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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

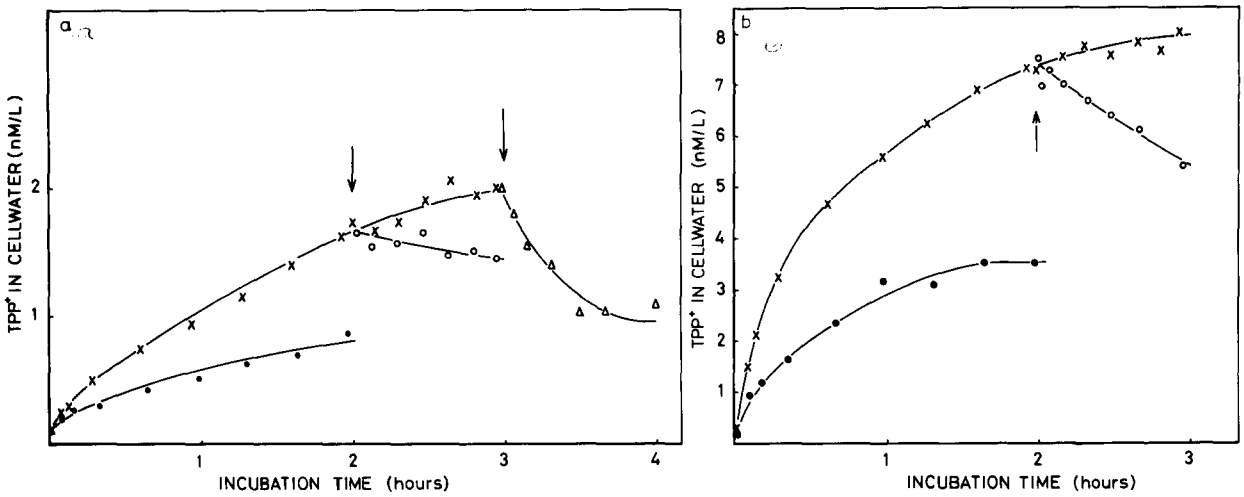

Fig. 1. TPP^+ uptake by resting cells and metabolizing cells and the effect of 2,4-dinitrophenol (100 μM) and KCl (50 mM). Metabolizing cells are starved cells which are pre-incubated (during 5 min) and incubated in the presence of 5% glucose (w/v). Resting cells are cells which are pre-incubated and 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-

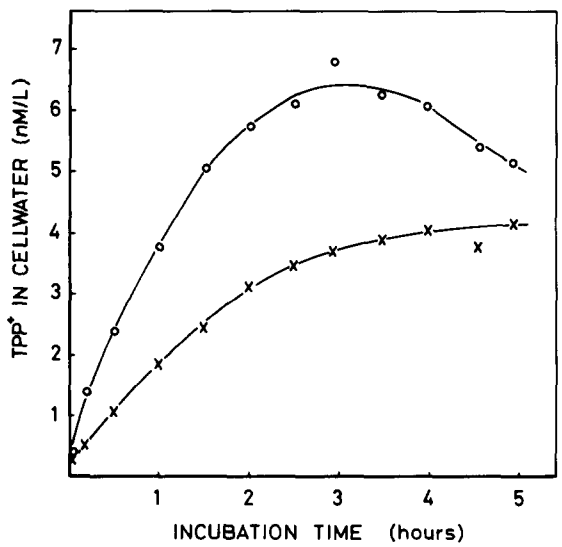

Fig. 2. Effect of $CaCl_2$ (1 mM) on TPP^+ uptake at pH 5.6 by metabolizing cells, which were pre-incubated during 5 min and incubated in the presence of 5% glucose (w/v). ×, without added $CaCl_2$; ○, with $CaCl_2$ added at $t=0$. Mean of duplicate experiments.

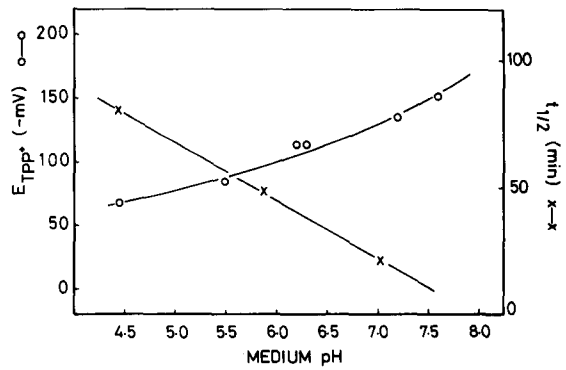

Fig. 3. Effect of the medium pH on the equilibrium potential of TPP^+ (E_{TPP}) (see Eqn. 1) and the half-maximal time constant ($t_{1/2}$). Metabolic cells (see legend to Fig. 1). Mean of triplicate experiments.

