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STUDIES ON PURINE TRANSPORT AND ON PURINE CONTENT IN VACUOLES ISOLATED FROM *SACCHAROMYCES CEREVISIAE*

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Summary

The transport of purine derivatives into vacuoles isolated from *Saccharomyces cerevisiae* was studied. Vacuoles which conserved their ability to take up purine compounds were prepared by a modification of the method of polybase-induced lysis of spheroplasts.

Guanosine > inosine = hypoxanthine > adenosine were taken up with decreasing initial velocities, respectively; adenine was not transported.

Guanosine and adenosine transporting systems were saturable, with apparent K_m values 0.63 mM and 0.15 mM respectively, while uptake rates of inosine and of hypoxanthine were linear functions of their concentrations.

Adenosine transport in vacuoles appeared strongly dependent on the growth phase of the cell culture.

The system transporting adenosine was further characterized by its pH dependency optimum of 7.1 and its sensitivity to inhibition by *S*-adenosyl-L-methionine.

In the absence of adenosine in the external medium, [^{14}C]adenosine did not flow out from preloaded vacuoles. However, in the presence of external adenosine, a very rapid efflux of radioactivity was observed, indicating an exchange mechanism for the observed adenosine transport in the vacuoles.

In isolated vacuoles the only purine derivative accumulated was found to be *S*-adenosyl-L-homocysteine.

Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoHCy, *S*-adenosyl-L-homocysteine; MES, 2(*N*-morpholino)ethane sulfonic acid; HEPES, *N*-2-hydroxymethyl piperazine-*N'*-2-ethane sulfonic acid.

Introduction

Yeast vacuoles are known to accumulate storage pools of various metabolites, such as amino acids [1], AdoMet [2] or allantoin [3]. Recently, the elaboration of methods for the isolation of intact vacuoles permitted the characterisation of systems specific for the vacuolar transport of arginine [4] and AdoMet [5].

Vacuolar accumulation of various purine compounds in yeast cells was observed by Svihla et al. [2] using ultraviolet microscopy. Further, the pulse and chase experiments of Cummins and Mitchison [6] suggested the existence of two distinct intracellular pools of purines: a main pool, characterised by a rapid turnover, and a slowly metabolized storage pool. Depending on the yeast species, the slowly metabolized pool consisted of either inosine [6] or adenine [7].

We have adapted the method described earlier [8] for the isolation of vacuoles competent in the transport of purines in order to investigate whether and by which mechanism various purine compounds susceptible to represent the storage forms are translocated in these organelles.

Materials and Methods

Strain and culture conditions

S. cerevisiae (strain LBG 1022, Institute of Microbiology, ETH, Zürich) was grown at 29°C in a synthetic minimal medium [9] supplemented with 10 mg/l each of adenine and uracile. Cultures were inoculated with $2 \cdot 10^7$ cells/l and harvested at $15 \cdot 10^9$ cells/l.

Chemicals

All purine compounds were products of Sigma. ^{14}C-labelled purines were obtained from C.E.A. (France). HEPES and MES were Calbiochemical products. Sorbitol and saccharose were purchased from Merck. Ficoll 400 was obtained from Pharmacia.

Standard buffered solutions comprised

Solution A. 0.6 M sorbitol, 10 mM MES-Tris at pH 6.0.

Solution B. 0.6 M saccharose, 10 mM HEPES-Tris, pH 7.0, and 25 g/l ficoll.

Solution C. 0.6 M sorbitol and 10 mM HEPES-Tris, pH 7.0.

Solution D. 5 vols. of solution C and 1 vol. of 0.6 M saccharose containing 10 mM HEPES-Tris, pH 7.0.

Preparation of spheroplasts

Cells (10 g of wet weight) were suspended for 15 min at 30°C in 50 ml of a pretreatment medium comprising 5 mM EDTA, 5 mM dithiothreitol, 0.6 M sorbitol, 100 mM Tris-HCl, pH 8 [10], then centrifuged and washed with solution A. The cell walls were lysed by incubation in 25 ml of solution A in presence of 1.25 g lyophilized snail enzyme (Industrie Biologique Française, Gennevilliers) for 30 min at 30°C. Released spheroplasts were separated from the lytic solution by centrifugation for 8 min at $3000 \times g$ through 20 ml of

solution B. Spheroplasts were pelleted and the supernatant was eliminated by aspiration.

