Expulsion of uracil and thymine from the yeast *Saccharomyces cerevisiae*: contrasting responses to changes in the proton electrochemical gradient

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The outflow of uracil from the yeast *Saccharomyces cerevisiae* is known to be relatively fast in certain circumstances, to be retarded by proton conductors and to occur in strains lacking a uracil proton symport. In the present work, it was shown that uracil exit from washed yeast cells is an active process, creating a uracil gradient of the order of -80 mV relative to the surrounding medium. Glucose accelerated uracil exit, while retarding its entry. DNP or sodium azide each lowered the gradient to about -30 mV, simultaneously increasing the rate of uracil entry. They also lowered cellular ATP content. Manipulation of the external ionic conditions governing $\Delta\bar{\mu}_{H^+}$ at the plasma membrane had no detectable effect on uracil transport in yeast preparations thoroughly depleted of ATP. It was concluded that uracil exit is probably not driven by the proton gradient but may utilize ATP directly. It is known that thymine is not normally absorbed by yeast. However, thymine expulsion was here observed during deamination of the substrate 5-methylcytosine in the presence of glucose. In the absence of glucose, or following ATP depletion, thymine uptake from the medium only occurred when $\Delta\bar{\mu}_{H^+}$ was dissipated, either by DNP or azide, or by manipulation of the external ionic environment. The yeast expelled absorbed thymine when $\Delta\bar{\mu}_{H^+}$ was restored to the physiological range. The properties of the system corresponded to those of an H^+ /thymine antiport that is distinct from the mechanism expelling uracil.

Keywords: uracil, thymine, yeast exit pumps, proton gradient, thymine antiport

INTRODUCTION

Yeasts accumulate diverse nutrients, including various purines and pyrimidines, by means of specific proton symports. These utilize the energy inherent in the proton gradient ($\Delta\bar{\mu}_{H^+}$) that the proton ATPase, encoded by the *PMA1* gene, establishes across the plasma membrane (Eddy, 1982; Serrano, 1991; van der Rest, 1995). Less is known about the role of $\Delta\bar{\mu}_{H^+}$ in the secretion, as opposed to the absorption, of yeast solutes. However, putative antiports have been recognized as open reading frames in the yeast genome, representing membrane proteins either explicitly associated with drug resistance or as base sequence homologues of such systems (Balzi & Goffeau, 1994; Schuldiner *et al.*, 1995; André, 1995). A second distinct group of membrane proteins utilize ATP directly to expel toxic agents (Decottignies *et al.*, 1994; André, 1995). The existence of other secretory mechanisms is shown by reports that when yeast

deaminates adenine (Roush & Saeed, 1960), guanine (Lahou, 1958) or cytosine (Grenson, 1969) the respective products hypoxanthine, xanthine and uracil accumulate outside the cells. Mutations leading to enhanced excretion of purines have also been described (Armitt & Woods, 1970). The four purines named above are all substrates of the cytosine-purine proton symport, the product of the *FCY2* gene (Foret *et al.*, 1978; Brèthes *et al.*, 1992). Whether uptake and exit of the purines is mediated by the same carrier is unclear. Uracil is not a substrate of the cytosine-purine symport and, furthermore, is known to be excreted by yeast strains of genotype *fur4* (*cyt p*) that lack a functional uracil proton symport (Grenson, 1969; Jund *et al.*, 1977). Jund *et al.* (1977) observed that the proton conductors 2,4-DNP and sodium azide (Seaston *et al.*, 1976) each retarded uracil efflux in these circumstances.

The present work was initiated in order better to define

the role of $\Delta\bar{\mu}_{\text{H}^+}$ in uracil efflux in *Saccharomyces cerevisiae*. First, the ability of the yeast to pump uracil out of the cells into the medium against a uracil concentration gradient was studied. Then the effect of varying $\Delta\bar{\mu}_{\text{H}^+}$ on the net outward movements of uracil was examined. For that purpose, yeast preparations were depleted of ATP in conditions where proton symports concentrate their solutes in proportion to the magnitude of $\Delta\bar{\mu}_{\text{H}^+}$ (Eddy, 1982; Eddy & Hopkins, 1996). Finally, a related series of experiments was done with 5-methyluracil (thymine). Thymine is not normally absorbed by *S. cerevisiae* (Grenson, 1969) but was here shown to be expelled from preparations fed glucose and 5-methylcytosine, a substrate of both the cytosine-purine symport (Chevallier *et al.*, 1975) and cytosine deaminase (Ipata & Cercignani, 1978).

METHODS

Yeast cultures. *S. cerevisiae* strain NC34Cy7 was isolated (A. A. Eddy & P. Hopkins, unpublished observations) as a spontaneous variant of strain NC34 *spa* (*fur1-8 rh fur4-6*) (Jund *et al.*, 1977) that grew faster than the parent strain on a glucose-mineral salts solid medium containing 5 mM cytosine as sole source of nitrogen for growth. The absence of the respective activities associated with uracil phosphoribosyl-transferase, ribohydrolase and uracil permease makes such strains especially suitable for studying uracil efflux (Jund *et al.*, 1977).

Yeast cultures (0.2 l) for experiments were grown aerobically at 30 °C, with constant agitation (150 r.p.m.) on a platform shaker (Gallenkamp, Orbital Incubator). The growth medium contained (l^{-1}): Oxoid Malt Extract (3 g), Difco Yeast Extract (3 g), glucose (10 g) and Difco Peptone (5 g). The yeast was harvested in exponential phase (OD_{615} 0.5–0.9; Cecil instruments CE 272 spectrophotometer), washed with water and stored as a suspension (20 mg dry wt ml^{-1}) on ice for up to 3 h.

For two sets of assays the yeast strain NC123-9B[pBH-15] that overexpresses the uracil proton symport was grown to early exponential phase in glucose-mineral salts solution (Hopkins *et al.*, 1988).

