Methylthioadenosine toxicity and metabolism to methionine in mammalian cells

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5'-Deoxy-5'-methylthioadenosine, a by-product of polyamine synthesis, can support the growth of Raji cells in a methionine-free medium, but not the growth of CCL39 cells, although these cells are also able to incorporate radiolabelled 5'-deoxy-5'-methylthioadenosine (MeSAdo) into methionine, S-adenosyl-L-methionine (AdoMet) and proteins [Christa, Kersual, Augé & Pérignon (1986) *Biochem. Biophys. Res. Commun.* **135**, 131–138]. We first tested the hypothesis of a toxic effect of MeSAdo in the conditions of growth experiments: we could not demonstrate any toxic effect of MeSAdo on the synthesis of macromolecules, nor any toxicity mediated by polyamines or pyrimidine starvation, and we found that the growth of CCL39 cells was strictly dependent on the supply of exogenous methionine. We then tried to determine whether the ability of CCL39 cells to metabolize MeSAdo to methionine and AdoMet was modulated by the proliferation state of CCL39 cells, which is dependent on the supply of exogenous methionine. Studies of the incorporation of radiolabelled MeSAdo show that: (i) the total synthesis of methionine from MeSAdo is twice as high in subconfluent cells (grown in 100 μ M-methionine) as in resting cells (cultured in 0 μ M-methionine); (ii) the incorporation into proteins does not parallel the total protein synthesis, and the methionine derived from MeSAdo mostly flows out of the cell; (iii) addition of methionine to resting cells immediately leads to a transient and marked increase in metabolism of MeSAdo to AdoMet, presumably reflecting the rapid replenishment of the AdoMet pool of the cells. Taken together, these results suggest that the methionine derived from MeSAdo is preferentially used to synthesize AdoMet rather than proteins, and that this synthesis of AdoMet depends on the ability of the CCL39 cells to grow, and hence on the supply of exogenous methionine. It is proposed that, in CCL39 cells, the metabolic pathway leading from MeSAdo (a by-product of polyamine synthesis) to methionine and to AdoMet (a precursor of polyamine synthesis) is part of a metabolic cycle the activity of which depends, like polyamine synthesis itself, on cell proliferation.

INTRODUCTION

5'-Deoxy-5'-methylthioadenosine (MeSAdo), a by-product of polyamine synthesis, has been demonstrated to be a potent inhibitor of cell proliferation (Pegg *et al.*, 1981) and to be metabolized to adenine (Kamatani & Carson, 1981) and methionine in mammalian (Backlund & Smith, 1981) and non-mammalian cells (Shapiro & Schlenk, 1980; Wang *et al.*, 1982; Yung *et al.*, 1982). In a previous study of the metabolism of MeSAdo in CCL39 cells (fibroblasts from lung of Chinese hamster), baby-hamster kidney cells and Raji cells, we have shown that only Raji cells were able to grow on this compound, although CCL39 and baby-hamster kidney cells were also able to incorporate radiolabelled MeSAdo into methionine (Met), S-adenosyl-L-methionine (AdoMet) and proteins (Christa *et al.*, 1986); this finding was thus similar to that described with homocysteine (Hcy) as a Met precursor, and named 'methionine dependence' by Hoffman & Erbe (1976). Since the incorporation studies mentioned above were performed on confluent cells that had grown in optimal concentrations of Met, we questioned whether the discrepancy between the results of growth experiments in a Met-free medium and those of studies with labelled compounds could be due (i) to an inhibitory effect of low concentrations of MeSAdo on

cell proliferation in a Met-free medium or (ii) to an effect of the culture conditions before the incorporation studies on the ability of the cells to metabolize MeSAdo to Met or AdoMet. These two hypotheses were tested in CCL39 cells.

MATERIALS AND METHODS

Cell lines

The Raji cell line (a human Burkitt's-lymphoma-derived cell line) was purchased from Flow Laboratories (Irvine, Scotland, U.K.), and routinely cultured in RPMI 1640 medium (Gibco, Grand Island, NY, U.S.A.). CCL39 cells (a line of female-Chinese-hamster lung fibroblast) and 8-aza-adenine-resistant GMA32-A1 and GMA32-A3 cells (isolated from CCL39, and deficient in both deoxycytidine kinase and adenine phosphoribosyl-transferase activities) were gifts from Dr. G. Buttin (Institut Pasteur, Paris, France). CL81, a cycloleucine-resistant clone isolated from Chinese-hamster ovary cells, was kindly provided by Dr. M. Caboche and Dr. P. Mulsant (Laboratoire de Génétique Cellulaire, I.N.R.A., Toulouse, France). Hamster fibroblasts were routinely cultured in Dulbecco's modified Eagle's medium (Eurobio, Paris, France). Media were routinely

Abbreviations used: AdoMet, S-adenosyl-L-methionine; Hcy, L-homocysteine; MeSAdo, 5'-deoxy-5'-methylthioadenosine.

