Uptake and utilization of 5'-methylthioadenosine by cultured baby-hamster kidney cells

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(Received 4 February 1982/Accepted 17 March 1982)

5'-Methylthioadenosine was taken up and immediately metabolized further by cultured baby-hamster kidney cells during the exponential phase of growth. The adenine moiety supplied the purine-nucleotide pool via the salvage pathway and was efficiently incorporated into nucleic acids. Catabolites of methylthioadenosine excreted by the cells included adenine, purinic compounds and metabolites of the ribose portion. 5'-Methylthiotubercidin had no significant effect on the cellular metabolism of methylthioadenosine, but greatly inhibited its uptake. *erythro*-9-(2-Hydroxy-3-nonyl)adenine had no effect on the uptake, but markedly interfered with the further utilization of methylthioadenosine after cleavage in the cells.

Mammalian cells produce 5'-methylthioadenosine as a by-product of polyamine biosynthesis and rapidly degrade it to 5-methylthioribose 1-phosphate and adenine (for references, see Williams-Ashman & Pegg, 1981). The further fate of methylthioadenosine is not well known. Owing to the wide distribution (Garbers, 1978) and high activity of methylthioadenosine phosphorylase, the concentration of methylthioadenosine remains fairly low in animal tissues (Seidenfeld *et al.*, 1980). Malignant cells lacking this enzyme excrete methylthioadenosine stoichiometrically with polyamine production (Kamatani & Carson, 1980). The efficient removal of methylthioadenosine from the cells probably has a significant physiological role, since the accumulation of this compound could seriously interfere with the synthesis of polyamines, especially spermine (Pajula & Raina, 1979). Indeed, methylthioadenosine has been reported to exert deleterious effects on growth processes characterized by marked changes in polyamine synthesis, e.g. the activation of human lymphocytes (Vandenbark *et al.*, 1980).

The present study was undertaken to clarify the fate and significance of methylthioadenosine in cellular metabolism and to determine whether moderate extracellular concentrations of methylthioadenosine had any effect on the proliferation of mammalian cells via cellular accumulation of this compound.

Experimental

Chemicals

Labelled methylthioadenosine was obtained by acid hydrolysis of labelled *S*-adenosyl-L-methionine

(Schlenk *et al.*, 1971), purified by adsorption chromatography on a Sephadex G-10 column (Wasternack & Reinbothe, 1972; Kajander & Raina, 1981) and stored in aqueous solution at -20°C . Radiochemical purity was checked by paper electrophoresis in 0.05 M-glycine/NaOH buffer, pH 11 (Kajander *et al.*, 1976), and by t.l.c. on precoated silica-gel 60 F-254 plates (E. Merck A.G., Darmstadt, Germany) developed with butan-1-ol/acetic acid/water (12:3:5, by vol.). Labelled *S*-adenosyl-L-methionine was synthesized (Pegg & Williams-Ashman, 1969a) from [8- ^{14}C]adenosine or from [U- ^{14}C]adenosine 5'-triphosphates obtained from The Radiochemical Centre, Amersham, Bucks., U.K., purified by Cellex-P chromatography (Eloranta *et al.*, 1976) and stored frozen in 1 M-HCl. Unlabelled methylthioadenosine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 5'-Methylthiotubercidin was synthesized as previously described (Raina *et al.*, 1981). Details of other chemicals and materials are given elsewhere (Kajander *et al.*, 1976; Eloranta *et al.*, 1976; Raina *et al.*, 1981).

Cell culture and assay methods

Baby-hamster kidney cells (BHK 21) were seeded on 50 mm-diameter plastic Petri dishes (4.5×10^5 cells/dish) and grown at 37°C for 24 h in 3 ml of Eagle's minimal essential medium supplemented with penicillin (200 units/ml) and 10% (v/v) foetal-calf serum (Tuomi *et al.*, 1980) before starting the experiments in 3 ml of fresh culture media supplemented with the compounds studied. After the experiment, growth media were removed and the

