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Uracil Transport in *Saccharomyces Cerevisiae*

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Summary. Uracil transport in *Saccharomyces cerevisiae* is mediated by a specific "permease" which does not recognize other pyrimidines such as uridine, cytosine, thymine, 2-hydroxypyrimidine or 5-amino-uracil; hypoxanthine and 6-amino-uracil slightly inhibit the uptake of uracil in a strain lacking cytosine permease activity. Wild type cells concentrate extracellular uracil before its transformation into UMP and subsequent incorporation into nucleic acids. A strain lacking UMP pyrophosphorylase and uridine ribohydrolase (strain *fur 1–8 rh*, in which the endogenous production as well as the utilization of uracil are lacking) is able to concentrate ^{14}C-2 uracil from the medium. At the same time no other ^{14}C-2 labelled compound could be detected in this strain, thus suggesting that the uptake of uracil in yeast occurs by active transport which is not coupled to the UMP pyrophosphorylase. The optimal pH of uracil uptake in standard growth conditions was 4.3. It was deduced from experiments performed on strain *fur 1–8 rh* with ^{3}H-5 and ^{14}C-2 uracil that the intracellular pool of uracil is recycled once the steady-state has been reached. First order kinetics with similar rate constants were observed for uracil efflux in strain *fur 1–8 rh* ($k\,\text{min}^{-1}=0.75\pm0.08$) as well as in the strain lacking uracil permease, *fur 1–8 rh fur 4–6* ($k\,\text{min}^{-1}=0.60\pm0.08$). The intracellular pool of ^{14}C-2 uracil can be chased in strain *fur 1–8 rh* by addition of ^{3}H uracil without inducing a large initial acceleration of the exit rate (the rate constant remained at 0.60). 2–4-dinitrophenol inhibits the uptake of uracil but also reduces the efflux of uracil in strain *fur 1–8 rh fur 4–6*. These data and the comparison with cytosine transport in the same organism support the hypothesis that, whereas uracil uptake is a "permease" mediated active transport, the efflux of uracil does not involve the uracil uptake "permease". A coefficient of permeability of $7.4\times10^{-7}\,\text{cm}\,\text{sec}^{-1}$ was calculated for uracil.

Uracil uptake in *Saccharomyces cerevisiae* can be described by a biphasic curve in a Lineweaver-Burk plot and mutants specifically impaired in uracil uptake have been isolated [6, 10, 12]. It has already been shown that a "uracil permease" is specifically involved in the uptake of uracil by *S. cerevisiae*, but the possibility that the uracil uptake is partly coupled to UMP pyrophosphorylase was not completely ruled out [1, 6, 7]. We therefore constructed a strain which was unable to convert uracil

into uridine 5′-monophosphate (5′-UMP) and also unable to generate uracil of catabolic origin. These two properties, as will be shown below, were combined in a recombinant strain of genotype *fur 1–8 rh*. Mutation *fur 1–8* is one of the numerous recessive allelic mutations at locus *fur 1* in *Saccharomyces cerevisiae* which renders the yeast cells resistant to 5-fluorouracil and several other fluorinated pyrimidines [6, 10, 12]. Mutation *rh* [6] corresponds to the loss of uridine ribohydrolase activity [3], which is the sole source of catabolic uracil in *S. cerevisiae* [6, 10]. In order to understand how uracil exit occurs from yeast cells and precisely what the role of the uracil permease is in this process, kinetics of uracil efflux were compared in strain *fur 1–8 rh* and in strain *fur 1–8 rh fur 4–6* which, in addition, is specifically impaired in uracil uptake.

Materials and Methods

Genetic Methods

Genetic analyses were made according to Mortimer and Hawthorne [13].

Organisms

The parent haploid strains of *Saccharomyces cerevisiae* used in this work were the isogenic wild type strains FL 100 (*a*) and FL 200 (α). The strain *fur 1–8 rh* was obtained from the diploid $\frac{fur\ 1\text{–}8\ rh}{+}\ \frac{+}{rh}$, which results from the cross of strain *fur 1–8* (α) by strain *rh* (*a*). Strain *fur 1–8* (α) derives from strain FL 100 (*a*) by mutagenesis, and strain *rh* (*a*) was a generous gift from Dr. Grenson (Univ. Libre de Bruxelles, CERIA, Fac. des Sciences, Microbiologie, Bruxelles 7).

Media

Yeast Nitrogen Base (YNB) (Difco) without amino acids but with ammonium sulfate (5 g/liter) and D-glucose (1%) was used as minimal medium. The complete growth medium was Difco Yeast Extract (1%), Difco Peptone (1%) and D-glucose (1%). Bacto Agar (2%) was used to solidify the media when nesessary.

Growth Measurements

Growth rates of cultures were measured with a Klett Summerson colorimeter with a blue filter (400–465 nm) for minimal medium and a green filter (500–570 nm) for complete medium. The cells were incubated at 30 °C on a New-Brunswick shaker. When grown on YNB medium, the cells are in exponential phase of growth up to ≃90 Klett units.

Measurements of Uptake and Exit of Uracil

All experiments were carried out at 30 °C with exponential phase cells (i.e., below 90 Klett units, blue filter) in minimal medium. For the measurement of the initial velocity of uptake, 1 ml of the culture was pipetted into a test tube containing the radioactive

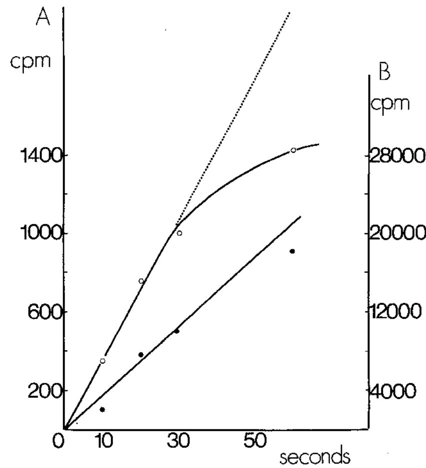

Fig. 1. Initial velocity of uptake of uracil in strain *fur 1–8 rh* as a function of time. The initial velocity of uptake of uracil was measured at different times in the presence of two concentrations of ^{14}C-2 uracil. Scale *A* corresponds to a concentration of 0.9×10^{-7} M uracil in the medium (o—o). Scale *B* corresponds to a concentration of 1.6×10^{-5} M uracil in the medium (●—●)