Chemical studies. Deamination of cytosine or 5-methylcytosine was studied as follows. The yeast (14 mg ml^{-1}), suspended in 10 mM Tris brought to pH 4.8 with citric acid (10 ml), was shaken for 2 min at 30 °C with 50 mM glucose. One of the above compounds was then added to a final concentration of 0.4 mM. Controls lacked added substrate. Samples (1.4 ml each) were taken at intervals up to 10 min and chilled rapidly to 0 °C. The yeast (A) was separated from the suspension medium (B) by centrifugation at $10^4 g$ for 0.5 min (Eppendorf microfuge, model 3200). For some purposes A was kept at 95 °C in water (0.7 ml) for 3 min. The cell suspension was then cooled on ice, centrifuged again and the extract (C) separated. The spectral behaviour of portions of B containing either 0.01 M HCl or 1 mM KOH was examined in the region 230–300 nm using a Beckman DU8 spectrophotometer. Authentic solutions of each substrate or its deamination product were similarly characterized. Other portions (0.1–1 ml) of B were examined by absorption chromatography on a column of polyacrylamide gel (Biogel P2, fine grade) as described by Uziel (1967). The column (30 cm $\times$ 1 cm) was eluted with water (0.8 ml min^{-1}) for up to 60 min, the eluate being monitored optically by passage through a Uvicord S (model

2138, LKB). In my hands authentic uracil or thymine reproducibly emerged in a volume of about 7 ml after about 30 min, whereas passage of cytosine or 5-methylcytosine was retarded by about a further 23 min. The presumptive uracil (or thymine) fractions obtained in this way from preparation B were concentrated *in vacuo* to give solution D (1 ml). Confirmation of the presence of uracil or thymine, respectively, was sought in two ways. (1) The absorption spectrum of D was observed in the UV region in the presence of 0.01 M HCl or 1 mM KOH. The results were compared with the spectral behaviour of various nucleic acid constituents reported by Dawson *et al.* (1969). (2) Portions of D were examined by TLC on cellulose containing a fluorescent indicator (Eastman Kodak). Solvents employed were methanol/HCl/water (70:20:10, by vol.), butanol/methanol/water/ammonia (60:20:20:1, by vol.) or butanol/water (86:14, v/v). Solutions of uracil, cytosine, uridine, thymine and 5-methylcytosine were routinely used as standards. They could be differentiated by the above combination of spectroscopic and chromatographic tests.

Fractionation of absorbed [$2\text{-}^{14}\text{C}$]-uracil. The yeast (80 mg dry wt) was suspended at 30 °C in 5 ml 6 mM [$2\text{-}^{14}\text{C}$]uracil (625 nCi ml^{-1} /23.1 kBq ml^{-1}), containing 5 mM KCl and 50 mM Tris brought to pH 4.8 with citric acid. Controls lacked added uracil. After 25 min a portion (1.2 ml) of the suspension was mixed with water (9 ml) at 0 °C. Glucose (25 mM final concentration) was added to the remainder. Three samples were taken during the next 5 min. The yeast in each sample was separated by centrifugation, washed with water (10 ml) at 0 °C and extracted with hot water as for extract C above. Portions (10 μl) of each extract were assayed for ^{14}C using a toluene/Triton-based scintillant (Hopkins & Eddy, 1985). Other portions were resolved by TLC using the above aqueous butanol solvent with uracil and uridine as standards. The chromatogram was subsequently cut into 10 sections, each of which was in turn assayed for ^{14}C by scintillation counting. These procedures were supplemented by column chromatography of the extracts (250 μl) as for solution B above. The solution (8 ml) containing the 'uracil' fraction emerging at about 30 min was concentrated to a small volume (1 ml). Portions were then assayed for ^{14}C and examined by TLC or spectrophotometrically. The proportion of the ^{14}C originally added to the column that emerged in the 'uracil' fraction was then computed. The hot water extract of yeast not exposed to uracil served as a control. It contained a mixture of nucleotides that eluted as a prominent peak before 30 min (compare Uziel, 1967) while the 30 min fraction itself contained uridine as the only UV-absorbing material detected by TLC. Extracts of yeast exposed to uracil behaved similarly except that both uracil and uridine were detected in the 30 min fraction. Because the ϵ_{290} values for uracil as compared with uridine are in the ratio 34/1 at pH 11, assay of the uracil content of the 30 min fraction was done at that pH. Thus, A_{290} at pH 11 was read against the corresponding fraction from the control to give the quantity ΔA_{290} . The ratio (^{14}C content ml^{-1})/ ΔA_{290} was then computed as a measure of the specific radioactivity of the uracil fraction. It was compared with the corresponding ratio for the uracil originally added to the yeast suspension.

Cellular ATP content. The yeast suspension (3 mg dry wt in 0.1 ml) was mixed with 0.5 ml 7.5% (w/v) perchloric acid and kept at 0 °C for up to 3 min. The solution was neutralized with 2 M KOH containing 0.5 M triethanolamine. The precipitate separating in 30 min at 0 °C was removed by centrifugation. Portions (20 μl) of the supernatant solution were assayed for ATP by measuring their chemiluminescence in the presence of

firefly extract (Sigma, FLE-250) as described by Stanley & Williams (1969). Light emitted in successive 10 s intervals was monitored in a Packard scintillation counter (model 3310), operating without its coincidence filter. The system was calibrated by addition of known amounts of ATP (10–200 pmol) as internal or external standards.

Transport assays. In order to load it with uracil the yeast (30 mg dry wt ml⁻¹) was suspended in 5 mM KCl, 50 mM Tris brought to pH 4.8 with citric acid. The suspension (2 ml) was held at 30 °C in a small flask (25 ml) and shaken (10 cm amplitude, 60 r.p.m.). Typically, uracil was then added to give a solution containing 3 mM [2-¹⁴C] uracil (160 nCi ml⁻¹/5.92 kBq ml⁻¹). Samples (150 µl each) collected during 20 min were each immediately mixed with 1.3 ml water at 0 °C in a microfuge tube and kept on ice. Corrections for extracellular binding of uracil were based on assays conducted throughout at 0 °C. Subsequent operations on the diluted samples were carried out at 4 °C in a cold room. After centrifugation at 10⁴ g for 1 min, the supernatant solution B, representing the extracellular solution, was separated from the cell pellet (A). The latter was washed with water (2 × 1.2 ml). The products A and B derived from each sample were each assayed for ¹⁴C, with appropriate attention to counting statistics (Eddy & Hopkins, 1996). The distribution of uracil between the yeast and suspension medium was calculated using a value of 1 ml cell water (450 mg dry wt)⁻¹ (Hopkins *et al.*, 1992). In computing the maximum gradient of uracil or thymine the yeast formed during energy metabolism, a correction was applied based on the observation that only about 68% of the cell water appeared accessible to these compounds.

