Cytosine accumulation as a measure of the proton electrochemical gradient acting on the overexpressed cytosine permease of *Saccharomyces cerevisiae*

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The magnitude of the proton gradient ($\Delta \mu^+$) driving solute accumulation in *Saccharomyces cerevisiae* has long been in doubt, principally because of the lack of an agreed method for assaying its electrical component, the membrane potential ($\Delta \psi$). In the present work, the size of the cytosine gradient ($\Delta \mu_{\text{cyt}}$) that the yeast generated was used as a measure of the driving gradient ($\Delta \mu^+$). The selected yeast lacked cytosine deaminase and overexpressed cytosine permease, a 1 H+/cytosine system. $\Delta \mu_{\text{cyt}}$ assayed in washed cell suspensions fermenting glucose and containing 0.5 or 50 mM KCl, was about 260 mV at pH 4 or 5, falling to about 194 mV at pH 7. As a first estimate, $-\Delta \mu^+$ was thus at least as large at the respective pH value. A 20 mM solution of the lipophilic cation tetraphenylphosphonium lowered $\Delta \mu_{\text{cyt}}$ to a value roughly equal to the magnitude of the pH gradient ($\Delta p\text{H}$). A mathematical model was used to correct the first estimates of $\Delta \mu^+$ for the effect of cytosine leakage outside the symport. In such a system, $\Delta \mu_{\text{cyt}}$ cannot exceed the equivalent ratio $V_{\text{max}}/K_m L$, where $V_{\text{max}}$ and $K_m$ are kinetic parameters of the symport and $L$ is the rate coefficient for leakage. The feasibility of assaying $\Delta \mu^+$ depends on it not being much larger than that ratio. The model was tested successfully against observations made with yeast preparations depleted of ATP. After correction, $-\Delta \mu^+$ during fermentation was estimated to be up to 25 mV larger than $\Delta \mu_{\text{cyt}}$ and at least 70 mV larger than previous estimates in the literature involving lipophilic cations. From a knowledge of $\Delta p\text{H}$, $\Delta \psi$ was in turn deduced and compared with the maximum methylamine gradient ($\Delta \mu_m$) the yeast formed. The results supported the claim in the literature that, at acid pH, $\Delta \mu_m$ is a measure of $\Delta \psi$.

**Keywords:** proton gradient, yeast, overexpressed yeast cytosine permease, yeast membrane potential

INTRODUCTION

The proton electrochemical gradient ($\Delta \mu^+$) generated by proton extrusion through the plasma membrane ATPase of *Saccharomyces cerevisiae* drives the accumulation of diverse nutrients into the yeast cytosol in symport with protons (Serrano, 1991). In contrast to other well-studied systems (Harold, 1986), the magnitude of this driving force in yeast is uncertain (Serrano, 1991). One component of $\Delta \mu_{\text{H}^+}$ relates to the pH difference ($\Delta p\text{H}$) between the yeast cytosol and the surrounding medium. It can be assayed satisfactorily in terms of the distribution of weak acids between the yeast cells and their environment (de la Peña et al., 1982; Krebs et al., 1983; Portillo & Serrano, 1989) or by NMR techniques (Van Aelst et al., 1991). The other component of the proton gradient, the membrane potential ($\Delta \psi$) is of uncertain magnitude, although its presence has been detected qualitatively by means of techniques based on ionophores, voltage-sensitive dyes (Hopkins et al., 1992), or lipophilic cations such as tetraphenylphosphonium (TPP) (Serrano, 1991).

Direct recording of $\Delta \psi$ by means of implanted microelectrodes has proved difficult with the relatively small...
cells of **Saccharomyces** (Conway, 1960; Bakker et al., 1986) but has been achieved with the larger cells of *Neurospora crassa* (Ballarin-Denti et al., 1994), *Endomyces magnusii* (Bakker et al., 1986), *Pichia homboldtii* (Hofer & Novacky, 1986) and *Chao australis* (Ritchie, 1982), as well as with "giant" cells of *Escherichia coli* (Felle et al., 1980). In all but the latter instance, Δψ was up to 100 mV more negative than was predicted from the Nernst potential corresponding to the equilibrium distribution of dilute solutions of a lipophilic cation between the cells and the environment. Ritchie (1982) plausibly attributed the discrepancy to the need for eukaryotic cells to expel such cations to prevent their accumulation in the mitochondria. The magnitude of diverse observations with *S. cerevisiae* (compare Balzi & Goffeau, 1991). Accordingly, the significance of the results obtained with *S. cerevisiae* in which the magnitude of Δψ was inferred indirectly from the TPP distribution is now doubtful (reviewed by Serrano, 1991; Ballarin-Denti et al., 1994).

In the present work, we have assayed Δψ(H+), ΔψH and thus, indirectly, Δψ, by studying the steady-state of cytosine uptake established by a strain of *S. cerevisiae* (NC 233-10B[pH4-9]) overexpressing the cytosine permease gene (FCY2) and lacking cytosine deaminase (Hopkins et al., 1988). According to Mitchell's chemiosmotic model, when cytosine leakage outside the cytosine permease is negligibly small, the maximum cytosine gradient [Δψcyt = RT ln([cytosine intracellular]/[cytosine extracellular])] the yeast forms equals −nΔψH, where n is the effective number of protons absorbed in symport with each molecule of cytosine.

