Tetraphenylphosphonium ion is a true indicator of negative plasma-membrane potential in the yeast *Rhodotorula glutinis*

Experiments under osmotic stress and at low external pH values

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In the yeast *Rhodotorula glutinis*, accumulation of the tetraphenylphosphonium ion (TPP⁺) was increased under conditions of osmotic stress, indicating a hyperpolarization of the negative membrane potential ($\Delta\psi$). The following observations were consistent with the occurrence of hyperpolarized $\Delta\psi$: (i) enhanced accumulation of glucosamine, the uptake of which is also driven by $\Delta\psi$; (ii) increased respiratory rate. The accumulation of TPP⁺ was gradually decreased by lowering the pH of cell suspensions. At pH values below 4.5, no TPP⁺ was taken up, but instead thiocyanate (SCN⁻) was accumulated, indicating a positive $\Delta\psi$. The pH-dependent influx of glucosamine followed the pattern of TPP⁺ accumulation both in the wild-type and in the nystatin-resistant mutant, M67, which displayed a negative $\Delta\psi$ down to pH 3. Thus TPP⁺ accumulation in *Rh. glutinis* reflected actual electrical potential difference across the plasma membrane.

The steady-state distribution of lipophilic cations TPP⁺, triphenylmethylphosphonium (TPMP⁺) and dibenzyltrimethylammonium (DDA⁺) has frequently been used to determine the electrical potential difference across biological membranes, such as the mitochondrial inner membrane (Bakeeva *et al.*, 1970; Grinius *et al.*, 1970) or the bacterial plasma membrane (Griniuvienė *et al.*, 1974; Shuldiner & Kaback, 1975; Zaritsky *et al.*, 1981). In eukaryotes, the use of lipophilic cations has been less successful. An interval of 2 h was necessary to reach the steady-state TPP⁺ accumulation in the green algae *Chlorella vulgaris* (Komor & Tanner, 1976) and *Chlorella emersonii* (Gimmler & Greenway, 1983); in *Saccharomyces cerevisiae* the corresponding interval was at least 3 h (Boxman *et al.*, 1982). Since intracellularly accumulated [¹⁴C]TPP⁺ was not exchanged against extracellular unlabelled TPP⁺ in *Ch. emersonii*, Gimmler and Greenway decided that TPP⁺ is unsuitable as a probe for electrical potentials. However, the yeast *Rhodotorula glutinis* was unusual, as the equilibration time did not exceed 20 min (Hauer & Höfer, 1978; Hauer *et al.*, 1981; Vacata *et al.*, 1981), and accumulated TPP⁺

was readily expelled from de-energized cells (Hauer & Höfer, 1978).

To obtain further evidence for TPP⁺ being a true indicator of the plasma-membrane potential in *Rh. glutinis*, we used three different experimental approaches: (i) cells were exposed to osmotic stress; (ii) the nystatin-resistant mutant, M67, which displayed distinctly lower plasma-membrane permeability for H⁺ (Künemund & Höfer, 1983) was used; and (iii) transport of glucosamine, a substrate of the H⁺-symport in *Rh. glutinis*, driven by $\Delta\psi$ (Niemietz *et al.*, 1981) was examined both in the wild-type and the mutant cells. On the basis of these experiments a possible dispersion of TPP⁺ in cells is discussed. A preliminary report of this work has already appeared (Höfer & Künemund, 1984).

Materials and methods

The obligatorily aerobic yeast *Rhodotorula glutinis* (*Rhodosporidium toruloides*), A.T.C.C. 26194 (= CBS 6681) and its mutant strain M67, A.T.C.C. 52636, were grown as previously described for 24 h (Heller & Höfer, 1975) and 38 h (Höfer *et al.*, 1982) respectively. The growth medium for *Rh. glutinis* M67 contained 150 µg of nystatin/ml. Before use, the yeast was washed by centrifugation (5 min at

Abbreviation used: TPP⁺, tetraphenylphosphonium ion.

