Proton stoichiometry of the overexpressed uracil symport of the yeast *Saccharomyces cerevisiae*

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Information about the molecular biology of the uracil carrier of *Saccharomyces cerevisiae* is now available but its properties as a symport are unexplored. We have now studied its proton stoichiometry at pH 6.5, 4.8 and 4.2, in a yeast strain over-expressing the symport and unable to metabolize uracil. After the depletion of cellular ATP, uracil uptake followed an approximately exponential time course to reach a plateau. Proton uptake was then indistinguishable from the basal rate shown in controls without added uracil. It was concluded that more than 87% of the uracil flux through the system was coupled to symported protons. Because the basal rate was a significant fraction of the total rate of proton uptake at pH 4.2 or 4.8, the ratio of the proton and uracil flows could not be defined uniquely during the initial near-linear phase of uptake. However, the

average rate of proton uptake during increasing time intervals up to 120 s was shown to be a linear function of the corresponding average rate of uracil uptake. This relationship, defining approx. 90% of the eventual uracil uptake, was used to deduce the mean proton stoichiometry as approx. 3 at pH 4.2, falling to near 2 at pH 6.5. The rate of uracil uptake increased 4-fold when the negative proton gradient ($\Delta\mu^H$) acting across the plasma membrane increased from 60 to 200 mV. The rate fell markedly after depolarization by KCl. The maximum uracil gradient ($\Delta\mu^S$) was less affected by depolarization. The ratio $\Delta\mu^S/\Delta\mu^H$ fell from approx. 2 to near 1 as $\Delta\mu^H$ increased in the above range. In contrast with the starved yeast, uracil accumulation during energy metabolism followed a pump-leak model.

INTRODUCTION

Facile uptake of uracil by wild-type strains of *Saccharomyces cerevisiae*, or by uracil auxotrophs, depends on the uracil permease [1,2] encoded by the *FUR4* gene [3], one of a small number of homologous yeast genes in the UPL group (reviewed in [4]). The primary structure [5], membrane topology [4] and mutant behaviour [6] of the uracil permease have been described but its properties as a symport [7] have received little attention. Uracil uptake in wild-type strains is too slow for a reliable assessment of the magnitude of any additional simultaneous proton uptake. However, after overexpression of the *FUR4* gene, preliminary work showed that uracil caused a net uptake of protons into yeast preparations depleted of ATP, although the stoichiometry was not established [7]. The work was done with a yeast strain carrying both the *fur1-8* and *rh* alleles to prevent the assimilation of exogenous uracil [2,7]. In the present study with the same yeast strain we have assayed the number of protons (n) absorbed in symport with each molecule of uracil, at three pH values in the physiological range from 6.5 to 4. Simple theory predicts that n governs the energy input into the system from the proton electrochemical gradient at the plasma membrane. $\Delta\mu^H$ is at least approx. –200 mV in the pH range 4–7 [8], whereas optimal uracil accumulation by the wild-type yeast is less than 100 mV [2], rising almost to 200 mV after overexpression of the uracil symport [7]. A limiting factor is the relatively fast exit of uracil, which occurs independently of the symport. This process exhibits a first-order rate constant of 0.6 min^{–1} [2,9] in the presence of glucose but is much slower after the depletion of cellular ATP [9]. Assay of the proton stoichiometry is done under the latter conditions and is complicated by the spontaneous leakage of protons into the yeast, especially at acid pH [10].

There are reasons for thinking that symport activity lowers the spontaneous proton leakage [10,11], a factor that can be analysed by considering the time course of proton uptake beyond the initial phase of quasi-linear substrate uptake [12].

Our observations on the magnitude of n also provide information about the tightness of coupling between the respective flows of protons and uracil through the system when the uracil distribution reached a steady state. The magnitudes of the proton gradient and the uracil gradient were then compared, to define the thermodynamic relations between these two parameters.

MATERIALS AND METHODS

Yeast cultures

The yeast strains NC123-9B[pBH-15][*fur1-8 rh fur4-6 leu2* (FUR4 LEU2)], RJ-25[*fur1-8 rh*] and RL31-1A[*fur1-8 rh dhul-1*] were used in a previous study, as was NC123-9B[pJDB] lacking FUR4 in the plasmid [7]. They were cultured aerobically at 30 °C in a glucose/mineral salts medium to early exponential phase ($D_{610} < 0.5$) [10]. The yeast was harvested, washed with water and stored on ice for up to 3 h.