In an earlier study with preparations of the same yeast depleted of ATP, in which Δψ was apparently governed by the Nernst potential of K+ ions, we concluded that n = 0 for cytosine was 1, both on the basis of the observed proton uptake with cytosine and on the approximate magnitude of the ratio Δψcyt/ΔψH (Hopkins et al., 1992). It thus follows that during energy metabolism the steady-state value of Δψcyt would be expected to equal −ΔψH if the equilibrium model is correct. In fact, cytosine leakage in a related yeast strain carrying the *fjy2* allele was small rather than zero, so that corrections have to be applied (Eddy et al., 1994). That approach is further developed here.

**METHODS**

**Yeast cultures.** The yeast strains NC 233-10B[pH4-9] [a, *fjy1*; *fjy2* ΔΔω 2*(FCY2 LEU2)*] and FLA42-2D [fjy1; fjy1] were cultured at 30 °C aerobically in a glucose/mineral salts medium to early exponential phase (OD660 < 0.4; Cecil Instruments CE 272 spectrophotometer) (Hopkins et al., 1992). The yeast was harvested, washed with water and stored on ice for up to 3 h (Eddy & Hopkins, 1985).

**Assay of proton absorption.** The procedures described by Eddy & Hopkins (1985) were employed. The yeast (36 mg dry wt) was suspended in 4.5 ml 25 mM Tris brought to pH 6.1 with MES. The cell suspension (final vol. 5 ml) was stirred continuously at 30 °C and antimycin (10 μg) and 2-deoxyglucose (25 μmol) were added to deplete cellular ATP (Eddy et al., 1970a). After 8 min, [14C]cytosine (0.33 μmol, 70 nCi/259 kBq) was added, the pH being recorded continuously. Three samples (each 100 μl) were taken during the next 3 min and subsequently a further four samples were taken at intervals of 20 s. Each was filtered through a wetted glass fibre filter (Whatman GF/C). The filtered yeast was washed at 4 °C with three successive portions (2 ml each) of water and its 14C content assayed. The rate of turnover of the system at 11 min was determined separately. To do this, cytosine (0.33 μmol) was added at 8 min followed by a tracer amount of [14C]cytosine at 11 min. Proton uptake was inferred by interpolation on the electrode traces displayed on a chart recorder. Cytosine typically caused a displacement of about 20 mm in 3 min, representing absorption of 130 n equivalents of protons and a pH change of about 0.025. Controls were run in which water was added instead of a solution of cytosine. Electrical drift during a typical experiment was negligible. Cytosine uptake followed an exponential time-course which was analysed using a non-linear least-squares fitting program, thereby allowing an estimate to be made of the cellular cytosine content at 180 s (see Table 2). Table 2 combines the results of three independent experiments in which cytosine uptake exhibited half-times of 62, 75 and 92 s and reached plateau values of 109, 134 and 162 nmol (36 mg dry wt)−1, respectively.

**Determination of Δψcyt or ΔψH**. Washed yeast cells (0.1 mg dry wt ml−1) were shaken at 30 °C for 20 min with access to air in the presence of 50 mM glucose, 50 mM or 1 mM KCl and selected buffer components. At pH 7, these comprised 50 mM HEPES adjusted with Tris; at pH 6, 50 mM MES with added Tris; at pH 6.5, 50 mM Tris adjusted with citric acid; at pH 4.5, 4 mM Tris with tartaric acid. [14C]Cytosine or [14C]hypoxanthine, typically at a final concentration of 0.4 μM (20 nCi ml−1/740 Bq ml−1), was then added and the system sampled (0.5 ml) at intervals up to 6 min. The yeast was filtered, washed at 4 °C and the filter was transferred to a counting vial to which 4 ml EcoScint (Mensura) was added. The vial was assayed for 14C, in most instances with a counting error of less than 1%. Portions (100 μl) of the cell suspension were likewise assayed to determine the total 14C content of the yeast. In that case, a dry glass fibre filter was included with the scintillant to obviate differential quenching corrections. For that purpose quenching was assessed in each sample by the channels ratio technique and was found to be constant. Under optimum conditions more than 90% of the 14C was eventually retained in the yeast. The residue in solution was estimated by difference. The validity of this procedure was checked in a series of assays in which the yeast concentration and cytosine concentrations were both varied (see Results). The factor of 450 mg dry wt (mg cell water)−1 was used in computing concentration gradients (Hopkins et al., 1992). When the effects of metabolic inhibitors (TPP or azide) were studied, these were routinely added 0.5 min before cytosine.

**Determination of ΔψH**. This was done in a similar manner to the assays of Δψcyt except that 1 mM KCl was used with 10 μM [14C]methylamine (40 nCi ml−1/148 kBq ml−1) and 0.3-μg yeast ml−1. Accumulation of methylamine reached a maximum in 1 h.