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supplemented with 10% (v/v) fetal-calf serum (Flow Laboratories), except for the studies of MeSAdo and Met incorporations which were performed in Met-free media supplemented with dialysed 10% (v/v) horse serum (Boehringer, Mannheim, Germany), which is devoided of MeSAdo phosphorylase activity. The cultures and the metabolic studies were performed under humidified air/CO₂ (19:1) at 37 °C; cells were counted in a Coulter counter (Coultronics, Margency, France).

Chemicals

[Me-¹⁴C]AdoMet (60 mCi/mmol) and [Me-¹⁴C]Met (60 mCi/mmol) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.); [6-³H]thymidine (29 Ci/mmol), [5-³H]uridine (27 Ci/mmol) and [3,4,5-³H]leucine (110 Ci/mmol) were from CEA (Saclay, France). [Me-¹⁴C]Methylthioribose was prepared by acid hydrolysis of [Me-¹⁴C]AdoMet by Dr. B. Estramareix (Institut de Chimie Multifonctionnelle, Orsay, France). [Me-¹⁴C]MeSAdo was prepared from [Me-¹⁴C]AdoMet by acid hydrolysis (Schlenk & Ehninger, 1964) and purified by reverse-phase h.p.l.c. on a μ Bondapak C18 Radial-Pak cartridge (Waters, Milford, MA, U.S.A.) with a three-step elution: isocratic elution by 0.02 M-KH₂PO₄, pH 4.2, for 15 min, followed by linear gradient elution (0–27% methanol in 0.02 M-KH₂PO₄, pH 4.2, in 70 min), then by isocratic elution by 27% methanol in water, for 35 min. AdoMet, MeSAdo, Met, uridine, putrescine, spermidine and spermine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Decarboxylated AdoMet was a gift from Dr. Keijiro Samejima (Josai University, Saitama, Japan).

Incorporations of thymidine, uridine and leucine

CCL39 cells were plated at 0.1×10^5 cells/cm² in six-well microtitre plates (Nunc, Roskilde, Denmark), cultured for 48 h, and pulsed for 1 h with [6-³H]-thymidine (1 μ Ci/well) or [5-³H]uridine (1 μ Ci/well), or [3,4,5-³H]leucine (2.5 μ Ci/well); the radioactivity in the acid-insoluble material was measured as described by Cartier *et al.* (1982).

Incorporations of MeSAdo and Met

In all the experiments described below, CCL39 cells were plated in six-well microtitre plates. Before the addition of labelled compounds, the cells were washed in Met-free medium; incorporations were performed for 3 h in 1 ml of medium. The media were collected and precipitated in 0.8 M-HClO₄; the cells were washed with Dulbecco's phosphate-buffered saline and extracted in 0.4 M-HClO₄. Since the acid extraction hydrolyses the 1-phosphate bond of methylthioribose 1-phosphate, methylthioribose was recovered instead of methylthioribose 1-phosphate. Acid extracts were neutralized with tri-n-octylamine/Freon (11:39, v/v) (Khym, 1975). Preliminary experiments have shown that the radioactivity incorporated into the acid-insoluble fraction corresponded mainly (~90%) to incorporation into proteins, and will be hereafter termed 'protein Met'. Samples of radioactive acid-soluble and acid-insoluble fractions, the latter dissolved in 0.1 M-NaOH, were measured in a liquid-scintillation spectrometer (Packard Tris-Carb 300) by using aqueous counting scintillant II (The Radiochemical Centre). The analysis of MeSAdo

