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POSSIBLE ENERGIZATION OF K^+ ACCUMULATION INTO METABOLIZING YEAST BY THE PROTONMOTIVE FORCE

BINDING CORRECTION TO BE APPLIED IN THE CALCULATION OF THE YEAST MEMBRANE POTENTIAL FROM TETRAPHENYLPHOSPHONIUM DISTRIBUTION

A.W. BOXMAN, J. DOBBELMANN and G.W.F.H. BORST-PAUWELS *

Department of Chemical Cytology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen (The Netherlands)

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Membrane potentials of yeast cells, *Saccharomyces cerevisiae*, calculated from the equilibrium distribution of tetraphenylphosphonium (TPP) between cell-water and medium should be corrected for a contribution due to binding of TPP to intracellular constituents. The magnitude of this correction depends upon the way in which it is determined. In cells permeabilized by boiling, cell-binding is much higher than in cells permeabilized by repeated freezing and thawing. The binding corrections are 75 ± 1 mV and 49 ± 7 mV, respectively. The binding correction obtained from TPP distribution between deenergized cells and medium is much lower and amounts to 19 ± 9 mV. The latter value is probably more reliable. It is supposed that permeabilization of the cells by boiling or repeated freezing and thawing unmasks potential TPP binding groups in the cell. The K^+ accumulation into anaerobically metabolizing yeast cells can be accounted for almost quantitatively by a cotransport of protons and K^+ ions if the lower binding correction is applied. This means that K^+ accumulation into the yeast cell may be driven by the sum of the protonmotive force and the membrane potential.

Introduction

K^+ can be accumulated into yeast to a very high extent. Accumulation ratios amounting to 10000 or more can be found [1,2]. Membrane potentials calculated from the equilibrium distribution ratio of TPP between cells and medium are much too low in order to account for the K^+ accumulation if K^+ uptake is a passive process. For this reason Goffeau et al. [1] hypothesized that the K^+ accumulation is coupled to ATP hydrolysis catalyzed by the yeast plasmamembrane ATPase. It is assumed that this ATPase functions as an electrogenic K^+/H^+ antiport. The possibility that a neutral K^+/H^+ antiport is involved [3] may also be considered. On the other hand there are several indications that K^+ uptake into yeast is driven by the membrane potential [4,5]. Recently we considered the possibility that K^+ uptake still consists of a passive process, namely of a cotransport of one proton and one K^+ ion [2]. Both in *Neurospora crassa* and in bacterial cells there are indications for the involvement of such a system [6,7]. We have now examined whether the distribution of K^+ between metabolizing cells and medium can be accounted for by such a model. Membrane potentials are calculated from the equilibrium distribution of the lipophilic cation TPP. Since TPP may be bound to intracellular constituents [8] we also examined to what extent TPP is bound to the yeast cells. Yeast cells with a low phosphate con-

* To whom correspondence should be addressed.
Abbreviations: TPP, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

tent are used in order to minimize possible internal sequestering of K^+ to polyphosphates [9].

Methods

4% (w/v) Yeast, *S. cerevisiae*, Delft 2, was aerated for 24 h in distilled ater, washed twice with distilled water and resuspended in buffer supplemented with 5% glucose. The buffer consisted of 45 mM Tris brought to the desired pH with either succinic acid or Hepes. The cells did not contain a detectable amount of polyphosphates as shown by ^{31}P-NMR. Uptake of ^{14}C-labelled TPP at 25°C was started after a 1 h preincubation of the cells with N_2 bubbling through the suspension. Uptake of TPP was studied as described in Ref. 10, i.e., by filtrating 1.8 ml yeast samples through filter papers and determining the radioactivity adhering to the papers. Where necessary, corrections for quenching were applied. Apparent membrane potentials were calculated from the equilibrium distribution ratio of TPP between the cell-water and the medium. The cell-water was taken as 1.52 ml/g dry weight [11].