Preparation of vacuoles

Spheroplasts were suspended in solution C to a final concentration of $2 \cdot 10^8$ per ml. To this suspension 0.1 mg DEAE-Dextran (dissolved in 50 µl of solution C) was added per ml and agitated for 1 min at 0°C. The DEAE-Dextran was then neutralized by adding an equal quantity of Dextran sulphate (dissolved in solution C). The suspension was made 50 mM with respect to glucose, incubated for 5—6 min at 30°C with vigorous shaking and then chilled to 0°C and diluted with 1 vol. of solution B.

The vacuoles were purified by the two steps method of Wiemken and Dürr [11] with slight modifications: 40 ml of the crude vacuolar preparation were layered in a centrifuged tube (4 × 11 cm) on the top of 25 ml of solution B, covered with 15 ml of solution C and centrifuged 1 h at 3000 × *g* in the swinging-bucket rotor of a K-63F Jouan centrifuge. The medium layer containing the vacuoles was harvested and the second step of purification was performed by diluting the vacuolar suspension 5-fold with solution C and centrifuging for 20 min at 2770 × *g*. The small pellet of vacuoles thus obtained was suspended in solution C at a concentration of about $1 \cdot 10^9$ vacuoles per ml. The yield of vacuoles was about 10—15% of the number of starting cells, as determined by counting in a hemacytometer. The purity of the vacuole fractions was routinely controlled by phase contrast microscopy.

Purine transport assay

The standard assay of purine uptake by isolated vacuoles was as follows: 0.5 ml comprising 0.6 M sorbitol, 0.75 mM radioactive [^{14}C]purine (1 Ci/mol), 20 mM HEPES-Tris buffer at pH 7.6, equilibrated at 25°C were added at zero time to 100 µl of the vacuoles suspension (prewarmed for 2 min at 25°C) and mixed. 1 ml of solution D equilbrated at 25°C, was then added carefully at the bottom of the radioactive mixture. The reaction was stopped by immersing the tube in an ice bath and centrifuging 10 min at 16 000 × *g* in the cold. In these conditions vacuoles sedimented in the bottom of the tube, while the radioactive incubation medium remained on the top and was eliminated by aspiration. The pellet of vacuoles was washed twice with 1 ml of solution D and centrifuged again under the same conditions as above. 80—90% of the initial number of vacuoles were recovered in the washed pellet. Vacuoles were then lysed by adding 0.2 ml of water and the aqueous mixture was transferred to the scintillation vial. The tube was rinsed with 0.2 ml of water and the radioactivity was counted using 12 ml of Bray solution in the Packard model 3310 scintillation counter.

Controls were made under the same conditions except that the incubation at 25°C was omitted. The radioactivity found in the controls was always in the limits of 5—20% of that found in the vacuoles incubated at 25°C.

Analysis of the cellular and vacuolar purine pools

(a) Preparation of vacuolar purine extract. The pellet of purified vacuoles containing $1 \cdot 10^9$ vacuoles was suspended in 1 ml of water and the suspension

was heated for 10 min in a boiling water bath in order to inactivate vacuolar enzymes, if any, which could metabolize the purines. Membrane debris were eliminated by centrifugation for 20 min in a Spinco ultracentrifuge at 10 000 × *g*. The supernatant was used for the assays.

Alternatively, the 10 min heating following the lysis of the vacuoles was omitted, or 1 M acetic acid was used instead of water for the lysis of vacuoles, followed by centrifugation, lyophilisation of the acetic supernatant and redissolution of the residue in 1 ml of distilled water.

(b) Preparation of cellular purine extract. Spheroplasts from 1 l culture were suspended in 15 ml of water and heated for 10 min in a boiling water bath. The suspension was centrifuged as described for vacuolar extract and the supernatant used for further analysis.

(c) Analysis of the ultraviolet-absorbing material. The fractions from Biogel P-2 column containing ultraviolet-absorbing material were pooled and lyophilized to dryness and the residue was redissolved in a minimal volume of water (0.1—0.2 ml). 10—20 µl of these solutions were deposited on thin layer cellulose chromatographic sheets with a ultraviolet fluorescent additive (Macherey-Nagel Co.). The developing systems were: (1) distilled water; (2) *n*-butanol/acetic acid/water (120 : 30 : 50); (3) 1 M HCl/isopropanol/ethanol (25 : 27 : 35.5).