**Intracellular pH.** This was assayed by measuring the accumulation of [14C]benzoic acid from a 34 μM solution (Kreb& et al., 1983). The yeast suspension was sampled in a fashion similar to that used in assaying cytosine uptake, the samples being washed for 4 s with three successive portions (2 ml each) of water at 4 °C. This procedure was found to reduce background adsorption of benzoate on the filters to zero. The sample 14C content was assayed to a statistically significant level relative to the background radioactivity. At an extracellular pH of 7, this
involved counting for 60 min and averaging the count exhibited by three independent samples.

**Vascular cytosine content.** Selective lysis of the plasma membrane was achieved by Cu²⁺ treatment (Ohsumi et al., 1988). Yeast cells that had accumulated cytosine for 2 or 3 min at pH 6.1 were collected by centrifugation and washed with water at 0 °C. They were then resuspended (0.1 mg dry wt ml⁻¹) at 30 °C in a solution containing 0.6 M sorbitol, 25 mM KH₂PO₄/K₂HPO₄ at pH 6, and 10 mM glucose and 0.2 mM CuCl₂, together with the same concentration of [¹⁴C]cytosine as remained in the medium when the cells were harvested. During the next 11 min, seven samples (0.5 ml each) were taken and the yeast was recovered by filtration. Alternate samples were washed at 4 °C with water or with 0.1 M potassium phosphate solution at pH 6 containing 0.6 M sorbitol. The difference between these two series represents the vascular cytosome released by hypotonic lysis.

**Computations**

Kinetic parameters of urea transport. The observations in Fig. 8 of Cooper & Sumrada (1975) show how the reciprocal of the initial velocity of urea uptake (V) varied with the reciprocal urea concentration ([S]⁻¹). The two kinetic components so revealed probably represent an H⁺/urea symport acting in parallel with a Aequorin expression in leak. We accordingly fitted the values of concentration, A = [S], to the equation

\[ V = V_{\text{max}}[S]/([S] + K_m) + L[S] \]

thereby obtaining the best values of \( V_{\text{max}}, K_m \) and L.

A mathematical model of the steady-state of solute accumulation. A steady-state of solute accumulation is reached when the net flow of solute through the symport equals its outflow through the leak. The relevant equations (Eddy et al., 1980), with the simplification outlined in Fig. 3 of Eddy et al. (1994), lead to a solution for the steady-state value of the intracellular substrate concentration, \( S_0 \), as the positive real root of a quadratic expression in \( S_0 \), namely

\[ A S_0^2 + B S_0 + C = 0 \]

Here

\[ A = \Theta H_2 \left(1 + \Theta^2\right) S_0 + \left(1 + \Theta^2\right) K_m \]

\[ B = \Theta \left(1 + \Theta^2\right) K_m H_o - \left(1 + \Theta^2\right) H_i S_0 \]

\[ C = \Theta \left(1 + \Theta^2\right) K_m H_o \left(S_0 + V_{\text{max}}/L + K_m\right) \]

The parameters \( K_m \) and \( V_{\text{max}} \) describe the initial rate of entry of the substrate through the symport from an external proton concentration \( H_o \) to an internal concentration \( H_i \); \( S_0 \) is the extracellular substrate concentration; \( L \) is the first-order rate constant describing the leak. The parameter \( \Theta \) equals exp (\( -\Delta F/2RT \)) where \( F, R \) and \( T \) have their conventional significance (\( F = 96485, R = 8.314, T = 303 \)). We thus computed \( \Delta \psi \) as \( (R T/F) \ln(S_o/S_i) \) in terms of the observables \( H_o, H_i, \Delta \psi, K_m, V_{\text{max}} \) and L.

At large values of \( \Delta \psi \), \( V_{\text{max}} \) becomes independent of \( \Delta \psi \) and substrate exit through the symport is negligible provided \( S_o \) is very small. \( \Delta \psi \) then reaches the maximum value equivalent to the dimensionless ratio \( V_{\text{max}}/K_m L \). This ratio is independent of further increases in the magnitude of \( \Delta \psi \).

**RESULTS**

**Effect of external pH and [K⁺] on cytosome accumulation from dilute solutions**

Cytosine uptake exhibited a \( V_{\text{max}} \) of about 26 nmol min⁻¹ (mg dry wt⁻¹) in assays of up to 10 s duration and a \( K_m \) of about 2 μM (Hopkins et al., 1992; Brethes et al., 1992). By integrating the rate equations we have now estimated that a steady-state of cytosome distribution would be established with a half-time of about 27 s in the presence of 0.6 μM cytosome and 0.1 (mg dry wt yeast) ml⁻¹. A series of 20 assays confirmed this prediction, an approximately steady-state concentration of cytosome being maintained in the yeast during the interval from 2 to 10 min after the addition of cytosome (see Methods). In ten assays at pH 5.1 with 0.5 mM extracellular KCl, the cytosome accumulation ratio ([cellular cytosome]/[extracellular cytosome]) did not vary significantly either with the yeast concentration, in the range 0.2-0.02 mg ml⁻¹, or with the final extracellular cytosome concentration, in the range 14-160 nM. The results thus reflect an authenticated distribution ratio. The mean value ± s.d. of \( \Delta \psi \) was 25 ± 6.8 kJ mol⁻¹ (ten assays), equivalent to 262 ± 7 mV. Seven further assays with 50 mM KCl gave a similar result, 260 ± 10 mV. We conclude that during energy metabolism the magnitude of \( \Delta \psi \) was almost independent of extracellular [K⁺] at pH 5.1.

