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TRANSPORT AND METABOLISM OF 5′-METHYLTHIOADENOSINE IN HUMAN ERYTHROCYTES

MARIA CARTENÌ-FARINA, FULVIO DELLA RAGIONE, GIOVANNA CACCIAPUOTI, MARINA PORCELLI and VINCENZO ZAPPIA

Department of Biochemistry (Second Chair), University of Naples, First Medical School, Via Costantinopoli 16, 80138 Naples (Italy)

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The transport and metabolism of 5′-deoxy-5′-*S*-methylthioadenosine have been studied in intact human erythrocytes. The sulfur nucleoside is rapidly accumulated into red cells and the extent of uptake largely exceeds the theoretical equilibrium between inner and outer compartment owing to its conversion into a non-permeable compound, namely 5-methylthioribose 1-phosphate. To characterize the nucleoside transport, phosphate-depleted erythrocytes, in which the methylthioadenosine metabolism is negligible, have been employed. The results indicate that: (i) the transport occurs via a facilitated-diffusion mechanism; (ii) the process is not energy-dependent and (iii) no specific cation is required. The kinetic analyses of both the transport and the metabolism show that the uptake of methylthioadenosine is a result of the tandem action of a transport step of high capacity (V_{max} = 604 ± 51 pmol / 10^6 cells per min) and low affinity (K_m = 3270 ± 321 μM) followed by a metabolic step of low capacity (V_{max} = 6.6 pmol / 10^6 cells per min) and high affinity (K_m = 30 μM). Furthermore, a substrate inhibition exerted by methylthioadenosine at high concentration (over 200 μM) on its specific phosphorylase is reported for the first time. Experiments performed with several analogs of the thioether indicate that the adenine amino group and the hydrophobic substituent at the 5′-position are critical for the transport carrier recognition. Adenine is the most powerful inhibitor of methylthioadenosine transport.

Introduction

Methylthioadenosine (5′-deoxy-5′-*S*-methylthioadenosine) is a naturally occurring sulfur-containing nucleoside ubiquitously distributed in mammalian tissues [1]. The molecule is formed from *S*-adenosylmethionine through multiple biosynthetic pathways [2,3]: in the eukaryotes it is produced from *S*-adenosyl-5′-(3)-methylthiopropylamine in stoichiometric amounts with spermidine and spermine [4,5], while a second pathway involves a direct cleavage of *S*-adenosylmethionine into methylthioadenosine and α-amino-γ-butyrolactone, through the action of a specific lyase [6,7].

Methylthioadenosine does not accumulate in normal tissues but is rapidly cleaved to adenine and 5-methylthioribose 1-phosphate by methylthioadenosine phosphorylase, an enzyme shown to exist in a variety of normal and transformed cells [8,9]. This enzyme is completely distinct from purine nucleoside phosphorylase [10] and at present no other catabolic pathways for methylthioadenosine are known, at least in mammalian cells.

In recent years increasing interest has been paid to methylthioadenosine mainly related to its antiproliferative activity [1,11,12] and much information has been accumulated on the in vitro inhibitory effect of the thioether on several enzymes catalyzing *S*-adenosylmethionine-dependent reac-

tions [2,13–16]. In addition a large number of structural analogs, also showing cytostatic, antiprotozoal and antiviral activity, have been synthesized [17–19].

Despite its biological importance, the mechanism of transport * of the nucleoside has been investigated only in few instances. A saturable system has been described in the protozoan *Ochromonas malhamensis* [20] and preliminary observations in isolated and perfused rat liver suggested that the thioether is actively taken up by that organ [21].

This paper reports studies on the uptake ** and metabolism of methylthioadenosine in human red cells and shows that phosphate-depleted erythrocytes (cells completely unable to degrade the thioether) represent an useful model for kinetic studies on the methylthioadenosine transport. Finally, the specificity of the carrier for methylthioadenosine related to that for bases and nucleosides is discussed.

Preliminary results have been already presented at the 11th Linderstrøm-Lang Conference [22].