Results

Stability of isolated vacuoles

The levels of uptake and reproducibility of the results described were obtained under the following conditions. (1) The temperature of the cell culture should not exceed 30°C. (2) The uptake rate of adenosine being dependent on the growth phase, cells should be harvested at the point of maximal uptake shown in Fig. 3. (3) The time of incubation at 30°C for the lysis of the cell wall and particularly of the plasmalemma should not exceed the limits of 30 and 6 min, respectively. (4) The concentration of the preparation at $1 \cdot 10^9$ vacuoles per ml was in our hands the one assuring the greatest stability of the vacuoles during the incubation at 25°C and a total preservation at 0°C during 5—6 h. Dilutions of vacuolar preparations in the isotonic sorbitol medium tend to accelerate the rupture of the vacuolar membrane. The 6-fold dilution used in our assay seems to be the highest limit.

Although after 24 h the microscopic aspect of the vacuoles seemed to remain unchanged, their stability at 25°C and their susceptibility to the dilution were altered.

Uptake rates of various purine compounds by isolated vacuoles

Fig. 1 shows the time course of the uptake of purine bases and purine ribosides by vacuoles. Guanosine was translocated with the greatest velocity, while adenosine uptake rate was about ten times lower. Inosine and hypoxanthine entered both at a rate intermediate between the uptake rates of the above two amino substituted ribosides. Surprisingly, adenine was not transported at all.

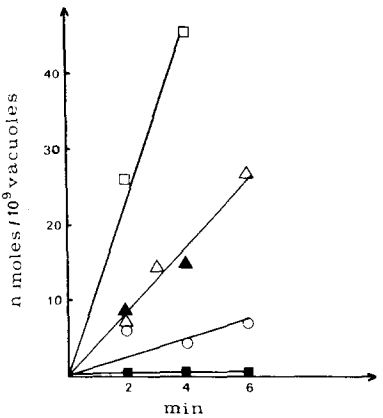


Fig. 1. Initial rate of purine compounds uptake by isolated vacuoles. Assays were performed as described in Methods and the reactions stopped at times indicated. ^{14}C-labelled purines were: guanosine (□——□); inosine (△——△); hypoxanthine (▲——▲); adenosine (○——○); adenine (■——■).

The uptake rates shown in Fig. 1 were not affected by the addition of 30 mM glucose or 10 mM sodium azide.

The rate of uptake was linear over a 4 min period. In most of our experiments the incubation was limited to 2 min. With longer incubation periods a decrease of radioactivity was observed in the vacuolar sediment, due to the bursting of vacuoles.

Dependence of the uptake rate on the concentration of the permeant

As seen in Fig. 2, the plots of reciprocal velocity of the uptake against the

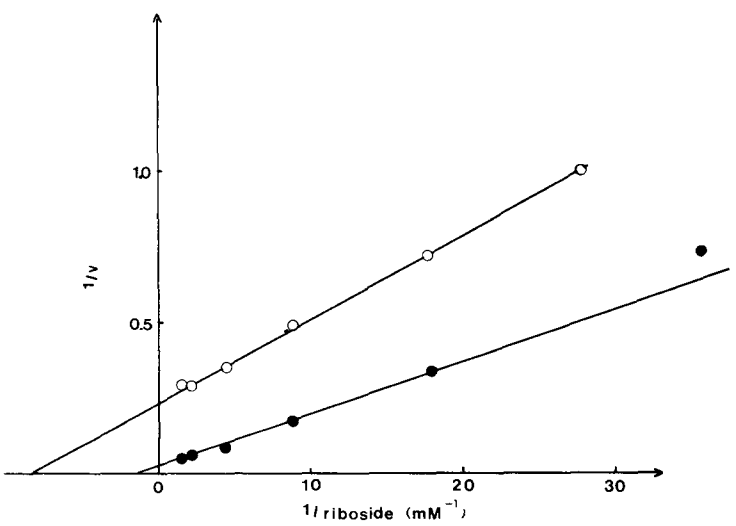


Fig. 2. Effect of external [^{14}C]adenosine and [^{14}C]guanosine concentrations on the initial uptake rate of these compounds by isolated vacuoles. Lineweaver-Burk plots. Assays were as described in Methods, except for adenosine (○——○) and guanosine (●——●) concentrations which were as indicated in the figure. v is expressed in $n$mol/10^9 vacuoles per min.

reciprocal of the permeant concentration for both guanosine and adenosine gave straight lines, corresponding to the classical Michaelis-Menten saturation kinetics and indicating apparent K_m values of 0.15 mM for adenosine and 0.63 mM for guanosine. On the other hand, both inosine and hypoxanthine uptake rates appeared to be linear functions of the permeant level in the medium up to 2 mM concentration.