A further set of assays showed that the magnitude of \( \Delta \psi \) observed in the presence of 50 mM KCl was almost the same at pH 4, 5 and 6, but declined outside this range (Table 1).

Hypoxanthine, an alternative substrate of the cytosome permease, accumulated to a similar extent to cytosome in cell preparations depleted of ATP during energy metabolism (Table 1).

**Magnitude of \( \Delta \psi \) and initial estimates of \( \Delta \omega \)**

Under the standard assay conditions (see Methods) the cellular pH, inferred from the distribution of benzoate, remained fairly constant in the range 6.3-6.6 when the extracellular pH varied from 4 to 7. The corresponding values of \( \Delta \psi \) are listed in Table 1 which further shows the values of \( \Delta \psi \) (\( \Delta \psi_{\text{un}} \)) predicted on the basis that \( \Delta \psi \) and \( \Delta \psi_{H^+} \) are equal in magnitude.

**Effects of azide and of tetrphenylphosphonium**

Azide strongly inhibited cytosome uptake, the accumulation ratio falling from about 2 × 10⁴-fold in the controls to three- to fourfold in the presence of 20 mM azide (Fig. 1a). Other observations (not shown) demonstrated that azide caused a fast efflux of radiolabelled cytosome, as did addition of 100 μM unlabelled cytosome.
Table 1. Parameters of the proton gradient (ΔpH) and the gradients of hypoxanthine (ΔΔH), cytosine (ΔΔcyt) and methylamine (ΔΔM) formed from dilute solutions of these substrates by yeast strain NC 233-1Ob, during glycolysis in the presence of 50 mM KCl at various pH values

<table>
<thead>
<tr>
<th>pH</th>
<th>ΔΔHpp (mV)</th>
<th>ΔΔcyt (mV)</th>
<th>ΔΔH (mV)</th>
<th>ΔΔcyt (mV)</th>
<th>Corrected parameter</th>
<th>ΔΔM (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>190</td>
<td>194 ± 5</td>
<td>-31 ± 3</td>
<td>225</td>
<td>194-228</td>
<td>219-265</td>
</tr>
<tr>
<td>6</td>
<td>246 ± 12</td>
<td>35 ± 4</td>
<td>211</td>
<td>253-299</td>
<td>≥ 230</td>
<td>≥ 187</td>
</tr>
<tr>
<td>5.1</td>
<td>241</td>
<td>258 ± 8</td>
<td>71 ± 1</td>
<td>260-286</td>
<td>113-139</td>
<td>143 ± 1</td>
</tr>
<tr>
<td>4</td>
<td>271</td>
<td>260 ± 4</td>
<td>142 ± 3</td>
<td>155-191</td>
<td>≥ 230</td>
<td>≥ 187</td>
</tr>
</tbody>
</table>

Our estimates of Δψ listed in Table 1 indicate that whereas the membrane potential was the major component of the proton gradient at pH 6 or 7, its contribution diminished as the pH fell until at pH 4 it was less than half the total driving force. In search of supporting evidence, we studied the magnitude of ΔΔcyt as a function of extracellular [TPP], in the expectation that sufficiently large amounts of this lipophilic cation would depolarize the yeast without changing ΔpH very much. Fig. 1(b) shows indeed that at pH 7 raising [TPP] to 20 mM progressively lowered ΔΔcyt to a mean value ±sd of 9 ± 10 mV (five assays), while at pH 5 a substantial cytosine gradient persisted in the presence of 20 mM TPP. Furthermore, Fig. 1(c) shows that accumulation of cytosine in the presence of 20 mM TPP was roughly proportional to the prevailing magnitude of ΔpH. The line of best fit exhibited a slope of 0.98, in satisfactory agreement with the corresponding behaviour of energy-depleted yeast (Hopkins et al., 1992) and with the assumption that Δψ was almost zero.

The assays of cellular pH used to construct Fig.1(c) and Table 1 showed that at pH 5 exposure to 20 mM TPP for 2-3 min increased cellular pH by 0.1-0.3 units in the presence of 50 mM extracellular KCl. To test whether TPP accelerated acid ejection by the yeast. R. Shaw assayed the rate of acid production at pH 4-8 on 11 occasions (personal communication). He found that the basal rate of 9-12 n mol min⁻¹ (mg dry wt yeast)⁻¹ indeed increased with [TPP] in the range 1-5 mM by up to 80%. The effect only persisted for about 2 min after the addition of the lipophilic cation. Subsequently, net acid formation slowed almost to zero for several minutes. We later noted that when the KCl concentration in our standard medium (see Methods) was lowered to 5 mM, 20 mM TPP at pH 4.8 caused a different and possibly deleterious response. There was a marked fall in cellular pH of 0.29, 0.49 and 0.57 units after 20, 40 and 60 s, respectively. The possibility of demonstrating the relationships shown in Fig. 1(b) and Fig. 1(c) thus appears to depend both on the presence of the larger concentration of KCl and on the limited duration of the exposure to TPP.