Materials and Methods

Chemicals. *S*-Adenosylmethionine was prepared by biosynthesis with yeast [23] and isolated by the procedure developed in this laboratory [24]; *S*-adenosyl-L-[*Me*-^{14}C]methionine (spec. act. 50 mCi/mmol) was supplied by the Amersham International (Amersham, Bucks., U.K.); methylthioadenosine and 5′-[*Me*-^{14}C]methylthioadenosine were prepared by acid hydrolysis of *S*-adenosylmethionine at pH 4.5 for 30 min at 100°C [25]; *S*-Adenosylhomocysteine and 5′-isobutylthioadenosine were purchased from Sefochem Fine Chemicals, Ltd., Israel. Tubercidin (7-deazaadenosine) was obtained from Calbiochem, AG, Lucerne, Switzerland. 5′-Methylthioinosine and 5′-isobutylthioinosine were prepared by established method [26] as were 5′-ethylthioadenosine [8] and 7-deazaadenine. 5′-Methylthiotubercidin was a generous gift of Dr. J.K. Coward [18]. All other chemicals were from Sigma Chemicals Co., St. Louis, MO, U.S.A.

Chemical and radiochemical purity of the above-mentioned compounds were checked by paper and thin-layer chromatography [16], high voltage electrophoresis [16] and HPLC [27].

Synthesis of 5-[Me-^{14}C]methylthioribose and 5-[Me-^{14}C]methylthioribose 1-phosphate. 5-Methylthioribose and 5-[*Me*-^{14}C]methylthioribose were prepared by acid hydrolysis of labeled and unlabeled methylthioadenosine in 1 M HCl at 100°C for 1 h. After the reaction, the mixture was adjusted to pH 8.8 with KOH and applied to an Affi-gel 601 column (1 × 4 cm) equilibrated with 0.25 M ammonium acetate (pH 8.8). Adenine was eluted with 10 ml of the same buffer and 5-methylthioribose with 15 ml 0.1 M acetic acid. This eluate was then concentrated under reduced pressure and the residue redissolved in H_2O. 5-Methylthioribose concentration was estimated by the orcinol method [28]; the absence of adenine and the radiochemical purity of labeled 5-methylthioribose were checked by HPLC analysis. 5-[*Me*-^{14}C]Methylthioribose 1-phosphate was obtained by the phosphorolytic cleavage of 5′-[*Me*-^{14}C]methylthioadenosine with methylthioadenosine phosphorylase purified from human placenta [29].

The assay mixture contained 50 nmol 5′-[*Me*-^{14}C]methylthioadenosine (6 μCi/μmol), 20 μmol potassium phosphate buffer (pH 7.4) and 30 μg purified enzyme in a final volume of 1 ml. After 1 h incubation at 37°C, the reaction was stopped with 2 ml ethanol and the precipitate was removed by centrifugation. To check the yield (usually about 80%), an aliquot (100 μl) was analyzed by the method of Pegg and Williams-Ashman [10]. The product of the reaction was isolated and identified by anion-exchange chromatography according to Cartenì-Farina et al. [30].

Preparation of human red cells. Erythrocytes were obtained from EDTA-treated human blood (20 μl 0.5 M EDTA (pH 7.4) for 5 ml) not more than 30 h after withdrawal. The whole blood was centrifuged for 10 min at 5000 × *g* and 4°C. The plasma and buffy coat were sucked off and then red cell pellet was resuspended in 5 vol. isotonic

* Transport, as used here, strictly defines the transfer of unmodified substance across cell membrane by a saturable carrier.

** Uptake, as used here, defines the intracellular accumulation of radioactivity after exposure of the cells to labelled substrate, regardless of metabolic conversion.

glucose/phosphate-buffered saline. The resuspended red cells were stored at 4°C until use.

To obtain phosphate-depleted cells, the erythrocytes were washed ten times in 50 vol. buffered saline (5 mM glucose/150 mM NaCl/5 mM Tris-HCl (pH 7.4)) (buffer A). The packed red cells were then resuspended in 5 vol. of the same buffer: 100 μl of this cell suspension corresponded to 20 μl erythrocytes and to $2 \cdot 10^8$ cells.

ATP-depleted cells were obtained by incubation for 4 h at 37°C in glucose-free buffer A.

Preparation of red cell membranes and cell-free extract. Red cell membranes were prepared according to Steck and Kaut [31].