Thymine uptake from a 0.9 mM solution of [1-¹⁴C]thymine (80 nCi ml⁻¹/2.96 kBq ml⁻¹) was studied at 30 °C in a similar fashion. Uptake was largest when Δp_H⁺ was deliberately lowered. This occurred, for instance, when glucose was absent and the yeast was suspended in a solution containing 50 mM KCl, 50 mM HEPES brought to pH 7.4 with Tris (see Results).

Uracil or thymine expulsion from yeast loaded with these compounds in the above manner was initiated by addition of 1 M glucose to a final concentration of 25 mM. In certain instances the uracil concentration was simultaneously raised from 3 mM to near 16 mM without changing the ratio ¹⁴C/¹²C. Yeast samples (0.15 ml) were taken at intervals and processed as above. In other instances, the yeast suspension was diluted 10-fold with buffered KCl containing 28 mM glucose in order to initiate uracil efflux. In that event, each cell sample (1 ml) was mixed with 9 ml of water at 0 °C before centrifugation. The diluted extracellular solution was separated and the cell pellet was washed. Both were assayed for ¹⁴C. In each experiment the radioactivity of the extracellular solution was virtually constant through the sample series and a mean value was used for the purpose of computing the uracil distribution (S_i/S_o) at each time point.

The validity of the above procedures for separating and washing the yeast samples was confirmed by reproducing the observations illustrated in Fig. 1 and Fig. 5 with larger specific radioactivities and the smaller amounts of yeast (~1 mg) that can be recovered on glass-fibre filters (Eddy & Hopkins, 1996 and unpublished work).

Transport as a function of Δp_H⁺. The effect of changing Δp_H⁺ on uracil and thymine transport in yeast preparations depleted of ATP was studied by manipulating extracellular pH and [K⁺] (Eddy & Hopkins, 1996). The buffered cell suspension (4 ml) at 30 °C contained antimycin (2 µg), 2-deoxyglucose

(10 µmol), yeast (45 mg dry wt) and 0.9 mM [2-¹⁴C]thymine (80 nCi ml⁻¹, 2.96 kBq ml⁻¹) or 3 mM [2-¹⁴C]uracil (160 nCi ml⁻¹, 5.92 kBq ml⁻¹). Buffers comprised 50 mM Tris brought to pH 4.4 or 4.8 with citric acid, 50 mM MES adjusted to pH 6.5 with Tris and 50 mM HEPES brought to pH 7.4 with Tris. [KCl] was usually 5 mM or 50 mM. Substrate uptake was initiated at the selected pH. After an interval of up to 15 min a portion (2 ml) of the cell suspension was centrifuged without prior cooling. The supernatant solution was carefully removed. The yeast pellet was resuspended in a selected second buffer solution. This contained 2.5 mM deoxyglucose and radiolabelled 3 mM uracil or 0.9 mM thymine. Both cell suspensions were sampled at intervals, each sample (0.15 ml) being mixed with water (1.3 ml) at 0 °C before centrifugation. The ¹⁴C content of the yeast and the corresponding extracellular solution were later assayed. The pH of the original yeast suspension and that of the resuspended cells was shown to vary little from its nominal value by the end of the experiment.

Proton absorption. These assays were done exactly as described by Hopkins *et al.* (1988).

Mathematical procedures. The aim was to describe the intracellular concentration of substrate (S_i) at time *t* as a function of the constant extracellular concentration (S_o) and the pseudo first-order rate constants for substrate entry (*k*) and exit (*k'*). It follows that

$$\frac{dS_i}{dt} = kS_o - k'S_i \quad (\text{Eqn 1})$$

Integration with respect to *t* gives the result

$$\ln\left(\frac{kS_o - k'S_i}{kS_o - k'S'}\right) = -k't \quad (\text{Eqn 2})$$

where S' is the value of S_i at time zero.

In one series of experiments the yeast was allowed to accumulate *S* progressively with time and S' was zero. Rearrangement of the above equation shows that in these circumstances

$$\frac{S_i}{S_o} = \frac{k}{k'}(1 - e^{-k't}) \quad (\text{Eqn 3})$$

In another series of experiments the yeast first accumulated *S* to a high level S' and then expelled it in response to the presence of glucose. By rearranging equation 2, these results were analysed as

$$\frac{S_i}{S_o} = \left(\frac{S'}{S_o} - \frac{k}{k'}\right)e^{-k't} + \frac{k}{k'} \quad (\text{Eqn 4})$$

Observations of S_i/S_o as a function of *t* were fitted to equations 3 and 4 by non-linear least squares fitting routines leading to estimates of S', k', and either *k* or the ratio *k*/k' and their respective errors.

RESULTS

Uracil uptake and efflux in relation to energy supply

When the yeast strain NC34Cy7 lacking the uracil proton symport was kept at pH 4.8 in the absence of glucose, uracil uptake at 30 °C from a 3 mM solution

Fig. 1. The time course of uracil absorption in the absence of glucose (○) and its subsequent expulsion after addition of glucose (●). The suspension of washed yeast cells at pH 4.8, 50 mM KCl was kept at 30 °C with 3 mM uracil for 35 min, a portion being separated at 16 min and supplemented with 25 mM glucose. Both suspensions were sampled at intervals and the ratio $[\text{uracil}]_i/[\text{uracil}]_o$ determined. The upper curve is represented by equation 3 with $k = 0.0544 \pm 0.0111$, $k' = 0.0909 \pm 0.017$. The lower line represents equation 4 with $k/k' = 0.0382 \pm 0.0039$; $S/S_o = 0.46$; $k' = 0.435 \pm 0.035$. Observations are based on one of several similar experiments summarized in Table 1.

occurred at a relatively low initial rate of about $0.4\text{--}0.6 \text{ nmol mg}^{-1} \text{ min}^{-1}$. After about 30 min net uptake ceased (Fig. 1, open symbols) when the mean

ratio ($\pm \text{SEM}$) $[\text{cellular uracil}]/[\text{extracellular uracil}]$ ($[\text{uracil}]_i/[\text{uracil}]_o$) observed in four experiments was 0.65 ± 0.03 . Addition of 25 mM glucose to a portion of the yeast suspension caused a rapid fall in the uracil content of the cells. Thus the yeast appeared to expel uracil against its own concentration gradient (Fig. 1, filled symbols). Cellular uracil, if uniformly distributed in the cellular water, fell to about 4 % of the extracellular concentration.