Assay of proton and uracil absorption

As described previously [8], the yeast (50 mg dry weight) was suspended in 4.5 ml of dilute buffer solution containing (1) 1.7 mM citric acid brought to pH 4.2 with Tris, (2) 2.5 mM Tris brought to pH 4.8 with citric acid, or (3) 1.7 mM Tris brought to

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pH 6.5 with Mes. The cell suspension (final volume 5 ml) was stirred continuously at 30 °C; antimycin (10 μ g) and 2-deoxy-glucose (25 μ mol) were added to deplete cellular ATP. After 4 min at pH 4 or 4.8, or 6 min at pH 6.5, [2- 14 C]uracil (typically 1.99 μ mol; 167 nCi) was added, the pH being recorded continuously. A series of samples (each 100 μ l) were taken during the next 300 s. Each sample was filtered through a wetted glass fibre filter (Whatman GF/C), the yeast was washed at 4 °C and its 14 C content was assayed with the precautions outlined by Eddy and Hopkins [8]. Within experimental error, uracil uptake occurred at a constant rate during the first 15 s, during which three samples could be taken without causing unacceptable disturbance to the pH record. Proton uptake at each sampling time was inferred by interpolation on the pH electrode trace, which was calibrated by the addition of 0.5 μ mol of HCl. Typically, at pH 4.8, 1.5 μ mol of acid was absorbed in 180 s, of which 0.8 μ mol was additional to the uptake that would have occurred had uracil not been added.

Uracil accumulation ratio during energy metabolism

The yeast suspension (0.5 mg dry weight/ml) at 30 °C contained 10 mM citric acid brought to pH 5 with Tris, 1 mM KCl, 100 mM glucose and [2- 14 C]uracil (up to 250 nCi/ml). The initial concentration of uracil was varied in the range 0.05 μ M to 10 mM. A series of samples (each 1 ml) were taken to define the steady state of uracil distribution, which was reached within 30 min. Each sample was filtered; the yeast was washed and assayed for 14 C (see above). Extracellular [uracil] was determined either by difference or by assaying the sample filtrates for 14 C. To compute the ratio of [cellular uracil] to [extracellular uracil], the cellular water content was assumed to be constant at 2.22 μ l/mg dry weight, the value determined for yeast strain NC123-9B[pBH15] at pH 4.8.

Intracellular pH

This was assayed by measuring the accumulation of [14 C]benzoic acid from a 3.4 μ M solution [8].

Mathematical procedures

Uracil uptake (U) at time t is represented as a first-order rate process, approaching an upper limit U^∞ , with rate constant k . Thus:

$$U = U^\infty(1 - e^{-kt}) \quad (1)$$

Case 1. No uracil leakage occurs. As described in [12], proton uptake in the presence of uracil is:

$$H = nU + \int_0^t B^t dt \quad (2)$$

where n is the proton stoichiometry of the carrier and B^t is the rate constant at time t for the basal flow of protons into the yeast outside the carrier. The carrier is assumed to function reversibly with the same proton stoichiometry for uracil entry and exit, so that the proton flow through the symport is zero in the steady state of uracil distribution. Making the assumption that B^t is a constant B in the presence of uracil, eqn. (2) rearranges to:

$$H/t = n(U/t) + B \quad (3)$$

Plotting H/t against U/t thus provides a means of estimating both n and B from the slope and intercept respectively. The primary variables represent the average rates of proton and uracil uptake respectively in the interval t .

Case 2. Uracil leaks out of the yeast independently of the symport, with a first-order rate constant L . Uracil entry by the leakage pathway is negligible. The magnitude of the correction (ϕ) at time t is:

$$n \int_0^t LU dt = tU^\infty Ln - (Ln/k)U \quad (4)$$

because ϕ is zero at zero time.

