium (14). On the other hand at this stage of knowledge we cannot explain why DES inhibits Rb^+ uptake for only 53%, whereas the Rb^+-stimulated Na^+ efflux is completely inhibited.

In *Escherichia coli* Robillard and Konings (19) found that oxidation-reduction reactions play an important role in transport processes. This may rehabilitate the old theories of Conway et al. (2,4) that the efflux of Na^+ is mediated by a single redox carrier system, which is coupled indirectly with either anion efflux or K^+ influx by transferring electrons between the Na^+ carrier and the two other carriers. In accordance with this hypothesis Conway et al. (2,3) found, that succinate efflux is reduced on adding K^+ whereas Na^+ efflux is stimulated. This is confirmed by us, see fig. (5). However, the recovery of anion efflux, observed approximately 20 min after the addition of K^+ cannot be explained (until now) by the carrier competition model of Conway.

In conclusion, Na^+ efflux from yeast cells into a K^+-poor medium is dependent upon metabolism. Our observations indicate that this Na^+ efflux may be due to either an electrogenic Na^+ pump, which is specific to Na^+ (model (4)) or to a Na^+-anion

symport (model (1)). A mechanism as proposed by Rodríguez-Navarro et al. (10,14), which consists of a non-electroneutral antiport of protons and Na^+ (model (7)) is less likely involved in Na^+ efflux. This also refers to a possible electroneutral H^+/Na^+ antiport (model (2)). In the presence of K^+ an additional Na^+ efflux seems to be involved consisting of a K^+/Na^+ antiport, which is presumably electroneutral and which is an active pump system.

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CHAPTER VIII

SUMMARY/SAMENVATTING

Much information about the way in which solutes are translocated across the membrane has been obtained from measurements and analysis of the relation between the initial uptake rate (v) into the cells and the concentration of the solute (s) being transported. Information about the way in which solute transport is energized is only scarcely abailable because of a lack of knowledge of the exact value of the membrane potential. In Chapter I it is described in which way the membrane potential might play a role in solute translocation.

In this thesis three aspects of solute transport have been examined for the yeast *Saccharomyces cerevisiae*. In Chapter II the influx rate of Rb^+ at high Rb^+ concentrations is investigated. In Chapters III V, VI and VII the role of the membrane potential in solute transport was examined. In Chapter IV the regulation of the Rb^+ influx rate during Rb^+ uptake was investigated.

Since there were indications that at high Rb^+ concentrations concave deviations in the Rb^+ Hofstee plot can be found the Rb^+ influx rate at high Rb^+ concentrations was examined more closely, see Chapter II. Depending upon the method of analysing the uptake data (linear or non-linear regression) either no deviations or concave deviations can be found in the Rb^+ Hofstee plot at high Rb^+ concentrations. It appeared that non-linear regression gave the most reliable results and it was concluded therefore that in the Rb^+ uptake in yeast probably three binding sites are involved simultaneously. The possibility that the third site is only apparent being a reflection of the decrease in the surface potential is also considered.

Because direct measurements of the membrane potential in *S.cerevisiae* yielded no reliable results, measurements of the membrane potential have to be relied to the use of lipophilic cations. In Chapter III it was studied whether the equilibrium distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) can be used to measure the membrane potential. The equilibrium potential of TPP^+ seems to reflect the membrane potential of yeast cells in a qualitatively correct way. On

changing the experimental conditions the equilibrium potential of TPP^+ varies according to the expected changes in membrane potential. There are, however, some disadvantages in the use of this probe. First, TPP^+ uptake has a slow response to changes in the membrane potential and secondly, TPP^+ may bind to cellular components giving rise to an overestimation of the negative membrane potential, see Chapter VI. The membrane potentials calculated from the equilibrium potential of TPP^+ need to be corrected with approximately 43 mV. We have applied our knowledge about the membrane potentials of the yeast for the examination of the energization of cation transport in more detail. In Chapters V, VI and VII some aspects of the energization of K^+, Rb^+ and Sr^{2+} uptake and Na^+ efflux have been examined.