Table 1. Effect of washing on uracil retention in strain *fur 1–8 rh*

Filter without cells, not washed	Filter without cells, washed with 10 ml water at 3 °C	Cells incubated for 30 sec and washed with									
		H_2O at 3 °C			H_2O at 30 °C			YNB at 3 °C		YNB at 30 °C	
		5 ml	10 ml	20 ml	5 ml	10 ml	20 ml	5 ml	10 ml	5 ml	10 ml
24,270 cpm	840 cpm	8,222	7,416	7,136	7,500	7,200	7,100	7,800	8,000	8,100	8,000

One ml of culture was incubated with ^{14}C uracil (0.5×10^{-6} M) for 30 sec; the incubation mixture was then filtered under vacuum on nitrocellulose discs Gelman, pore size 1.2 μ, and the cells were washed as indicated. Results are the average of three measures.

solution ($\simeq 5$ μCi/μmole) of the ^{14}C-2 uracil, shaken manually for 30 sec (*see* Fig. 1), filtered through a membrane nitrocellulose filter (Gelman, pore size 1.2 μm or Sartorius, pore size 0.45 μm). The Sartorius filters had a lower adsorption level of ^{14}C-2 uracil and were therefore utilized when available. After filtration, cells were quickly washed with 10 ml of cold water. Variations in washing procedure did not lead to important differences in uracil uptake measurements (Table 1).

The radioactivity of the dried filters was measured in a PPO-toluene mixture (5 g PPO/liter toluene) with a Beckman scintillation spectrometer LS 50. In the same way, the efflux of ^{14}C-2 uracil from cells preloaded with ^{14}C-2 uracil was estimated by measuring the loss of radioactivity from the cells shifted to uracil-free minimal medium. For the

estimation of the intracellular concentration, a total cellular volume of 8×10^{-6} ml for 1 ml of haploid cell culture at an optical density of 1 unit was adopted (measured with Klett photometer, blue filter). This cell volume includes vacuoles, mitochondria and cell walls. It was defined as the difference between the packed cell volume and the intercellular space. The intercellular space was calculated by measuring the dilution of dextran blue in the extracellular water. A similar value of cellular space was obtained by calculating the mean volume of a wild type haploid cell assumed to be an ellipsoid of 39.8 μ^3. Thus the volume of the cells contained in 1 ml of culture of 1 Klett unit absorbance (2×10^5 cells) is 8×10^{-6} ml. The cell surface utilized to determine the permeability coefficient was also calculated on the basis that the haploid yeast cell is an ellipsoid (long axis 4.6 μ, short axis 4.04 μ) whose mean surface is 56.49 μ^2; the surface of the cells contained in 1 ml of culture with an O.D. of 1 unit Klett is, therefore, 0.112 cm^2.

Preparation of Cell Free Extracts

Yeast cells grown in liquid minimal medium (YNB) were harvested by centrifugation ($5000 \times g$), washed and resuspended in Tris or phosphate buffer (0.05 M; pH 7.4). The cells were broken with glass beads (0.5 mm diameter) in a Braun disintegrator. The supernatant obtained by centrifuging the broken cells and glass beads ($20{,}000 \times g$; 15 min) was used as enzyme source. Small molecules were removed by filtering the extract on a G25 Sephadex column.

Enzyme Assays

Uridine hydrolase: The uridine hydrolase activity was measured using the technique described by Carter [3].

Uridine kinase and uridine monophosphate pyrophosphorylase (UMPpase): To measure the activities of UMPpase and uridine kinase, we used a modification of the reaction mixtures described by Sköld and Reichard [16]. The medium for uridine kinase was: ^{14}C-2 uridine 8 mM (100,000 cpm/μmole); $MgCl_2$ 40 mM; ATP 20 mM (pH 6.8); crude extracts (in phosphate buffer 20 mM) containing 0.1 mg proteins; final volume 0.28 ml. The reaction mixture used to assay UMPpase contained: ^{14}C-2 uracil 1 mM (specific activity: 64,500 cpm/μmole); phosphoribosylpyrophosphate (PRPP) 0.32 mM; Tris buffer (pH 8.1) 100 mM and 0.02 ml cell free extracts ($\simeq 0.2$ mg proteins); glutathione (100 mM) was usually added. The final volume of the reaction mixture was 0.19 ml. The uridine kinase as well as the UMP pyrophosphorylase catalyzed reactions were stopped by addition of urea (7 M); samples of the reaction mixture (0.020 to 0.050 ml) were transfered into tubes containing 2.5 ml urea 7 M at 0 °C. The contant of the tubes was filtered on discs of AE 81 or DEAE cellulose paper and washed with 20 ml of a solution of urea (7 M) and 10 ml water at 0 °C. UMP, which is the product of these reactions, remained fixed on the cationic discs, whereas uracil and uridine were eluted. The filters were dried and their radioactivity was measured in a PPO-toluene scintillation liquid. About 50% of the UMP synthetized was usually fixed on the filters. The coefficient of retention of UMP on the discs was determined in each experiment by adding traces of a known quantity of ^{14}C UMP in a control reaction mixture containing "cold" uracil. In these experimental conditions, the enzymatic activity remained linear during 8 to 12 min. UMPpase and uridine kinase activities were unstable and more than 50% of their activities were lost after 24 hr at 4 °C.

Pyrimidine Pool Measurements

The free pyrimidine compounds (uracil, uridine, UMP, etc.) were extracted with ice cold trichloracetic acid 5% (TCA 5%) from cells in exponential phase of growth. Usually, 2 to 10 ml of a culture labelled with ^{14}C-2 uracil were concentrated on a nitrocellulose disc Millipore HAWP (porosity 0.45 μ, diameter 47 mm). The cells were washed with cold water (2 × 10 ml) and then extracted with 0.5 ml TCA 5% at 0 °C. The radioactivity of such an extract can be measured directly by adding 10 ml "Aquasol" (NEN) and 2 ml of 5% TCA to the extract in the counting vial. When separation of uracil from its derivatives (UMP, UDP, etc.) was required, 10 μl of the TCA soluble extracts were applied to a polyethylen-imine (PEI) cellulose thin layer plate (Merck) where uracil and uridine are separated from each other and from the nucleotides (which stayed at the origin) by migration in distilled water. UMP, UDP, and the group (UTP+CTP) are separated in a second run in the same direction with 0.5 M Cl Li. The powder of the corresponding spots is scrapped off and eluted with 2 ml HCl 0.05 at 37° during 1 hr. The radioactivity of the eluate is measured in the Aquasol mixture already described.