Uptake of TPP in non-metabolizing yeast was determined as follows: 2% (w/v) yeast was aerated for 24 h in distilled water, washed and resuspended in 45 mM Tris-succinate, pH 6.4, supplemented with 15 mM deoxyglucose and 15 μM antimycin for 18 h with nitrogen bubbling through the suspension. Then 0.1 mM nystatin with or without 50 μM CCCP was added and 1 h later both 250 mM KCl and radioactive TPP were added. Uptake of TPP was followed for 7 h. Efflux of TPP from non-metabolizing yeast cells was studied with cells treated in a similar manner. The cells, however, were preloaded with ^{14}C-labelled TPP during the 18-h incubation with deoxyglucose and antimycin. After addition of nystatin, efflux was studied by determining at appropriate times both the radioactivity of the supernatant of the yeast cells and the radioactivity of the yeast cells sampled on filter papers. 1 h after the addition of nystatin 250 mM KCl was added and efflux was determined during the 7 h.

Permeabilized cells were obtained either by repeated (ten times) freezing and thawing 40% (w/v) cells or by boiling the cells for 1 min. Distribution of labelled compounds between permeabilized cells or intact cells and the medium was determined by adding carrier-free radioactive compound to 3 or 5% (w/v) cells and by measuring the radioactivity of 1 ml of the supernatant (AS) obtained after spinning down the cells and by measuring the radioactivity of the residue (AR) obtained after carefully removing the supernatant. The dry weight of the residue (DW) as well as the wet weight of the yeast (WW) were determined. The water content (WC) was calculated from $WC = WW - DW$. The experiments were carried out in 50 mM Hepes buffer brought to the desired pH with Tris and in the presence of 250 mM KCl.

The amount of non-solvent water of intact cells per gram dry weight (V_i) was calculated from the distribution of [^{14}C]mannitol added in tracer amounts between the yeast residue and the supernatant:

$$AR/AS \cdot DW = WC/DW - V_i \quad (1)$$

The Donnan ratio for monovalent cations between cell walls and medium (r_{cw}) was calculated from the distribution of $^{86}Rb^+$ between intact non-metabolizing cells and the medium:

$$AR/AS \cdot DW = WC/DW - V_i + V_{cw}(r_{cw} - 1) \quad (2)$$

V_{cw} is the volume of the water space of the cell wall per gram dry weight. This value was taken to be equal to 0.23 V_i, corresponding with 10% of the wet weight of the cells as described in Ref. 12.

The Donnan ratio of a monovalent cation between the interiors of permeabilized cells and the medium (r_i) was calculated from the distribution of $^{86}Rb^+$ between permeabilized cells and medium:

$$AR/1.5AS \cdot DW = WC/1.5DW + V_{cw}(r_{cw} - 1) + V_i(r_i - 1) \quad (3)$$

The dry weights were multiplied by 1.5, since after permeabilizing the cells, the dry weight was reduced by a factor of 1.5. The adsorption coefficient for binding of TPP to the cell wall was calculated from the distribution of TPP between intact non-metabolizing cells and medium at 0°C in order to prevent as much as possible uptake of TPP into the cells. The distribution is given by:

$$AR/AS \cdot DW = WC/DW - V_i + V_{cw}((f_{cw} + 1)r_{cw} - 1) \quad (4)$$

The adsorption coefficient of cell-water for binding of TPP to intracellular constituents (f_i) was calculated from the distribution of TPP between permeabilized cells and medium as follows

$$AR/1.5AS \cdot DW = WC/1.5DW + V_{cw}((f_{cw}+1)r_{cw}-1) + V_i((f_i+1)r_i-1) \quad (5)$$

The Donnan ratio of a monovalent cation between cells treated with nystatin and the medium was calculated from the distribution of $^{86}Rb^+$ between cell-water and medium from cells preincubated for 17 h with 15 mM deoxyglucose and 15 μM antimycin and an additional hour also with 0.1 mM nystatin. Then 250 mM KCl was added together with carrier-free ^{86}RbCl. r_i was calculated from:

$$AR/AS \cdot DW = WC/DW + V_{cw}(r_{cw}-1) + V_i(r_i-1) \quad (6)$$

pH_i was determined as described in Ref. 11. After washing the cells and then destroying them with nitric acid, the K^+ concentration of the medium and that of the cells was determined by means of flame spectrophotometry.