5000g) three times in distilled water and aerated for 4h as a 5% (wet wt./v) suspension. The uptake of pentose or [14 C]glucosamine and $S^{14}CN^-$ was measured intracellularly by membrane filtration (Heller & Höfer, 1975; Niemietz *et al.*, 1981). Uptake of [3 H]TPP $^+$ was determined by measuring the radioactivity in the supernatant (Hauer & Höfer, 1978), using the emulsifier scintillator 299 TM and scintillation spectrometer B 400 (both of Packard, Frankfurt, Germany). All experiments were carried out in Tris/citric acid buffer (0.02 mol/litre; pH as indicated in the Figure legends) at 25°C. Hyper-osmotic medium was produced by adding the appropriate amount of solid sorbitol, lactose or melibiose to the yeast suspension 1 min before the start of the experiment. Oxygen was measured at room temperature with a Clark-type electrode (Beckman Instruments).

[3 H]Tetraphenylphosphonium chloride (160 MBq/mol) was from Hoechst AG, Frankfurt, Germany; [U- 14 C]glucosamine (2-deoxy-2-amino-D-glucopyranose, 2.0 TBq/mol) and [14 C]thiocyanate (potassium salt, 2.1 TBq/mol) were from Amersham Buchler, Braunschweig, Germany. All other reagents were of analytical grade from Merck, Darmstadt, Germany.

Results and discussion

Cells exposed to an osmotic stress respond by decreasing the osmotically free space. With sorbitol as an osmotically active substance, Klöppel & Höfer (1976) demonstrated a decrease in intracellular water volume in *Rh. glutinis* by up to 60% at 0.8 osmol/kg. Kotyk & Höfer (1965) reported a close relationship between the amount of monosaccharide accumulated and the intracellular water volume, manipulated by NaCl. However, the accumulation of TPP $^+$ was enhanced when cells were exposed to sorbitol, melibiose or lactose at 0.6 osmol/kg (Table 1). These substances were used because, in *Rh. glutinis*, sorbitol is taken up only very slowly (Klöppel & Höfer, 1976), and there is no detectable uptake of melibiose and lactose (Janda & von Hedenström, 1974). Table 1 also shows that pentose accumulation was decreased markedly by the hyperosmotic conditions, the cell shrinkage corresponding to that described by Klöppel & Höfer (1976).

The enhanced accumulation of TPP $^+$ under conditions of osmotic stress indicated a hyperpolarization of the plasma membrane. This interpretation was supported by measuring the accumulation of another cationic substance, namely glucosamine (2-deoxy-2-amino-D-glucose; $pK_a = 7.75$). The uptake of glucosamine is mediated by the monosaccharide carrier and driven by the membrane potential (Niemietz *et al.*, 1981).

Fig. 1 demonstrates that glucosamine accumulation parallels that of TPP $^+$ (Table 1). Furthermore, the higher energy demand for osmotically induced hyperpolarization was associated with enhanced cell respiration (Fig. 1).

Acidification of the cell suspension brought about a rapid and complete outflow of TPP $^+$ (Hauer & Höfer, 1978; Künemund & Höfer, 1983).

Fig. 1. Effect of osmotic stress [sorbitol (▨) or lactose (▤) at 0.6 osmol/kg] on steady-state distribution of glucosamine (GlcNH $_2$) and on endogenous cell respiration in wild-type cells

Yeast (8mg dry wt.·mol $^{-1}$) was suspended in Tris/citrate buffer (pH 7.5, 0.02 mol/litre)/glucosamine (1 mmol/litre). Results are mean percentages of the control (□) [57 nmol of GlcNH $_2$ /mg dry wt. ($n = 2$) and 9.6 ± 1.2 nmol of O $_2$ /mg dry wt.·min ($n = 4$). No correction was made for the decrease of the intracellular water volume.

Table 1. Effect of sorbitol, melibiose or lactose (each 0.6 osmol/kg) on steady-state cellular content of TPP+ and of two pentoses in the wild strain

Yeast suspensions (8 mg dry wt · ml⁻¹) in Tris/citric acid buffer (0.02 mol/litre), pH 7.5, were used for the TPP⁺-uptake experiments ([³H]TPP⁺, 160 MBq/mol). The cellular steady-state content of pentoses was determined under the same conditions, except at pH 6.0. Results are means ± S.E.M. No correction was made for the osmotically induced decrease of the intracellular water volume.

