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SALVAGE OF 5'-DEOXY-5'-METHYLTHIOADENOSINE AND L-HOMOCYSTEINE INTO METHIONINE IN CELLS CULTURED IN A METHIONINE-FREE MEDIUM : A STUDY OF "METHIONINE-DEPENDENCE"

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The metabolism of the two methionine precursors, L-homocysteine and 5'-deoxy-5'-methylthioadenosine was compared to the ability of these compounds to support cell growth in a Met-free medium, in the following mammalian cell lines : Raji, CCL 39 and BHK cells. These three cell lines metabolized L-homocysteine and 5'-deoxy-5'-methylthioadenosine into methionine, S-adenosyl-L-methionine and proteins. However there was a discrepancy between metabolic and growth studies : Raji cells could grow on L-homocysteine and on 5'-deoxy-5'-methylthioadenosine, BHK cells could grow on L-homocysteine but not on 5'-deoxy-5'-methylthioadenosine, and CCL 39 cells could not grow either on L-homocysteine or on 5'-deoxy-5'-methylthioadenosine. The metabolism of exogenous methionine, andof methionine endogenously synthesized from 5'-deoxy-5'-methylthioadenosine was studied in CCL 39 and Raji cells, incubated with 25 µM [methyl-^{14}c] methionine + 25 µM 5'-deoxy-5'-methylthioadenosine or 25 µM [methyl-^{14}c] 5'-deoxy-5'-methylthioadenosine + 25 µM methionine : there was no difference between the metabolism of exogenous and endogenous methionine in either type of cell. Our results indicate that i) "methionine dependence" initially described for L-homocysteine [Hoffman, R.M. and Erbe, R.W. (1976) Proc. Natl. Acad. Sci. USA 73, 1523] , can also be observed with the other precursor of methionine, ie 5'-deoxy-5'-methylthioadenosine ; ii) "methionine-dependence" can not be considered as the inability of a cell to grow on methionine endogenously synthesized from a precursor, but depends on the precursor used, and possibly, on a toxic effect of the precursor in the absence of methionine.

Two metabolic pathways are known to result in the formation of methionine (Met) in mammalian cells : one is the methylation of L-homocysteine (Hcy) by methyltetrahydrofolic acid in the presence of vitamin B_{12}, or by betaine ; the other one is the salvage of the ribose moiety of the purine nucleoside 5'-deoxy-5'-methylthioadenosine (MTA). Hoffman and

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ABBREVIATIONS: AdoMet, S-adenosyl-L-methionine; MTA, 5'-deoxy-5'-methylthioadenosine; Met, L-methionine; Hcy, L-homocysteine.

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Erbe (1) have reported that some cell lines ("methionine-dependent") were unable to grow when Met was replaced by Hcy in spite of the fact that they were perfectly able to synthesize Met from Hcy ; we thus hypothesized that a similar phenomenon may be observed with the other precursor of Met, i.e. MTA. In the present work, we studied the ability of three mammalian cell lines to grow on MTA or Hcy and to synthesize Met from these two precursors.

MATERIAL AND METHODS

Chemicals. [Methyl-^{14}C] S-adenosyl-L-methionine(60 mCi/mmol), [Methyl-^{14}C] methionine (60 mCi/mmol) and 5- [methyl-^{14}C] tetrahydrofolic acid (58 mCi/mmol) were purchased from the Radiochemical Center (Amersham, U.K.) ; [6-^{3}H] thymidine (1 Ci/mmol) was from CEA (Saclay, France). [Methyl-^{14}C] MTA was prepared from [methyl-^{14}C] S-adenosyl-L-methionine by acid hydrolysis (2) . MTA, Met, 2-ketomethylthiobutyrate, S-adenosyl-L-methionine (AdoMet), cyanocobalamin, folic acid, L-homocysteine thiolactone, were from Sigma (St Louis, MO). Pork trypsine was from Choay (Paris, France). The baby hamster kidney cell line, BHK-21 (C13) (BHK), the Raji cell line (a human Burkitt's lymphoma derived cell line), and the fetal calf serum (FCS) were purchased from Flow Laboratories (Irvine, Scotland). CCL 39, a line of female chinese hamster lung fibroblast was a gift of Dr. M. Buttin (Institut Pasteur, Paris, France). Met-free media were reconstructed RPMI 1640 (Gibco, Long Island NY, USA) and Dulbecco's modified Eagle's media (Eurobio, Paris, France).