Chemicals and Labelled Compounds

Yeast Nitrogen Base, the Yeast Extract and the Peptone are Difco products. The ^{14}C-2 uracil and uridine and the ^{3}H-5 uracil were purchased from C.E.A., France.

Results

Characterization of Strain fur 1–8 rh

Mutation *fur 1–8*, like other recessive mutations at locus *fur 1* on *Saccharomyces cerevisiae* abolishes the UMP pyrophosphorylase activity as demonstrated by enzyme assay in vitro (Table 2). The loss of UMP pyrophosphorylase activity in these mutants is correlated with a high resistance level to 5-fluorouracil (5 FU, 10^{-2} M) and to 5-fluorouridine (5 FUR, 10^{-3} M). The resistance to 5 fluorouridine results from the splitting of a large proportion of 5 fluorouridine into 5 fluorouracil and ribose by uridine ribohydrolase [3] which is the sole catabolic enzyme of pyrimidine nucleosides detected in *S. cerevisiae* [6, 10]. A recombinant strain *fur 1–8 rh* was isolated from a "tetratype" tetrad after meiosis of the diploid $\frac{fur\ 1\text{–}8\ \ +}{+\ \ \ rh}$. This tetrad was composed of four spores with respective genotypes: (1) *fur 1–8 rh*; (2) *fur 1–8* +; (3) +*rh*; (4) + +. Spore 1 of genotype *fur 1–8 rh* was recognized by its resistance to 5 FU (10^{-3} M) and its sensitivity to 5 FUR (10^{-4} M) from spore 2 (parental genotype *fur 1–8 rh*+), which is resistant to 5 FU (10^{-3} M) and 5 FUR (10^{-3} M), the two other spores not being resistant to those analogues. Enzyme assay with cell free extracts (Table 2) demonstrated clearly the absence of uridine ribohydrolase activity in strain *fur 1–8 rh* (spore 1) and the presence of this enzyme in strain *fur 1–8* + (spore 2).

Table 2. Characterization of uracil metabolism in the wild type strain and in mutant strains

Strains	Growth response on			Ribo-hydro-lase	Uridine kinase	UMP pase	Uracil incor-porated into N.A.
	5 FU (2× 10^{-3} M)	5 FUR (3+ 10^{-2} M)	(10^{-3} M)				
Wild type Fl 100 (a)	–	–	–	11	8.7	2.2	1
fur 1–8	++	++	++	≃8	8	0	0.002
fur 1–1	++	++	++	11	8	0	0.002
rh	–	–	–	0	/	/	/
fur 1–8 rh	++	–	–	0	8	0	undetect-able

The enzymic activities are expressed in nanomoles of substrate transformed per min per mg of protein of cell free extracts. Proteins are measured by biuret reaction. Incorporation of ^{14}C-2 uracil was determined by measuring the TCA insoluble radioactivity in cells grown on 2×10^{-3} M uracil during four generations; the reference value of incorporation of uracil in the wild type corresponds to a replacement of about 80% of the pyrimidines of cellular origin by the ^{14}C-2 uracil supplied in the medium.

Abbreviations used: 5 FU: 5 fluorouracil; 5 FUR: 5 fluorouridine; ribohydrolase: uridine ribohydrolase or uridine nucleosidase; UMPpase: UMP pyrophosphorylase; N.A.: nucleic acids.

Kinetics of Labelling of the Intracellular Uracil and Uracil Nucleotides in the Wild Type Strain and in Strain fur 1–8 rh

|^{14}C-2| labelled uracil was added to exponentially growing cultures of wild type and *fur 1–8 rh* cells. Samples of culture were removed at different times and extracted with TCA 5%, and the soluble material was analyzed by thin-layer chromatography on PEI cellulose as described under *Materials and Methods* (Fig. 2). Free uracil was always the only labelled material found in the acido-soluble extract of strain *fur 1–8 rh*. In the experiment described in Fig. 2, it was found that the intracellular uracil was concentrated 40 times, the extracellular concentration of uracil being 0.45×10^{-6} M. The radioactivity of TCA-insoluble material and of pyrimidine nucleotides represents in each case less than 10^{-7} M uracil equivalents. This demonstrates that no significant synthesis of UMP is occuring from exogenously supplied uracil in strain *fur 1–8 rh*. In the wild type cells, uracil is also concentrated ($Si/Se \simeq 10$), but after two min incubation, the major TCA-soluble radioactivity migrated with the spot of UMP and the spot corresponding to the bulk of (UDP

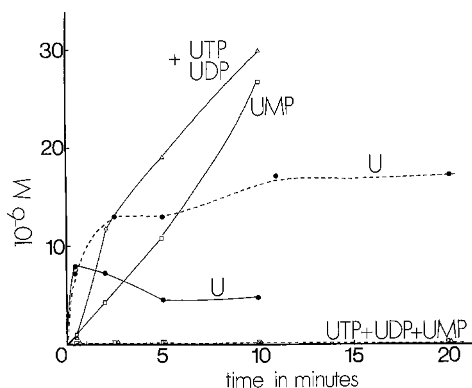

Fig. 2. Evolution of ^{14}C labelling of uracil and uracil derivatives in strain *fur 1–8 rh* and wild type FL 100. Strains *fur 1–8 rh* and FL 100 (wild type) were incubated with ^{14}C-2 uracil (0.45×10^{-6} M). Samples of 2 ml of culture were analyzed at different times (extraction of the cells with 5% trichloracetic acid, separation of the TCA soluble compounds on PEI cellulose (*(see Materials and Methods)*. The dotted lines correspond to strain fur *fur 1–8 rh* and the solid lines to the wild type strain. In strain *fur 1–8 rh*, the ratio *Si/Se* (for uracil) was 40 at steady state and the intracellular concentration of ^{14}C labelled nucleotides was less than 10^{-7} M

+UTP+CTP). After 10 min incubation of the wild type cells with 0.45 $\times 10^{-6}$ M ^{14}C-2 uracil, 3×10^{-4} M uracil was found in the TCA-insoluble material, whereas the external concentration was 0.42×10^{-6} M at this time.