and Met incorporations was conducted by h.p.l.c.; the h.p.l.c. apparatus included a Varian model 5000 pump (flow set at 1 ml/min), a model 440 fixed-wavelength (254 nm) absorbance detector (Waters) connected to an Icap 10 peak-integrator/calculator (Delsi, Paris, France) and a continuous-flow radioactivity monitor (Flow-one model HP; Radiomatic Instruments and Chemical Co, Tampa, FL, U.S.A.). When the radioactivity of the acid-soluble cell extracts was too low (<10000 c.p.m.) to be monitored in continuous flow, 1 ml fractions were collected, added to 5 ml of aqueous counting scintillant II and counted for radioactivity. Either of the two following h.p.l.c. chromatographic systems was used. (a) Reverse-phase chromatography on a μ Bondapak C18 Radial-Pak cartridge, with isocratic elution by 0.02 M-KH₂PO₄, pH 6.00, for 30 min, followed by a linear gradient elution (0–40% methanol in 0.02 M-KH₂PO₄, pH 5.6, in 10 min). The retention times of Met sulphoxide, Met, methylthioribose, AdoMet, MeSAdo sulphoxide and MeSAdo were 9, 12, 22, 27, 47 and 54 min respectively. The radioactivities of Met sulphoxide and MeSAdo sulphoxide were expressed as Met and MeSAdo respectively. (b) Ion-exchange chromatography on a Partisil-10 SCX column (Whatman, Maidstone, Kent, U.K.), which allowed the separation of AdoMet and decarboxylated AdoMet, was adapted from Zimmerman *et al.* (1979): first there was an isocratic elution by 0.005 M-KH₂PO₄, pH 2.2, for 15 min, followed by a linear gradient elution (0–40% 0.5 M-KH₂PO₄, pH 3.5, in 45 min). The retention times of methylthioribose, Met or Met sulphoxide, MeSAdo or MeSAdo sulphoxide, AdoMet and decarboxylated AdoMet were 4, 12, 29, 47 and 67 min respectively. Peak identities of MeSAdo, AdoMet and decarboxylated AdoMet were confirmed by co-elution with standards and by analysis of u.v. spectra with a HP1040A spectrophotometric detector (Hewlett-Packard, Corvallis, OR, U.S.A.).

Enzyme activities

Cells were disrupted by rapid freeze-thawing, diluted in 0.05 M-KH₂PO₄, pH 7.2, containing 0.004 M-dithiothreitol and centrifuged in an Ependorff Microfuge (10000 rev./min for 4 min) to remove cell debris. MeSAdo phosphorylase activity was measured as previously described (Christa *et al.*, 1983), and methionine adenosyltransferase activity was assayed by the method of Caboche (1977), modified as follows: the incubation mixture contained, in a total volume of 0.2 ml, 0.15 M-Pipes (dipotassium salt), pH 7.2, 0.067 M-MgCl₂, 0.010 M-ATP (dipotassium salt) and 67 μ M (0.2 μ Ci) [*carboxy*-¹⁴C]Met. After 15, 30 or 45 min incubation at 37 °C, 0.05 ml samples were transferred into 0.025 ml of 0.3 M-HClO₄. The resulting precipitate was removed by centrifugation; 0.02 ml samples of the supernatant were applied to phosphocellulose (P81) paper strips (Whatman), and AdoMet was separated from Met and ATP by descending chromatography in water, by the method of Öztürk *et al.* (1983).

AdoMet-pool determination

After 72 h of culture in various Met and MeSAdo concentrations, the cells were trypsin-treated (0.5 g/l; 10 min at 37 °C) (CCL39 and CL81 cells) or centrifuged (300 g for 6 min) (Raji cells). CCL39 and CL81 cells (about 20×10^6 for cultures in 100 μ M-Met) and Raji cells

Table 1. Effects of various additions on the proliferation of CCL39 cells in a Met-free medium

CCL39 cells were plated at 0.1×10^5 cells/cm² in a Met-free medium containing the indicated effectors. After 72 h of culture, the cells were counted in a Coulter counter. The results are those of one representative experiment. Similar results were obtained in three other experiments.

Additions	$10^{-6} \times \text{Cell no.}$
None	0.13
Met (100 μM)	3.6
MeSAdo (15 μM)	0.19
MeSAdo (15 μM) + putrescine (10 μM)	0.17
MeSAdo (15 μM) + spermidine (10 μM)	0.17
MeSAdo (15 μM) + spermine (10 μM)	0.17
MeSAdo (15 μM) + spermidine (10 μM) + spermine (10 μM)	0.17
MeSAdo (15 μM) + uridine (50 μM)	0.15

(about 40×10^6 for cultures in 100 μM) were washed in cold Dulbecco's phosphate-buffered saline (pH 7.2) and extracted in 0.4 M-HClO₄ containing [*Me*-¹⁴C]AdoMet (0.2 nmol, 0.55 nCi) in order to evaluate the recovery and to locate precisely the peak fraction of AdoMet. HClO₄-soluble cell extracts were neutralized with tri-*n*-octylamine/Freon (Khym, 1975) and pre-chromatographed through a column of Dowex AG 1X4 (100–200 mesh; formate form), eluted with water to remove u.v.-absorbing contaminants, then analysed by h.p.l.c. on a μ Bondapak reverse-phase C18 Radial-Pak cartridge, which was eluted with 0.02 M-KH₂PO₄, pH 4.8, for 30 min. AdoMet was quantified by integration of the *A*₂₅₄.