The time course of the uptake of uracil, as well as its subsequent exit, conformed approximately to the exponential relationships represented by the respective symbols in Fig. 1. In the former instance, the entry rate coefficient k was 0.054 min^{-1} and the exit rate coefficient k' , 0.091 min^{-1} (equation 3, Methods), whereas in the presence of glucose k was 0.017 min^{-1} and k' was 0.43 min^{-1} (equation 4, Methods). Table 1 summarizes a series of observations showing that the presence of glucose on average lowered the magnitude of k by a factor of at least 4, simultaneously increasing k' by a factor of about 4. Application of the mathematical model thus appeared to show that during net uracil efflux the effective entry coefficient of uracil was only $0.01\text{--}0.03 \text{ min}^{-1}$ (Table 1). To confirm this, the initial rate of entry of uracil in the presence of glucose was assayed directly. It corresponded to a mean value of k of 0.010 , in fair agreement with the former estimate. The value of k was independent of $[\text{uracil}]_o$ in the range $0.03\text{--}30 \text{ mM}$ (not shown).

Table 1 further shows that the rate constant for uracil

Table 1. Effect of glucose or ATP depletion on the first-order rate constants of uracil entry (k) and exit (k')

The value of k was independent of $[\text{uracil}]_o$ in the range $0.03\text{--}9 \text{ mM}$ (not shown). Assays were done at 30 °C, pH 4.8 with 5 mM or 50 mM KCl. [Glucose] was 25 mM. Antimycin ($0.04 \mu\text{g mg}^{-1}$) and 2.5 mM deoxyglucose were present when ATP was to be depleted. $[\text{Uracil}]_o$ was 3 mM in the assay of k . For k' , it was 3 mM when uracil moved against its own concentration gradient (uphill) or 0.3 mM when exit occurred down the concentration gradient (downhill). For the latter purpose the loaded cell suspension was diluted 10-fold (see Methods). Values of k were determined either (1) from the initial linear phase of uracil uptake with time, or (2) by applying equations 3 or 4 in the approach to the steady state (see Methods). Values of k' were based on the second procedure. Estimates are means $\pm \text{SE}$ based on n independent determinations.

Conditions	KCl (mM)	<i>n</i>	<i>k</i> (min ⁻¹)	<i>k'</i> (min ⁻¹)
Glucose absent	5	5	0.069 ± 0.005	0.108 ± 0.021
ATP depleted	5	3	0.058 ± 0.007	0.085 ± 0.008
Glucose present	5	3	$0.018 \pm 0.006^*$	
Glucose present	5	9	$0.010 \pm 0.002^\dagger$	
Exit downhill, glucose absent	5	3		0.150 ± 0.015
Exit downhill, glucose present	5	3		0.554 ± 0.030
Exit uphill, glucose present	5	5		$0.475 \pm 0.030^\ddagger$
Exit uphill, glucose present	50	6	$0.027 \pm 0.009^*$	0.592 ± 0.122

* Entry rate during net uracil expulsion.

† Initial rate of net uptake.

‡ Chi-square tests indicated expulsion was initially delayed.

Fig. 2. The quasi steady state concentration of cellular uracil reached in three yeast preparations kept with glucose and 16 mM uracil after each had been loaded with a different amount of uracil. Four yeast preparations were kept at 30 °C, pH 4.8 with 5 mM KCl and, respectively, 3 mM (○, ●), 0.6 mM (■) or 0.2 mM (△) uracil of the same specific radioactivity for about 30 min. At time zero one preparation (○), serving as a control, was transferred to a similar buffered solution containing 16 mM uracil, again of the same specific radioactivity. The other three preparations were transferred at time zero to 25 mM glucose at pH 4.8 containing 16 mM uracil, 5 mM KCl and sampled at the indicated times (see Methods). In the final steady state reached in the presence of glucose $[\text{uracil}]_i/[\text{uracil}]_o$ was about 0.04. Observations are representative of three similar experiments (see also Table 2).

exit was approximately the same in the 'uphill' as in the 'downhill' mode.

Magnitude of the uracil gradient established in the presence of glucose

The steady state established after 25 min in the presence of glucose and 3 mM uracil was assumed above to represent the condition where the rates of entry and exit of uracil were equal. An alternative view would be that a small fraction of the cellular uracil was sequestered and unavailable for efflux. To examine this matter further, three yeast preparations were initially loaded with about 0.1 mM, 0.5 mM or 2 mM cellular uracil. Glucose was then added to each, together with about 16 mM uracil. Fig. 2 shows that the same steady state was reached in the three preparations, although uracil was initially accumulated by two of them even in the presence of glucose. The constant level reached in the presence of uracil thus represented a dynamic steady state. The gradient of uracil formed in three such experiments with 16 mM extracellular uracil was similar to that formed with 0.3 mM or 3 mM uracil outside (Table 2). Control assays conducted at 0 °C instead of 30 °C showed that a very small amount of uracil was retained by the cell samples under these conditions, representing a level of radioactivity approximately equal to the natural background radioactivity of samples

without added ^{14}C . The mean correction of $0.0133 \pm 0.003(5)$ was subtracted from the values of $[\text{uracil}]_i/[\text{uracil}]_o$ listed in Table 2. It was insignificant in most other circumstances.