cells were rapidly washed twice with 5 ml of cold phosphate-buffered saline (6.8 g of NaCl, 1.69 g of Na_2HPO_4 and 0.2 g of KH_2PO_4 in 1 litre) containing 0.2% (w/v) bovine serum albumin, detached with a rubber 'policeman' (the dishes being kept on ice) and collected by centrifugation. Acid-soluble radioactivities were determined from cold-trichloroacetic acid extracts of the cell pellets. RNA was hydrolysed in 500 μl of 1 M-KOH for 1 h at 37°C. Protein and DNA were then precipitated by adding 50 μl of 10 M-HCl and 100 μl of 50% (w/v) trichloroacetic acid and leaving the mixture in an ice bath for several hours before collecting the precipitate by centrifugation and washing with 300 μl of 5% (w/v) trichloroacetic acid. RNA was determined from a diethyl ether-extracted supernatant (Ashwell, 1957) and DNA from a hot- HClO_4 extract of the precipitate (Giles & Myers, 1965). After DNA extraction, the remaining precipitates were dissolved in 300 μl of 0.1 M-NaOH and used for protein assay (Spector, 1978). All the fractions and extracts were stored frozen. Radioactive compounds present in the media deproteinized by treatment with 8% (w/v, final concn.) trichloroacetic acid were fractionated by Sephadex G-10 chromatography (Wasternack & Reinbothe, 1972; Kajander & Raina, 1981). The compounds used as standards were well separated and were eluted from the column in the following order: AMP, inosine, hypoxanthine, uric acid, adenosine, methylthioadenosine and adenine. Portions of the media and of acid-soluble fractions of the cell pellets were also analysed by t.l.c. (see above) and paper electrophoresis both in 0.05 M-glycine/NaOH, pH 11 (Kajander *et al.*, 1976) and in 0.1 M-sodium citrate, pH 3.5 (Eloranta *et al.*, 1976). Marker compounds containing the purine ring were located under u.v. light, ribose by using anisidine spray (Mukherjee & Srivastava, 1952), and methionine by using a ninhydrin spray (Raina *et al.*,

1967). Distribution of radioactivity was determined from paper strips as described by Kajander *et al.* (1976), and from thin-layer plates by scraping out and extracting the silica-gel fractions thoroughly with 1.5 ml of 10 M-HCl and counting the radioactivity of 1 ml portions of the extracts in 10 ml of Bray's scintillant (Eloranta *et al.*, 1976).

Results and discussion

Methylthioadenosine has been reported to be actively transported into isolated rat liver by a high-affinity permease specific for thioethers (Zappia *et al.*, 1978a). However, uptake and subsequent metabolism of methylthioadenosine have not previously been studied in any mammalian cell lines. The rate of methylthioadenosine uptake in proliferating BHK 21 cells, shown in Tables 1 and 2, appears to be an order of magnitude higher than that of methylthioadenosine production by methylthioadenosine phosphorylase-deficient human leukaemic-cell lines (Kamatani & Carson, 1980) and closely corresponds to the rate of adenine uptake, but is much lower than the rate of adenine uptake in isolated rat hepatocytes (Smith *et al.*, 1977). The uptake of labelled methylthioadenosine was calculated from the radioactivity incorporated into cells and into metabolites accumulating in the culture medium, assuming that the excretion of methylthioadenosine by the cells is negligible. Since methylthioadenosine was stable in both fresh and conditioned media at 37°C for 24 h (see the legends to Tables 1 and 3), the radioactivity found in other extracellular compounds (Tables 1 and 3) obviously resulted from the cellular utilization of labelled methylthioadenosine and subsequent excretion of the metabolites. An active transport mechanism is favoured by the findings that the rate of methylthioadenosine uptake remained fairly con-

Table 1. *Uptake and utilization of methylthio[$\text{U-}^{14}\text{C}$]adenosine by exponentially growing BHK 21 cells*
BHK 21 cells were incubated in the presence of 50 μM -methylthio[$\text{U-}^{14}\text{C}$]adenosine (sp. radioactivity 5.2 Ci/mol) for the time periods indicated and as described in the Experimental section. Medium incubated without cells for 24 h contained 97.2% of the total radioactivity in methylthioadenosine. The percentages given in the Table for extracellular catabolites of methylthioadenosine have been corrected by using this value.

	Incubation time (h) ...	6	12	24
Growth parameters				
Protein (mg/culture)		0.228	0.360	0.573
RNA (μg /culture)		29.8	41.4	59.3
Distribution of radioactivity (%)				
Extracellular catabolites		3.40	9.75	30.4
Cellular radioactivity		1.60	5.27	17.2
DNA		0.18	1.00	3.36
Methylthioadenosine uptake				
nmol/culture		7.50	22.5	71.4
nmol/h per mg of protein		5.48	5.22	5.19
nmol/h per μg of RNA		0.04	0.05	0.05

Table 2. *Effect of methylthiotubercidin and erythro-9-(2-hydroxy-3-nonyl)adenine on the uptake and utilization of [8-¹⁴C]methylthioadenosine by exponentially growing BHK 21 cells*