^{14}C-labelled TPP, [^{14}C]mannitol, ^{22}NaCl and ^{86}RbCl were purchased from Amersham International, U.K. Nystatin and deoxyglucose were from Sigma, St. Louis, MO, U.S.A., CCCP was from Aldrich, Milwaukee, WI, U.S.A., and antimycin was from Boehringer, Mannheim, F.R.G. Nystatin was dissolved in propane-1,2 diol and CCCP in ethanol. Final concentrations of solvent did not exceed 1% (v/v).

Results

Before determining the membrane potentials of the yeast cell, we first examined to what extent TPP is bound to cellular constituents. The distribution of TPP between permeabilized cells and medium is governed by several factors. At first TPP is accumulated into the cell walls. This accumulation depends upon both the Donnan potential between cell walls and medium and upon the binding capacity of the cell wall for TPP. Sec-

TABLE I

VALUES APPLIED IN THE CALCULATION OF THE BINDING CORRECTIONS DUE TO TPP BINDING TO INTRACELLULAR CONSTITUENTS

V_i is the amount of cell-water per gram dry weight. r_{cw} and r_i are the Donnan ratios for $^{86}Rb^+$ between the cell-wall space and the medium and the cell interior and the medium, respectively. $E_{D,cw} = -(F/RT)\ln r_{cw}$ and $E_{D,i} = -(F/RT)\ln r_i$ are the corresponding Donnan potentials expressed in mV. f_{cw} and f_i are the adsorption coefficients of TPP referring to the cell wall and the cell interior, respectively. $E_{corr} = (F/RT)\ln(1+f_i)$ in mV is the correction term to be applied in the calculation of the membrane potential from the equilibrium distribution of TPP between cells and medium. All experiments are carried out in the presence of 250 mM KCl at pH_0 6.5. Intact cells are non-metabolizing cells with which uptake of $^{86}Rb^+$ into the cell walls for the determination of the Donnan potential between cell wall and medium and of ^{14}C-labelled TPP for the binding of TPP to the cell walls is studied. Deenergized cells are non-metabolizing cells in which the efflux and uptake of TPP into the cell inner is studied in the presence of 0.1 mM nystatin, 15 μM antimycin and 15 mM deoxyglucose with or without 50 μM CCCP (see also Fig. 1). Permeabilized cells are cells permeabilized by either boiling or 10-times freezing and thawing (10×(fr.+th.)). E_M is the apparent membrane potential in mV calculated from the rates of TPP efflux and influx according to Eqn. 7. Figures between brackets are standard deviations.

	Intact cells	Permeabilized cells		Deenergized cells
		boiling	10×(fr.+th.)	
V_i	1.35(0.09)			
r_{cw}	1.83(0.24)			
$E_{D,cw}$	−15.4 (3.61)			
f_{cw}	2.38(0.55)			
E_M				−28.7 (5.3)
r_i		1.14(0.05)	1.17(0.06)	1.46(0.19)
$E_{D,i}$		−3.34(1.14)	−4.00(0.22)	−9.7 (3.5)
f_i		17.6 (0.4)	5.7 (1.4)	2.1 (0.6)
E_{corr}		75 (1)	49 (7)	19.0 (8.8)

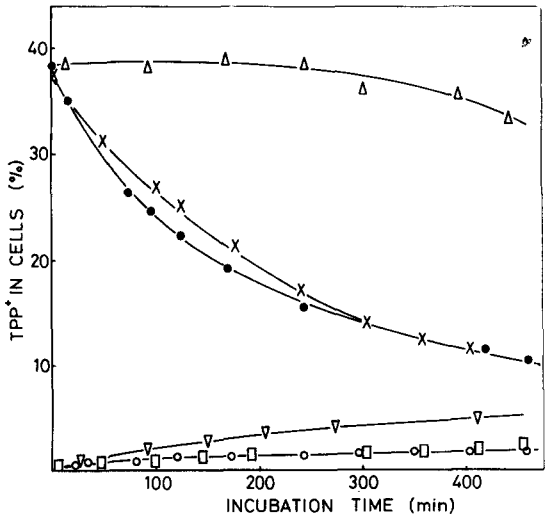

Fig. 1. Uptake and efflux of TPP in non-metabolizing cells in the presence of 250 mM KCl. Effect of nystatin and CCCP. Uptake (▽, □, ○); efflux from cells preloaded with radioactive TPP (△, x, ●); no extra addition (△, ▽); 0.1 mM nystatin (●, ○); 0.1 mM nystatin and 0.05 mM CCCP (□, x). The experiments are carried out at pH_0 6.5 in the presence of 15 μM antimycin and 15 mM deoxyglucose.