Culture. Raji cells were grown on RPMI 1640 medium supplemented with 10 % fetal calf serum, and the proliferation was evaluated by [^{3}H] thymidine incorporation (3) . CCL 39 and BHK cell lines were maintained in Dulbecco's supplemented with 10 % FCS ; cells were counted in a Coulter counter. For salvage experiments, Met-free media were used and FCS was dialysed for 6 hours at 4°C against 3 changes of 154 mM NaCl. For CCL 39 and BHK cells MTA salvage experiments were made in the presence of 50 µM uridine to palliate the toxic effect of adenine. For Hcy requirement experiments, Met-free media were supplemented with 100 µM folic acid and 8 µM cyanocobalamin. The effectors were added at culture initiation.

For the metabolic studies, CCL 39 and BHK cells were plated at 10^{6} cells/well in 6-well microtiter plates and grown for 48 h to obtain about 4.10^{6} cells/well. The medium was then removed and the cells washed with Met-free medium. Raji cells were washed in Met-free medium and plated at 2.10^{6} cells/ml just before experiments. Incorporation of 25 µM radiolabeled Met or MTA was conducted during 6 h at 37°C in 1 ml of Met-free medium. The incorporation of 5- [methyl-^{14}C] tetrahydrofolic acid (34 µM) was conducted for 24 h at 37°C in 1 ml of a Met-free medium supplemented with 100 µM Hcy thiolactone and 8 µM cyanocobalamin. After labeling, the cells were cooled on ice, washed with ice-cold Dulbecco's PBS and extracted in 0.4 N cold PCA. Nucleic acids were solubilized by heating the acid precipitate at 90°C for 15 min in 1 ml of 5 % TCA. The pellets were hydrolysed in 6 N HCl for 24 h at 110°C. Radioactivity of the acid-soluble fraction and of the hydrolysate was measured in a Packard liquid scintillation spectrometer. Aliquots of the acid extracts were neutralized with tri-N-octylamine/freon (4) , then evaporated and analysed by either of 2 chromatographic systems ; a) a paper chromatography on Whatman 3MM in n-butanol/acetic acid/water (5/1/4) separated AdoMet, Met, Met sulfoxide and MTA (the Rf values were respectively 0.06, 0.44, 0.18, 0.62) ; after visualization (UV light and Ninhydrin spray), the spots were cut out and counted ; b) a HPLC chromatography on a µ Bondapak C18 column, with isocratic elution

by 20 mM KH_2PO_4, pH 4.2, during 15 min, followed by linear gradient elution (0 % to 40 % methanol in 20 mM KH_2PO_4, pH 4.2, in 80 min) ; 1 ml fractions were collected and counted. The retention times of AdoMet, Met, Met sulfoxide, MTA, methyltetrahydrofolic acid were respectively 22, 12, 9, 103, 90 min. The counts of Met and Met sulfoxide were added and expressed as Met. The identification of AdoMet, Met, Met sulfoxide was verified by a third chromatographic system on a μ Bondapak C18 column with the following steps : (1) isocratic elution by 20 mM KH_2PO_4 + 5 mM heptanesulfonic acid, pH 2.5 during 10 min, (2) linear gradient elution (20 mM KH_2PO_4 20 mM + 5 mM heptanesulfonic acid, pH 2.5 to 100 % KH_2PO_4 20 mM pH 6,0) in 5 min, (3) isocratic elution by 20 mM KH_2PO_4, pH 6,0, during 25 min, (4) linear gradient elution (0 to 40 % methanol in 20 mM KH_2PO_4, pH 6,0) in 30 min. The retention times of AdoMet, Met, Met sulfoxide and MTA were respectively 66, 34, 15 and 79 min. Total cellular protein measurements were done as described by Lowry et al. (5) .