Addition of ϕ to both sides of eqn. (2) gives:

$$H/t = n(1 - L/k)U/t + B + LnU^\infty \quad (5)$$

H/t and U/t are thus linearly related in Case 2 but the slope and intercept of the graph differ from those in Case 1. Furthermore the leakage of uracil in the steady state of its distribution leads to a net uptake of protons through the symport at a rate of nLU^∞ . It follows from eqns. (3) and (5) that uracil leakage lowers the apparent value of n by nL/k , where:

$$nL/k = (\text{steady-state proton flow})/kU^\infty \quad (6)$$

The uracil steady state

Eddy and Hopkins [8] describe a mathematical model of the steady state of uracil distribution in terms of S_i and S_o , the respective cellular and extracellular concentrations and the following parameters: (1) H_o and H_i , the respective extracellular and intracellular proton concentrations; (2) the rate constant L for solute leakage; (3) K_m and $V_{\max}$ for the initial rate of uracil entry through the symport; (4) a parameter Θ equal to $\exp(-\Delta\psi/F/2RT)$. Here $\Delta\psi$ is the plasma membrane potential and F , R and T have their conventional significance.

The steady-state value of S_i is the positive real root of the quadratic expression:

$$AS_i^2 + BS_i + C = 0 \quad (7)$$

We consider the general case in which the rate coefficient for uracil entry other than through the symport equals αL , α being an arbitrary parameter. When $\alpha = 1$, leakage occurs by simple diffusion [8]; when $\alpha = 0$, uracil efflux outside the symport is by active transport by a mechanism that has yet to be defined [9]. The coefficients of eqn. (7) are:

$$\begin{aligned} A &= \Theta H_i [2S_o(1 + \Theta^2) + (1 + \Theta)^2 K_m] \\ B &= (1 + \Theta)^2 [\Theta^2 K_m H_o (S_o + K_m) + K_m H_i V_{\max}/L] - \\ &\quad \alpha \Theta S_o H_i [2S_o(1 + \Theta^2) + (1 + \Theta)^2 K_m] \\ C &= -\Theta^2(1 + \Theta)^2 K_m H_o S_o [V_{\max}/L + \alpha(S_o + K_m)] \end{aligned}$$

We confirmed that when $\alpha = 1$, A , B and C are consistent with the alternative formulations given in [8].

The above equations are based on a symport with unit proton stoichiometry. We show below that $n \geq 2$ for the uracil symport. It initially proved satisfactory to take this into account simply by replacing H_o , H_i and Θ by H_o^2 , H_i^2 and Θ^2 respectively for the purpose of computing the numerical values of A , B and C .

RESULTS AND DISCUSSION

In the assay illustrated in Figure 1 the yeast preparation depleted of ATP at 30 °C absorbed uracil at an initial rate of approx. 9 nmol/s per 50 mg dry weight from a 0.4 mM solution lightly buffered at pH 4.8. Uracil uptake [U in eqn. (3)] followed an approximately exponential time course to a limit of 530 nmol per 50 mg yeast dry weight with a rate constant of 0.017 s $^{-1}$ (●). Proton uptake [H in eqn. (3)] was simultaneously observed for 300 s (○) and compared with the basal proton uptake into

Figure 1 Time course of proton and uracil uptake at pH 4.8 and 30 °C by preparations of NC123-9B[pBH-15] overexpressing the uracil permease

Yeast (50 mg dry weight) was depleted of ATP in the presence of deoxyglucose and antimycin. The addition of 0.4 mM [14 C]uracil at zero time initiated the uptake of uracil (●) and of protons (○). Observations are mean values based on three assays with a single yeast culture. Error bars represent the S.E.M. where this was larger than the symbol itself. The line drawn through the uracil data corresponds to eqn. (1) with k at 0.017 s^{-1} and U^∞ at 530 nmol. The dotted line represents the mean proton uptake that occurred in three controls to which no uracil had been added.

controls lacking added uracil (Figure 1, dotted line). In assays with five independent yeast cultures, the mean rate of proton uptake into the cell suspension (50 mg) when uracil uptake had reached a plateau was indistinguishable from the corresponding rate of proton uptake of $2.4 \pm 0.3\text{ nmol/s}$ (given as mean $\pm$ S.E.M.) into the controls without added uracil (see Figure 1). We conclude that proton flow through the symport in the steady state of uracil distribution was less than the uncertainty in the estimates, namely approx. 0.65 nmol/s per 50 mg at pH 4.8. The leakage rate in the steady state was thus relatively small in comparison with the control proton leakage rate and smaller still than the initial rate of proton uptake with uracil, which averaged 23 nmol/s in 14 assays. Similar behaviour was observed in three assays at pH 6.5, in which the basal proton flow was $0.8 \pm 0.6\text{ nmol/s}$. At pH 4.2 the basal proton flow was $6.0 \pm 0.8\text{ nmol/s}$ and a steady state of uracil uptake was not reached during the assays.