ondly, TPP is accumulated into the cell inner. This accumulation depends upon the Donnan potential between cell inner and medium, and upon the binding capacity of the cell constituents. The various factors are calculated as described under Methods. Table I shows that the Donnan ratio of monovalent cations between cell wall and medium is larger than that between cell interior and of permeabilized cells and medium. There is an appreciable binding of TPP to the cell walls. The adsorption coefficient for binding of TPP to the intracellular constituents depends upon the way in which the cells are permeabilized. Permeabilization of the cells by boiling gives rise to a much greater binding than permeabilization by repeated freezing and thawing. Repeated freezing and thawing renders the cells completely permeable to small molecules [11].

Apparently the adsorption capacity of the yeast cells for TPP depends upon the way in which the cells are permeabilized. Therefore, we have tried to determine the adsorption coefficient of TPP in cells which are still intact but which are deenergized as much as possible. Residual metabolism of cells exhausted by aeration is stopped by adding both deoxyglucose and antimycin [13]. The diffusion potential between cells and medium is reduced by equalizing both the K^+ concentrations of the cell (250 mM) and the medium, and pH_i and pH_o (pH 6.5). These cells show a greatly reduced uptake of TPP. However, efflux of TPP from cells loaded overnight with carrier-free labelled TPP is negligibly small (see Fig. 1). The efflux of TPP can be increased by adding the uncoupler CCCP (data not shown). Addition of nystatin, which increases the K^+ permeability of the yeast plasmamembrane enhances the TPP efflux still more and also decreases the influx rate. CCCP does not have a significant effect upon influx and efflux rates in the presence of nystatin. Even after an incubation period of 7 h, no equilibrium distribution of TPP between cells and medium is reached. The apparent membrane potential (E_M), however, can be calculated from the quotient of the efflux rate constant (k_{out}) and the influx rate constant (k_{in}) as follows:

$$E_M = (RT/F) \ln(k_{out}/k_{in}) \quad (7)$$

These rate constants are calculated from the rates of influx (v_{in}) and efflux (v_{out}) as follows:

$$v_{in} = k_{in}TPP_o \quad (8)$$

$$v_{out} = k_{out}TPP_i - k_{in}TPP_o \quad (9)$$

v_{in} is the initial rate of influx of TPP added together with 250 mM KCl 1 h after the addition of nystatin ± CCCP, and v_{out} is the net efflux rate of TPP from cells preloaded with labelled TPP during 1 night and refers to the moment that 250 mM KCl is added. TPP_o and TPP_i are the TPP concentrations in the medium and in the cell-water, respectively. The value of E_M is approximately the same in the presence or absence of 50 μM CCCP. The mean value is given in Table I. This value should still be corrected for the contribution of the Donnan potential in order to calculate the correction term to be applied for binding of TPP to intracellular constituents. The Donnan potential is calculated from the distribution of $^{86}Rb^+$ between the cell inner and the medium. Because of the presence of nystatin the cells are permeable to Rb^+. Rb^+ will be distributed passively between cells and medium. The Donnan potential is −9.7

mV (inside negative). On accounting for this Donnan potential the correction term for binding of TPP becomes 19 mV (see also Table I).