DNA was assayed by a modification of the method of Erwin *et al.* (1981), by using the Hoechst 33258 fluorescent dye. Protein was determined as described by Lowry *et al.* (1951), with prior dissolution of HClO₄-insoluble fractions in 0.1 M-NaOH.

RESULTS

Toxicity of low concentration of MeSAdo on cells cultured on low or zero Met concentrations

We have previously reported that CCL39 cells were unable to grow on low (15 μM) MeSAdo concentration, i.e. a concentration which is not toxic for cells cultured at high (100 μM) Met concentrations (Christa *et al.*, 1984). We first tested the hypothesis that a positive effect of MeSAdo on cell growth (via the biosynthesis of Met) may be counterbalanced by an inhibitory effect of low concentration of MeSAdo on cell growth at low Met concentration. The synthesis of macromolecules was not affected, since MeSAdo (15 μM) did not decrease either the uptake of labelled thymidine, uridine or leucine or the synthesis of DNA, in cells cultured for 48 h in normal (100 μM -Met), low (10 μM -Met) or Met-free media (results not shown). The hypothesis of an inhibitory effect on specific metabolic pathways was tested indirectly, from the ability of various supplementations to restore the cell growth in a Met-free medium supplemented with 15 μM -MeSAdo (Table 1). It has been previously shown that MeSAdo is an inhibitor of polyamine biosynthesis (Pajula & Raina, 1979; Hibasami *et al.*, 1980). Table 1 shows that the addition of polyamines (10 μM -putrescine, -spermidine or -spermine, each in the presence of 1 mM-aminoguanidine to prevent their oxidation to toxic metabolites by fetal-calf serum) had no effect on cell growth.

In preliminary experiments, we had found that adenine was highly toxic for CCL39 cells (50 μM inhibited the growth in optimal conditions by 50 %); since MeSAdo can be metabolized to adenine by MeSAdo phosphorylase, we hypothesized that MeSAdo toxicity might be mediated by adenine. We first tested the ability of adenine phosphoribosyltransferase-deficient clones selected from CCL39 cells, such as GMA32-A1 and GMA32-A3, to grow on MeSAdo in a Met-free medium. Although these cells were resistant to adenine toxicity (the proliferation was not inhibited by 50 μM -adenine), they did not grow on MeSAdo. Secondly, because previous

Table 2. AdoMet intracellular concentrations in CCL39, CL81 and Raji cells

CCL39 and CL81 cells were plated at 0.1×10^5 cells/cm² and Raji cells at 2×10^5 cells/ml in media containing various extracellular Met and MeSAdo concentrations. After 72 h of culture, AdoMet intracellular concentrations were determined as described in the Materials and methods section. Results are the means $\pm$ s.d. for *n* independent cultures; N.D., not determined.

Growth conditions	Cells...	AdoMet (nmol/mg of protein)		
		CCL39	CL81	Raji
Met (100 μM)		1.02 ± 0.19 <i>n</i> = 12	1.66 ± 0.15 <i>n</i> = 5	0.75 ± 0.15 <i>n</i> = 11
Met (25 μM)		0.47 ± 0.10 <i>n</i> = 6	1.00 ± 0.11 <i>n</i> = 3	0.13 ± 0.02 <i>n</i> = 4
Met (10 μM)		0.32 ± 0.04 <i>n</i> = 3	1.02	0.07 ± 0.01 <i>n</i> = 3
Met (10 μM) + MeSAdo (15 μM)		0.49 ± 0.04 <i>n</i> = 4	0.98, 0.75	0.12 ± 0.01 <i>n</i> = 3
MeSAdo (15 μM)		0.14 ± 0.04 <i>n</i> = 4	N.D.	0.06 ± 0.01 <i>n</i> = 3
Met (0)		0.14 ± 0.04 <i>n</i> = 3	N.D.	N.D.

Fig. 1. Effect of increasing Met concentrations on the proliferation of CCL39 cells in media containing 15 μ M-MeSAdo

CCL39 cells were plated at 0.1×10^5 cells/cm², and proliferation was evaluated after 72 h of culture by cell counting on a Coulter counter. Similar results were obtained in another experiment. □, Met alone; ■, Met + MeSAdo.

studies had showed that the toxicity of adenine could be corrected by uridine (Debatisse & Buttin, 1977; Archer *et al.*, 1985), we tested the ability of uridine to restore cell proliferation: Table 1 shows that the addition of uridine (50 μ M) did not allow the growth of CCL39 cells. It should be pointed out that uridine is also a means to supply the cells with ribose phosphate (Wice & Kennell, 1982): the lack of effect of uridine thus also argues against the hypothesis of an arrest of cell growth via a shortage of the production of ribose 5-phosphate, as suggested by Boss & Pilz (1985).