Thus, only guanosine and adenosine seemed to enter into the vacuoles by a saturable process, suggesting a mediated transfer of these purines in the vacuole.

Because of the high K_m value found for guanosine and the low solubility of this riboside, further characterization of a mediated vacuolar transport was performed with adenosine as a substrate.

Identification of labelled products in loaded vacuoles

Vacuoles were incubated for 5 min with 0.5 mM [^{14}C]adenosine and centrifuged, and the pellet was treated as described in Methods for the preparation of vacuolar extract. The supernatant of lysed vacuoles was chromatographed on the Biogel P-2 column in the same conditions as described in Fig. 7. The radioactivity was eluted in a single peak in the elution volume corresponding to adenosine. It contained 88% of the radioactivity absorbed by the vacuoles. The homogeneity of the radioactive compound and its identity with an authentic sample of adenosine was confirmed by thin-layer chromatography. This result shows that adenosine is not metabolized by isolated vacuoles. Analysis of vacuoles loaded with [^{14}C]inosine led to the same conclusion.

Dependence of the adenosine uptake on the growth phase

Fig. 3 illustrates the evolution of the initial uptake rate of adenosine during the two generations of growth preceding the onset of the stationary phase. A

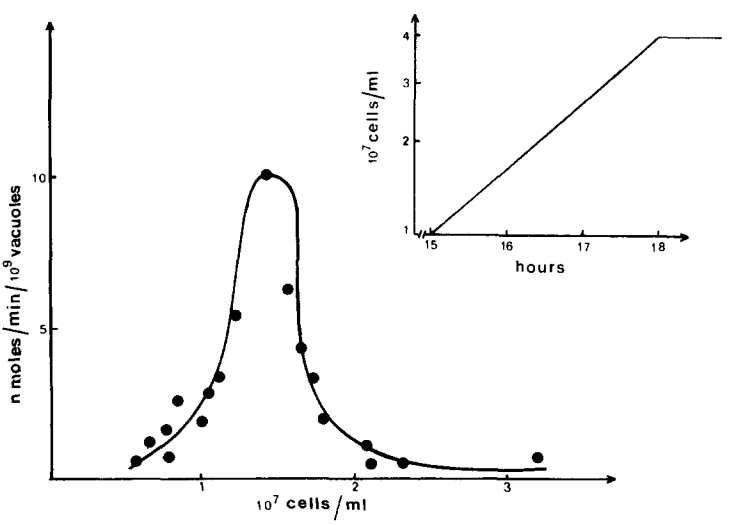


Fig. 3. Dependence of the initial vacuolar uptake rate of adenosine on the growth phase of the culture. Cultures were stopped at cellular concentrations indicated in abscissa. Isolated vacuoles were prepared and the uptake rate of adenosine assayed as described in Methods. The part of the growth curve concerned with the experiment is reported in the insert.

5-fold increase of the uptake was observed during the 15th and 16th hours of the culture, with a return to the initial value afterwards. Analogous depedence of the rate on the growth phase was already observed for the uptake of adenine, guanine and hypoxanthine by *S. cerevisiae* cells [12].

pH dependency and specificity of the vacuolar transport of adenosine

In order to assess the existence of a mediated transport of adenosine across the vacuolar membrane, the pH dependency of the transport and the sensitivity towards some purine compounds were studied. Fig. 4 illustrates the dependence of the adenosine uptake on the pH of the medium in the limits of the reported stability of the vacuolar membrane [4,5]. Within these limits an optimum uptake of adenosine appeared at pH 7.1.

The sensitivity of the system transporting adenosine towards analogues is shown in Table I. Among the purine derivatives tested, only AdoMet appeared as a potent inhibitor. At saturating (0.6 mM) concentration of adenosine, 0.25 mM AdoMet inhibited the uptake by 63%, while *S*-adenosyl-L-homocysteine (AdoHCy), the demethylated analogue of AdoMet, had a very small effect at a concentration ten times higher (2.5 mM). Adenine, though it was not taken up at all by the vacuoles, slightly inhibited the uptake of adenosine. On the other hand, a 30% stimulation by 2.5 mM inosine was observed in most of our experiments. Guanosine, hypoxanthine and AMP were without any effect.