From the results of uptake experiments of TPP in metabolizing cells carried out at varying pH_o values, steady-state TPP accumulation ratios are determined. Decreasing the concentration of TPP applied twice did not lead to a significant change in the TPP accumulation ratio neither at pH_o 4.5 nor at pH_0 7.5 (data not shown). This shows that at the low TPP concentrations applied (0.5 μM) saturation of intracellular TPP binding groups is not involved. Fig. 2 shows the pH_0 dependence of the calculated membrane potentials obtained after application of the correction term accounting for binding of TPP to intracellular constituents. The membrane potentials become increasingly negative on increasing pH_0. The membrane potentials are much less negative than the K^+ Nernst potential (E_K) calculated from the distribution of K^+ between cell-water and medium as follows:

$$E_K = -(RT/F) \ln K_i/K_o \quad (10)$$

K_i and K_o are the K^+ concentration in the cell-water and medium, respectively. Possible differences in the value of the activity coefficient of the cell-water and the medium are neglected. The K^+ Nernst potential hardly depends upon pH_0. If one proton is cotransported with one K^+ ion the following relation will apply at equilibrium:

$$E_K = E_M + E_P \quad (11)$$

E_P is the protonmotive force expressed in millivolt equivalents, Fig. 2 shows that $E_M + E_P$ does not differ much from E_K. At pH_0 4.45 and 6.00 the differences are not mathematically significant, whereas at pH_0 6.84 and 7.30 the differences are 41 ± 23 mV and 38 ± 18 mV, respectively. The values are given with standard deviations.

We have also calculated the number of protons (n) which should be cotransported with one K^+ ion in order to account for the accumulation of K^+ found. n is given as follows:

$$n = (E_K - E_M)/E_P \quad (12)$$

n varies from 1.06 to 1.36 depending upon pH_0 (see Table II). Only at pH_0 6.84 and 7.30 does n differ significantly from 1. When no binding correction is applied in the calculation of the membrane potential, n does not differ significantly from 1 at pH_0 6.0 and higher, and is significantly lower than 1 at pH 4.45.

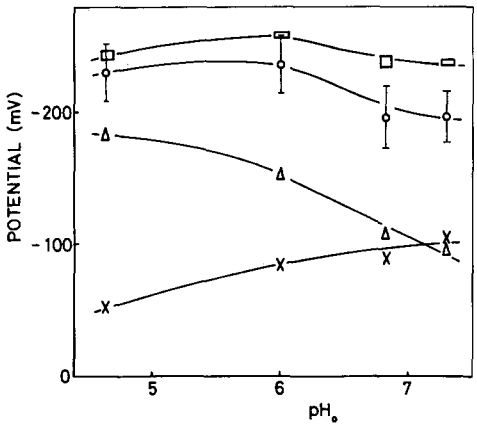

Fig. 2. pH_0 dependence of the K^+ Nernst potential, the membrane potential, the protonmotive force and the sum of the membrane potential and the protonmotive force. □, K^+ Nernst potential, the height of the symbol equals twice the standard deviation; ×, the membrane potential; △, the protonmotive force expressed in mV equivalents; and ○, the sum of the protonmotive force and the membrane potential, the height of the bars indicates the standard deviation. The values of the membrane potentials and the protonmotive force are corrected for intracellular binding of TPP by adding 19 mV to the values calculated.

TABLE II

STOICHIOMETRY OF PROTON-K^+ COTRANSPORT

The number of protons which should be cotransported with one K^+ ion is calculated by means of Eqn. 12. No binding means that no correction is applied for binding of TPP to intracellular constituents and binding means that a correction of 19 mV is applied for binding of TPP. Figures between brackets are standard deviations.

pH_0	n (no binding)	n (with binding)
4.45	0.87(0.06)	1.06(0.12)
6.00	0.91(0.08)	1.15(0.19)
6.84	1.03(0.12)	1.39(0.25)
7.30	0.98(0.06)	1.36(0.22)

Discussion

There are various indications that TPP can bind to cell constituents. Bakker [15] showed that the

partition coefficient of TPP between bacterial cells and medium greatly exceeds that of Rb^+. From his results a correction term to be applied in the calculation of the membrane potential from the equilibrium distribution of TPP amounting to 37 mV can be calculated. This value, however, is still overestimated, since no correction for binding of TPP at the outside of the cell membrane is applied. This is also true for the binding correction calculated by Maloney and Hansen [16] amounting to 47 mV. This value should, in addition, be corrected for the contribution of the Donnan potential. From the adsorption coefficient of TPP to intracellular bacterial constituents determined by Lolkema et al. [17] a correction term of 42 mV can be calculated. This value should also be corrected for the Donnan potential between cells and medium. The accumulation of TPP into the yeast *Rhodotorula gracilis* is much greater than that of TPMP [18]. Binding of TPMP to bacterial cells is negligible [19]. If this is also true for yeast cells, then the difference in membrane potential calculated from the equilibrium distribution of TPP and TPMP represents the correction term to be applied in the calculation of the membrane potential from the TPP distribution. This value is 24 mV for *R. gracilis* [18]. Unfortunately uptake of TPMP into the yeast cells used by us proceeds via the thiamin carrier [10] instead of by way of a simple diffusion process. The value of 24 mV found with *R. gracilis* is of the same order of magnitude as the value calculated in this publication from TPP uptake in deenergized cells, namely 19 mV. These values are somewhat lower than those found for bacterial cells but as pointed out above, the values found in bacterial cells are mostly still too high.