BHK 21 cells were incubated in the presence of 45 μ M-[8-¹⁴C]methylthioadenosine (sp. radioactivity 5.61 Ci/mol) with or without 1 mM-methylthiotubercidin (MTT) or 20 μ M-erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) for 24 h, as described in the Experimental section. The cultures were duplicates, which were combined for analysis. The proliferation rate during the 48 h growth period was observed by measuring the accumulation of DNA and RNA in separate control cultures devoid of methylthioadenosine 4, 24 and 48 h after seeding. Percentages of extracellular catabolites have been corrected for the radioactive impurities of labelled methylthioadenosine (see the legend for Table 3). Abbreviation used: n.d., not detectable.

	Control	MTT (1 mM)	EHNA (20 μ M)
Growth parameters (μ g/culture)			
DNA	10.7	5.6	7.6
RNA	57.8	37.5	45.3
Distribution of radioactivity (%)			
Extracellular catabolites	16.00	n.d.	25.94
Cellular radioactivity	37.50	7.81	23.52
Acid-soluble	7.65	1.70	7.78
RNA	23.68	4.72	12.93
DNA	6.17	1.39	2.81
Acid-soluble/RNA	0.32	0.36	0.60
Acid-soluble/DNA	1.24	1.22	2.77
RNA/DNA	3.84	3.40	4.60
Methylthioadenosine uptake			
nmol/culture	72.23	10.54	66.77
nmol/h per μ g of DNA	0.28	0.08	0.37
nmol/h per μ g of RNA	0.05	0.01	0.06

stant throughout the 24 h experimental period in spite of decreasing extracellular concentration (Table 1) and that this uptake was markedly inhibited by a nucleoside analogue, 5'-methylthiotubercidin (Table 2). Cultured lymphocytes have been reported to take up methylthioadenosine only during active growth (Vandenbark *et al.*, 1980).

The distribution pattern of radioactivity derived from [8-¹⁴C]methylthioadenosine (Table 2) differs in an interesting way from that of methylthio[U-¹⁴C]adenosine (Table 1). In terms of the initial cell number, the accumulation of RNA, as well as the rate and amount of methylthioadenosine uptake, the 24 h experiments described in Tables 1 and 2 (control) can be considered identical. Since the radioactivity of methylthio[U-¹⁴C]adenosine is equally distributed between the adenylyl and the methylthioribosyl moieties that are split apart by the specific phosphorylase, the results suggest that all the cellular radioactivity, most of which was incorporated into nucleic acids, was derived from the adenylyl moiety of methylthioadenosine. Radioactivity from the ribosyl portion of the *adenosine*-U-¹⁴C-labelled compound seemed to be excreted entirely into the growth medium. It may also be asked whether the ribosyl portion ever entered the cell during the uptake process. Some reports indicate that, at least in rat liver extracts, the methylthioribose moiety of methylthioadenosine is con-

verted into methionine by an unknown mechanism (Edwards *et al.*, 1977; Backlund & Smith, 1981). However, we could not find any incorporation of radioactivity from methylthio[U-¹⁴C]adenosine into methionine or into cellular proteins. The extracellular compound(s) formed from the ribosyl portion remained unidentified.

Most of the acid-soluble cellular radioactivity derived from [8-¹⁴C]methylthioadenosine resided in nucleotides and deoxynucleotides (Table 3). It can be calculated from the results shown in Table 2 that approx. 80% of the purine bases incorporated into DNA during the 24 h experiment originated from labelled methylthioadenosine. Considering the marked turnover of RNA, decreasing the specific radioactivity of cellular purine pools, 50 μ M-methylthioadenosine in the medium seemed to satisfy the purine needs of proliferating BHK 21 cells. It thus appears that the accumulation of adenine nucleotides (resulting from the uptake and catabolism of labelled methylthioadenosine) blocked purine synthesis *de novo* and made methylthioadenosine the sole source of both adenine and guanine nucleotides and deoxynucleotides via an efficient purine-salvage pathway. The inability of *erythro*-9-(2-hydroxy-3-nonyl)adenine, a potent inhibitor of adenosine deaminase (EC 3.5.4.4) (Newby, 1981), to cause adenosine accumulation (Table 3) suggests that in BHK 21 cells, and probably in rat hepatocytes as

Table 3. Fate of [8-¹⁴C]methylthioadenosine in BHK 21 cell culture in the presence of methylthiotubercidin (MTT) or erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA)

Distribution of radioactivity in the culture medium (M) as well as in the acid-soluble pool of cells (A-S) was analysed as described in the Experimental section. The results are expressed as a percentage of the total radioactivity found in M or A-S pools respectively. Fresh medium incubated without cells for 24 h contained 1.3% of the total radioactivity in adenosine, 3.0% in adenine and the rest in methylthioadenosine. The distribution pattern of radioactivity in the conditioned media (M) or in the fresh medium was not changed by incubation at 37°C for an additional 24 h period, ruling out the presence of methylthioadenosine-degrading activities in any of the media. For other details, see the legend for Table 2.