Characterization of absorbed uracil

Using the procedures outlined in the Methods section, samples of the hot water extract of yeast that had absorbed 6 mM ^{14}C uracil for 25 min and then been exposed to 25 mM glucose were fractionated by TLC and on polyacrylamide gel. During TLC the main radioactive component of the extracts migrated with uracil. The recovery of radioactivity in the 'uracil' fraction from eight samples fractionated on polyacrylamide varied from 88 to 96%, with a mean value $\pm$ SD of $92 \pm 4\%$. Furthermore, the mean ratio (radioactivity $\text{ml}^{-1}/\Delta A_{290}$) was $(1.34 \pm 0.18) \times 10^4$ for the 'uracil' recovered from the column (see Methods). The corresponding ratio for the uracil originally added to the yeast was 1.345×10^4 . Uracil was absent from extracts prepared from yeast that had not been exposed to uracil. These observations in combination show that the cellular pool of ^{14}C indeed represented uracil as such.

Effect of ATP depletion or proton conductors on uracil transport

Yeast preparations depleted of ATP in the presence of antimycin and 2-deoxyglucose absorbed uracil with a rate constant of 0.058 min^{-1} , similar to the mean value of 0.069 observed when the metabolic inhibitors were omitted (Table 1). Likewise, the presence of 0.25 mM DNP had no significant effect on the rate constant of uracil entry assayed in the absence of glucose (not shown). In contrast DNP, azide or CCCP each accelerated uracil uptake in the presence of glucose (Table 2). The maximum rate approached that observed in the absence of glucose. A more detailed study of the dependence of the increase in k on [azide], at six selected concentrations, showed that the effect was half maximal at $0.11 \pm 0.03 \text{ mM}$.

Fig. 3(a) illustrates the time course of uracil expulsion in the presence of 20 mM azide, a concentration sufficient to produce the maximum effect on uracil entry. Whereas in the controls the ratio $[\text{uracil}]_i/[\text{uracil}]_o$ fell to about 0.05, the ratio with azide present was about 0.18. The corresponding values of the entry (k) and exit (k') rate coefficients (Table 2) show that azide increased the magnitude of k but left k' unchanged. The fourfold effect on k was similar to that observed on the initial rate of uracil entry.

Other observations summarized in Table 2 show that, in comparison with the controls, DNP and FCCP each also eventually hindered uracil expulsion. Thus, the steady value of $[\text{uracil}]_i/[\text{uracil}]_o$, reached after about 10 min in the presence of glucose, was near 0.2 or 0.1 with the respective proton conductors present, whereas it fell to about 0.04 in the controls. Fig. 3(b) shows that the time

Table 2. The maximum gradient of uracil distribution reached during glycolysis in the presence of 0.3 mM, 3 mM or 16 mM uracil: effect of proton conductors on this distribution and on uracil entry

Uracil distribution was determined as for Figs 1 or 2. On three out of twelve occasions the maximum gradient was not maintained for more than 6 min in the presence of glucose. Observations like those in Fig. 1 were used with equation 4 of Methods to determine the best value of k/k' , the asymptotic distribution. Alternatively, the minimum value of $[\text{uracil}]_i/[\text{uracil}]_o$ (maximum gradient) was determined by inspection of observations like those in Fig. 2. In both cases, the amount of uracil binding to the yeast at 0 °C was subtracted (see text). A further correction was based on the circumstance that, after ATP depletion, k/k' was about 0.68 (Table 1). This was taken to mean that uracil penetrated no more than that fraction of the cellular water. Thus the true minimum value of $[\text{uracil}]_i$ was $(0.68)^{-1}$ times its apparent value. Mean corrected values of $k/k' \pm \text{SE}$, based on n independent assays, were used to compute confidence limits ($\pm 2\text{SE}$) for the magnitude of the negative uracil gradient the yeast formed ($-RT/F \ln([\text{uracil}]_i/[\text{uracil}]_o)$). The effect of a proton conductor supplement on the magnitude of k was studied in two circumstances: (1) in terms of the initial rate of uracil entry from a 3 mM solution, pH 4.8, 5 mM KCl, 25 mM glucose; (2) in the approach to the steady state, when k' was also determined (see Fig. 3). [KCl] was increased to 50 mM for that purpose, to minimize loss of cellular K^+ ions.

[uracil] _o (mM)	[KCl] (mM)	Supplement	n	k (min ⁻¹)	k' (min ⁻¹)	Corrected value of:	
						k/k'	Negative uracil gradient (mV)
0.3	5	None	3			0.0197 ± 0.0110	> 83
3	5	None	4			0.0368 ± 0.0044	81–93
16	5	None	3			0.0562 ± 0.0055	70–81
3	50	Control*	6	0.027 ± 0.009	0.592 ± 0.122	0.0475 ± 0.0106	70–95
3	50	20 mM azide*	5	0.117 ± 0.029	0.609 ± 0.133	0.2636 ± 0.0140	32–38
3	5	Control†	5	0.0112 ± 0.002			
3	5	1 mM azide†	3	0.050 ± 0.003			
3	5	20 mM azide†	2	0.046			
3	5	0.25 mM DNP†	2	0.036			
3	5	0.1 mM CCCP‡	3	0.0530 ± 0.006			
3	50	Control	3			0.0412 ± 0.0176	67–133
3	50	0.2 mM DNP	4			0.160 ± 0.023	41–57
3	50	0.1 mM FCCP‡	2			0.093 ± 0.012	56–70
3	5	None*	5			0.0394 ± 0.004	80–90

* Observations in first minute omitted on basis of chi-square tests showing delayed uracil exit.

† Initial rates.

‡ Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were added as ethanolic solutions.

course of uracil expulsion followed a complex oscillatory pattern in the presence of DNP, which was not amenable to analysis by equation 4 (see Methods). Similar behaviour occurred in three other experiments. It is apparent that DNP had little effect on the initial net rate of uracil expulsion. Table 2 shows further that significant gradients of uracil concentration formed even in the presence of the proton conductors.

Cellular ATP content

Table 3 summarizes observations on the cellular ATP content in yeast preparations like those used in the foregoing studies. After starvation in the presence of uracil, glucose addition increased the ATP content by about 14% to a fairly steady level that was maintained for 5 min. Even in the presence of glucose, 10 mM azide or 0.5 mM DNP caused a progressive fall in ATP

content. Addition of 2-deoxyglucose and antimycin lowered cellular ATP to about 3% of the level maintained with glucose present.