Uracil Uptake in Strain fur 1–8 rh, Wild Type and fur 1–8 rh fur 4–6

Uracil uptake was compared in *fur 1–8 rh* and wild type strains in exponential phase of growth. Measurements of 30 sec were performed since nearly all the labelled intracellular material was still in form of free uracil. During this time the uptake velocities of uracil remained linear within the range of concentrations used in this assay (*see* Figs. 1 and 2). This comparison (Fig. 3) showed that uracil uptake was not significantly different in these two strains in the range of low concentrations corresponding to the high affinity function. In the range of high concentrations of uracil, which corresponds to the low affinity function, the results were less meaningful due to the scattering of the data. This scattering may be explained by the fact that radioactivity adsorbed on the nitrocellulose discs was twice that taken up by the cells. Uptake of uracil was also measured on a strain *fur 1–8 rh fur 4–6*, the mutation *fur 4–6* being allelic with the already described *fur 4–1* mutation [10] which is itself allelic with the "uracil permease-less" (ura-p) mutation described

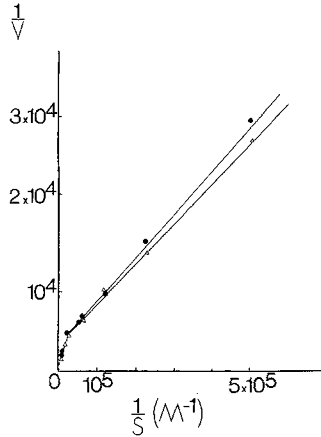

Fig. 3. Uptake of ^{14}C uracil in wild type strain and in mutant strain *fur 1–8 rh*. Initial velocities of uptake of uracil as a function of external concentration, were measured in 30-sec assays and the results are plotted according to Lineweaver-Burk. S is the concentration of uracil in the medium (in M) and V the number of moles per liter of cell water taken up in 30 sec. (△—△) corresponds to wild type strain FL 100, and (●—●) to strain *fur 1–8 rh*. The K_m of the high affinity function is $\simeq 1.9 \times 10^{-5}$ M in both strains and the V_{max} (M/min in intracellular space) of the same function is about 0.8×10^{-3} in both strains. The K_m of the low affinity function is about 2×10^{-4} M, and the V_{max} 2.6 $\times 10^{-3}$ M/min in both strains

by M. Grenson [6]. After sporulation of the diploid $\frac{fur\ 1\text{–}8}{+}\ \frac{rh}{+}\ \frac{+}{fur\ 4\text{–}6}$, uptake of uracil was measured on two strains having the phenotype "*fur 1–8 rh*" issued from the same tetrad. In one of them, which corresponds to recombinant *fur 1–8 rh fur 4–6*, uracil transport was drastically reduced. Addition of the uncoupler 2–4 DNP (10^{-3} M) [17] in the assay tube inhibited the uptake in *fur 1–8 rh* by more than 80%. As a control, the initial uptake velocity of cytosine in strain *fur 1–8 rh* was inhibited to 98% by the same concentration of DNP.

Effect of pH on the Uptake of Uracil

The optimal pH for uracil uptake in the wild type strain was between 4.3 and 4.5 (Fig. 4) which is also the optimal zone of pH for uptake of cytosine, the standard pH of the growth medium YNB being 4.6.

Uptake of Uracil in the Presence of other Pyrimidines

The effect of adenine, hypoxanthine, cytosine, thymine, 2-hydroxypyrimidine, 5-amino-uracil and 6-amino-uracil on the initial velocity of

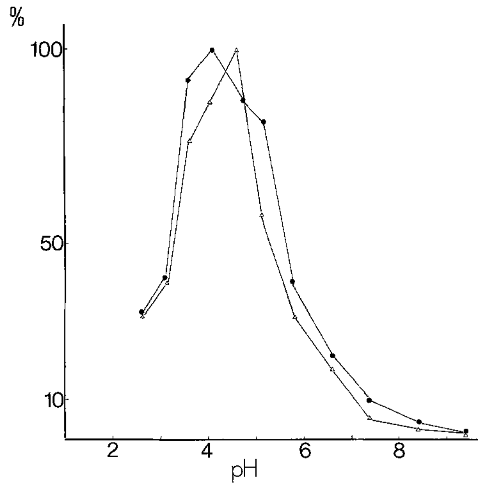

Fig. 4. Effect of pH on the uptake of uracil and cytosine in the wild type strain (FL 100). Concentrations of 1.96×10^{-6} M ^{14}C-2 cytosine and 1.19×10^{-6} M ^{14}C-2 uracil were used. For uptake assays, exponential phase cells were filtered and resuspended in a solution containing 10% glucose and "McIlvaine" buffer at the wanted pH (according to D.D. Perrin, Boyd Dempsey, Buffers for pH and Metal Ion Control, Chapman & Hall, London, 1974). The uptake activities are expressed in percentage of the maximal activity. ●—●: uracil. △—△: cytosine

Table 3. Constant of the cytosine and uracil transport systems

	Cytosine	Uracil
Intracellular concentration reached	10^{-2} M	$\simeq 0.5 \times 10^{-3}$ M (2×10^{-3} M)[a]
K_m (M)	2.5 (± 0.80) $\times 10^{-6}$	High affinity system: 1.16 (± 0.45) $\times 10^{-5}$ Low affinity system: 1.56 (± 0.60) $\times 10^{-4}$
V_{max} (in moles taken up per liter of intracellular space per min)	1.72 (± 0.42) $\times 10^{-3}$	High affinity system: 0.42 (± 0.30) $\times 10^{-3}$ Low affinity system: 2.05 (± 0.70) $\times 10^{-3}$
Coefficient of permeability[b]	5.7×10^{-8} cm sec^{-1}	7.4×10^{-7} cm sec^{-1}

[a] If the cells are grown on cytosine, the intracellular pool of uracil which comes mostly from the deamination of cytosine, can reach higher concentration when the wild type FL 100 is grown on 2×10^{-3} M ^{14}C-cytosine, the intracellular concentration of uracil was 2×10^{-3} M.

[b] The coefficient of permeability was determined from the rate constants of uracil and cytosine exit in the respectively deficient permease-less cells.

See ref. 4 for data concerning cytosine.

uptake was investigated in a strain lacking cytosine-permease activity (strain *fcy* 2–3). In this strain the uptake of cytosine, adenine, and hypoxanthine is grossly impaired [4] and, thus, transinhibition [5, 8] of the entry of uracil by substrates of the cytosine-permease is very unlikely. At concentrations as high as 10^{-4} M, hypoxanthine and 6-amino-uracil slightly inhibited the uptake of 4×10^{-6} M uracil, while the other compounds tested were ineffective (uridine, thymine, cytosine, adenine, 2-hydroxypyrimidine and 5-amino-uracil). The effect of hypoxanthine is interesting since this compound is a substrate of the cytosine permease in *Saccharomyces cerevisiae* [4, 14].