Radioactive compound	Additions ...	Radioactivity (% of total)					
		None		1 mM-MTT		20 µM-EHNA	
		M	A-S	M	A-S	M	A-S
Methylthioadenosine		68.0	0.0	96.8	0.0	60.3	0.0
Adenine		8.7	2.2	1.1	1.6	20.0	1.4
Adenosine		2.1	0.7	0.5	1.2	2.1	1.6
Adenosylmethionine		0.0	1.6	0.0	1.5	0.0	1.0
Allantoin/hypoxanthine		21.2	9.1	1.6	9.8	17.9	6.7
Nucleotides/deoxynucleotides		0.0	86.4	0.0	85.9	0.0	89.3
Radioactivity of the pool (% of total in culture)		62.5	7.7	92.2	1.7	76.5	7.8

well (Smith *et al.*, 1977), the reaction catalysed by adenosine kinase (EC 2.7.1.20) is quantitatively much more important than that catalysed by adenosine deaminase. Clearly, AMP was not further converted into adenosine in sizeable amounts, but was further metabolized via ADP and IMP, the latter giving rise both to guanine nucleotides and to purinic catabolites. Although hypoxanthine and allantoin could not be separated by the methods used, it is more probable that the cells excreted allantoin, as that is known to be the final catabolite of purine metabolism in mammals other than higher primates (Smith *et al.*, 1977).

Labelled methylthioadenosine could not be detected in the cellular pool under any conditions (Table 3), which might be explained by a very efficient degradation system that continued to function inside the cell during the washing and detachment procedures. However, it is also possible that methylthioadenosine is not taken up intact. Methylthiotubercidin, a competitive inhibitor of methylthioadenosine phosphorylase (Coward *et al.*, 1977; Zappia *et al.*, 1978b; Ferro *et al.*, 1979), at a concentration 20-fold that of methylthioadenosine, was unable to cause any cellular accumulation or change in the metabolism (Table 3), but markedly inhibited the uptake (Table 2) of methylthioadenosine. The role of the phosphorylase in the elimination of cellular methylthioadenosine has been clearly demonstrated in human leukaemic cell lines (Kamatani & Carson, 1980). Other enzymes important in nucleoside metabolism, e.g. adenosine deaminase, AMP deaminase (EC 3.5.4.6) and purine-nucleoside phosphorylase (EC 2.4.2.1), do not use methylthioadenosine as a substrate (Pegg & Williams-

Ashman, 1969b). Therefore it seems likely that the phosphorylase either was inefficiently inhibited by the methylthiotubercidin taken up by the cells or was not involved in the cleavage of methylthioadenosine. The former is supported by the fact that the uptake of [8-¹⁴C]methylthioadenosine was accompanied by a concomitant release of labelled adenine from the cells (Table 3). This release was further potentiated in the presence of 20 µM-erythro-9-(2-hydroxy-3-nonyl)adenine. Since this compound did not change the distribution pattern of the acid-soluble cellular radioactivity (Table 3), it probably increased adenine excretion by a mechanism not involving inhibition of adenine phosphoribosyltransferase (EC 2.4.2.7).

In conclusion, 50 µM-methylthioadenosine was taken up and immediately utilized by proliferating BHK 21 cells at a rate sufficient to supply most, if not all, of the purine pool. Whereas the ribosyl portion was efficiently excluded from the cells, the adenylyl moiety flowed via AMP and the purine-salvage cycle to DNA, RNA and finally most likely to allantoin, the latter being then excreted into the culture medium. The capacity of the cellular degradation mechanism greatly exceeded that of the uptake mechanism, making it unlikely that extracellular concentrations of methylthioadenosine could cause accumulation of this nucleoside in the cells.

This work was supported by grants from the Sigrid Jusélius Foundation, the Medical Research Council of the Academy of Finland and the Cancer Society of Finland, J. K. Paasikivi Fund. The skilful technical assistance of Miss Päivi Kivistö and Mr. Juha Taskinen is gratefully acknowledged.

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