Effect of alterations in extracellular pH and [K⁺] on uracil transport

Yeast preparations were allowed to accumulate uracil from a 3 mM solution for 40 min at pH 7.4 in the presence of 50 mM KCl. The ratio $[\text{uracil}]_i/[\text{uracil}]_o$ was then about 0.7. The yeast was then transferred to a new solution at pH 4.8 containing 1 mM KCl, 3 mM uracil. Alternatively, uracil uptake was assayed at pH 4.8 without prior exposure to pH 7.4. Whether or not the yeast was depleted of ATP in the presence of deoxyglucose, no significant expulsion of uracil occurred in five experiments as a result of increasing $\Delta\mu_{\text{H}^+}$ either (1) by lowering both $[\text{K}^+]$ and the pH sim-

Fig. 3. Effects of 20 mM azide or 1 mM DNP on uracil expulsion in the presence of glucose. The yeast was loaded with uracil from a 3 mM solution at pH 4.8, 50 mM KCl for 29 min (see Methods). Azide or DNP was added, followed by 25 mM glucose 1 min later (time zero). Samples were taken at the indicated intervals. Controls were run without a proton conductor. (a) Azide (○) and paired controls (●); observations are means $\pm$ SEM of five independent experiments. The rate coefficients deduced by applying equation 4 of Methods are summarized in Table 2. (b) DNP (○) and controls (●); observations are from one of four similar assays.

ultaneously, or (2) by lowering $[K^+]$ from 50 mM to 2.5 mM at pH 7.4. The significance of this negative result is enhanced by the contrasting behaviour shown by thymine in similar circumstances (see below).

Studies with thymine

Expulsion of thymine during deamination of 5-methylcytosine. In order to test whether the yeast could excrete thymine, it was fed 5-methylcytosine under the conditions outlined in the Methods section. Parallel tests were made using cytosine as a control, when the uracil resulting from deamination is known to accumulate in the medium (see Introduction). In the latter instance, progress of the reaction leads to changes in the spectral characteristics of the supernatant solution obtained by centrifugation of the cell suspension. In the presence of

Table 3. Percentage reduction in cellular ATP content under various conditions

Yeast preparations were starved for 20 min aerobically at pH 4.5, 5 mM KCl in the presence of 3 mM uracil, at 30 °C. Proton conductors were then added 1 min before 25 mM glucose (see Methods). Observations are mean values $\pm$ SEM based on four independent yeast cultures. The cellular ATP content in six preparations containing glucose was 1.66 ± 0.05 nmol (mg dry wt) $^{-1}$. This was taken as 100%. ATP depletion occurred in the presence of antimycin ($0.04 \mu\text{g mg}^{-1}$) and 2.5 mM deoxyglucose.

Conditions	Relative ATP content (%)
Control, with 25 mM glucose	100 ± 3
Glucose absent	87 ± 7
ATP depleted	3 ± 1
0.5 mM DNP, 2 min	23 ± 2
0.5 mM DNP, 4 min	13 ± 2
10 mM azide, 2 min	42 ± 9
10 mM azide, 4 min	26 ± 3

0.01 M HCl, the UV absorption spectrum of cytosine exhibited a peak at 274 nm whereas uracil showed similar ϵ values peaking at 260 nm. Interconversion of the two compounds thus leads to progressive replacement of the one peak by the other. The corresponding wavelengths for 5-methylcytosine and thymine were 282 nm and 263 nm, respectively. It was shown in this way in each of three experiments that the yeast converted more than 90% of the added cytosine to extracellular uracil during 10–25 min at 30 °C in the presence of glucose. Catabolism of uracil thus seemed to be negligible during that interval. Likewise, added 5-methylcytosine was replaced in similar yield by a compound that absorbed maximally at about 263 nm. After fractionation of the suspension medium on the polyacrylamide gel, the UV-absorbing material behaved like thymine both (1) during TLC in two solvents and (2) with respect to its detailed spectral characteristics (see Methods). These observations show that the yeast can excrete thymine formed endogenously.

Conditions leading to thymine uptake. Preliminary observations confirmed earlier literature showing that the amount of thymine absorbed from a 1 mM solution during 20 min at pH 4.8 in the presence of glucose was very small ($[thymine]_i/[thymine]_o \sim 0.01$). Omission of glucose scarcely affected the rate, unlike the corresponding behaviour of uracil (Table 1). These circumstances suggested that thymine entry might be hindered by the prevailing proton electrochemical gradient. To test this hypothesis uptake was assayed at pH 6.5 or at pH 7.4 with 50 mM KCl present, conditions that would lower $\Delta\mu_{H^+}$ (Eddy & Hopkins, 1996). Thymine was then absorbed significantly faster, at a rate of about $0.15 \text{ nmol mg}^{-1} \text{ min}^{-1}$ (rate coefficient 0.067 min^{-1}) which is similar to the rate of uracil entry under the same conditions. The observations illustrated

Fig. 4. Time course of thymine uptake and efflux in response to a decrease (↓) or increase (↑), respectively, in $\Delta\bar{\mu}_{H^+}$, caused by DNP or by alterations in extracellular pH and $[K^+]$. The procedures used to transfer the yeast from one solution to another are described in Methods. The presence throughout of deoxyglucose and antimycin ensured that the cellular content of ATP was about 2.7% of the value during glycolysis. Observations are based on the behaviour of one washed cell preparation, the two series of transfers (solid and dashed lines, respectively) with sampling being completed in 2 h. Individual effects were reproduced on at least two other occasions (see Table 4). In the first series (---) the yeast was kept with 0.9 mM thymine at pH 4.8, 5 mM KCl for 30 min (▼). Meanwhile, at 9 min a portion was transferred to 0.9 mM thymine, pH 6.5, 50 mM KCl (△). At 21 min another portion was supplemented with 0.2 mM DNP, pH 4.8, 5 mM KCl (■). In the second series (—) thymine uptake was initiated at pH 6.5, 50 mM KCl (○). At 14 min one portion was transferred to pH 4.8, 5 mM KCl, 0.9 mM thymine (●). A second portion was transferred to a similar solution containing 0.2 mM DNP (▲). The transient fall in thymine content at 15 min (▲) was observed in two other assays. The arrow associated with each line indicates whether an increase or decrease of $\Delta\bar{\mu}_{H^+}$ occurred when the yeast was transferred to the new medium.