Kajander *et al.* (1986) have shown that an adenosine kinase-deficient subclone of the R1.1 murine T-lymphoma cell line containing elevated AdoMet pools and increased methionine adenosyltransferase activity was cross-resistant to adenine and several adenine nucleosides, including MeSAdo. To test the hypothesis that CCL39 cells may be specifically sensitive to MeSAdo in culture conditions leading to low AdoMet concentrations, this compound was measured in these cells and in another hamster fibroblast cell line (CL81). Although these cycloleucine-resistant cells have an increased AdoMet pool at low Met concentration (Table 2, and Caboche & Mulsant, 1978) they were unable, like CCL39 cells, to grow on MeSAdo in a Met-free medium, which suggests that there is no relationship between the postulated MeSAdo toxicity and the cell content of AdoMet.

Taken together, all these results failed to demonstrate any toxicity, which may explain the inability of CCL39 cells to proliferate on MeSAdo. Lastly, Hoffman & Erbe (1976) have shown that some Met-dependent cells were unable to grow on Hcy alone, but grew rapidly on Hcy in the presence of otherwise limiting amounts of Met. To test a similar permissive effect of Met on the growth of

Table 3. Metabolism of [*Me*-¹⁴C]MeSAdo in resting and subconfluent CCL39 cells

CCL39 cells were plated at 0.2×10^5 cells/cm² and cultured either in Met-free medium or in the same medium supplemented with 100 μ M-Met. After 48 h of culture, resting cells (Met-free medium) and subconfluent cells (100 μ M-Met) were washed in Met-free medium, then incubated for 3 h with [*Me*-¹⁴C]MeSAdo (15 μ M; 0.5 μ Ci/well) as described in the Materials and methods section. Each point is the mean $\pm$ S.D. for three independent experiments.

	MeSAdo incorporated (pmol/3 h per mg of protein)	
	Resting cells	Subconfluent cells
AdoMet in the acid-soluble fraction	48 $\pm$ 5	131 $\pm$ 27
Met in the acid-soluble fraction	76 $\pm$ 9	99 $\pm$ 55
Extracellular Met	3148 $\pm$ 644	5734 $\pm$ 991
Protein Met	4265 $\pm$ 283	8998 $\pm$ 990
Total Met	7537 $\pm$ 908	14962 $\pm$ 1378
Proteins (μ g/well)	85 $\pm$ 27	392 $\pm$ 25

CCL39 cells cultured in 15 μ M-MeSAdo, the culture medium was supplemented with increasing Met concentrations (Fig. 1). Such a permissive effect was not found with MeSAdo, since very small amounts of Met, such as 1 or 5 μ M, did not allow the proliferation of CCL39 cells on 15 μ M-MeSAdo. Furthermore, there was no additive effect between 15 μ M-MeSAdo and greater Met concentrations, in the range 10–100 μ M. In fact, Fig. 1 demonstrates that the growth is strictly dependent on the supply of exogenous Met.

Regulation of MeSAdo metabolism by exogenous Met

The second hypothesis that we tested was that the biosynthesis of Met from MeSAdo may be dependent on the ability of CCL39 cells to proliferate.

Incorporation of [*Me*-¹⁴C]MeSAdo in resting and proliferating CCL39 cells. Our previous studies, demonstrating the inability of CCL39 cells to grow on MeSAdo, were conducted in cells which were plated at 0.1×10^5 cells/cm² in a Met-free medium supplemented with MeSAdo; the proliferation was evaluated by cell counting after 72 h of culture. On the contrary, the experiments demonstrating the incorporation of MeSAdo into Met, AdoMet and proteins were conducted in subconfluent cells that had grown for 48 h at high Met concentrations (200 μ M), and were subsequently washed in a Met-free medium and then incubated with the radiolabelled precursor. Since the discrepancy between the results of the experiments of proliferation and those of incorporation could be due to these different culture conditions, we tried to determine if MeSAdo metabolism was coupled to the proliferation state of CCL39 cells, which depends on the concentration of exogenous Met in the culture medium before incorporation studies. Thus we compared MeSAdo metabolism to Met between cells which had been plated at 0.2×10^5 cells/cm² for 48 h either in 100 μ M-Met (optimal growth condition) to