Magnitude of corrections for uracil leakage

The above rate of proton leakage of less than 0.65 nmol/s corresponds to a decrease nL/k in the apparent stoichiometry as defined in eqn. (6). A mean k value of 0.022 s^{-1} was obtained in 14 assays in which U^∞ averaged 490 nmol per 50 mg of yeast dry weight. The aforesaid decrease was therefore expected to be less than 0.060. We consider this quantity to be small enough to be ignored relative to the overall slopes of 2 or more obtained by plotting H/t against U/t (see below). We accordingly used eqn. (3) rather than eqn. (5) to analyse our data. The observed rate of proton leakage corresponded to a first-order rate constant for uracil leakage of less than 0.032 min^{-1} . The latter value should be compared with the mean rate coefficient for uracil exit in the steady state, which was determined as $0.448 \pm 0.041\text{ min}^{-1}$ (mean $\pm$ S.E.M.) in five other assays with starved yeast. Less than 0.032/0.448, or 7%, of the uracil flux was therefore not in symport with the proton flux.

Table 1 Upper and lower estimates of the ratio (n) of the initial rate of proton uptake to the initial rate of uracil uptake under five conditions

The upper estimate (mean $\pm$ S.E.M.) in each case assumes that all the observed proton uptake occurred through the uracil symport [B in eqn. (3) is zero]. The lower estimate assumes that the proton uptake through the symport equalled the total proton uptake minus that in the controls without added uracil (B equals the rate in the control). N is the number of independent assays.

pH	Extracellular [K^+] (mM)	Stoichiometry, n		N
		Upper value	Lower value	
4.2	1	3.079 ± 0.135	2.217 ± 0.171	3
4.8	1	2.301 ± 0.188	2.062 ± 0.180	5
4.8	50	3.363 ± 0.088	2.276 ± 0.039	3
6.5	1	2.394 ± 0.133	2.279 ± 0.235	5
6.5	50	2.247 ± 0.215	2.226 ± 0.222	5

Proton slippage through the amplified uracil carrier in the absence of uracil

If protons slip through the uracil carrier in the absence of uracil itself, the above estimates of the basal proton flow B outside the carrier are too large. To explore this issue, the mean ($\pm$ S.E.M.) basal rate of proton uptake in the absence of uracil in strain NC123-9B[pBH15] with the amplified level of carrier activity was compared with the basal rate in strain NC123-9B[pJDB] lacking the *FUR4* gene product. The former quantity at pH 4.8 averaged $2.11 \pm 0.18\text{ nmol/min}$ per mg dry weight in nine assays, whereas the latter quantity averaged $1.42 \pm 0.25\text{ nmol/min}$ in four assays. The difference, $0.69 \pm 0.34\text{ nmol/min}$, between them was significant in a one-tailed t test ($P \sim 0.025$). A similar difference was observed when glutamate was used as a source of nitrogen for growth. We assume that the difference is due not to adventitious factors but to proton uptake through the uracil carrier in the absence of extracellular uracil. The increase in basal proton flow of 0.69 nmol/min per mg with the enhanced expression of *FUR4* is approx. 5.7% of the maximum flow of uracil through the system in the same units. On that interpretation the basal proton flow taking place outside the carrier is thus smaller than its apparent value B by 0.69 nmol/min per mg. As our observations do not show whether the same proton flow through the carrier persists in the steady state of uracil distribution, our best estimate of uracil slippage is therefore that it lies between 0% and 13% of the uracil flux.