in Fig. 4 show that lowering the pH to 4.8 and simultaneously lowering $[K^+]$ to 5 mM expelled thymine against its own concentration gradient, from preparations depleted of ATP in the presence of 50 mM KCl, pH 6.5. Correspondingly preparations kept at pH 4.8 with 5 mM KCl excluded thymine until they were transferred to the more alkaline buffer. The presence of 0.2 mM DNP at pH 4.8 resulted in an even larger uptake of thymine than occurred at pH 6.5. Table 4 shows how the maximum uptake ratio varied with the ionic conditions or when either 2 mM azide or 0.2 mM DNP was added. Azide increased the ratio 11.9 ± 2.0 times,

whereas DNP increased it 5.7 ± 1.6 times (series 2). Increasing $[K^+]$ up to 50 mM, in order to lower $\Delta\psi$, raised the ratio 2.7 ± 0.6 times at pH 7.4 (series 4) and 2.2 ± 0.3 times at pH 4.4 (series 1). Simultaneously raising $[K^+]$ and the pH increased the ratio 8.7 ± 1.1 times (series 1, series 2). Clearly, the above observations support the hypothesis that thymine expulsion was driven by $\Delta\bar{\mu}_{H^+}$.

Effect of glucose on thymine accumulation at pH 7.4 with 50 mM KCl. Addition of glucose to a yeast preparation that had accumulated thymine for 30 min at pH 7.4 resulted in its rapid expulsion (Fig. 5). Accumulated uracil was also expelled under these conditions. The mean value $\pm$ SEM of the rate coefficient for thymine exit (k') observed in three such experiments was $1.406 \pm 0.136 \text{ min}^{-1}$, about three times larger than k' for uracil. In the presence of glucose the thymine accumulation ratio fell to a mean value $\pm$ SEM of 0.048 ± 0.015 .

DISCUSSION

Jund *et al.* (1977) gave no values for the uracil entry coefficient k but concluded that the rate constant for uracil exit (k') from a strain of yeast closely related to the one used in the present work was 0.60 min^{-1} . That estimate agrees well with the corresponding value in Table 1. Jund *et al.* (1977) incubated the yeast for 30–45 min in a minimal medium containing glucose, NH_4^+ and uracil, in order to load the cells with uracil. The ratio $[\text{uracil}]_i/[\text{uracil}]_o$ was then about 0.44 (Fig. 8, Jund *et al.*, 1977). Ratios of that magnitude are near those expected if the distribution of uracil was governed by facilitated diffusion, and only hint at the presence of a mechanism actively transporting uracil out of the yeast. In order to initiate uracil efflux, the loaded cells were transferred to minimal medium lacking uracil. The foregoing sequence of events contrasts with the behaviour illustrated in Fig. 1 of the present work. Here, the yeast absorbed uracil in the absence of glucose and NH_4^+ , afterwards expelling most of it when glucose was added. Whether uracil was absorbed or expelled depended on its distribution relative to a quasi equilibrium value, of the order of 0.04, reached during about 4 min (Fig. 2). After exposure of the yeast to glucose for 30 min, the 'equilibrium' value itself tended to increase substantially (A. A. Eddy & P. Hopkins, unpublished work), a circumstance that may be relevant to the interpretation of Fig. 8 of Jund *et al.* (1977). Irrespective of that issue, our observations (Figs 1, 2) show clearly that uracil exit is an active process, expelling uracil against its own concentration gradient. The maximum uracil gradient formed was about -80 mV relative to the external medium.

The systems expelling uracil and thymine differ in various ways. While both were driven by glucose metabolism, imposition of an inwardly directed proton electrochemical gradient, after depletion of cellular ATP, led to expulsion of thymine but not of uracil. The respective exit mechanisms thus appear to be distinct and to utilize different sources of energy.

Table 4. Accumulation and expulsion of thymine, but not uracil, in response to changes in $\Delta\mu_{H^+}$

The assays were based on the circumstance that, after depletion of cellular ATP, lowering extracellular pH in the range 7–4 increased $\Delta\mu_{H^+}$, whereas lowering extracellular K^+ in the range 50–1 mM increased the negative membrane potential $\Delta\psi$ (Eddy & Hopkins, 1996). Alternatively, the addition of 2 mM azide or 0.2 mM DNP lowered $\Delta\mu_{H^+}$. The cell suspensions contained antimycin ($0.04 \mu\text{g mg}^{-1}$) together with 2.5 mM deoxyglucose to ensure that the cellular ATP content was small (see Table 3). The preparations were kept at 30 °C, pH 4.5, 6.5 or 7.4 with 50 mM, 5 mM, 2.5 mM or 1 mM KCl (solution 1 as specified below). The cell suspension also contained either 0.9 mM thymine or 3 mM uracil labelled with ^{14}C (see Methods). Substrate uptake was followed for up to 40 min as appropriate (see Fig. 4). A portion of the cell suspension in solution 1 was separated and the yeast transferred to a new ionic environment, containing labelled thymine or uracil (solution 2). Uptake of the pyrimidine was followed for a further 10–30 min. The maximum uptake occurring in each solution is shown below.

	Composition of solution 1	Maximum uptake ratio*	Composition of solution 2	Maximum uptake ratio*	Result of transfer†
Thymine					
Series 1	pH 7.4, 50 mM KCl	0.572 ± 0.076	pH 4.4, 2 mM NaCl	0.052 ± 0.007	E
			pH 4.4, 2 mM Na azide	0.587 ± 0.098	
			pH 4.4, 50 mM KCl	0.118 ± 0.025	E
Series 2	pH 4.5, 5 mM KCl	0.066 ± 0.017	pH 4.5, 5 mM KCl + 0.2 mM DNP	0.374 ± 0.039	U
Series 3	pH 6.5, 50 mM KCl	0.462 ± 0.098	pH 4.5, 5 mM KCl	0.083 ± 0.022	E
			pH 4.5, 5 mM KCl + 0.2 mM DNP	0.658	U
Series 4	pH 7.4, 2.5 mM KCl	0.212 ± 0.020			
	pH 7.4, 50 mM KCl	0.539 ± 0.020			
Uracil					
Series 5	pH 7.4, 2.5 mM KCl	0.783 ± 0.040			
	pH 7.4, 50 mM KCl	0.722 ± 0.038	pH 4.5, 1 mM KCl	0.682 ± 0.181	

* Mean value of the ratio $[S]_i/[S]_o$ based on three independent determinations, or two where no value of the SEM is given.