In Chapter V the effect of inhibitors of the yeast plasma membrane ATPase upon cation transport and membrane potential was examined. The main results can be summarized as follows:

a) Inhibitors of the yeast plasma membrane ATPase induce K^+ efflux from the cells. The effect of low concentrations of inhibitors upon K^+ movements point to all-or-none effect which probably differ for the various inhibitors applied and which will seriously complicate the interpretations of the results.
b) Due to all-or-none effect, provoked by the various inhibitors, the direction of the effects of the inhibitors (stimulation or inhibition) upon Rb^+ or Sr^{2+} uptake and upon the membrane potential as estimated from the TPP distribution may be different.
c) Upon addition of the inhibitors no changes in cell pH are detected. This means that changes in the influx rates are only a reflection of changes in the membrane potential irrespectively whether the energization is performed by the pmf of by the membrane potential alone.

In Chapter VI it has been investigated whether the K^+ uptake represents secondary active transport or not. Convincing evidence was obtained that the membrane potential is not large enough to account for the observed K^+ accumulations. Also a co-transport of one K^+ ion with one proton has been excluded,

provided that the binding of TPP^+ in intact cells is of the same magnitude as the binding of TPP^+ in permeabilized cells. The energy available for a cotransport of one K^+ ion with two protons is approximately high enough to account for the observed K^+ accumulations, but kinetical arguments for this type of transport have never been reported. It is concluded, that K^+ uptake may proceed as a secondary active transport consisting of a cotransport of one K^+ ion with two protons or as a primary active transport consisting of a K^+ uptake mechanism which is directly energized by ATP.

In Chapter VII some aspects of Na^+ efflux from yeast have been examined. It appeared that the Na^+ efflux is metabolism-dependent and that both the Na^+ efflux rate and Na^+ steady-state distribution are not dependent upon changes in medium pH. This means that Na^+ efflux according to a neutral H^+/Na^+ or non-electroneutral H^+/Na^+ type of exchange is not very likely. As far as Na^+ efflux in a K^+-poor medium is considered we conclude that Na^+ efflux may be due to a Na^+-anion symport mechanism or to an electrogenic Na^+-pump, although for the latter model no evidence exists until now. Na^+ efflux into a K^+-rich medium proceeds against the electrochemical Na^+ gradient and is probably due to an electroneutral K^+/Na^+ exchange mechanism.

Although many studies have dealt with the monovalent cation transport mechanism itself, much less is known about the regulation of the monovalent cation uptake. In Chapter IV the regulation of the Rb^+ influx rate during Rb^+ uptake has been examined. It appeared that the Rb^+ influx rate decreased during Rb^+ uptake and that a so-called "constant-pump and leak" mechanism is not operating. It has been excluded that the decrease in the Rb^+ influx rate is due to changes in the rate of glycolysis, changes in the cellular osmotic value, depolarisation of the cell membrane or to a decrease in the pmf. The decrease in the rate of Rb^+ influx can be explained by assuming that the Rb^+ carrier is an allosteric carrier of which the conformational state depends upon the cellular cation content. According to this hypothesis, during Rb^+ uptake the state of the carrier changes, which results in a decreased influx rate.

In dit proefschrift zijn drie aspecten bestudeerd van de wijze waarop stoffen over de cel membraan van de gist *Saccharomyces cerevisiae* kunnen worden getransporteerd. In Hoofdstuk II is de initiële opname snelheid van Rb^+ bij hoge Rb^+ concentraties bestudeerd. In de Hoofdstukken III, V, VI and VII is nagegaan wat de rol van de membraan potentiaal bij het transport van verschillende stoffen is. In Hoofdstuk IV is gekeken naar de regulatie van de Rb^+ opname snelheid tijdens de Rb^+ opname.

Omdat er aanwijzingen waren dat er bij hoge Rb^+ concentraties concave afwijkingen in de Hofstee plot van de Rb^+ opname gevonden kunnen worden, is er nader gekeken naar de initiële Rb^+ opname snelheid bij hoge Rb^+ concentraties. De verkregen resultaten zijn afhankelijk van de manier waarop de opname gegevens werden geanalyseerd. Het blijkt dat, als de opname gegevens geanalyseerd worden m.b.v. niet-lineaire regressie, de meest betrouwbare resultaten verkregen worden. Wij hebben dan ook geconcludeerd dat de Rb^+ opname in gist beschreven kan worden door een mechanisme met drie bindingsplaatsen, die gelijktijdig werken. Mogelijk is de derde bindingsplaats slechts schijnbaar en wordt de concave afwijking in de Hofstee plot bij de hoge Rb^+ concentraties veroorzaakt door een daling in de oppervlakte potentiaal.

