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# PITFALLS IN THE MEASUREMENT OF MEMBRANE POTENTIAL IN YEAST CELLS USING TETRAPHENYLPHOSPHONIUM

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**The uptake of the lipophilic cation tetraphenylphosphonium ($Ph_4P^+$) by *Saccharomyces cerevisiae* was measured using yeast grown on glucose and harvested either at the logarithmic or at the stationary phase of growth. When yeast was collected at the stationary phase, $Ph_4P^+$ uptake proceeded steadily during several hours until an equilibrium was reached. When yeast was collected in the logarithmic phase of growth, a biphasic uptake was observed. The second phase of uptake began when the glucose of the incubation medium had been exhausted. From experiments in the presence of cycloheximide or chloramphenicol it is concluded that the second phase of $Ph_4P^+$ uptake is dependent on the synthesis of some protein(s) repressed by glucose but unrelated with the existence of functional mitochondria. The addition of compounds which collapse the membrane potential provokes an efflux from the yeast cells of the $Ph_4P^+$ accumulated both during the first phase and the second phase of uptake. It is concluded that accumulation of $Ph_4P^+$ in yeast cells is a complex process and that $Ph_4P^+$ cannot be used to give a quantitative measure of the yeast plasma membrane potential.**

## Introduction

Living cells are able to produce and maintain an electric potential across their membranes. In *Neurospora*, it has been shown, using microelectrodes, that different kinds of external perturbations result in a depolarization of the plasma membrane and in an increase of intracellular cAMP [1]. It has been suggested that a similar process occurs in yeast [2] although measurements of the membrane potential were not carried out. To investigate this point further, a reliable method for the measurement of the membrane potential was sought.

Most yeasts are too small to allow the use of a direct procedure involving microelectrodes [3] and therefore indirect methods have to be employed. These indirect techniques, however, are not without problems. Procedures based in changes in fluorescence of yeast suspensions equilibrated with cyanine dyes yield only qualitative results [4,5]. The measurement of the distribution of lipophilic cations at both sides of the plasma membrane is not reliable when the cations are taken up by a specific transport system, as reported for dibenzyldimethylammonium that crosses the yeast membrane through the thiamine carrier [6]. It has been reported [7] that the lipophilic cation tetraphenylphosphonium ($Ph_4P^+$) is not translocated via the thiamine transport system, and it was concluded that this cation, if applied at low concentrations, could be used to measure the membrane potential of *Saccharomyces cerevisiae*. We have found, however, that the method still shows unexpected com-

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plications and present in this paper results indicating that $Ph_4P^+$ cannot be used to obtain a quantitative measure of the membrane potential in yeast.

## Material and Methods

*Yeast strain and growth conditions.* *Saccharomyces cerevisiae* X2180 was grown on a rich medium with 1% yeast extract, 2% peptone and 2% glucose (YPD) and collected in the exponential (5–10 mg yeast wet weight/ml) or the stationary (25–30 mg yeast wet weight/ml) phase of growth.

*Measure of tetraphenylphosphonium uptake.* The yeast was harvested by centrifugation, washed twice with 45 mM Tris-succinate buffer (pH 7), and resuspended in the same buffer at a cell concentration of 20 mg (wet weight)/ml. The uptake of $Ph_4P^+$ was started by the addition of 5 nmol (33 $\mu$Ci/$\mu$mol) [U-$^{14}$C]$Ph_4P^+$ (Amersham) per ml incubation medium. At different times 100 $\mu$l samples were taken, received on 10 ml ice-cold 20 mM $MgCl_2$ [6] filtered by suction through GF/C Whatman glass-fiber filters and washed with 10 ml of ice-cold water. The filters were dried under an infrared lamp, suspended in toluene-based scintillation fluid and radioactivity was determined in a liquid scintillation counter. A correction was made for the radioactivity retained on the filters using samples in duplicate without yeast cells. To calculate the intracellular concentration of $Ph_4P^+$ it was accepted that 1 gram wet yeast contains 0.6 ml cell sap [8].

*Glucose determination.* Glucose in the medium was assayed enzymatically as described in Ref. 9.

## Results and Discussion

The uptake of the lipophilic cation $Ph_4P^+$ by *S. cerevisiae* collected in the stationary phase and suspended in buffer at pH 7 proceeds at a very slow rate (Fig. 1a). At pH 4 the equilibration time is similar but the final accumulation level is lower (12 $\mu$M at 5 $\mu$M external concentration of $Ph_4P^+$).When the yeast is preincubated with glucose there is an increase in the initial rate of uptake and the concentration of $Ph_4P^+$ reached at equilibrium is greater (Fig. 1b). The slow rate of uptake observed in these experiments is similar to