Cell-free extract was obtained by hemolysis of 250 μl resuspended red cells in 2 ml H_2O. After addition of 250 μl 1 M potassium phosphate buffer (pH 7.4) and 200 μl 40 mM dithiothreitol, the mixture was centrifuged at $15000 \times g$ for 30 min. The supernatant was employed to investigate methylthioadenosine phosphorylase activity.

Methylthioadenosine phosphorylase assay. Methylthioadenosine phosphorylase was assayed by measuring 5-[*Me*-^{14}C]methylthioribose 1-phosphate released from 5'-[*Me*-^{14}C]methylthioadenosine [29]; ion-exchange chromatography [10] or reversed-phase HPLC (see the corresponding section) were employed for the separation of the two molecules.

All the assays were conducted under conditions in which the reaction rate was linear with respect to the time and enzyme concentrations.

Uptake experiments. In the standard procedure, 100 μl of cell suspension were placed in a 37°C Dubnoff incubator with gentle shaking; after 5 min preincubation, 150 μl radioactive methylthioadenosine (prewarmed at 37°C) was added. The uptake was quickly stopped at the indicated times by addition of 2 ml ice-cold phosphate-buffered saline. The sample was then immediately centrifuged for 30 s at $5000 \times g$ at 0°C; the supernatant was poured off and the cell pellet was washed with 2 ml phosphate-buffered saline at 0°C. After centrifugation, the pellet was hemolyzed with 2 ml distilled water at 0°C and the proteins were precipitated with 0.4 ml 0.3 M trichloroacetic acid. The time between the addition of phosphate-buffered saline and trichloroacetic acid precipitation was under 200 s. In the experiments employing phosphate-depleted red cells, phosphate-buffered saline was substituted by buffer A. After trichloroacetic acid treatment, the sample was centrifuged for 10 min at $15000 \times g$ and 1 ml supernatant was mixed with 4 ml InstaGel in the scintillation vials and counted in Tri-Carb liquid scintillation spectrometer.

The possible loss of methylthioadenosine during the time required for washing, investigated with erythrocytes preloaded with labeled methylthioadenosine, was less than 3%.

Zero time uptake was routinely determined by the addition of stopping solution to the cells immediately followed by the addition of labeled methylthioadenosine. The calculated value, corresponding to the amounts of radioactivity trapped in the extracellular space of the pellet, was usually less than 0.05% of the total radioactivity.

Analysis of transport data. Initial zero-trans velocities (i.e., the initial velocities of the transport from side 1 of the membrane to side 2 when the zero-time concentration, at side 2, of the molecule transported is negligible) were calculated according to [32,33].

Determination of methylthioadenosine and its metabolites by high-performance liquid chromatography. Intracellular and extracellular ^{14}C-labeled metabolites were identified by a newly developed high performance liquid chromatographic-isotopic methodology. A Beckman liquid chromatograph (model 324) and a Perkin-Elmer liquid chromatograph (model LC 65T, series 2), both equipped with an ultraviolet detector operating at 254 nm, were used. Aliquots varying from 20 μl to 150 μl of sample were injected into the columns via a model 70-10 sample injector valve (Rheodyne).

Protein determination. Protein concentration was estimated by the method of Lowry et al. [34] using crystalline serum albumin as standard.

Radioactivity measurement. Radioactivity was measured in a TriCarb liquid scintillation spectrometer (Packard, model 460C) equipped with an absolute radioactivity analyzer. The scintillation liquid was InstaGel (Packard). Quenching was corrected by external standardization.

Results and Discussion

Uptake of 5'-[Me-^{14}C]methylthioadenosine

The uptake of 5'-[*Me*-^{14}C]methylthioadenosine into human erythrocytes has been investigated as a function of time, at three concentrations of the thioether, as reported in Fig. 1: dotted lines represent the theoretical intracellular concentrations of methylthioadenosine, at equilibrium between inner and outer compartment. These values have been calculated on the basis of intracellular water space of human red cells, corresponding to 12.91 ± 0.25 μl for 20 μl packed erythrocytes [35].

The complex pattern (Fig. 1) suggests the presence of a multi-step process; in fact, the significant difference between the theoretical equilibrium and the experimental data is indicative of a membrane transport coupled to or followed by metabolic step(s). Furthermore, the decline of total uptake at longer times, especially observable at low concentrations of methylthioadenosine, suggests a subsequent escape of labeled metabolite(s) from the cells.