Wil men meer inzicht verkrijgen over de wijze waarop het transport van stoffen van energie wordt voorzien, dan is kennis van de membraan potentiaal onontbeerlijk, zie Hoofdstuk I. Aangezien de membraan potentiaal van gist niet direct kan worden bepaald met behulp van microelectroden, is gezocht naar een indirecte methode. In Hoofdstuk III is nagegaan of de verdeling van de lipofiele quarternaire phosphoniumbase TPP^+ gebruikt kan worden om de membraan potentiaal te meten. Van dit kation wordt aangenomen, dat het ten gevolge van zijn lipofiele eigenschappen in staat is zich te verdelen tussen het cytoplasma en het medium volgens de Nernst-vergelijking. Het blijkt, dat de evenwichtspotentiaal van TPP^+ de membraanpotentiaal op een kwalitatief juiste wijze weergeeft. Er zijn echter ook nadelen verbonden aan

het gebruik van TPP^+ voor meting van de membraan potentiaal. In de eerste plaats verandert de evenwichts potentiaal van TPP^+ maar langzaam als de membraan potentiaal veranderd. In de tweede plaats kan TPP^+ aan cel bestanddelen binden waardoor de membraan potentiaal wordt overschat, zie Hoofdstuk VI. Dit laatste houdt in, dat de membraan potentiaal met ongeveer 43 mV moet worden gecorrigeerd. De methode van de membraan potentiaal bepaling is toegepast in de Hoofdstukken V, VI en VII, waarin enige aspecten van de energetisatie van de K^+, Rb^+ en Sr^{2+} opname en Na^+ uittree zijn bestudeerd.

In Hoofdstuk V is het effect van remmers van het plasma membraan ATPase op het kationen transport en de membraan potentiaal nagegaan. De belangrijkste resultaten kunnen als volgt worden samengevat:

a) Remmers van het membraan ATPase induceren K^+ uittree. Het effect van lage remmer concentraties wijst op een alles-of-niets effect wat betreft K^+ uittree, en bemoeilijkt de interpretatie van de resultaten aanzienlijk.
b) Door dit alles-of-niets effect is het effect van de verschillende remmers of de Rb^+ en de Sr^{2+} opname, en op de membraan potentiaal moeilijk te interpreteren.
c) Na toevoeging van de remmers worden er geen veranderingen van de cell pH gemeten. Dit betekent dat veranderingen in de opname snelheden alleen kunnen komen door veranderingen in de membraan potentiaal, ongeacht of het transport wordt geenergetiseerd door de proton drijvende kracht (pmf) of alleen door de membraan potentiaal.

In Hoofdstuk VI is nagegaan hoe de K^+ opname geënergetiseerd kan worden. Het blijkt dat de membraan potentiaal niet groot genoeg is om de gevonden K^+ verdeling te verklaren. Wij concluderen in dit Hoofdstuk, dat de K^+ opname geënergetiseerd kan worden door een cotransport van één K^+ ion met twee protonen ofwel dat de K^+ opname direct van energie wordt voorzien door ATP, hetzij via een electrogeen danwel via een electroneutraal mechanisme.

In Hoofdstuk VII zijn enige aspecten van de Na^+ uittree in gist bestudeerd. Het blijkt dat de Na^+ uittree afhankelijk is

van het metabolisme van de cel en dat de Na^+ uittree en Na^+ verdeling onafhankelijk zijn van veranderingen van de medium pH. Dit betekend, dat Na^+ uittree via hetzij een electroneutraal of niet-electroneutraal H^+/Na^+ antiport mechanism minder waarschijnlijk is. Wij concluderen dat de Na^+ uittree in een K^+-arm medium verklaard kan worden via een Na^+-anionen symport dan wel via een electrogene Na^+-pomp, hoewel voor dit laatste model geen direct bewijs bestaat. Na^+ uittree in een K^+-rijk medium gaat tegen de electrochemische Na^+ gradient in en kan verklaard worden door een electroneutraal K^+/Na^+ antiport mechanisme.