Relationship between Intracellular and Extracellular Concentration of Uracil at Steady-State

The steady-state concentration of the intracellular pool (Si) of uracil, is reached 10 min after the cells are incubated with ^{14}C-2 uracil. At low external concentration of uracil in the medium, the steady-state ratio Si/Se (measured after 16-min incubation) is high and it decreases if Se becomes high. It was observed that uracil is hardly concentrated by the cells when its concentration in the medium is greater than 0.3×10^{-3} M (Fig. 5). In the range of low external concentrations of uracil in the medium, the steady-state obeys relation $k \cdot Si = \frac{V_{max} Se}{K_m + Se}$ (18), where V_{max} (Fig. 6) and K_m are the constants of the uracil high affinity uptake function. Initial velocities of uptake of uracil, measured in the same experiments, were proportionnal to the internal steady-state concentration of uracil (Fig. 6). It was found that $Vi = k' Si$ and k' (min^{-1}) $= 0.75 \pm 0.08$ and that k (min^{-1}) $= 0.70 \pm 0.11$. These results are discussed further (*see Discussion*).

Important variations in the rate of uptake and in the steady-state ratio Si/Se were observed (up to 100%) in standard conditions. Similar variations were reported by Polak *et al.* [14] in cytosine transport in the same species *S. cerevisiae*. These fluctuations may reflect the inherent instability of such systems far from equilibrium.

Pool of Uracil at Steady-State

^{14}C-2 label of the pool of uracil was followed in strain *fur* 1–8 *rh*, which was grown on 11×10^{-6} M ^{14}C-2 uracil. At steady-state, the intracellular concentration of uracil was 2.7×10^{-4} M, and the con-

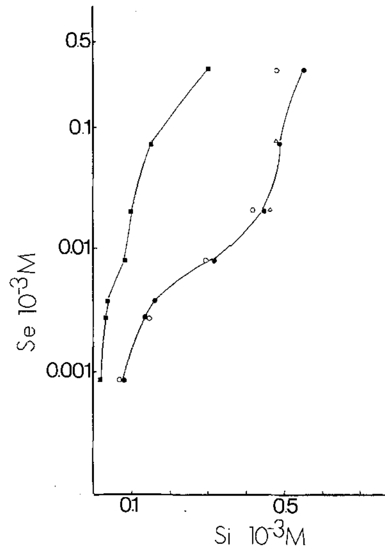

Fig. 5. Intracellular concentration of uracil in strain *fur 1–8 rh* as a function of the concentration of uracil in the medium and of time. Cultures of 10 ml of strain *fur 1–8 rh* where incubated with different concentrations of ^{14}C-2 uracil. Samples of 2 ml of culture were analyzed at different times to determine the intracellular concentrations of uracil (*Si*) which were plotted logarithmically against the corresponding extracellular concentrations of substrate (*Se*). ■—■: incubation of 20 sec; o—o: incubation of 5 min; △: incubation of 12 min; ●—●: incubation of 16 min

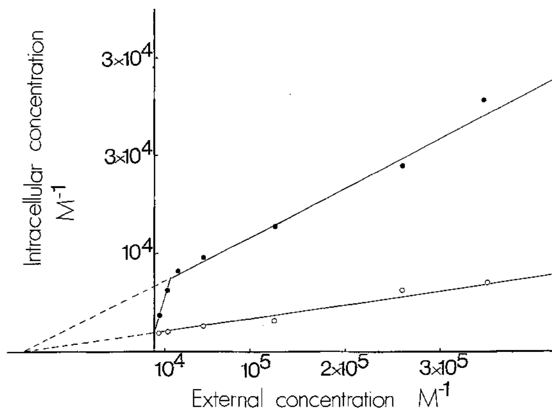

Fig. 6. Measurement of steady-state concentration of the internal ^{14}C-2 uracil in strain *fur 1–8 rh*. The intracellular concentration of ^{14}C-2 uracil was measured after incubation of the cells with different concentrations of uracil. The reciprocals of the intracellular concentrations reached after 20 sec incubation (●—●) and after 16 min incubation (o—o) were plotted against the reciprocal of the external concentrations of uracil. The internal concentrations are expressed in moles per liter of intracellular space. In this experiment, the K_m of high affinity was 0.75×10^{-5} M, the corresponding V_{max} was 1.5×10^{-3} (M/min)

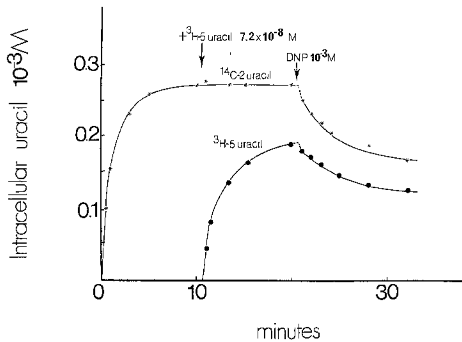

Fig. 7. Recycling of the pool of uracil at steady-state in strain *fur 1–8 rh*. Effect of 2–4 dinitrophenol on the level of the pool of uracil. *—*: Strain *fur 1–8 rh* was incubated on minimal medium supplemented with 11×10^{-6} M ^{14}C-2 uracil (specific activity: 9.19 $\times 10^{6}$ cpm/µmole). Samples of 1 ml of culture were taken at different times, filtered through a nitrocellulose disc, washed with 2×10 ml ice-cold water, dried and counted in the standard way. ●—●: At time 10 min 30 sec, 7.2×10^{-8} M ^{3}H-5 uracil (specific activity: 90×10^{6} cpm/µmole) was added to the culture. The ^{3}H and the ^{14}C radioacitivities were measured on the same samples. At time 20 min 30 sec, 2–4 dinitrophenol (2–4 DNP) (10^{-3} M) was added to the culture and the radioacitivity of the cells was measured as before addition of the drug

centration of uracil in the medium was 10.7×10^{-6} M. Traces of ^{3}H-5 uracil (7.2×10^{-8} M) were then added to the culture. It was observed (Fig. 7) that the uptake velocity of ^{3}H-5 uracil was slower than the uptake velocity of ^{14}C-2 uracil measured at time zero and that the steady-state concentration of ^{3}H-5 uracil was slightly lower than the ^{14}C-2 uracil concentration. It is difficult to regard as significant such differences mainly for two reasons. Firstly, the efficiency of measuring the two isotopes ^{14}C and ^{3}H is different when they are trapped inside the cells; secondly, the steady-state is often accompanied by fluctuations as stated previously.