Theoretical and experimental basis for correcting estimates of ΔΔH

Testing the tightness of coupling between ΔΔH and ΔΔcyt

Imperfect coupling might occur either because the symport mechanism itself 'slips' (Booth, 1988; King & Wilson, 1990), or because the accumulated cytosine leaks out of the yeast outside the symport with a rate constant ±SEM of 0.061 ± 0.012 min⁻¹ (three assays) (Hopkins et al., 1992). A steady distribution of cytosine is then associated with a continuing net uptake of protons. In the circumstances illustrated in Table 2, the postulated proton leakage into the 36 mg dry wt yeast was expected to be in the range 8.5-12.8 nmol min⁻¹ if the symport mechanism itself was tightly coupled with unit stoichiometry. This quantity is very small, being at the limits of detection, bearing in mind the buffering characteristics of the system (see Methods). However, in an attempt to detect it, we depleted yeast preparations of ATP, allowing them to accumulate radiolabelled cytosine from a 67 μM solution for 180 s, by which time ΔΔcyt was about 104 mV. At 180 s the yeast maintained a fast influx of cytosine of 163 nmol min⁻¹, the net uptake rate being about 10% of this. Table 2 shows that the observed proton leakage was very small and indeed not clearly different from either zero or the expected proton leakage outside the symport. We conclude that (1) the latter process was too slow to be
Proton electrochemical gradient of yeast

10 20
[Azide] (mM)

-40 0 80 160

[APH (mV)]

Fig. 1. Effects of sodium azide or TPP on the cytosine gradient \(\Delta \psi_{\text{cyt}}\) formed by washed yeast cells in the presence of 50 mM glucose, 50 mM KCl at various pH values. \(\Delta \psi_{\text{cyt}}\) was computed as \(\Delta \psi = (RT/F) \ln([\text{cyt}]/[\text{cyt}])\). (a) Azide at pH 5.1, observations (○) are means of two to five determinations. (b) TPP at pH 5.1 (●) or pH 7 (○); one of five such experiments. (c) Residual magnitude of \(\Delta \psi_{\text{cyt}}\) in the presence of TPP, at pH 4, 5.1, 6 or 7, depicted as a function of the pH gradient at the plasma membrane. The mean value of each variable is shown with its SD, mostly based on three observations.

detected unambiguously in these assays, and that (2) the fast turnover of cytosine in the symport itself was strictly coupled to proton turnover, as conventional chemiosmotic models demand (Booth, 1988).

Comparison of the theoretical and observed effects of substrate leakage on the accumulation ratio. From a knowledge of (1) the \(K_m\) and \(V_{\text{max}}\) of the symport with respect to cytosine or hypoxanthine concentration, (2) the first-order rate constant for the leakage pathway, and (3) \(pF_{\text{out}}, pF_{\text{in}}\) and \(\Delta \psi\) we computed the expected magnitude of \(\Delta \psi_{\text{cyt}}\) or \(\Delta \psi_{\text{hyp}}\) as a function of \(\Delta \psi_{\text{H}^+}\) (see Methods). The predictions of the mathematical model were then compared with the behaviour of a series of yeast preparations depleted of ATP at various pH, \(\Delta \psi\) being equated with the Nernst potential for diffusion of K⁺ ions across the plasma membrane (Hopkins et al., 1992).

In one set of comparisons, cytosine accumulation at pH 5.6 attained 152 and 67 mV when \(\Delta \psi_{\text{H}^+}\) was \(-168\) and \(-62\) mV, respectively (Hopkins et al., 1992). The corresponding predicted values were 149 and 60 mV, a satisfactory agreement. In another eight assays [cytosine], was either in the range 5–16 μM or near 350 μM. \(\Delta \psi_{\text{cyt}}\) varied systematically with increasing cytosine concentration from 142 to 36 mV, in good agreement with the model. Fig. 2 illustrates analogous observations made with hypoxanthine.

Fig. 3 summarizes various other observations made with hypoxanthine at pH 4, 5 or 6·5, with 0·8 or 50 mM K⁺, and either strain FL 442-2D carrying a single copy of FCY2, or strain NC 233-1 Ob[pII4-91 over-expressing the cytosine permease. There was again satisfactory agreement between the observed and the predicted behaviour.

Fig. 3 also illustrates an important property of the system as regards the numerical difference between \(\Delta \psi_{\text{H}^+}\) and \(\Delta \psi_{\text{hyp}}\). The difference is quite small in the range of \(\Delta \psi_{\text{H}^+}\) up to 150 mV, but increases systematically thereafter (Eddy et al., 1994). Indeed, the model computations show that \(\Delta \psi_{\text{hyp}}\) (or \(\Delta \psi_{\text{cyt}}\)) cannot exceed the critical value \(V_{\text{max}}/K_mL\) however large \(\Delta \psi_{\text{H}^+}\) becomes. Large increases of \(\Delta \psi_{\text{H}^+}\) in the range above about 250 mV cause only relatively small increases in \(\Delta \psi_{\text{hyp}}\) up to the limiting value (Fig. 3).