Estimates of n from initial rates of proton and uracil uptake

The mean ($\pm$ S.E.M.) initial rate of uracil uptake from a 0.4 mM solution during the first 15 s of exposure to that compound was $10.11 \pm 0.61(5)\text{ nmol/s}$ per 50 mg dry weight. The proton uptake was $23.26 \pm 1.2\text{ nmol/s}$ per 50 mg. Of this, $20.84 \pm 1.31\text{ nmol/s}$ represented proton flow in excess of the basal control value. The corresponding upper and lower estimates of the proton stoichiometric ratio (n) at pH 4.8 were therefore 2.301 ± 0.188 and 2.062 ± 0.180 . Similar assays were done (1) in the presence of 50 mM KCl to depolarize the yeast [8], (2) at pH 6.5 and (3) at pH 4.2. Table 1 compares the five pairs of estimates at the three pH values. The upper and lower estimates at each pH diverged as the pH decreased and the basal proton flow in the controls increased. The lower estimates are consistent with a constant value of n near 2, whereas the upper estimates range between about 2.0 and 3.5.

Figure 2 Linear relationships between the average rate of proton uptake (H/t) in time t and the corresponding average rate of uracil uptake (U/t) at each of three pH values

Results like those in Figure 1 were used to test eqn. (3). The plots were found to be linear for values of t up to approx. 120 s. Observations at pH 4.2 (○) were made with three independent yeast cultures. Observations at pH 4.8 (●) were made at 10 values of t with 13 yeast cultures. They were pooled to give means $\pm$ S.E.M. of H/t based on between six and thirteen assays at each value of U/t . The S.E.M. in U/t as depicted was smaller than the symbol itself. Observations at pH 6.5 in the presence of 50 mM KCl (×) were based on three yeast cultures. A fourth set at pH 6.5, involving five other cultures, is not depicted as the results overlapped with the observations at pH 4.8; they are included in Table 2. The slope of each straight line equals the value of the proton stoichiometry n at that pH (Table 2). The intercept on the ordinate equals the value of the basal proton flow B in the presence of uracil (Table 2).

Estimates of n obtained from the time course of uracil absorption

Eqn. (3) shows that, provided that n and B are both constant, H/t is a linear function of U/t . Figure 2 (●) tests that relationship at pH 4.8 with pooled data obtained with 13 yeast cultures. The observations were made at 10 intervals of up to 120 s. They show no significant departure from linearity (variance ratio F of 0.19959 with $P < 0.001$, as described in [13]). Observations made after 120 s deviated significantly from the trend line (results not shown). Table 2 lists the values of B and n obtained by fitting eqn. (3) to the above results and to other similar observations. Except at pH 4.8 in the presence of 50 mM KCl, the magnitude of the effective proton leakage rate, B , in the presence of uracil was not significantly different from zero. The corresponding values of n given in Table 2 ranged from near 2 to 3. The detailed

statistical analysis showed that lowering the pH progressively increased the value of n in that range.

Comparison of the upper estimates in Table 1, which are based on the assumption that the effective basal proton flow, B , was zero, shows that these are in fact similar to their counterparts in Table 2. This behaviour relates to the circumstance that the unconstrained value of B emerging from the fitting routine was also near zero. In contrast, the lower estimates in Table 1 are based on the assumption that the effective basal proton flow was the same as that in the absence of uracil. At pH 4.2 and 4.8 the lower estimates are smaller than their counterparts in Table 2. We conclude that, although these lower estimates might imply that n was fairly constant near 2, that hypothesis fails to account for the stoichiometry shown in the assays up to 120 s provided that B is constant. The constancy of B indeed seems to be implied by the linear relationships depicted in Figure 2. However, if this assumption is nevertheless rejected and n is set at 2, the variation of the function $H - 2U$ with time represents the changes in the putative basal proton flow. Reference to eqn. (2) shows that the latter flow equals $\int_0^t B' dt$. We found that at pH 4.2 and 4.8 B' would on that basis decrease systematically in the interval up to 120 s (results not shown). Although such behaviour might be attributed to progressive cellular acidification, the absence of an effect of uracil on the proton flow at 300 s (Figure 1) refutes that explanation. We accordingly reject the hypothesis that $n = 2$ at the two lower pH values.

A series of assays at pH 4.8 was done with 12.5 or 50 μ M uracil. The total proton displacements per mol of uracil absorbed were similar to those described above.