In order to test for the occurrence of a surface adsorption, the uptake of methylthioadenosine was also measured over an interval from 3 to 15 s (inset in Fig. 1): the uptake is linear only for 9 s and its slope extrapolates close to the point of origin. The latter finding excludes the possibility of any significant surface adsorption.

The intracellular and extracellular compartments have been analyzed for their content of methylthioadenosine and its metabolites at different time intervals of exposure to 120 μM methylthioadenosine [22]. At 30 s, approximately equimolar amounts of 5-methylthioribose 1-phosphate and methylthioadenosine are detectable in the inner compartment, while in the outer compartment only methylthioadenosine is present. After 5 min, a significant increase in the cellular concentration of 5-methylthioribose 1-phosphate was observable, while 5-methylthioribose did not accumulate in the red cells. This compound is, on the contrary, present in large amount in the medium and probably represents the metabolite escaping from the cells. It is interesting to note that 5-methylthioribose 1-phosphate is not detectable in the outer compartment, confirming the general view that the plasma membrane is poorly permeable to phosphorylated compounds [36,37].

Fig. 2 compares the total uptake with the levels of intracellular thioethers over a 60 min interval.

These results suggest that methylthioadenosine

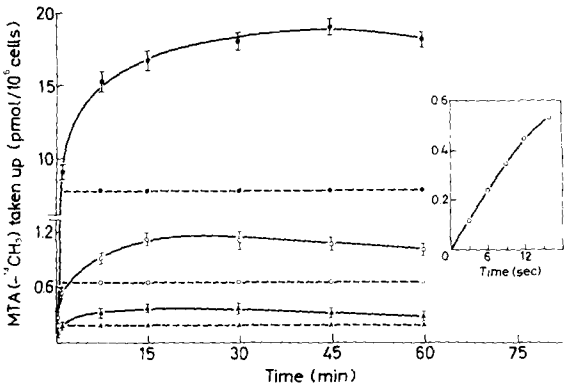

Fig. 1. Time course of 5'-[*Me*-^{14}C]methylthioadenosine (MTA) uptake by human erythrocytes in phosphate-buffered saline at 37°C. Each value represents the average from triplicate samples; dotted lines refer to the theoretically calculated intracellular methylthioadenosine concentration at equilibrium. Methylthioadenosine concentrations: 120 μM (●); 9.8 μM (○); 2.7 μM (▲). The inset reports the uptake of 9.8 μM methylthioadenosine at short times. For the experimental conditions see Materials and Methods.

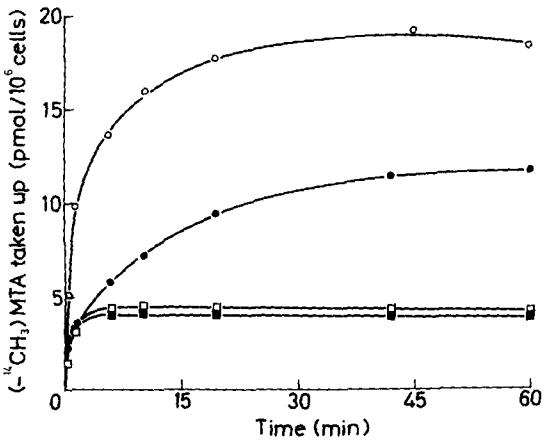

Fig. 2. Time courses of accumulation of labeled methylthioadenosine (MTA), 5-methylthioribose and 5-methylthioribose 1-phosphate after exposure of red cells to 120 μM 5'-[*Me*-^{14}C]methylthioadenosine. At the times indicated, cells were assayed by HPLC as described under Materials and Methods. ○——○, Total; ●——●, methylthioribose 1-phosphate; □——□, methylthioribose; ■——■, methylthioadenosine.

is rapidly taken up and metabolized in human red cells. Since membrane preparations are unable to metabolize labeled methylthioadenosine, the possibility of a group translocation mechanism has to be excluded.

To evaluate whether the cytosolic phosphorolytic cleavage of methylthioadenosine is the rate-limiting step in the uptake process, the kinetic parameters of methylthioadenosine phosphorylase from soluble fraction of human erythrocytes were studied at various concentrations of the two substrates.