Inferring \(\Delta \psi_{\text{H}^+}\) from the value of \(\Delta \psi_{\text{cyt}}\) observed during energy metabolism. We concluded above that when the magnitudes of \(\Delta \psi\), \(\Delta \psi_{\text{H}^+}\) and therefore \(\Delta \psi_{\text{H}^+}\) were known, the observed value of \(\Delta \psi_{\text{cyt}}\) (or \(\Delta \psi_{\text{hyp}}\)) formed by the starved yeast agreed with the value predicted from the mathematical model. In Table 1 we predict the values of \(\Delta \psi\) and \(\Delta \psi_{\text{H}^+}\) needed to maintain a given value of \(\Delta \psi_{\text{cyt}}\) when \(\Delta \psi\) and \(pF_{\text{out}}\) are known. The appropriate values of \(V_{\text{max}}\) and \(K_m\) are now those that apply to the metabolizing yeast cells. As \(\Delta \psi_{\text{H}^+}\) approached the critical limit referred to above, uncertainty in the estimation of \(\Delta \psi_{\text{H}^+}\) increased. In general, \(\Delta \psi_{\text{H}^+}\) was at least about \(-235\) mV at pH 4 or 5 and possibly \(-30\) mV larger. It decreased by up to 20% at pH 7.
Table 2. Assay of proton slippage in the approach to the steady-state of cytosine distribution maintained by yeast preparations depleted of ATP

The yeast (36 mg dry wt) was starved for 8 min at pH 6.1, kept with 66 μM cytosine for a further 190 s and sampled for cytosine content and proton absorption at the stated intervals (see Methods). Basal proton absorption was assayed in a similar cell suspension to which no cytosine was added. Observations from three independent experiments were combined to give mean values for the respective cytosine uptake and total proton absorption [nmol (36 mg dry wt)⁻¹]. Slippage was computed in terms of the changes taking place after 180 s. The first aim was to compare the observations with the behaviour expected if each cytosine molecule crossed the cell membrane with one proton (zero slippage). Total proton uptake minus cytosine uptake was therefore computed in order to determine the deviation from the hypothesis. The effect of cytosine leakage outside the carrier is a measure of the deviation from this hypothesis. The observed proton leakage (A−B) at each time point was averaged to give quantity A (see Methods). Table 1 shows that the maximum methylamine gradient the yeast formed at the same pH value (see Methods). Table 1 shows that ∆ψ and Δψ were of similar though not identical magnitude.

<table>
<thead>
<tr>
<th>Time with cytosine (s)</th>
<th>180</th>
<th>190</th>
<th>210</th>
<th>230</th>
<th>250</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean cytosine uptake (nmol)</td>
<td>120</td>
<td>127</td>
<td>130</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td>Mean proton uptake (nmol)</td>
<td>127</td>
<td>144</td>
<td>156</td>
<td>172</td>
<td></td>
</tr>
<tr>
<td>Total proton uptake−cytosine uptake (A) (nmol)</td>
<td>0</td>
<td>6.9±0.7</td>
<td>17.1±4.5</td>
<td>25.5±6.2</td>
<td>46.3±7.4</td>
</tr>
<tr>
<td>Basal proton uptake (B) (nmol)</td>
<td>0</td>
<td>5.2±0.8</td>
<td>13.5±0.9</td>
<td>24.7±2.1</td>
<td>36.9±5.7</td>
</tr>
<tr>
<td>Net proton slippage (A−B) (nmol)</td>
<td>0</td>
<td>1.7±1.6</td>
<td>3.7±4.6</td>
<td>0.8±4.1</td>
<td>4.7±6.3</td>
</tr>
</tbody>
</table>

Fig. 2. Hypoxanthine accumulation ratio (∆ψhyp), formed by yeast preparations depleted of ATP, as a function of extracellular [hyp]. As a test of the mathematical model, experimental values (●) were compared with the predicted values (continuous line) in the range up to 75 μM hypoxanthine. The measured parameters of the model were: H, 1 μM; H, 0.5 μM; Khyp, 6.7 μM; Θ, 1802; Vmax, 3739 nmol min⁻¹ ml⁻¹; L, 0.079 min⁻¹. The observations were made with nine cell preparations from cultures grown on four occasions. About 40 mg starved yeast (Hopkins et al., 1992) was put with 200, 400 or 600 nmol hypoxanthine for up to 150 s at pH 6, 30 °C.

Further tests of the mathematical model. Cooper & Sumrada (1975) have reported that yeast strains defective in urea metabolism concentrate urea reversibly about 200-fold, probably by a proton symport dependent on the DUR3 gene product (ElBerry et al., 1993) that operates in parallel with a facilitated diffusion pathway. Our analysis of Fig. 8 of Cooper & Sumrada (1975) showed that Vmax and Km for the active transport system were 3030 nmol ml⁻¹ min⁻¹ and 7.78 μM, respectively, whereas the first-order rate constant of the leak coefficient was 1.40 min⁻¹ (see Methods). The critical ratio Vmax/KmL is thus equivalent to 147 mV, which defines the maximum value of the urea gradient. On the assumption that the relevant parameters of ∆ψ+ at pH 4 were the same as those determined in the present work (Table 1) and that one proton is absorbed with each urea molecule, we estimated that urea accumulation would reach 132 mV from a 5.5 μM solution and 139 mV from a 2.4 μM solution. The values reported in Fig. 4 of Cooper & Sumrada (1975) are about 131 mV in both instances, an impressive agreement. As a corollary, we note that the smallness of the critical ratio means that this urea gradient would be formed at any value of −∆ψ+ exceeding about 160 mV. This behaviour underlines the advantage of using the cytosine system to...
that the vacuole and other intracellular compartments about 10% of the absorbed cytosine was retained by the contents of cell samples washed with water as intervals up to 10 min at 30 °C in the presence of cupric ions, glucose and sorbitol (Ohsumi et al., differences after exposure for 2, 5, 8 or 10 min, respectively, between the progressively diminishing cytosine for concentrated [14C]cytosine for spectrophotometric assay the much larger proton gradients that are in fact produced.