Variation of initial rate of uracil uptake with the ionic conditions

The initial rate of uracil uptake from a 0.4 mM solution increased from approx. 2.1 nmol/s per 50 mg yeast dry weight at pH 6.5 in the presence of 50 mM KCl to approx. 8.8 nmol/s at pH 4.8 with 1 mM KCl (Table 3, series 1). Raising the concentration of K^+ depolarizes the starved yeast, ideally by 102 mV when a Nernst relationship applies [8]. In the present study 50 mM KCl retarded the initial rate of uracil uptake by approx. 55 % at pH 4.8 and by approx. 70 % at pH 6.5. Lowering the pH from 6.5 to 4.2 tended to increase the rate. Thus the initial rate increased roughly in parallel with the magnitude of $\Delta\mu^H$ (Table 3).

Uracil gradient as a function of the ionic conditions

The mean maximum accumulation of uracil from a 0.4 mM solution decreased slightly on depolarization from the equivalent

Table 2 Proton stoichiometry (n) and basal proton flow in the presence of uracil (B) and their respective S.E.M. values deduced from observations in the period up to 120 s under five ionic conditions

The linear plots in Figure 2 defined the respective slopes and intercepts corresponding to n and B in eqn. (3). B refers to 50 mg of yeast dry weight. The basal rate of proton uptake (mean $\pm$ S.E.M.) per 50 mg of yeast in the absence of uracil is shown for comparison. N is the number of pairs of values of H/t and U/t analysed. Statistical analyses were as described in [13].

pH	Extracellular [K^+] (mM)	N	n	B (nmol/s)	Proton leakage in control (nmol/s)
4.2	1	15	$3.098 \pm 0.239^*$	0.259 ± 1.371	5.97 ± 0.83 (3)
4.8	1	58	$2.468 \pm 0.095^{\dagger\dagger}$	-0.054 ± 0.555	2.42 ± 0.36 (5)
4.8	50	13	$2.940 \pm 0.128^{\ddagger}$	1.128 ± 0.335	4.07 ± 0.19 (4)
6.5	1	27	$2.249 \pm 0.173^{\S}$	0.379 ± 0.796	0.80 ± 0.37 (5)
6.5	50	25	$2.347 \pm 0.290^{\S}$	-0.387 ± 0.445	0.20 ± 0.25 (5)

* Larger than at pH 4.8 ($P < 0.001$).

$\dagger$ Larger than at pH 6.5 ($P < 0.001$).

$\ddagger$ More than 2 ($P < 0.001$).

$\S$ Not more than 2 ($P \sim 0.1$).

Table 3 Initial rate of uracil uptake and the maximum uracil gradient ($\Delta\mu^S$) formed by a starved preparation of NC123-9B[pBH15] as functions of the proton gradient ($\Delta\mu^H$)

The preparations were maintained at 30 °C under the specified ionic conditions, with the selected initial uracil concentration, as described for Figure 1. The magnitude of $\Delta\mu^H$ was estimated from the sum of (1) ΔpH assayed with 3.4 μM benzoic acid and (2) $\Delta\mu^K$, the gradient of K^+ ions between the yeast and the surrounding medium [8]. In series 1, $\Delta\mu^H$ refers to estimates at the time at which uracil was added. In series 2, the estimates refer to the steady state of uracil distribution reached 2–5 min later. Initial rates (means $\pm$ S.E.M.) of uracil uptake are based on three to nine independent assays with 50 mg yeast dry weight. Gradients (means $\pm$ S.E.M.) were assayed with three or four independent yeast cultures. For the coupling ratio, $\Delta\mu^H$ was assayed at uracil steady state. The differential coupling ratio (see text) is the ratio of the change in $\Delta\mu^S$ brought about by depolarizing the yeast with 50 mM KCl (NaCl serving as control) to the corresponding change in $\Delta\mu^H$; this ratio takes into account the small change in ΔpH that ensued.