Steady-state uptake	Control content (nmol/mg dry wt.)	Content (% of control) in the presence of:		
		Sorbitol	Melibiose	Lactose
TPP ⁺ (20 μmol/litre)	1.56 ± 0.1 (n = 10)	138 ± 7 (n = 6)	103 ± 1 (n = 7)	131 ± 9 (n = 4)
D-Xylose (2 mmol/litre)	206 ± 23 (n = 6)	66 ± 15 (n = 4)	69 ± 10 (n = 4)	49 (n = 1)
L-Arabinose (10 mmol/litre)	362 ± 49 (n = 6)	59, 52 (n = 2)	53 ± 6 (n = 4)	33, 38 (n = 2)

Fig. 2. pH-dependency of the electrical potential difference, Δψ, across the plasma membrane of wild-type cells of *Rh. glutinis*

Δψ was calculated by means of the Nernst equation from the steady-state distribution of either 1 μM-TPP⁺ (●, for negative Δψ) or 0.5 μM-SCN⁻ (○, for positive Δψ), as described in the text. Yeast (8 mg dry wt · ml⁻¹) was suspended in Tris/citrate buffer (0.02 mol/litre) at the given pH. The steady-state distributions of TPP⁺ and SCN⁻ were achieved during 20 and 10 min respectively. The vertical bars give the S.D.; the small numbers against the data points are the numbers of independent measurements.

TPP⁺ accumulation in the wild-type cells of *Rh. glutinis* gradually decreased when cells were suspended in buffers with increasing H⁺ concentration. No detectable uptake of TPP⁺ occurred at pH 4.5 and below (Fig. 2; see also Hauer *et al.*, 1981). This is due to the increasing diffusion of H⁺ into the cells as the pH gradient across the plasma membrane increases (Höfer *et al.*, 1983a). Consequently, at pH values below 4.5, a membrane potential, which is positive-inside, is to be expect-

Fig. 3. pH-dependency of the membrane potential and of the initial velocity of glucosamine transport in the mutant M67

The membrane potential (Δψ, ○) was determined with TPP⁺ as in Fig. 2; measurement of initial velocity (v₀, ●) of glucosamine transport is described in the Methods and materials section. Yeast (8 mg dry wt · ml⁻¹) was suspended in Tris/citrate buffer (0.02 mol/litre). The dotted line indicates the pH-limit below which no uptake of either TPP⁺ or glucosamine could be measured in wild-type cells (Hauer *et al.*, 1981; Niemietz *et al.*, 1981). Results are means ± S.E.M. (bars) for six experiments.

ed. Indeed, the lipophilic anion thiocyanate (SCN⁻) was accumulated by *Rh. glutinis* cells only when suspended in buffers of pH below 4.5 (Fig.

2). The smooth transition of the membrane potential from negative values at pH > 4.5, as indicated by TPP⁺ accumulation, to positive values at pH < 4.5, as indicated by SCN⁻ accumulation, demonstrates the suitability of both TPP⁺ and SCN⁻ as true indicators of the actual electrical potential difference across the plasma membrane of *Rh. glutinis*.

The plasma membrane of the nystatin-resistant mutant of *Rh. glutinis*, M67, possesses a distinctly lower permeability for H⁺. Correspondingly, the mutant accumulated TPP⁺ also at pH values below 4.5 (Höfer *et al.*, 1983). In order to prove that this TPP⁺ accumulation reflected a negative $\Delta\psi$, the carrier-mediated influx of glucosamine in the mutant was tested in a broad pH range from pH 7 down to pH 3.8 (Fig. 3). The influx of glucosamine in wild-type cells was closely associated with the actual size of $\Delta\psi$, which is the driving force of the process, being zero at pH 4.5 and below (no TPP⁺ accumulation) (cf. Niemietz *et al.*, 1981). As shown in Fig. 3, the influx of glucosamine in the mutant was still about one-third of the maximum at as low a pH as 3.8 and corresponded to the actual driving force at this pH as indicated by TPP⁺ accumu-

lation. Hence, it seems unlikely that a non-specific pH-dependent binding could have simulated a $\Delta\psi$ -dependent accumulation of TPP⁺.