**Cytosine accumulation into the yeast vacuole.** Unlike allantoin or arginine, urea is not concentrated to a significant extent in the yeast vacuole, so that urea transport can be treated as accumulation into a single compartment (Zacharski & Cooper, 1978). The question arose whether our estimates of ΔpH+ were complicated by a similar phenomenon. To answer this the plasma membrane was selectively lysed using the procedure outlined in the Methods section. Yeast preparations that had concentrated [14C]cytosine for 3 min were lysed for intervals up to 10 min at 30 °C in the presence of cupric ions, glucose and sorbitol (Ohsumi et al., 1988). In assays involving three separate yeast cultures, we found no differences after exposure for 2, 5, 8 or 10 min, respectively, between the progressively diminishing cytosine contents of cell samples washed with water as opposed to buffered osmolyte solutions. In either case about 10% of the absorbed cytosine was retained by the yeast after 10 min exposure to cupric ion. We conclude that the vacuole and other intracellular compartments sensitive to osmotic lysis contained insignificant amounts of radiolabelled cytosine.

**DISCUSSION**

Solute accumulation driven by the proton gradient acting across the plasma membrane can only be used to assay ΔpH+ itself in certain restricted circumstances. The four criteria are as follows. (1) The solute is not metabolized to a significant extent during the assay, is not further concentrated in other cellular organelles and is not toxic. The ready exchangeability and release of the [14C]cytosine absorbed by the yeast strain NC.233-10B[pIII-4-9], together with the verification of its chemical nature after extraction from a closely related yeast strain (Chevallier et al., 1975), as well as the absence of a significant vacuolar pool, shows that the cytosine symport meets the foregoing requirements. (2) Other properties that need to be established, in general, are the proton coupling stoichiometry of the system (Hopkins et al., 1992), its reversibility and its ability to bring the proton gradient near to equilibrium with the solute gradient. Equilibrium is most likely to be achieved when [solute]out << Ks. In the present context, the ready reversibility of the cytosine symport (Chevallier et al., 1975; Hopkins et al., 1992; present work) contrasts with the apparent irreversibility of amino acid absorption (Seaston et al., 1976; Opekara et al., 1993). (3) The kinetic characteristics of any leak pathways (Chevallier et al., 1975; Hopkins et al., 1992) are used to define their effects on substrate accumulation as expressed in a mathematical model. The kinetic model used in the present work leads to definition of the critical parameter \( V_{max}/K_m L \). Its magnitude defines the range of values of ΔpH+ within which substrate accumulation increases monotonically with ΔpH+. In that connexion, we concluded that the carrier system for cytosine provides a better basis for assaying ΔpH+ than the urea system described by Cooper & Sumrada (1975). Experimental support for the mathematical model is based on Figs 2 and 3 and is described in the text. (4) A further criterion is that the solute is not actively transported out of the cell. While a variety of exit pumps have been recognized in yeasts (Balzi & Goffeau, 1991), there is no reason to think that they affect the distribution of cytosine or hypoxanthine.

The estimates of ΔsH+ used in connexion with Fig. 3 depend on the assumption that the yeast behaved like a K+ ion-electrode after depletion of ATP (Hopkins et al., 1992). We note in particular that if ΔsH+ is in fact more positive than the potential corresponding to ΔpH+, for instance as a result of significant proton leakage into the yeast, then ΔsH+ would exceed the value permitted by the model. In this connexion, the consistency between the new observations and the model, as well as the behaviour observed in the presence of TPP (Fig. 1c), support our earlier contention that both cytosine and hypoxanthine exhibit a coupling stoichiometry (ΔpH+/ΔpH+) of one proton per solute molecule (Hopkins et al., 1992). This is despite the fact that whereas the ratio H+ absorbed/cytosine absorbed is about 1 in initial rate assays, the corresponding ratio for hypoxanthine is consistently larger. Nevertheless, during energy metabolism (Table 1) or after depletion of ATP.
(Hopkins et al., 1992), a given preparation of the yeast concentrated dilute solutions of cytosine or hypoxanthine to similar extents. The alternative substrate 5-fluorocytosine exhibits even larger discrepancies with respect to the two stoichiometrical ratios (Hopkins et al., 1992). We term that phenomenon ‘pseudochannelling’ (Eddy et al., 1994).