Fig. 2. Correlation between growth rate and MeSAdo phosphorylase activity of CCL39 cells

CCL39 cells were plated at 1×10^5 cells/25 cm² (N_0) in complete Dulbecco's modified Eagle's medium, supplemented with 10% horse serum. At designated times, cells were trypsin-treated, counted (N), and MeSAdo phosphorylase activity was assayed as described in the Materials and methods section. Points are means of duplicate determinations. Similar results were obtained in two other experiments.

obtain subconfluent cells, or in a Met-free medium to obtain resting cells. The results presented in Table 3 show that the total incorporation of MeSAdo into Met (proteins + AdoMet + intracellular Met + extracellular Met) was decreased by 50% in CCL39 cells cultured in the absence of Met, and that, quantitatively, the difference was mainly due to a difference in the incorporation of MeSAdo into extracellular Met and proteins; the incorporation of MeSAdo into AdoMet was also decreased by more than 50% in CCL39 cells cultured in the absence of Met.

Because the metabolism of MeSAdo was higher in subconfluent CCL39 cells than in resting cells, we investigated the variations during cell proliferation of (a) the activity of MeSAdo phosphorylase, which catalyses the first step of MeSAdo metabolism, and (b) the incorporation of [$Me-^{14}C$]MeSAdo.

MeSAdo phosphorylase activity has been previously shown to be induced in rat and human lymphocytes stimulated by lectins (Christa *et al.*, 1983; Ferro *et al.*, 1979), and in mammalian cells during their cell cycle (Sunkara *et al.*, 1985). Fig. 2 shows that the activity reaches a peak during the exponential growth of CCL39 cells, a result similar to that of Sunkara *et al.* (1985).

To determine the variation of MeSAdo metabolism during cell proliferation, the cells were plated at 1×10^5 cells/cm² for 24 h in Met-free medium; then 100 μ M-Met was added to start the proliferation. Incorporations of 15 μ M-[$Me-^{14}C$]MeSAdo were performed at 0, 3, 12, 24 and 48 h after the addition of exogenous Met, in 100 μ M-Met in order not to modify Met concentration during the 3 h of incorporation (Fig. 3a). The results show that the total synthesis of Met from MeSAdo increased with cell proliferation, as measured by the increase in proteins. However, there was no increase in the radioactivity incorporated into proteins, and the Met synthesized from MeSAdo mostly flowed out of the cell. When no exogenous Met was added after the 24 h of pre-culture in Met-free medium, the total synthesis of Met from MeSAdo remained constant (Fig. 3b). Interestingly, the incorporation of MeSAdo into AdoMet showed a pattern quite different from that into proteins. (i) During the first hours after Met addition there was a high incorporation of radioactivity into AdoMet: the incorporation at zero time, performed in 100 μ M exogenous Met, was 3.8 times that performed in the absence of exogenous Met (Fig. 3b) (ii) This exogenous Met-induced increase of the incorporation of MeSAdo into AdoMet was only precocious, since this incorporation sharply decreased to reach a plateau 24 h after the addition of Met.

Biosynthesis of AdoMet from MeSAdo. The fact that the incorporation of MeSAdo into AdoMet was de-

Fig. 3. Metabolism of [$Me-^{14}C$]MeSAdo during CCL39 cell proliferation

CCL39 cells were plated at 1×10^5 cells/cm² for 24 h in a Met-free medium, then 100 μ M-Met was added (Fig. 3a, arrow), or no addition was made (Fig. 3b). The incorporation of [$Me-^{14}C$]MeSAdo (15 μ M; 0.5 μ Ci/well) into AdoMet (Δ), protein Met ($\blacksquare$), total Met (AdoMet + intracellular Met + extracellular Met + protein Met; $\square$) was performed for 3 h, at the indicated time after Met addition; results are in pmol/3 h per mg. $\blacktriangle$, Total proteins (μ g). Points are the means of duplicate cultures.

Table 4. Effect of exogenous Met and endogenous Met (MeSAdo-derived Met) on the repression of methionine adenosyltransferase activity (MAT) in CCL39 cells

CCL39 cells were plated at 0.4×10^5 cells/cm² and cultured for 20 h in the indicated growth conditions. Specific methionine adenosyltransferase activity was assayed as described in the Materials and methods section. Results are means $\pm$ S.D. for triplicate determinations.