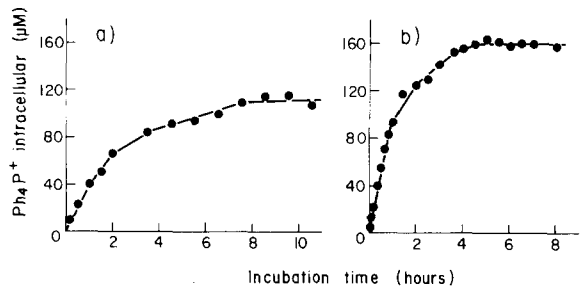


Fig. 1. Effect of glucose on $Ph_4P^+$ uptake by cells in the stationary phase of growth. Yeast was grown on YPD and collected during the stationary phase of growth. (a) Cells were incubated at 30°C in 45 mM Tris-succinate buffer (pH 7) during 15 min before the addition of 5 $\mu$M $Ph_4P^+$. (b) Cells were incubated at 30°C in 45 mM Tris-succinate buffer (pH 7) containing 2% glucose (w/v) during 60 min before the addition of 5 $\mu$M $Ph_4P^+$.

that reported by Boxman et al. [7]. However, in other laboratories equilibration times of 1–2 h have been found [10,11]. Since the experimental conditions were not very different it could be concluded that the rate of uptake of $Ph_4P^+$ is strongly dependent on the yeast strain used.

Experiments have also been carried out with yeast collected in the exponential phase of growth. As it can be seen in Fig. 2, in these conditions the uptake of $Ph_4P^+$ proceeds in two phases. In the absence of added glucose (Fig. 2a) only a very small amount of $Ph_4P^+$ is taken up in the first phase, after about one hour a steady slow uptake is observed and a new equilibrium is reached after 8 h. In the presence of glucose (Fig. 2b) the amount of $Ph_4P^+$ accumulated in the first phase is greater but otherwise the kinetics of uptake are very similar. These results are in accordance with those reported by De la Peña et al. [12]. However, those authors carried out only short-term experiments and therefore could not observe the second phase of uptake.

The biphasic uptake can be interpreted as the result of rapid unspecific binding of $Ph_4P^+$ to the cell membrane followed by a slow incorporation into the cell. To check this possibility we tested the effect of some positively charged compounds on the kinetics of $Ph_4P^+$ uptake. Choline and $Mg^{2+}$ were chosen since they have been reported not to affect the membrane potential [6,13]. As it can be seen in Table I, both $Mg^{2+}$ and choline decreased

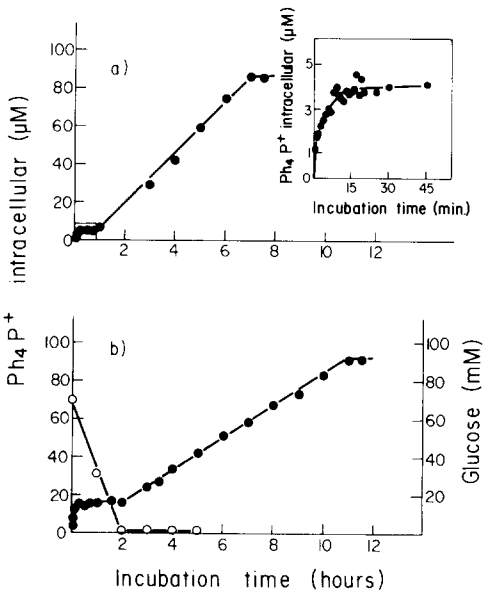


Fig. 2. Effect of glucose on $Ph_4P^+$ uptake by cells in the exponential phase of growth. Yeast was grown on YPD and collected during the exponential phase of growth. (a) Cells were incubated as in Fig. 1a. (b) Cells were incubated as in Fig. 1b. ●——●, $Ph_4P^+$ uptake; ○——○, glucose concentration in the medium.

the amount of $Ph_4P^+$ incorporated into the cell during the first phase. This could mean that $Ph_4P^+$ binds to negatively charged groups on the outside of the membrane and that cations like $Mg^{2+}$ and choline act by blocking these groups. We have found however that the addition of compounds which collapse the membrane potential like carbonylcyanide *m*-chlorophenylhydrazone (CCCP), 2,4-dinitrophenol or KCl provokes an efflux from the yeast cells of the $Ph_4P^+$ accumulated during the first phase (Table I). This fact implies that $Ph_4P^+$ uptake during the first phase is really dependent on the membrane potential and suggests that the effect of the cations is due to a depolarization of the plasma membrane. It should be observed that if the $Ph_4P^+$ incorporated into the cell in this first phase is assumed to give a true measure of the membrane potential, this potential would not exceed 30 mV, far lower than the values reported for other systems, from 60 mV in lymphocytes [14] to 180 mV in *E. coli* [15].

TABLE I

ACCUMULATION OF $Ph_4P^+$ IN YEAST CELLS IN DIFFERENT CONDITIONS

Cells were collected during the exponential phase of growth and incubated at 30°C in 45 mM Tris-succinate buffer (pH 7) containing 2% glucose (w/v) during 60 min before the addition of 5 μM $Ph_4P^+$ (standard conditions). Choline chloride and $MgCl_2$ were added 7 min before $Ph_4P^+$, while the addition of CCCP, 2,4-dinitrophenol (DNP) and KCl was made when the equilibrium of the first phase of $Ph_4P^+$ uptake was attained.