Series	[Uracil] (μM)	pH	[K^+] (mM)	[Na^+] (mM)	Initial rate of uracil uptake (nmol/s)	$-\Delta\mu^H$ (mV)	$\Delta\mu^S$ (mV)	Coupling ratio, $\Delta\mu^S/\Delta\mu^H$	Differential coupling ratio
1	400	4.2	1	0	7.90 ± 0.18	205 ± 1	—	—	—
	400	4.8	1	0	7.29 ± 0.47	186 ± 5	76 ± 6	0.41	—
	400	4.8	1	50	8.83 ± 0.65	184	77 ± 2	0.42	—
	400	4.8	50	0	3.26 ± 0.87	98 ± 3	59 ± 6	0.33	—
	400	6.5	1	0	7.02 ± 0.31	127 ± 5	66 ± 4	0.55	—
	400	6.5	50	0	2.12 ± 0.10	46 ± 12	37 ± 4	0.80	—
2	40	4.8	1	50	—	184 ± 5	184 ± 4	1.00 ± 0.04	—
	40	4.8	50	0	—	98 ± 3	167 ± 9	1.70 ± 0.10	0.20
	20	5.7	1	50	—	149 ± 4	181 ± 5	1.21 ± 0.05	—
	20	5.7	50	0	—	62 ± 5	141 ± 3	2.28 ± 0.18	0.46

of approx. 77 mV to 59 mV at pH 4.8 and from 66 to 37 mV at pH 6.5 (Table 3, series 1). However, the gradients of uracil that formed were considerably larger when the uracil concentration was decreased to one-tenth or less (Table 3, series 2). In that way, extracellular [uracil] at the peak of uracil accumulation was decreased from 250 μM or more to the optimal levels near 1 μM , below the K_m of the system (5 μM [1]; 11.6 μM [2]). Coincidentally $\Delta\mu^S$ increased to near 180 mV at pH 4.8 in the controls containing 50 mM Na^+ and to a level 17 mV lower in the depolarizing solutions containing 50 mM K^+ . At pH 5.7 $\Delta\mu^S$ was 181 mV and depolarization lowered it by 40 mV. The relative lack of response to a putative depolarization of 102 mV is the more striking in a system in which $n \geq 2$.

The steady state of uracil distribution reached with 20 μM uracil and 50 mM KCl at pH 5.7 was studied both by loading the yeast in the presence of KCl and by depolarizing yeast already loaded with uracil. In four assays this revealed that at pH 5.7 the net outflow of uracil after depolarization was relatively slow, with a first-order exit coefficient of $0.036 \pm 0.014 \text{ s}^{-1}$ (mean $\pm$ S.E.M.). This circumstance makes it difficult to establish the value of $\Delta\mu^S$ at low uracil concentrations above pH 6, when $\Delta\mu^H$ is small.

$\Delta\mu^S/\Delta\mu^H$

We concluded above that at pH 4.8 less than approx. 13 % of the uracil flow through the carrier at the steady state of uracil distribution was not coupled to symported protons. In other words, coupling exceeded 87 %. In the familiar case in which true thermodynamic equilibrium is reached, coupling is perfect and both $\Delta\mu^S/\Delta\mu^H$ and the differential $\partial(\Delta\mu^S)/\partial(\Delta\mu^H)$ equal n . Somewhat similarly, when coupling is only 87 %, a so-called ‘static head’ is reached and a theorem of irreversible thermodynamics indicates that the aforesaid ratios equal $0.87n$ [14]. In reality the observed ratios of $\Delta\mu^S$ to $\Delta\mu^H$ were not in general in the range $0.87n$ to n (Table 3). Only when [uracil] was small and the yeast was depolarized were ratios near 2 observed. The observations made with the lower range of uracil concentrations show that $\Delta\mu^S/\Delta\mu^H$ decreased as the magnitude of $\Delta\mu^H$ itself increased. Furthermore the effect of depolarization expressed in the corresponding differential ratio was conspicuously small. We con-

clude that at $\Delta\mu^H$ above approx. -100 mV, uracil accumulation was constrained below the thermodynamic limits by kinetic factors other than slippage, even at concentrations well below the K_m with respect to [uracil] (Table 3). The behaviour of the uracil symport is in sharp contrast with our findings with the cytosine symport, in which $\Delta\mu^S$ and $\Delta\mu^H$ were almost linearly related in the range of the latter variable up to -200 mV and were nearly in thermodynamic equilibrium.