The Lineweaver-Burk plot for methylthioadenosine, at saturating concentration of phosphate, is shown in Fig. 3: the apparent K_m value for methylthioadenosine, graphically calculated from the linear portion of the curve, is 30 μM, with a V_{max} value of 6.6 pmol/10^6 cells per min. Furthermore, a significant substrate inhibition is observable at high concentrations of the thioether (over 200 μM).

It is important to note that this inhibition could not be ascribed to product inhibition (i.e., adenine).

At present it is not known whether the reported substrate inhibition is only observable employing methylthioadenosine phosphorylase from red cell extract or whether it represents a general property of cellular extract. The understanding of this point could be of interest, expecially in view of a pharmacological use of this nucleoside. In this respect, the 70% inhibition exerted by 200 μM methylthioadenosine on the growth rate of transformed mouse fibroblasts [12] can be possibly ascribed to the reduced activity of the phosphorylase. The decrease of inhibition of cell growth at extended incubation times and at low methylthioadenosine concentrations [12] supports this view.

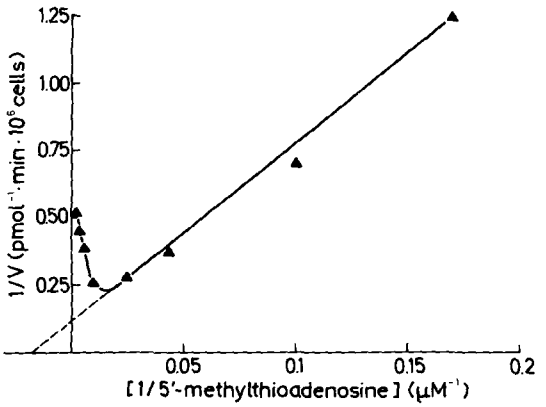

Fig. 3. Lineweaver-Burk plot of methylthioadenosine phosphorylase from human erythrocytes. The assay was performed as indicated in Materials and Methods except that methylthioadenosine was added at the given concentrations. The graphically estimated K_m for methylthioadenosine is 30 μM.

In order to investigate the effect of phosphate on methylthioadenosine cleavage, methylthioadenosine phosphorylase activity from phosphate-depleted erythrocytes was assayed. From the double-reciprocal plot (not reported) a quite high K_m value for phosphate has been calculated (K_m = 2.1 mM). No degradation of the thioether is observable in the absence of exogenous phosphate, suggesting a strict dependence of the enzyme activity on phosphate ions.

This result is confirmed in vivo when the phosphate-depleted red cells are exposed to 120 μM methylthioadenosine. No significant amount of 5-methylthioribose 1-phosphate is detectable in the intracellular space (data not reported).

The results indicate that phosphate-depleted erythrocytes represent an appropriate model to investigate methylthioadenosine transport without interference with metabolic events.

Kinetics of methylthioadenosine transport in phosphate-depleted erythrocytes

Fig. 4 illustrates typical overall time courses of zero-trans influx of methylthioadenosine in phosphate-depleted human erythrocytes. The patterns obtained demonstrate the rapidity of methylthioadenosine transport, in that nearly complete equilibrium between intracellular and extracellular compartment is attained within 30 s up to 100 μM extracellular concentration.

The mathematically obtained $V_{1,2}^{zt}$ values [32,33] are plotted in terms of [S]/V vs. [S], as reported in Fig. 5A, and an apparent K_m of 3270 $\pm$ 321 μM and a V_{max} of 604 $\pm$ 51 pmol/10^6 cells per min have been calculated. The linearity of pattern is indicative of a saturable system. Similar kinetic parameters are obtained employing ATP-depleted red cells, suggesting that methylthioadenosine entry is not energy dependent.