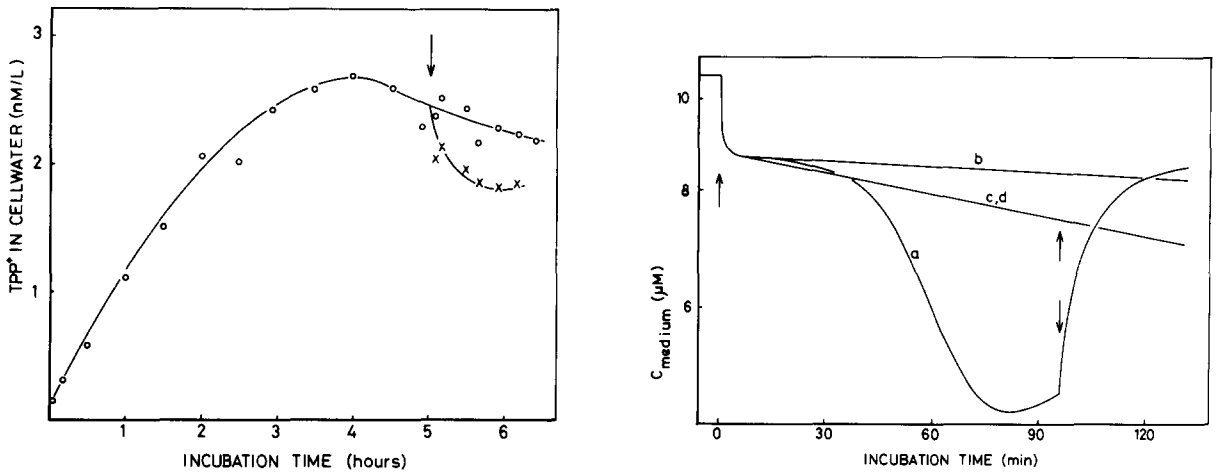

Fig. 4. Effect of 3 mM iodoacetic acid on TPP^+-accumulation by metabolizing cells at pH 4.5. Pre-incubation (5 min) and incubation were carried out in the presence of 5% glucose (w/v). In order to prevent exhaustion of the glucose, each 1.5 h extra glucose was added to a final concentration of 2% (w/v). 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)
$0.18 \cdot 10^{-3}$	$6.8 \cdot 10^{-4}$	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 I, 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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References

1 Vacata, V., Kotyk, A. and Sigler, K. (1981) Biochim. Biophys. Acta 643, 265–268
2 Eddy, A.A., Philo, R., Earnshaw, P. and Brocklehurst, R. (1977) in Biochemistry of Membrane Transport, FEBS Symp. Vol. 42 (Semenza, G. and Carafoli, E., eds.), pp. 250–260, Springer-Verlag, Berlin
3 Barts, P.W.J.A., Hoeberichts, J.A., Klaassen, A. and Borst-Pauwels, G.W.F.H. (1980) Biochim. Biophys. Acta 597, 125–136
4 Komor, E. and Tanner, W. (1976) Eur. J. Biochem. 70, 197–204
5 Bakker, E.P. and Harold, F.M. (1980) J. Biol. Chem. 255, 433–440
6 Kashket, E.R., Blanchard, A.G. and Metzger, W.C. (1980) J. Bacteriol. 143, 128–134
7 Felle, H., Porter, J.S., Slayman, C.L. and Kaback, H.R. (1980) Biochemistry 19, 3585–3590
8 Tokuda, H., Nakamura, T. and Unemoto, T. (1980) Biochemistry 20, 4198–4203
9 Serrano, R. (1980) Eur. J. Biochem. 105, 419–427
10 Hauer, R. and Höfer, M. (1978) J. Membrane Biol. 43, 335–349
11 Kotyk, A. (1980) in Cell Compartmentation and Metabolic Channeling (Nover, L., Lynen, F. and Mothes, K., eds.), pp. 63–74, Elsevier/North-Holland Biomedical Press, Amsterdam
12 Kotyk, A. and Michaljanicová, D. (1978) Folia Microbiol. 23, 18–26
13 Shinbo, T., Kamo, N., Kurihara, K. and Kobatake, Y. (1978) Arch. Biochem. Biophys. 187, 414–422
14 Lombardi, F.J., Reeves, J.P. and Kaback, H.R. (1973) J. Biol. Chem. 248, 3551–3565
15 Borst-Pauwels, G.W.F.H. and Jager, S. (1969) Biochim. Biophys. Acta 172, 399–406
16 Cockburn, M., Earnshaw, P. and Eddy, A.A. (1975) Biochem. J. 146, 705–712
17 Iwashima, A., Wakabayashi, Y. and Nose, Y. (1977) J. Bacteriol. 131, 1013–1015
18 Borst-Pauwels, G.W.F.H. (1981) Biochim. Biophys. Acta 650, 88–127
19 Slayman, C.L., Lu, C.Y.-H. and Shane, L. (1970) Nature 226, 274–276
20 Slayman, C.L. (1965) J. Gen. Physiol. 49, 69–92
21 Heinz, E., Geck, P. and Pietrzyk, C. (1975) Ann. N.Y. Acad. Sci. 264, 428–441