† U, E, appreciable net uptake or exit, respectively, of thymine after transfer to solution 2.

Fig. 5. Comparison of thymine and uracil expulsion in the presence of glucose at pH 7.4, 50 mM KCl. The yeast was loaded either with uracil (●) from a 3 mM solution or with thymine (○) from a 0.9 mM solution. Glucose, deoxyglucose and antimycin were absent in both cases. After 30 min at 30 °C, glucose was added to 25 mM. The ratio of the cellular concentrations of uracil or thymine to the corresponding extracellular concentration is shown as a function of time after glucose addition. The observations were made in one of three similar experiments. Mean values of the exit coefficients are given in the text.

After depletion of ATP, the membrane potential is mainly governed by diffusion of K^+ ions across the plasma membrane (Eddy & Hopkins, 1996). That the pH gradient and the membrane potential each contributed to the energy driving thymine out of the yeast is shown by the results listed in Table 4. Furthermore, the presence of azide or DNP, by short-circuiting the proton gradient, had the expected effect of allowing a net uptake of thymine to occur in circumstances where thymine was expelled from the controls. The foregoing behaviour indicates that the flow of thymine across the cell membrane is coupled to the proton flow in an antiport relationship. The ready reversibility of the putative thymine carrier in these experiments recalls the similar behaviour of the cytosine carrier which Eddy (1982) contrasted with the seeming irreversibility of the general amino acid proton symport (compare van der Rest *et al.*, 1995).

The maximum inward thymine gradient the yeast developed was of the order of 25-fold or -84 mV . Such a gradient is considerably smaller than the likely magnitude of $\Delta\mu_{H^+}$ at pH 4.5 with 2 mM K^+ present, namely -220 mV (Eddy & Hopkins, 1996). Interpretation of the difference is complicated (1) by the possibility that small amounts of thymine sequestered in

the yeast may lead to underestimation of the thymine gradient and (2) by lack of information about the number of routes of entry and exit of thymine (compare Eddy & Hopkins, 1996). It is interesting that the native uracil proton symport (encoded by the gene *FUR4*) failed, even when overexpressed (see Methods), to catalyse proton absorption in the presence of 1 mM thymine (unpublished work). The yeast thus discriminates between uracil and thymine both on entry and on exit.

After depletion of ATP, the rate of uracil uptake was similar to the optimal rate of thymine uptake observed when $\Delta\bar{\mu}_{\text{H}^+}$ was relatively small. Glucose caused the rate coefficient k for uracil entry to fall from about 0.06 to about 0.01 min⁻¹. Simultaneously k' for uracil exit increased from about 0.1 min⁻¹ to at least 0.4 min⁻¹ (Table 1). The simplest view is that both changes involve the same mechanism, namely a uracil exit pump. Azide and DNP each increased k and restricted the ability of the yeast to expel uracil, without however stopping active transport altogether. Indeed DNP scarcely affected the initial net exit of uracil against its own concentration gradient. It seems unlikely therefore that the actions of these two compounds on uracil exit were due to their effects on $\Delta\bar{\mu}_{\text{H}^+}$ as such. Absence of a correlation with $\Delta\bar{\mu}_{\text{H}^+}$ is further shown by the fact that K_i for the variation in uracil entry rate with [azide] was 0.11 ± 0.03 mM, much lower than the K_i of 1.95 ± 0.30 mM for the effect of azide on $\Delta\bar{\mu}_{\text{H}^+}$ as reflected in the cytosine accumulation ratio (Fig. 1a, Eddy & Hopkins, 1996). Because DNP and, to a lesser extent, azide lowered the cellular ATP content (Table 4), it is possible that the net rate of uracil exit was governed by that factor rather than $\Delta\bar{\mu}_{\text{H}^+}$.

Jund *et al.* (1997) also observed that DNP and azide each lowered both the rate and extent of uracil efflux, which in the presence of the drug tended towards an asymptotic value. Exit coefficients of about 0.1 min⁻¹ and 0.3 min⁻¹, respectively, were obtained dependent on whether the value of the asymptote was included in or subtracted from [uracil]_i. While the foregoing behaviour broadly parallels the observations shown in Fig. 3 of the present work, there is the important difference that, whereas Jund *et al.* (1977) assayed the rate uracil moved out of the yeast cells down its own concentration gradient, uracil was expelled into a higher concentration in most of this work.

The main conclusions to be drawn from the present study of uracil expulsion are as follows. (1) The mechanism can expel uracil against its own concentration gradient. (2) The response to the presence of the proton conductors probably means that uracil efflux is not driven by the proton electrochemical gradient. (3) That conclusion is reinforced by the lack of response to changes in $\Delta\bar{\mu}_{\text{H}^+}$ brought about by altering the ionic conditions after ATP depletion. (4) The requirement for glucose may mean that the system utilizes ATP as such, although an additional regulatory role for glucose cannot be ruled out. Studies with everted yeast membrane vesicles might help to resolve some of these issues.

Recent elegant work describes a nucleotide triphosphatase of the yeast plasma membrane that appears to catalyse efflux of various drugs (Decottignies *et al.*, 1994). A similar nucleotide triphosphatase may drive uracil exit. Loss of this function, by genetic manipulation or mutational events, would be expected to impair growth with cytosine as a sole source of nitrogen for growth. Nothing is known at present about the genes governing uracil and thymine secretion, the discovery of which may perhaps be facilitated by plans to prepare deletion mutants of individual open reading frames representing putative yeast membrane proteins of unknown function (André, 1995; Oliver, 1996).

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