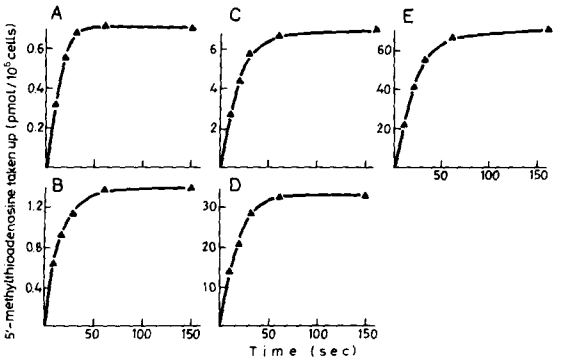

Fig. 4. Zero-trans influx of methylthioadenosine in phosphate-depleted red cells. Time courses of radioactivity uptake at 10 (A), 20 (B), 100 (C), 500 (D) and 1000 (E) μM 5'-[*Me*-^{14}C]methylthioadenosine (1000 cpm/μl, irrespective of concentration) were determined as indicated in Materials and Methods. The zero-trans rate equation was fitted to the experimental data whereby time (t) and extracellular methylthioadenosine concentration (S_1) were treated as independent variables and intracellular concentration ($S_{2,t}$) as a dependent variable. $V_{1,2}^{zt}$ values: A, 2.17; B, 4.34; C, 21.1; D, 101; E, 164 pmol/10^6 cells per min.

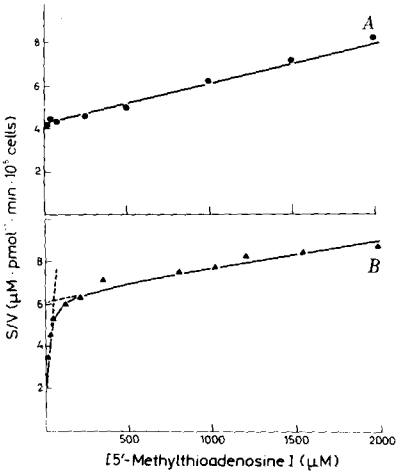

Fig. 5. A, Kinetic analysis of transport of methylthioadenosine in phosphate-depleted red cells. Initial velocity values were mathematically calculated as reported in Materials and Methods and in the caption of Fig. 4. Slope and intercepts were obtained with least-squares method (ry, $\bar{y} = 0.9981$). B. Woolf plot of methylthioadenosine uptake in untreated red cells. Initial velocity values were estimated by graphical approximation as discussed in the Results and Discussion section. Slope and intercepts were obtained by linear regression.

Methylthioadenosine uptake by untreated red cells: tandem operation of transport and phosphorylation

In Fig. 6 are compared the time courses of the uptake of methylthioadenosine by normal and phosphate-depleted erythrocytes at two concentrations of the thioether. At 20 μM methylthioadenosine, the uptake in presence of phosphate is apparently linear up to 9 s (see inset) and the influx largely exceeds that of phosphate-depleted cells.

These data suggest that at low concentrations of the thioether, the initial velocity values in untreated cells, graphically estimated, reflect the metabolism besides the transport. Conversely, at high concentrations (i.e., 550 μM), where methylthioadenosine phosphorylase is completely saturated ($K_m = 30$ μM), the uptake depends almost exclusively on the transport rate, as indicated by the overlapping of the two curves, even at short times. These findings are in agreement with the biphasic pattern observed in Fig. 5B, where the Woolf plot of the uptake of methylthioadenosine in untreated human erythrocytes is depicted.

The reported plot indicates the presence of two saturable systems and allows us to extrapolate two K_m values. The high apparent K_m value (K_m = 3270 μM), obtained for methylthioadenosine concentrations above 500 μM, strictly corresponds to that calculated for the transport system in phos-

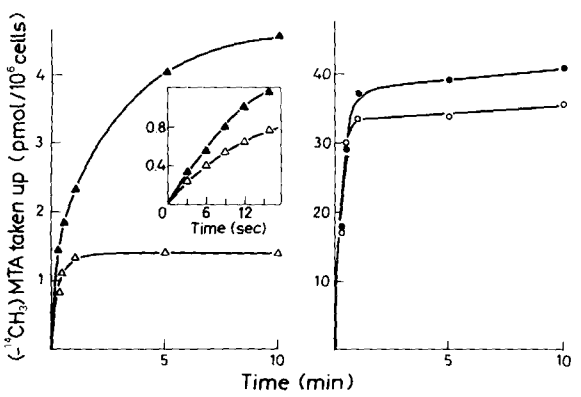

Fig. 6. Time courses of methylthioadenosine (MTA) uptake in untreated (▲——▲, ●——●) and phosphate-depleted (△——△, ○——○) red cells incubated with 20 μM (left) and 550 μM (right) 5'-[*Me*-^{14}C]methylthioadenosine. The experimental procedure is described under Materials and Methods. The inset shows the uptake of 20 μM methylthioadenosine at short times.

phate-depleted cells. The low apparent K_m value (58 μM), estimated for low concentrations of the thioether, largely reflects the in vivo saturation of methylthioadenosine phosphorylase, even if it does not represent a direct measurement of the in situ enzymatic kinetic constants. These results allow us to predict the steady-state levels of the molecule after its cell entry.