The values for the electrical potential difference across the plasma membrane, $\Delta\psi$, were calculated by means of the Nernst equation for diffusion potentials from the accumulation data of both the cation TPP⁺ and the anion SCN⁻:

$$\Delta\psi = \pm 59 \log(c_o/c_i)$$

in mV at 25°C (c_o/c_i are the extracellular and intracellular ion concentrations, + and - correspond to the cation and anion respectively). However, the intracellular distribution of TPP⁺ somewhat complicates the interpretation of uptake data in eukaryotes. Mitochondria, having their own negative potential across the inner membrane, would be expected to accentuate TPP⁺ accumulation. The following observations seem to rule out the possibility that, in *Rh. glutinis*, TPP⁺ is absorbed by mitochondria to any appreciable extent. (1) Acidification of the suspension medium induced an immediate and complete efflux of TPP⁺ from preloaded wild-type cells (Fig. 4a). This treatment could not have affected the

Fig. 4. Outflow of TPP⁺ and of glucosamine (GlcNH₂) from preloaded wild-type cells induced by either acidification of the cell suspension to pH 4.0 (a) or by nystatin (5 μmol/litre) (b)

Rh. glutinis cells (8 mg dry wt. · ml⁻¹) were preloaded with [³H]TPP⁺ (1.2 μmol/litre) for 20 min or with glucosamine (1 mmol/litre) for 60 min in Tris/citrate buffer (0.02 mol/litre), pH 7.0. At the arrows, either HCl (H⁺) or nystatin was added. In the experiment with nystatin and glucosamine, the pH was 5.0. ○, ●, Intracellular TPP⁺ concentration ([TPP]_i); △, ▲, intracellular glucosamine concentration ([GlcNH₂]_i); open symbols, controls without additions; closed symbols, after the respective additions. The results given are representative of those obtained in three experiments.

mitochondria, since the intracellular pH decreased from 7.5 to 7.2 at the most (Höfer *et al.*, 1983b), so that the distribution of any TPP^+ held in mitochondria would be unaffected by the change. Moreover, a semi-logarithmic plot of the efflux of TPP^+ was linear (not shown), indicating that the ion was not compartmented. (2) The polyene antibiotic nystatin, which makes non-specific aqueous pores by interacting with plasma-membrane sterols (Finkelstein & Holz, 1973), depolarizes the plasma membrane potential (Hauer & Höfer, 1978) but not that of mitochondria (von Hedenström & Höfer, 1979). When applied to preloaded cells, nystatin produced a similar efflux of accumulated TPP^+ (Fig. 4b).

It is difficult to explain where TPP^+ is dispersed when not interacting with mitochondria. One possible explanation could be that TPP^+ does not enter the cytosol but is absorbed in a strictly $\Delta\psi$ -dependent amount in the hydrophobic plasma membrane itself. This location would also explain the rather slow efflux of TPP^+ induced by nystatin when compared with that of other ions or neutral molecules (shown for glucosamine in Fig. 4b), which all are freely dispersed in the cytosol. Of course, the present results do not provide any conclusive evidence for this interpretation. However, if this is a true interpretation, there are necessarily errors in calculating values of the membrane potential by means of the Nernst equation from data of TPP^+ accumulation. Nevertheless, TPP^+ is a suitable, although possibly only a semi-quantitative, indicator of negative plasma-membrane potentials in *Rh. glutinis* and also perhaps in other eukaryotes.

To sum up, the above conclusion is supported by following observations. (1) The continuous transition from TPP^+ accumulation (indicating negative $\Delta\psi$) at pH values above 4.5 to SCN^- accumulation (indicating positive $\Delta\psi$) at pH values below 4.5; at pH 4.5, $\Delta\psi = 0$ (no accumulation of either TPP^+ or SCN^-). (2) Enhanced TPP^+ accumulation after hyperpolarization of negative $\Delta\psi$ in hyperosmotic media. (3) Quick and complete efflux of TPP^+ on acidification of the cell suspension or on adding nystatin or uncouplers (Hauer & Höfer, 1978). (4) Rapid equilibration and exchangeability of TPP^+ taken up [not shown here; $t_{1/2}(\text{exchange}) = 22 \text{ min}$].

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