Nevertheless, it can be deduced from these data that the steady-state results from a continuous recycling of uracil. These data also show that the intracellular pool of uracil did not strongly inhibit the uptake of uracil.

Efflux of Uracil in Strain fur 1–8 rh and fur 1–8 rh fur 4–6

Several mutants of pyrimidine metabolism such as *fur 1* and *fur 2* [6, 10] mutants excrete uracil, but not other pyrimidines; thus the excretion of uracil has a physiological importance for yeast cells in some conditions. It was observed in the experiment described in Fig. 8 that

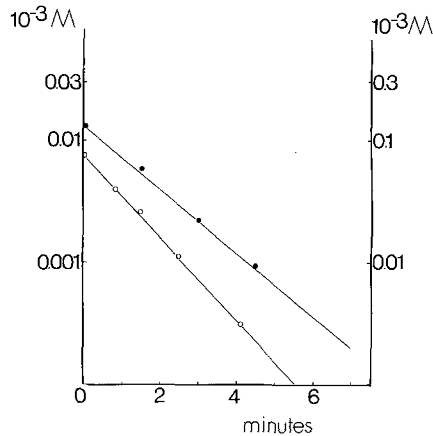

Fig. 8. Exit of uracil in strain *fur 1–8 rh* and *fur 1–8 rh fur 4–6*. The cells were incubated for 30 min in minimal medium supplemented with ^{14}C-2 uracil. Uracil concentration in the medium was 0.27×10^{-5} M for strain *fur 1–8 rh* and 0.3×10^{-4} M for strain *fur 1–8 rh fur 4–6*. The cells were then filtered and resuspended in minimal medium (YNB, 12 ml) free of uracil. Sampling and measurement of the cellular radioactivity were performed at the indicated times. The variations of intracellular concentration of ^{14}C-uracil were plotted on the ordinate (the radioactivity of the cells at steady-state was substracted from the crude values of cellular radioacitivity measured at each time). The left side ordinate axis corresponds to strain *fur 1–8 rh fur 4–6* (●—●) and the ordinate on the right side corresponds to strain *fur 1–8 rh* (○——○). In this experiment, the rate constant of uracil exit was 0.75 in strain *fur 1–8 rh* and 0.60 in strain *fur 1–8 rh fur 4–6*

exit of ^{14}C-2 uracil from strains *fur 1–8 rh* and *fur 1–8 rh fur 4–6* is a first order process; the rate constant ($k\,\mathrm{min}^{-1}$) being 0.60 ± 0.08 for the permeaseless strain and 0.75 ± 0.05 for the other strain. In the experiment described in Fig. 8, the presence of the uracil permease in strain *fur 1–8 rh* is reflected by the concentration ratio at time zero, $Si/Se \simeq 19$ *vs.* $Si/Se \simeq 0.75$ in strain *fur 1–8 rh fur 4–6*.

Chase of ^{14}C-2 Uracil from the Cellular Pool by ^{12}C or ^{3}H-5 Uracil

As described above, when strain *fur 1–8 rh* preloaded with ^{14}C-2 uracil was shifted to minimal medium free of uracil, we observed an exponential loss of the cellular uracil. By following the kinetics of this exit, it appeared that the intracellular ^{14}C-uracil reached a new steady-state concentration. Nonradioactive uracil or ^{3}H-5 uracil (10^{-3} M) was then added in the culture. A first order exit of the intracellular ^{14}C-uracil was observed, with a rate constant ($k\,\mathrm{min}^{-1}$) of 0.70 in the case with "cold" uracil and of 0.64 in the chase with ^{3}H-5 uracil (Fig. 9). In the

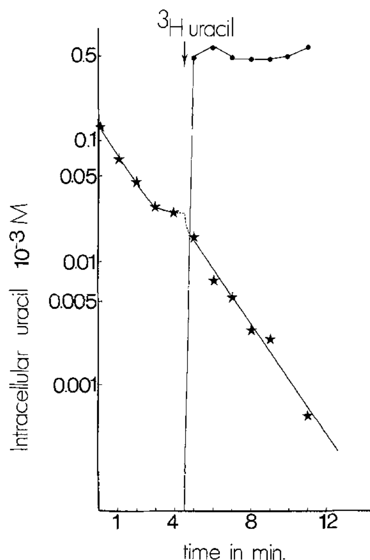

Fig. 9. "Chase" of the pool of ^{14}C-2 uracil by ^{3}H-5 uracil. Strain *fur 1–8 rh* was incubated in minimal medium (YNB) with 10^{-4} M ^{14}C-2 uracil (specific activity: 5.05 $\times 10^{6}$ cpm/μmole) for 45 min. The cells were then filtered through a nitrocellulose disc (Gelman) washed with 10 ml minimal medium and resuspended in 20 ml of this medium. At time 4 min 30 sec, 1×10^{-3} M ^{3}H-5 uracil (specific activity: 5.5×10^{6} cpm/μmole) was added to the medium. At the indicated times, samples of 2 ml of culture were withdrawn. The cells were filtered, washed with 10 ml ice-cold water and extracted with 2 ml TCA (5% w/v) to solubilize the intracellular uracil. The radioactivity of the TCA extract was then measured in presence of 13 ml "Aquasol" (NEN). The rate constants (after subtraction of the asymptote) were 0.50 (without chase) and 0.64 in the chase by ^{3}H uracil. ★: ^{14}C radioactivity; ●—●: ^{3}H radioactivity

later experiment, a large influx of ^{3}H-5 uracil accompanied the efflux of the ^{14}C-2 uracil, but there is no direct evidence for an initial accelerative phase in the ^{14}C-2 uracil chase.