In fact the rate of phosphorolytic cleavage may or may not approach the rate of transport, depending on the concentration of exogenous methylthioadenosine. At physiological level of the thioether (below 5 μM), the initial velocity values of transport represent the limiting step of methylthioadenosine influx and only small amounts of methylthioadenosine are detectable intracellularly. Conversely, when the cells are exposed to cytostatic concentrations of the thioether (above 200 μM), the initial velocity of transport largely exceeds that of methylthioadenosine phosphorylase, and the cellular pool of the free nucleoside approaches the concentration of methylthioadenosine in the medium.

These observations appear relevant in the evaluation of the mechanism of cytostatic action of the thioether and its analogs.

Requirements and specificity of methylthioadenosine transport systems

Transport experiments performed with an isotonic medium in which Na^+ was substituted by K^+, Li^+ or Mg^{2+}, respectively (data not reported) demonstrated that the transport rate does not depend on specific cations.

The specificity of methylthioadenosine transport has been examined by testing various compounds (i.e., thioethers, bases and nucleosides) as inhibitors of methylthioadenosine influx in phosphate-depleted red cells. As reported in Table I, none of the nucleosides tested (adenosine, 7-

TABLE I

EFFECT OF METHYLTHIOADENOSINE ANALOGS AND RELATED COMPOUNDS ON THE UPTAKE OF 5'-[*Me*-^{14}C]METHYLTHIOADENOSINE BY HUMAN ERYTHROCYTES

The transport of methylthioadenosine was measured in phosphate-depleted red cells; the unlabelled inhibitors were added simultaneously with labelled substrate. The incubation time was 20 s. Experimental details are described under Materials and Methods.

Compound	Concentration of compound (μM)	Methylthioadenosine concentration (μM)	Inhibition (%)
Nucleosides			
Adenosine	1000	10	4
Inosine	1000	10	2
Guanosine	1000	10	4
Xanthosine	1000	10	2
7-Deazaadenosine	1000	10	3
Bases			
Adenine	250	500	21
	1000	500	58
	2000	500	91
	20	10	35
	100	10	60
7-Deazaadenine	1000	10	42
4-Aminopyrazolo-3,4-pyrimidine	1000	10	31
Guanine	1000	10	53
Various			
AMP	1000	10	–
5-Methylthioribose	1000	10	–
S-Adenosyl-L-homocysteine	1000	10	–

deazaadenosine, inosine, guanosine or xanthosine) is inhibitory on methylthioadenosine influx when added at a concentration of 1 mM.

Since the K_m value for the nucleoside carrier in red cells is approx. 500 μM [32,38], these results suggest that methylthioadenosine transport is not mediated by the nucleoside carrier.

On the other hand, a significant inhibitory effect was obtained employing several purines as inhibitors of the rate of thioether entry (Table I). Adenine, among the bases assayed, appears to be the most potent inhibitor of methylthioadenosine transport, a 58% inhibition being observable at 1 mM concentration (Table I). The effect exerted by adenine suggests that the two compounds compete for a common carrier (which could be the carrier responsible for the base influx).

When several thioethers were examined in the same conditions (1 mM inhibitor, 500 μM methylthioadenosine) (data not shown), 5'-isobutylthioadenosine, 5'-ethylthioadenosine and 5'-methylthiotubercidin were inhibitors of methylthioadenosine entry (over 30%); on the other hand, 5'-methylthioinosine and 5'-isobutylthioinosine appear ineffective, suggesting some relevance of 6-NH_2 in the interaction of the thioether with the carrier protein.

These results are in good agreement with those reported on the specificity of the uptake of 5'-isobutylthioadenosine in chick embryo fibroblasts [39].

Finally the data on the carrier specificity allow us to formulate a new hypothesis on the mechanism of the cytostatic effect of methylthioadenosine and its analogs, in that their action could also be ascribed to some interference with the cellular transport of adenine and of the bases in general.

Acknowledgments

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