The values of \( \Delta \psi \) listed in Table 1 are larger than those based on the use of lipophilic cations, which range from -55 to -130 mV at pH 4.5 and from -80 to -130 mV near pH 7 (de la Peña et al., 1982; Borst-Pauwels, 1981; Kotyk & Georgi, 1991). Borst-Pauwels (1981) concluded that \( \Delta \psi_{\text{cyt}} \) fell from -206 mV at pH 4.5 to -120 mV at pH 7-5, a similar fall from -150 to -90 mV being noted by de la Peña et al. (1982). Comparison with Table 1 shows that the lower estimates of \( \Delta \psi_{\text{cyt}} \) in the literature are mainly due to underestimation of \( \Delta \psi_{\text{H}^+} \). Values of \( \Delta \psi_{\text{H}^+} \) of -300 mV or more have been found for \( N.\ crassa \) hyphae where the magnitude of \( \Delta \psi \) is known unambiguously (Slayman, 1987).

We examined whether by assuming perfect coupling of cytosine and its co-substrate in the carrier we had seriously underestimated \( \Delta \psi_{\text{cyt}} \). Using the experimental lower limit for the stoichiometry of 0:92 (Hopkins et al., 1992) a value of 243 mV for \( \Delta \psi_{\text{cyt}} \) required \( \Delta \psi_{\text{H}^+} \) to be -290 mV rather than -267 mV as for the perfectly coupled carrier. Raising the upper limit of the range of each estimate of \( \Delta \psi_{\text{cyt}} \) given in Table 1 by about 20 mV would thus allow for that possibility.

There is very rough agreement between the magnitudes of \( \Delta \psi \) and \( \Delta \psi_{\text{M}} \) for methyamine at the three pH values studied (Table 1 and text). Bearing in mind the fact that the two parameters were not assayed in identical circumstances, this behaviour supports the hypothesis that methyamine accumulation is based on a uniport and equilibrates with \( \Delta \psi \) (Bogonez et al., 1983, although see Vallejo & Serrano, 1989). We estimate from the values of \( \Delta \psi_{\text{M}} \) and \( \Delta \psi_{\text{H}^+} \) reported in Bogonez et al. (1983) that at pH 3.5 and 5.1 \( \Delta \psi_{\text{H}^+} \) for their yeast strain was, respectively, -248 and -242 mV, in general agreement both with our observations and with those made by Roos et al. (1977) with another yeast at pH 5-6. The gene MEP1, representing the low-affinity mode of ammonia uptake, as opposed to the one of higher affinity studied by Bogonez et al. (1983), has recently been cloned (Marini et al., 1994). Characterization of methyamine uptake in strains overexpressing the methyamine carriers might help to define the role of \( \Delta \psi \) in the process.

We have shown that at pH 5 raising extracellular [K+] from 0.5 to 50 mM scarcely changed the magnitude of \( \Delta \psi_{\text{cyt}} \) or \( \Delta \psi_{\text{cyt}} \) maintained during energy metabolism. The system nevertheless responded to the presence of TPP in a manner showing that \( \Delta \psi \) represented an important component of \( \Delta \psi_{\text{H}^+} \) (Fig. 1). Thus, changing [K+]out appeared to have little effect on the magnitude of \( \Delta \psi_{\text{H}^+} \). In contrast, raising [K+]out systematically lowered \( \Delta \psi_{\text{cyt}} \) after ATP depletion (Hopkins et al., 1992). Analogous behaviour was shown with respect to glycine absorption through the general amino acid permease (Eddy et al., 1970a, b). One can speculate that during energy metabolism the magnitude of \( \Delta \psi \) is mainly governed by the proton pump, whereas after energy depletion K+-selective channels open and \( \Delta \psi \) depends on a K+-diffusion potential. Bertl et al. (1993) describe the gating characteristics of outward rectifying K+-selective channels of about 20 pS conductance which they suggest allow efflux of K+ ions in response to membrane depolarization during secondary active transport. The channels also respond to changes in cytosolic [Ca2+] or in the prevailing transmembrane gradient of K+ ions. Whether these three factors alone acting on the 20 pS channels can account for the apparent changes in K+ conductance that accompany ATP depletion in our preparations is by no means clear. Possibly covalent modification of the system is also required.

Kotyk (1994) has challenged the assumption that during energy metabolism the bulk values of the components of \( \Delta \psi_{\text{H}^+} \) govern the kinetics, as opposed to the thermodynamic parameters, of secondary active transport in yeast (Sanders et al., 1984). He argues instead that the symports respond to the local concentration of protons at the sites in the membrane of their extrusion by the proton pump (Kotyk & Dvorakova, 1992; Kotyk, 1994). Certain difficulties with that point of view are outlined by Eddy et al. (1994). Kotyk (1994) observed linear increases in the rate of amino acid or adenine uptake as the rate of extracellular acidification increased, but only a weak negative correlation with the apparent magnitudes of \( \Delta \psi \) or \( \Delta \psi_{\text{H}^+} \). Because Slavik & Kotyk (1984) assayed \( \Delta \psi \) using TPP and assayed \( \Delta \psi_{\text{H}^+} \) by a method that fails to detect a sharp change in \( \phi \) pH between the yeast cytosol and its environment, we question the need to introduce the concept of local gradients as their observations may not reflect the true bulk parameters of the proton gradient.

REFERENCES


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