The Lineweaver-Burk plot of the adenosine concentration versus uptake rate kinetics in the absence and in presence of fixed concentrations of AdoMet (Fig. 5) indicated a competitive type of inhibition by this analogue.

Efflux of adenosine from isolated vacuoles

Further information relative to the translocation mechanism of adenosine across the vacuolar membrane was obtained by the study of the efflux of this compound from previously loaded vacuoles. Isolated vacuoles were loaded with [^{14}C]adenosine as described in Fig. 6 to an intravacuolar concentration of

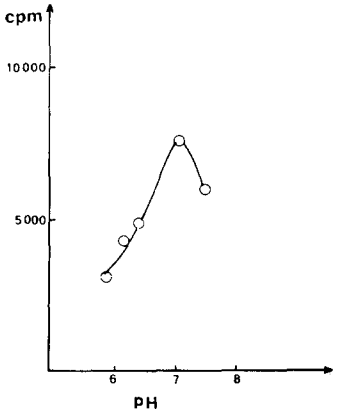


Fig. 4. Effect of pH on the initial uptake rate of adenosine by isolated vacuoles. Buffers were prepared by fitting a 200 mM Hepes solution to desired pH with 1 M Tris solution. The pH indicated correspond to the pH values of the assay mixtures described in Methods containing 20 mM Hepes-Tris buffers.

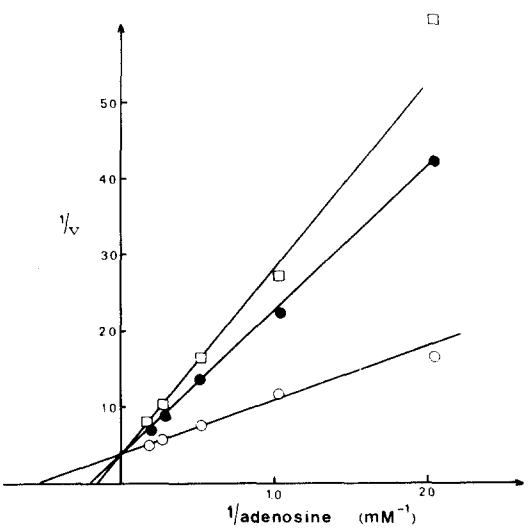
TABLE I

EFFECT OF ANALOGUES ON THE UPTAKE OF ADENOSINE BY ISOLATED VACUOLES

Assay was as described in Methods, except for adenosine concentration which was 0.6 mM.

Analogue	Concentration (mM)	Activity (%)
None	—	100
Inosine	2.5	130
Guanosine	2.5	100
Hypoxanthine	2.5	100
Adenine	2.5	80
AMP	5.0	100
AdoHCy	2.5	75
AdoMet	2.5	0
AdoMet	0.25	37

0.3 mM. When these vacuoles were resuspended in an isotonic, adenosine free medium, 96% of the radioactivity was sedimentable after 7 min of incubation. However, when 0.5 mM nonradioactive adenosine was added to the external medium, a very rapid outflow of [^{14}C]adenosine was observed for about 2 min,



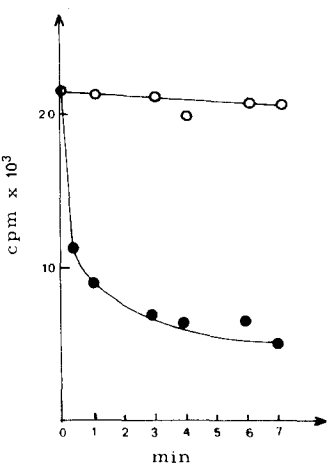


Fig. 5. Effect of external [^{14}C]adenosine concentration in the absence or in the presence of fixed concentrations of AdoMet, on the initial vacuolar uptake rate of adenosine. Lineweaver-Burk plot. Assays were as described in Methods except for the AdoMet content which was: 0 mM (○———○); 0.125 mM (●———●); 0.250 mM (□———□). v is expressed in cpm × 10^{-5}.