Growth conditions	MAT activity (nmol/h per mg of protein)	Growth conditions	MAT activity (nmol/h per mg of protein)
Met (10 μ M)	26 $\pm$ 2	MeSAdo (15 μ M)	22 $\pm$ 2
Met (25 μ M)	15 $\pm$ 3	Met (10 μ M) + MeSAdo (15 μ M)	17 $\pm$ 1.5
Met (50 μ M)	10 $\pm$ 1	Met (10 μ M) + MeSAdo (40 μ M)	18 $\pm$ 1
Met (75 μ M)	10 $\pm$ 2	Met (10 μ M) + MeSAdo (65 μ M)	23 $\pm$ 1
Met (100 μ M)	5.5 $\pm$ 0.5	Met (10 μ M) + MeSAdo (90 μ M)	18 $\pm$ 2

creased in resting CCL39 cells, and stimulated by exogenous Met (Fig. 3a), led us to focus on the metabolism of AdoMet in CCL39 cells. Coalson *et al.* (1982) have shown that Met-dependent cells were deficient in utilizing endogenous Met (synthesized from the other precursor of Met, i.e. Hcy), for AdoMet synthesis. They proposed that this could be due to a difference in the enzymic activity catalysing the synthesis of AdoMet, methionine adenosyltransferase. However, we found the same enzyme activity in Raji and CCL39 cells cultured in 100 μ M-Met (5.2 ± 0.5 and 5.5 ± 0.5 nmol/h per mg of protein respectively), in accordance with the results of Oden *et al.* (1983), who found no difference in methionine adenosyltransferase activity between Met-dependent and -independent cells.

Previous studies by Caboche (1977) have shown that exogenous Met exerts a potent repression on the synthesis of methionine adenosyltransferase, presumably via an

increase in the concentration of AdoMet itself. To characterize such a repression with Met and with its precursor, MeSAdo, we compared methionine adenosyltransferase activity in CCL39 cells cultured for 20 h in media with various (Met+MeSAdo) concentrations. Our results show that Met repressed, and MeSAdo did not repress, the methionine adenosyltransferase activity, which also suggests that the production of AdoMet from MeSAdo is decreased in CCL39 cells cultured in Met-free medium (Table 4). We thus compared intracellular AdoMet concentrations in Raji and CCL39 cells as a function of the concentrations of extracellular Met and MeSAdo added to the culture medium for 72 h of culture (Table 2). In Raji cells the content of AdoMet was the same when the cells were cultured in 15 μ M-MeSAdo or 10 μ M-Met, whereas in CCL39 cells the content of AdoMet of cells cultured in 10 μ M-Met was twice that of cells cultured in 15 μ M-MeSAdo, indicating that in the absence of exogenous Met these cells less efficiently metabolize MeSAdo to Met than do Raji cells.

Our previous results on MeSAdo metabolism on CCL39 cells cultured in optimal conditions have shown that the addition of exogenous Met markedly inhibited the incorporation of MeSAdo into proteins, but only moderately that into AdoMet (Christa *et al.*, 1986). When CCL39 cells were cultured in the absence of Met for 48 h, exogenous Met similarly inhibited the incorporation of MeSAdo into proteins (Fig. 4). As reported above (Fig. 3a), exogenous Met increased the incorporation of MeSAdo into AdoMet; only at high Met concentration (200 μ M) was an effect of isotopic dilution observed. We also tested the ability of 100 μ M-MeSAdo to inhibit the incorporation of 15 μ M-[Me-¹⁴C]Met into proteins and AdoMet. Table 5 shows that 100 μ M-MeSAdo inhibited the incorporation of exogenous Met into proteins by only 25%, and did not decrease the incorporation into AdoMet, in CCL39 cells cultured in either 0 or 100 μ M-Met for 48 h before incorporation studies. The increase in incorporation into decarboxylated AdoMet in the presence of 100 μ M-MeSAdo is a finding similar to that reported by Kubota *et al.* (1985).

DISCUSSION

Our previous studies revealed that CCL39 cells could not grow on MeSAdo in a Met-free medium, although they were able to incorporate radiolabelled MeSAdo into Met, AdoMet and proteins (Christa *et al.*, 1986). Because

Fig. 4. Effect of exogenous Met on the incorporation of [Me-¹⁴C]MeSAdo in resting CCL39 cells

CCL39 cells were plated at 0.2×10^5 cells/cm², and cultured for 48 h in a Met-free medium, then washed in a Met-free medium. The incorporations of [Me-¹⁴C]MeSAdo (15 μ M; 0.5 μ Ci/well) into AdoMet (●), intracellular Met (○), total Met (▲) and protein Met (△) were performed for 3 h in the presence of the indicated concentrations of Met. Points are the means of duplicate cultures. Similar results were obtained in two independent experiments.