Both with yeast cells and bacteria the apparent binding capacity of the cells depends upon the way in which the cells are treated. Permeabilization of bacterial cells by boiling them gives rise to a greater adsorption of TPP than permeabilization with for example toluene [17]. In yeast cells boiling also increases the adsorption capacity greatly. Probably the rather gentle method of permeabilization of the cells by repeated freezing and thawing still gives rise to an increase in the adsorption coefficient, since the adsorption coefficient of TPP found with these cells is larger than the one calculated from the experiments with deenergized cells. Since the latter method is less invasive than the methods based on permeabilization of the cells, the binding correction obtained by this method will be more reliable.

Apparently the rate by which TPP distributes across the yeast cell membrane is greatly reduced by the addition of 250 mM KCl (see Ref. 20). This may be attributed, at least partly, to a reduction in the surface potential [21]. Because of this reduction in the rate of TPP fluxes across the yeast cell membrane, establishment of an equilibrium distribution of TPP between cells and medium takes a very long time.

The dependences of both the membrane potential and the protonmotive force upon pH_0 are in accordance with earlier findings [22–24]. The membrane potential is not high enough in order to account for the steady-state distribution of K^+ between cells and medium according to a model of passive distribution of K^+ without coupling to any other fluxes across the cell membrane. In addition, the dependence of the K^+ Nernst potential upon pH_0 is quite different from the dependence of the membrane potential upon pH_0. The K^+ Nernst potential hardly depends upon pH_0, whereas the membrane potential becomes more negative on increasing pH_0. In order to account for the high K^+ accumulation ratios Goffeau et al. [1] suggested that K^+ uptake into the yeast is driven by an electrogenic K^+/H^+-antiport mediated by the plasmamembrane ATPase of the yeast. Until now, however, there is no direct evidence that the membrane ATPase is also a transport ATPase [25]. Coupling of K^+ influx to H^+ influx provides an alternative model for the energization of K^+ accumulation. K^+ uptake is then driven by the sum of the membrane potential and the protonmotive force. This model is also considered to be involved in bacterial K^+ transport [7]. It would be a new example for the energization of solute transport by way of the protonmotive force [26]. If no binding correction is applied in the calculation of the membrane potential the sum of the membrane potential and the protonmotive force is high enough in order to account for the K^+ accumulation ratios found at all pH_0 values applied. Thus, to the values of $E_M + E_P$ in Fig. 2, -38 mV should be added. On applying the binding correction derived from the experiments with deen-

ergized cells, the sum of the membrane potential and the protonmotive force is still high enough in order to account for the K^+ accumulation at $pH_0 \leqq 6.0$, but at higher pH_0 values this sum is significantly lower than the K^+ Nernst potential. In accordance with this, the value of n, the number of protons which should be cotransported with one K^+ ion, is significantly higher than one above pH_0 6.0 (see Table II). It might be possible that part of the cellular K^+ is bound to cell constituents [9,27] and that therefore, the K^+ Nernst potentials are overestimated. In addition, the possibility that the binding correction applied by us is still too high should not be overlooked. Van den Broek et al. [29] showed for the yeast *Saccharomyces fragilis* that the equilibrium distribution of the sugar fucose can be accounted for almost quantitatively by a symport of protons and the sugar when no binding correction is applied in the calculation of the membrane potential from the TPP distribution. On the other hand, application of the binding correction found by us would lead to a discrepancy between the accumulation ratios found and the protonmotive force calculated.

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