| Conditions | Intracellular $Ph_4P^+$ in the first phase of uptake (μM) |
|---|---|
| Standard conditions | 13 |
| + 100 mM choline chloride | 5 |
| + 20 mM $MgCl_2$ | 3 |
| After addition of | |
| 0.1 mM CCCP | 4 |
| 2.0 mM DNP | 3 |
| 200 mM KCl | 2 |

The second phase of uptake does not begin until the glucose from the medium has been exhausted (Fig. 2b). This could indicate that this uptake is dependent on a system which is repressed by glucose and which does not become functional until the glucose concentration drops to a very low level. To test this idea we tried the effect of cycloheximide on $Ph_4P^+$ uptake. As shown in Fig. 3, in the presence of cycloheximide no second phase of uptake is observed up to 29 h although the glucose from the medium has been practically used up after 10 h. This indicates that

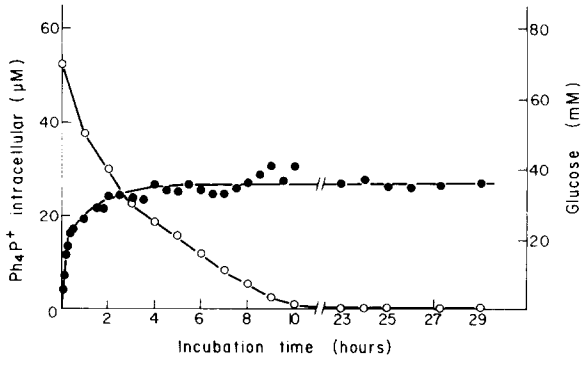


Fig. 3. Effect of cycloheximide on $Ph_4P^+$ uptake. Cells were collected during the exponential phase of growth and incubated at 30°C in 45 mM Tris-succinate buffer (pH 7) containing 2% glucose (w/v) and 10 μg/ml cycloheximide during 60 min before the addition of 5 μM $Ph_4P^+$. ●——●, $Ph_4P^+$ uptake; ○——○, glucose concentration in the medium.

$Ph_4P^+$ uptake depends on the synthesis of some protein(s) repressed by glucose. It could be thought that these protein(s) are mitochondrial. In this case $Ph_4P^+$ uptake would be driven by the potential of the mitochondrial membrane as has been described for lymphocytes [14]. However, we have found that the second phase of uptake is not blocked by incubation of the cells with oligomycin and antimycin at concentrations that do not allow growth on ethanol. Moreover, the second phase of uptake is not supressed in the presence of chloramphenicol. It appears, therefore, that the second phase of $Ph_4P^+$ uptake is dependent on the synthesis of some protein(s) repressed by glucose but unrelated with the existence of functional mitochondria. Experiments with a petite mutant show that $Ph_4P^+$ uptake proceeds also in two phases, but that $Ph_4P^+$ accumulation is strongly reduced (5 $\mu$M $Ph_4P^+$ in the first phase and 17 $\mu$M $Ph_4P^+$ in the second). The reason for the reduction in the first phase is not clear but the reduction in the second phase could be interpreted as inhibition of protein synthesis after glucose exhaustion due to ATP depletion [16].

At the present time the nature of the protein(s) that mediate an increased uptake of $Ph_4P^+$ has not been established. Any protein binding $Ph_4P^+$, a specific transport system or proteins which allow accumulation in an intracellular compartment, like the vacuole, would be possible candidates. The fact that the addition of CCCP, of $K^+$ (in the presence of glucose), or even an acidification of the medium provoke an efflux of the accumulated $Ph_4P^+$ (Fig. 4) indicates that in any case the uptake of $Ph_4P^+$ by the yeast is associated with some form of membrane potential.

From the results described it can be concluded that accumulation of $Ph_4P^+$ in yeast cells is a complex process which depends on multiple factors, some of them not well characterized. Although depolarization of the yeast membrane is usually followed by an efflux of the $Ph_4P^+$ accumulated, it is clear that uptake of $Ph_4P^+$ cannot be used to give a quantitative measure of the plasma membrane potential of yeast cells.

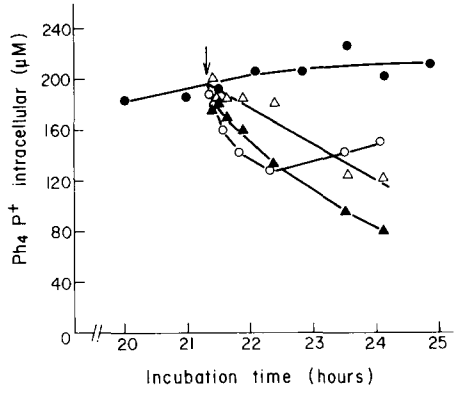


Fig. 4. Effect of depolarizing agents on the second phase of $Ph_4P^+$ accumulation. Cells were collected during the exponential phase of growth and incubated at 30°C in 45 mM Tris-succinate buffer (pH 7) containing 2% glucose (w/v) during 60 min. 5 $\mu$M $Ph_4P^+$ was added and cells were incubated until the second equilibrium was reached. At the time indicated by the arrow the following additions were made: ○———○, 100 $\mu$M CCCP; △———△, 2% glucose (w/v) +0.2 M KCl; ▲———▲, 6 M HCl to give pH 2.5: ●———●, control.

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