Table 5. Effect of MeSAdo on the incorporations of [*Me*-¹⁴C]Met in resting and subconfluent CCL39 cells

CCL39 were plated at 1×10^5 cells/cm², and cultured for 48 h either in Met-free medium or in the same medium supplemented with 100 μ M-Met. Resting and subconfluent cells were washed in a Met-free medium, then incubated for 3 h with [*Me*-¹⁴C]Met (15 μ M or 100 μ M; 0.5 μ Ci/well), in the absence or in the presence of 100 μ M-MeSAdo. Results are means $\pm$ s.d. for triplicate cultures.

[<i>Me</i> - ¹⁴ C]Met	Metabolite	MeSAdo...	Met incorporated (pmol/3 h per mg of protein)			
			Resting cells		Subconfluent cells	
			0	100 μ M	0	100 μ M
15 μ M	AdoMet		235 $\pm$ 50	454 $\pm$ 32	91 $\pm$ 12	126 $\pm$ 9
	Decarboxylated AdoMet		31, 35	162, 180	12, 14	27, 28
	Protein Met		12691 $\pm$ 254	7766 $\pm$ 121	6501 $\pm$ 842	4221 $\pm$ 375
100 μ M	AdoMet		2357 $\pm$ 6	2180 $\pm$ 148	478 $\pm$ 49	448, 433
	Decarboxylated AdoMet		135, 184	361, 340	37, 38	76, 70
	Protein Met		20424 $\pm$ 1137	17333 $\pm$ 964	17570 $\pm$ 796	13044 $\pm$ 296

of the known toxicity of high concentrations of MeSAdo on various cell lines cultured in optimal Met concentrations, we surmised that this paradox may be explained by a toxicity of low MeSAdo concentrations for cells cultured in low or zero Met concentrations. However, in these conditions we could not demonstrate any toxic effect of MeSAdo on the synthesis of macromolecules, nor any toxicity mediated by polyamine or pyrimidine starvation. In fact, the growth was strictly dependent on the supply of exogenous Met, and it was not affected by the addition of 15 μ M-MeSAdo at any Met concentration.

Since our previous experiments on incorporation of radiolabelled MeSAdo had been performed in subconfluent CCL39 cells which had grown in optimal concentrations of Met (Christa *et al.*, 1986), we questioned whether these results could be validly extrapolated to cells cultured in a Met-free medium (that is, in culture conditions that do not allow the growth of cells). Indeed, our results show that the synthesis of Met from MeSAdo in subconfluent cells (cultured in 100 μ M-Met) was twice that of resting CCL39 cells (cultured in Met-free medium). Thus the total synthesis of Met from MeSAdo is dependent on cell proliferation, which is dependent on the supply of exogenous Met.

We further investigated the utilization of the Met synthesized from MeSAdo for protein synthesis, on the one hand, and for AdoMet synthesis, on the other. Whereas 100 μ M-MeSAdo inhibited by only 25% the incorporation of radiolabelled Met into proteins, exogenous Met markedly inhibited the incorporation of MeSAdo into proteins (100 μ M-Met inhibited this incorporation by 90%); moreover, in these conditions the incorporation of MeSAdo into proteins was not correlated with the total protein synthesis, and the MeSAdo-derived Met mostly flowed out of the cell. Our findings concerning the metabolism of MeSAdo into AdoMet are quite different. The addition of Met to resting Met-deprived cells immediately led to a transient and marked increase in the incorporation of MeSAdo into AdoMet, presumably reflecting the rapid replenishment of the AdoMet pool of these cells. Taken together, these results suggest that the MeSAdo-derived Met is poorly used for protein synthesis, and, on the contrary,

is readily used for AdoMet synthesis. Like the total synthesis of Met from MeSAdo, this synthesis of AdoMet from MeSAdo depends on the ability of the cells to grow, and hence on the supply of exogenous Met. If one considers that AdoMet is a metabolic precursor, and MeSAdo a potentially toxic by-product, of polyamine synthesis, it may be proposed that the pathway AdoMet $\rightarrow$ MeSAdo $\rightarrow$ Met $\rightarrow$ AdoMet could be a metabolic cycle allowing both the detoxication and the salvage of MeSAdo. The apparent paradox raised, in some cell lines, by a metabolic pathway of Met synthesis unable to support the growth of cells in a Met-free medium would be explained by the fact that the activity of this salvage pathway depends on cell proliferation, like the synthesis of polyamine itself (reviewed by Pegg, 1986).

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