Fig. 6. Efflux of [^{14}C]adenosine from preloaded vacuoles. 5 ml of prewarmed uptake medium (specific radioactivity: 2 Ci/mol) were added to 3 ml of a prewarmed vacuolar preparation containing $2 \cdot 10^9$ vacuoles/ml. The suspension was incubated for 5 min at 25° C and the loaded vacuoles were then divided into two parts in conical tubes, centrifuged and washed as described in Methods. The intravacuolar concentration of [^{14}C]adenosine was calculated to be 0.3 mM. For this, the vacuolar volume was determined by means of inulin-carboxyl ^{14}C. One sample of vacuoles loaded with [^{14}C]adenosine (○———○) was suspended in 2.5 ml of prewarmed 0.6 M sorbitol buffered by 20 mM Hepes-Tris buffer at pH 7.1, the other in 2.5 ml of the same sorbitol solution made 0.5 mM in respect to nonradioactive adenosine (●———●). The suspensions were incubated at 20° C. Aliquots of 0.5 ml were taken as indicated and sedimentable radioactivity determined as described in Methods.

suggesting an exchange mechanism for the translocation of this riboside across the vacuolar membrane.

Analysis of cellular and vacuolar purine pools

The characteristics of the vacuolar transport of adenosine reported here, which indicate a nonconcentrative exchange mechanism, are very similar to those reported for the transport of arginine in vacuoles of *S. cerevisiae* [4]. However, in contradiction to the observed stoicheiometric exchange mechanism for the uptake of arginine in the isolated vacuoles, analysis of the intracellular arginine distribution showed that the bulk of this amino acid is accumulated in the vacuole of this yeast over the concentration gradient [1].

In order to verify whether similar vacuolar accumulation of some purine compounds occurs in vivo, analysis of the vacuolar purine content, in comparison to cellular purine content, was carried out. The separation of purine derivatives from the ultraviolet-absorbing material of vacuolar extracts (prepared as described in Methods) was performed by chromatography on a polyacrylamide Biogel P-2 column [13]. Fig. 7 shows this separation into three distinct peaks. Only the peak eluted between 50 and 68 ml had a spectrum characteristic of purines. The two first peaks, not completely identified, contained, however, important amounts of polyphosphates. Spectral characteristics of the third peak were very close to those of the adenosine, but its elution pattern was significantly different. The content of this peak, after concentration and thin-layer chromatography in the three systems as described in Methods, appeared homogeneous under ultraviolet light and cochromatographed with an authentic sample of *S*-adenosyl-L-homocysteine. The identity with AdoHCy was further confirmed by a positive ninhydrine reaction of the ultra-

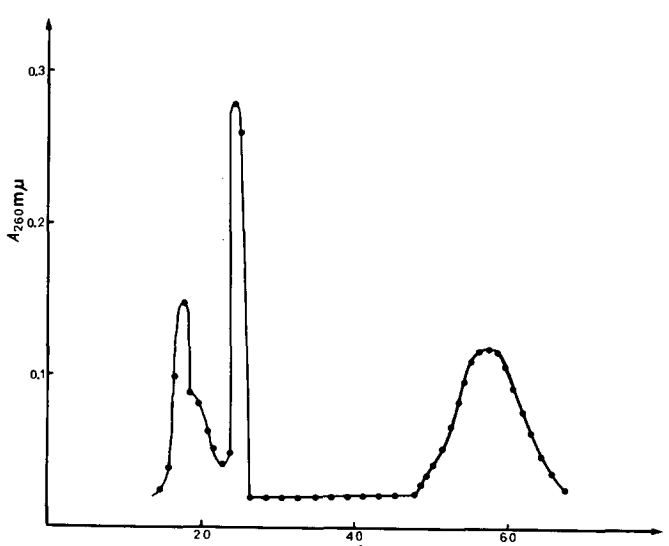


Fig. 7. Elution profile of the vacuolar ultraviolet-absorbing material from the Biogel P-2 column. At 0°C, 0.2 ml of the vacuolar extract (see Methods) was applied on a Biogel P-2 column (1.5 × 30 cm), equilibrated and eluted with distilled water at the rate of 18 ml/h. 1 ml fractions were collected.

violet-absorbing spot identical to the control AdoHCy spot as well as a positive reaction with the chloroplatinate reagent characteristic for sulphides [14]. Finally, the determination of ribose in the third peak from Biogel P-2 established a 1 : 1 stoicheiometry with adenosine, determined by absorbance at 258 nm.

All these results lead to the conclusion that *S*-adenosyl-L-homocysteine is the only purine derivative identifiable in the vacuolar extract. Its intravacuolar concentration was calculated to be about 2 mM.