Hoewel er veel bekend is over het monovalente kationen opname mechanisme is er maar weinig bekend over de wijze, waarop de monovalente kationen opname wordt gereguleerd. In Hoofdstuk IV is de regulatie van de initiële Rb^+ opname snelheid tijdens de Rb^+ opname bestudeerd. Het blijkt dat de Rb^+ opname snelheid afneemt tijdens de Rb^+ opname en dat een z.g. "pomp en lek" mechanisme niet werkzaam is. Het is uitgesloten dat de afname in de Rb^+ opname snelheid veroorzaakt wordt door veranderingen in de snelheid van glycolyse, veranderingen in de osmotische waarde van de cel, depolarisatie van de cel membraan of door een afname van de pmf. Door aan te nemen, dat de Rb^+ carrier een allosterische carrier is, kan de afname van de Rb^+ opname snelheid verklaard worden. Volgens deze hypothese verandert tijdens de Rb^+ opname de conformatie toestand van de carrier door een toename van de kationen concentratie in de cel, waardoor de Rb^+ opname snelheid afneemt.

CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 17 juni 1954 te Velp (Gld). Na het behalen van het diploma HBS-b aan het Rhedens Lyceum te Velp, studeerde hij scheikunde aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S_2) werd in juli 1974 afgelegd. Het doctoraalexamen met als hoofdvakken Farmacochemie (o.l.v. Prof. Dr. J.M. van Rossum en Dr. T.D. Yih) en Biofysische Chemie (o.l.v. Prof. Dr. G.A.J. van Os en Dr. S.H. de Bruin) werd in oktober 1977 afgelegd.

Van 1 november 1977 tot 1 mei 1982 was hij als wetenschappelijk medewerker verbonden aan het Laboratorium voor Chemische Cytologie. In deze periode werd het in dit proefschrift beschreven onderzoek verricht, en werd daarnaast een bijdrage geleverd aan het onderwijs aan biologie studenten. Thans is hij op zoek naar een andere werkkring.

In september 1975 is hij getrouwd met Elja Vonk. Hun zoon Olaf werd in september 1978 geboren, hun dochter Sanne in april 1981.

STELLINGEN

I

De stelling van Rodríguez-Navarro et al. dat de Li^+ efflux in gist gedreven wordt door de pmf is onvolledig.

Rodríguez-Navarro, A., E.D. Sancho and C. Pérez-Lloveres (1981) Biochim. Biophys. Acta 640, 352-358

Dit proefschrift

II

Het is een onjuiste veronderstelling van Bianchi et al., dat de Rb^+ verdeling in gist een indicatie is voor de membraanpotentiaal.

Bianchi, M.E., M.L. Carbone and G. Lucchini (1981) Plant Science Letters 22, 345-352

Dit proefschrift

III

De interpretatie van resultaten, verkregen met remmers van het plasmamembraan ATPase van gist dient met grote voorzichtigheid te gebeuren.

Borst-Pauwels, G.W.F.H., A.P.R. Theuvenet and A.W. Boxman (1983) Proc. FEMS Symposium, in press, Pushchino, USSR

IV

De binding van TPP^+ in gedeenergetiseerde *Rhodopseudomonas sphaeroides* cellen, berekend door Lolkema et al., is niet correct en dient gecorrigeerd te worden voor de Donnan potentiaal.

Lolkema, J.S., K.J. Hellingwerf and W.N. Konings (1982) Biochim. Biophys. Acta 681, 85-94

V

In studies met radioactieve tracers heeft het de voorkeur de resultaten weer te geven in dpm (desintegrations per minute) in plaats van in cpm (counts per minute), tenzij de efficientie duidelijk wordt aangegeven.

VI

De toevoeging van "wilde extracten" aan cosmetische producten doet het ergste vrezen voor de flora.

VII

Haast je als je tijd hebt, dan heb je tijd als je haast hebt.

VIII

Het gezegde "wat niet weet, wat niet deert" is niet van toepassing op het illegaal storten van chemisch afval.

IX

Het moet voorkomen worden dat werkloze academici buiten kennis raken.

A.W. Boxman

Nijmegen, 19 mei 1983