Uracil accumulation during energy metabolism as a function of *FUR4* expression

During energy metabolism, uracil accumulation by wild-type yeast is at most approx. 100 mV [2]. Jund et al. [2] showed that the dependence of the accumulation ratio on uracil concentration could be explained in terms of a pump acting in parallel with an independent leak. To test this concept further we compared the accumulation of uracil in the wild-type strain (RJ25) with that in the strains RL31-1A and NC123-9B[pBH-15], which overexpress the carrier 12-fold and 75-fold respectively. The maximum accumulation ratios that the three yeasts established were 13.3 ± 0.9 (67 mV), 262 ± 20 (145 mV) and 1293 ± 139 (187 mV), on the basis of at least four observations in each instance. Figure 3 shows that over a wide range of substrate concentrations the uracil accumulation conformed approximately to the pump-leak model described by eqn. (7) with the values of the parameters specified in the legend to Figure 3. Our observations thus support the interpretation of Jund et al. [2] of the wild-type behaviour, according to which the leak of 0.59 min^{-1} [9] is available for both entry and exit of uracil. We examined by means of eqn. (7) whether an exit leak that was not available for uracil entry [α of eqn. (7) set at zero] was consistent with the observations on the three yeast strains. Although that assumption did not affect the predicted accumulation of uracil from the most dilute solutions, it failed to describe the accumulation from solutions of concentration 1 mM or more, especially with the wild-type yeast strain. We conclude that a bidirectional leak is required to account for the behaviour of the energized yeasts.

A leak coefficient of 0.59 min^{-1} is at least 10-fold the estimate of less than 0.06 min^{-1} for the starved yeast preparations as discussed in connection with Figure 1 and later. The similarity

Figure 3 Steady-state uracil accumulation as a function of extracellular uracil concentration during energy metabolism at pH 5 by three yeast strains differing in permease activities: comparison with the magnitude of [cellular uracil] predicted by a mathematical model in which uracil leakage outside the permease occurs by facilitated diffusion

The experimental observations were made with yeast strain NC123-9B[BH-15] (+), for which $V_{\max}$ was 11766 nmol/min per ml of cell water; with yeast RL31-1A (O), for which $V_{\max}$ was 1839; and with yeast RJ-25 (●), for which $V_{\max}$ was 156.9. The continuous lines with each set of data are the theoretical predictions based on eqn. (7). The computations were done with nine regularly spaced values of the abscissa. Each line represents a fifth-order polynomial fitted to the predicted ordinates. K_m was 11.6 μM and Θ^2 was 256, with H_0^2 and H_1^2 being respectively 62.4 (μM)² and 0.102 (μM)² [8]. The value of the leak coefficient was 0.59 min⁻¹ [9] with α set at 1. $\Delta\mu^H$ was 228 mV.

between the maximum values of $\Delta\mu^s$ shown in the assays illustrated in Figure 3 and those in Table 3 thus seems fortuitous. On the one hand, starvation virtually eliminated uracil leakage; on the other it lowered $V_{\max}$ from approx. 26.1 nmol/min per mg dry weight to approx. 9.4 nmol/min per mg. However, we found that the observed values of $\Delta\mu^s$ for strain NC123-9B [pBH15] were smaller than those computed with eqn. (7). (1) It seems relevant that the uracil carrier becomes phosphorylated on insertion into the yeast plasma membrane [15]. Starvation might subsequently lead to dephosphorylation of the carrier, changing its kinetic behaviour. We are not aware of any direct evidence

supporting this possibility. (2) In general, starvation lowers the cytosolic pH by up to approx. 0.5 units [8]. For the glycine symport the rate and extent of glycine uptake became progressively smaller as the cellular pH fell by amounts that scarcely affected $\Delta\mu^H$ [16]. It seems possible that the uracil symport behaves similarly. A detailed study of the carrier kinetics would be required to test that speculation, preferably in a system in which the compositions of the solutions on both sides of the cell membrane were well defined.

The effects of changing extracellular $[\text{K}^+]$ as opposed to $[\text{Na}^+]$ on the rate and extent of uracil accumulation strengthen other evidence that the system is electrogenic *in vivo* [7], like both the yeast glycine symport [11,16] and the cytosine symport [7,8]. Nevertheless a partly reconstituted preparation of the last-named system failed to respond to changes in $\Delta\psi$ but accumulated cytosine weakly in response to a pH gradient [17]. This discrepancy remains to be resolved.

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