When the ultraviolet-absorbing material extracted from spheroplasts was chromatographed on the Biogel P-2 column, two overlapping peaks appeared at 260 nm in those volumes between 46 and 68 ml corresponding to the elution of purine ribosides and purine bases. After concentration and analysis by thin-layer chromatography, both peaks appeared heterogeneous under ultraviolet observation. The first peak contained mainly inosine and adenosine with two small complementary spots corresponding to hypoxanthine and AdoHCy; the second peak contained mainly hypoxanthine but also visible spots of adenosine and AdoHCy. Although the incomplete separation of these products on the Biogel P-2 column prevented their quantitative estimation, the qualitative difference between the two cellular and vacuolar purine pools appeared clearly. Although appreciable amounts of inosine, hypoxanthine and adenosine exist in the cells, they do not accumulate in the vacuoles.

Discussion

Among the purine compounds susceptible to represent purine storage forms and supposed to be localised inside the yeast vacuoles, only the two amino-substituted ribosides, namely guanosine and adenosine, seem to be taken up in isolated vacuoles of *S. cerevisiae* by a mediated process. The linear relationship between the concentrations and the uptake rates of inosine and hypoxanthine suggests either that these compounds cross the vacuolar membrane by simple free diffusion or that the operative K_m of their transport system is very high.

The adenosine transport system was further characterized as a facilitated diffusion process according to the following criteria: (1) saturability of the uptake rate; (2) inhibition by structural analogues; and (3) existence of the *trans*-effect (Fig. 6). This effect corresponds to an accelerative exchange diffusion, which, according to Stein [15], occurs when the rate of outward transfer of permeant is accelerated by inward transfer of the same or related permeant present on the opposite membrane face.

An interesting feature of the vacuolar uptake of adenosine is its strong dependence on the growth phase. During a very delimited period of time, a 5-fold increase in the uptake of adenosine takes place (Fig. 3). The possibility of a correlation between the maximum uptake rate and a minimum vacuolar pool size of adenosine is discussed below.

Similar to vacuolar transports of arginine and AdoMet [4,5], the uptake of purines described here was energy independent.

Although the pool of purine bases and ribosides extracted from the spheroplasts was formed mainly by inosine, hypoxanthine and adenosine, none of

these compounds was found in extracts from isolated vacuoles. However, a definitive conclusion about the intracellular localization of purine storage pools should await further studies. Indeed, it is possible that the variations in adenosine uptake rate shown in Fig. 3 could reflect the reciprocal of its intravacuolar concentration. In this case, the growth phase corresponding to the maximal vacuolar uptake rate of adenosine, phase at which the cells were harvested, would coincide with a minimal intravacuolar concentration of adenosine or other ribosides. Similar fluctuations in cellular pools of inosine and adenosine have already been observed during the growth of *Schizosaccharomyces pombe* cells, where two distinct peaks in the concentration of these two ribosides were measured at the beginning of the logarithmic growth and at the onset of the stationary phase. The minimum concentration of these two compounds appeared precisely at the stage at which the cells, used for the experiments described here, were harvested (Reichert, U. and Nagy, M., unpublished).

The vacuolar localization of AdoHCy that we have demonstrated in *S. cerevisiae* grown in the absence of this compound or of its methylated analogue (AdoMet), provides valuable information concerning the metabolism of these two sulfonium derivatives. Indeed, contradictory information concerning the intracellular and intravacuolar accumulations of these compounds has been reported.

Nakamura and Schlenk [16] observed a rapid uptake and a rapid degradation of extracellular AdoHCy which did not accumulate in the cells in opposition to extracellular AdoMet which was accumulated in vacuoles of a *S. cerevisiae* mutant derepressed for the uptake of AdoMet. However, these observations were made on cells in non-growing conditions.

On the other hand, Knudsen et al. [17] working with an adenine auxotroph of *S. cerevisiae* have shown that AdoMet was taken up at a constant rate and used as a source of purines for growth, while AdoHCy was taken up after a lag period and was used more slowly. According to them, the lag observed in the assimulation of external AdoHCy could be correlated with the synthesis of adaptive enzymes. The localisation of AdoHCy, but not of AdoMet we have shown in vacuoles, may reflect a similar situation. During the logarithmic growth of the cells in absence of external AdoMet and AdoHCy, the biosynthetic AdoMet is demethylated in numerous transmethylation reactions to AdoHCy, which — in the absence of adaptive splitting enzymes — is stored as such in the vacuoles.

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