Effect of 2–4 Dinitrophenol and Sodium Azide on the Efflux of Uracil

The rate constant of uracil exit was measured in the presence of 2–4 DNP (10^{-3} M) or in the presence of sodium azide (10^{-2} M) in strain *fur 1–8 rh fur 4–6* (Fig. 10). Cells preloaded with ^{14}C-2 uracil were shifted to uracil-free minimal medium containing either 2–4 DNP (10^{-3} M) or sodium azide (10^{-2} M), or no drug (control). DNP as well as sodium azide decreased significantly the rate of exit of uracil as shown in Fig. 10 (k was 0.36 in presence of DNP, and 0.29 in presence of sodium azide; in the control, k was 0.60). The effect of DNP was also measured in strain

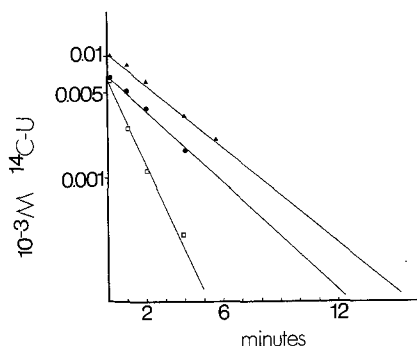

Fig. 10. Effect of 2–4 DNP and sodium azide on the uracil exit in strain *fur 1–8 rh fur 4–6*. The cells were incubated 45 min with 0.14×10^{-4} M ^{14}C-2 uracil (specific activity: 5.05 $\times 10^6$ cpm/μmole). After filtration on nitrocellulose discs (porosity 0.6 μm) the cells were divided into three parts and shifted to minimal medium containing: (1) ●—●: 2–4 DNP (10^{-3} M), (2) ▲—▲: sodium azide (10^{-2} M), or (3) no drug □—□ (control). At the indicated times, samples of 1 ml of culture were filtered and their radioactivity measured. The final asymptote was subtracted in each case and the resulting differences were plotted *vs.* time. The observed slopes were: 0.60 for control, 0.29 in presence of 2–4 DNP, and 0.36 in presence of sodium azide

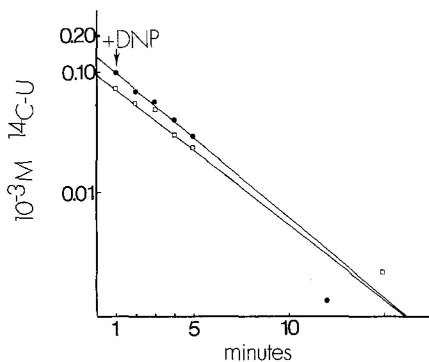

Fig. 11. Effect of 2–4 DNP (10^{-3} M) on the pool of uracil at steady-state in strain *fur 1–8 rh*. Strain *fur 1–8 rh* was incubated on minimal medium supplemented with 11×10^{-6} M ^{14}C-2 uracil (specific activity: 9.1×10^6 cpm/μmole) at time zero, and with 7.2×10^{-8} M ^{3}H-5 uracil (specific activity: 90×10^6 cpm/μmole) at time 10 min. At time $t = 20$ min, 2–4 DNP (10^{-3} M) was added to the culture and the radioactivity of the cells was measured as before addition of the drug. The final asymptote (i.e., the radioacitivity of the sample at time 35 min) was subtracted from each value and the differences were plotted on the ordinate. The rate constant of ^{14}C-uracil exit (slope of curve ●—●) was 0.32 and for ^{3}H-5 uracil was 0.37 (slope of curve □—□)

fur 1–8 rh (*see* Figs. 7 and 11). An exponential exit of uracil was observed immediately following the addition of DNP to the culture, the rate constant being 0.34 (min^{-1}). This result might be explained by a double

effect of DNP on uracil transport [19]. The first and important effect of DNP is to inhibit the uptake of uracil, the second is to lower the rate of exit of uracil.

Discussion

The results of the present paper demonstrate that the uptake of uracil by *Saccharomyces cerevisiae* occurs by "active transport" which is not coupled to UMP pyrophosphorylase activity. A different situation is described in *E. coli* and *S. typhimurium*, where the transporrt of free bases is mediated by membrane-associated phosphoryltransferase that converts the bases into their nucleoside monophosphates [1, 7, 9]. In contrast with these results, membrane vesicles of *S. typhimurium* lacking the phosphoribosyltransferase activities were recently described [15] and participation of adenine phosphoribosyltransferase activity in the uptake of adenine in *E. coli* has also been contested [2]. The transport of uracil in *S. cerevisiae* for low concentrations of uracil in the medium may be described as a "pump and leak" model [18], in which the entry is clearly a carrier mediated process [11, 20, 21] but where the mechanism of efflux is not elucidated. At steady state, efflux is equal to influx and Eq. (1) may be applied (as shown in Fig. 6)

$$kSi = \frac{V_{max} Q Se}{K_m + Se} \quad (1)$$

where Si is the intracellular steady state concentration, Se the external steady state concentration, K_m the Michaelis Menten constant of the uptake system; Q is the energy coupling coefficient and "$V_{max} Q$" the experimentally measured V_{max}. This equation indicates that the rate of efflux at steady state is proportional to the intracellular concentration of uracil Si. The value of k (0.70 ± 0.08) calculated from Eq. (1) is very similar to the rate constants of uracil exit obtained experimentally whether or not the cells had a functional system of uracil uptake (0.75 ± 0.05 and 0.60 ± 0.08). In the hypothesis that some carrier is involved in the efflux process, its affinity for intracellular uracil (K_{mi}) is expected to be such as $K_{mi} > 0.45 \times 10^{-3}$ M which is the value corresponding to the highest intracellular concentration of uracil observed in strain *fur 1–8 rh*. In *S. cerevisiae*, the uracil transport system is much less efficient than cytosine transport system. The steady state ratio Si/Se is 1,900 for cytosine *vs.* $\simeq 40$ for uracil, the external concentration of substrate being

Table 4. Values of the rate constants[a] of uracil and cytosine exit in different conditions

Strains used	Drugs present in the medium	Cytosine	Uracil
fur 1–8 rh and *fcy 1–1*[b] (the permeases for uracil and cytosine are functional)	None	0.37[e]	0.45 ± 0.10[f] 0.75 ± 0.08[g]
	+ 2–4 DNP 10^{-3} M	Accelerated efflux followed by first order exit: 0.40 → 0.040[f]	no import accelerated efflux is induced: 0.073[f] 0.34[g]
fur 1–8 rh fur 4–6 and *fcy 1–1 fcy 2–3*[c] (the permeases for uracil and cytosine are not functional)	None	0.042 ± 0.004[f]	0.48 ± 0.14[f] 0.60 ± 0.08[g]
	+ 2–4 DNP 10^{-3} M	0.026[f]	0.11[f] 0.36[g]
	+ azide Na 10^{-2} M	0.026[f]	0.11[f] 0.29[g]
	+ NEM[d] 10^{-3} M	0.030[f]	0.18[f] 0.39[g]

[a] The values of the rate constants are expressed in min^{-1}.
[b] Strain *fcy 1–1* is not able to deaminate cytosine which is accumulated by this strain if cytosine is available in the medium [4, 10].
[c] Strain *fcy 1–1 fcy 2–3* is lacking both the cytosine deaminase and the cytosine permease activities.
[d] Nem is the abreviation for N-ethyl-maleimide, an SH group reagent.
[e] The value was calculated [4] from steady-state and initial velocity data for cytosine uptake in strain *fcy 1–1*.
[f] [g] correspond to two estimations of the rate constant of exit of the pyrimidines: in [g] the rate constants were calculated after subtraction of the asymptote and in [f] the asymptote was not subtracted (the value of the asymptote corresponds to the remaining radioactivity of the cells at steady-state; in [f] this value was not subtracted from the crude values of cellular radioactivity).

See ref. 4 for data concerning cytosine.

0.37×10^{-5} M in both cases. The rate constant of uracil exit, which is tenfold higher than the rate constant of cytosine exit in cytosine permeaseless cells may explain this difference.

The biphasic curve of uracil uptake in *S. cerevisiae* is still not completely understood. More accurate data are required to determine which are respectively the parts of passive and facilitated diffusion in uracil uptake within the range of high uracil concentrations in the medium. Besides the mutations at locus *fur 4*, two other mutations involved in the transport of uracil have been characterized recently. These mutations were selected as uracil auxotrophs which are able to grow on uracil in the presence of an inhibitory concentration of dihydrouracil. One of

these mutations (DHU_2) is dominant, and the other (dhu_1) is recessive, the strain showing tenfold increase of the rate of uracil uptake. The existence of these different classes of mutations suggests that in *S. cerevisiae* the transport of uracil is subject to a more complicated system of genetic regulation than cytosine transport since all known mutations affecting cytosine transport are allelic.

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References

1. Berlin, R.D., Stadtman, E.R. 1966. A possible role of purine nucleotide pyrophosphorylase of purine by *Bacillus subtilis*. *J. Biol. Chem.* **211**:2679
2. Burman, S. Roy, Visser, D.W. 1975. Transport of purines and deoxyadenosine in *Escherichia coli*. *J. Biol. Chem.* **250**:9270
3. Carter, C.E. 1957. Partial purification of a nonphosphorylytic uridine nucleosidase from yeast. *J. Am. Chem. Soc.* **73**:1508
4. Chevallier, M.R., Jund, R., Lacroute, F. 1975. Cytosine permeation in *Saccharomyces cerevisiae*. Characterization of the cytosine permease. *J. Bacteriol.* **122**:629
5. Cuppoletti, J., Segel, I.H. 1974. Transinhibition kinetics of the sulfate transport system of *Penicillium notatum*: Analysis based on an Iso Uni. Uni velocity equation. *J. Membrane Biol.* **17**:239
6. Grenson, M. 1969. The utilization of exogenous pyrimidines and the recycling of uridine 5′-phosphate derivatives in *Saccharomyces cerevisiae*, as studied by means of mutants affected in pyrimidine uptake and metabolism. *Eur. J. Biochem.* **11**:249
7. Hochstadt-Ozer, J., Stadtman, E.R. 1971. The regulation of purines utilization in *Bacteria*. *J. Biol. Chem.* **246**:5312
8. Hunter, D.R., Segel, I.H. 1973. Control of the general amino acid of *Penicillium chrysogenum* by transinhibition and turnover. *Arch. Biochem. Biophys.* **154**:387
9. Jackman, L., Hochstadt, J. 1976. Characterization of hypoxanthine and guanine uptake into isolated membrane vesicles of *Salmonella tiphimurium*. *J. Bacteriol.* **126**:312
10. Jund, R., Lacroute, F. 1970. Genetic and physiological aspects of resistance to 5-fluoropyrimidines in *Saccharomyces cerevisiae*. *J. Bacteriol.* **102**:607
11. Kepes, A. 1973. Trois classes de systèmes de transport chez les bactéries. *Biochimie* **55**:693
12. Lacroute, F., Slonimski, P. 1964. Etude physiologique des mutants resistants au 5-fluorouracil chez la levure. *C.R. Acad. Sci.* **258**:2172
13. Mortimer, R.K., Hawthorne, D.C. 1966. Genetic mapping in *Saccharomyces cerevisiae*. *Genetics* **53**:165
14. Polak, A.M., Grenson, M. 1973. Evidence for a common transport system for cytosine, adenine and hypoxanthine in *Saccharomyces cerevisiae* and *Candida albicans*. *Eur. J. Biochem.* **32**:276
15. Rader, R.L., Hochstadt, J. 1976. Involvement of membrane-associated nucleoside phosphorylases in the uptake and the base-mediated loss of the ribose moiety of nucleosides by *Salmonella typhimurium* membranes vesicles. *J. Bacteriol.* **128**:291

16. Reichard, P., Skold, O. 1963. Pyrimidine synthesis and breakdown. *In:* Methods in Enzymology S.P. Colowick and N.O. Kaplan, Editors Vol. 6, pp. 177–197. Academic Press, New York
17. Slater, E.C. 1966. Oxydative phosphorylation. *In:* Comprehensive Biochemistry M. Florkin and E.H. Stotz, Editors Vol. 14, p. 327. American Elsevier, New York
18. Stein, W.D. 1967. The movement of molecules across cell membranes. *In:* Theoretical and Experimental Biology. J.F. Danielli, editor. Vol. 6, Chap. 4 and 6. Academic Press, New York
19. Surandra, P. Verma, Schneider, H., Smith, I.C.P. 1973. Organizational changes in phospholipid multilayers induced by uncouplers of oxydative phosphorylation: A spin label study. *Arch. Biochem. Biophys.* **154**:400
20. Wilson, T.H., Kashket, E.R., Kusch, M. 1972. Energy coupling to lactose transport in *E. coli. In:* The Molecular Basis of Biological Transport. Academic Press, New York
21. Winkler, H.H., Wilson, T.H. 1966. The role of energy coupling in the transport of β-galactosides by *E. coli. J. Biol. Chem.* **241**:2200