RESULTS

Growth of cell lines in Met-free media

To compare the quantitative importance of the various Met biosynthetic pathways, we measured the cellular proliferation in a Met-free medium supplemented with different Met precursors (Table I). The proliferation of CCL 39 cells reached a plateau at 50 μM Met (Fig. 1A). The Met keto analog, 2-ketomethylthiobutyrate, could support the growth of the cells, whereas MTA, and Hcy (even after preincubation in a complete medium supplemented with 8 μM cyanocobalamin for 4 days), could not. BHK cells could grow on Hcy and on 2-ketomethylthiobutyrate (but less efficiently than on Met), but not on MTA (Fig. 1B), and Raji cells could grow on MTA and on Hcy (Table I and (3)).

Table I: Effects of different Met precursors on the proliferation of CCL 39, Raji and BHK cells in a Met-free medium

Effectors		Cell lines		
		CCL 39 percent of control	Raji percent of control	BHK percent of control
Met	100 μM	100	100	100
KMTB*	100 μM	95	96	25
MTA	15 μM	5	95	5
MTA	100 μM	5	40	5
Hcy + Vitamin B_{12} + Folic acid	100 μM + 8 μM + 100 μM	4	100	35

* KMTB : 2-ketomethylthiobutyrate

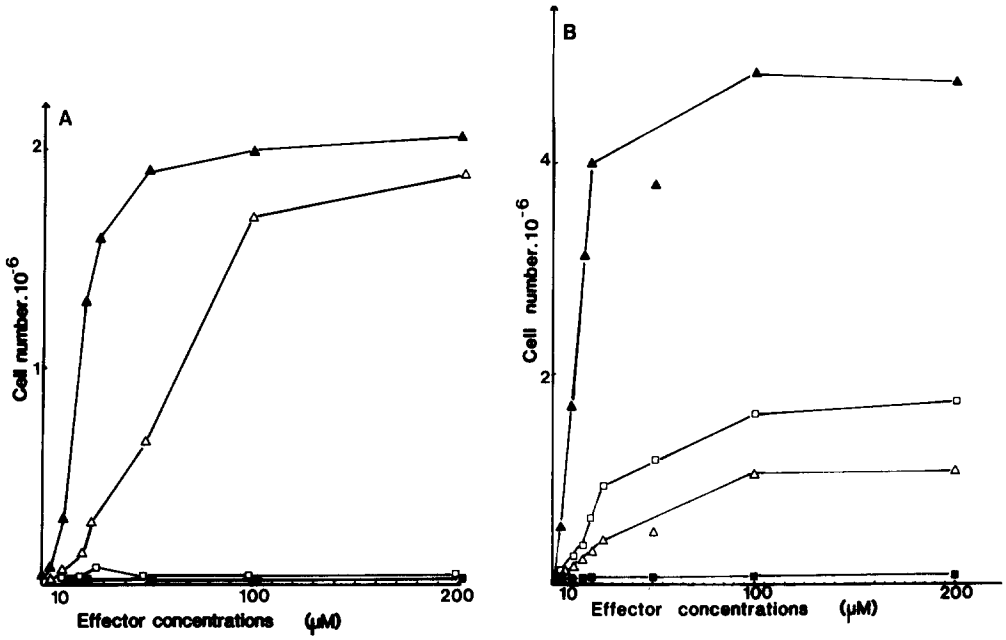

Fig. 1 Effect of Met (▲), 2-ketomethylthiobutyrate (△), MTA (■), Hcy (□), on CCL 39 (**A**) and BHK (**B**) fibroblasts proliferation. CCL 39 cells and BHK were plated respectively at 1.10^5 and 2.10^5 for 96 hours in a Met-free medium containing the indicating effectors. Cell number was determined as described in "Material and Methods"

Metabolism of 5- [methyl-^{14}C] tetrahydrofolic acid in CCL 39 and Raji cells

In order to determine why CCL 39 cannot proliferate when Hcy replaces Met, we compared the incorporation of radioactivity of 5- [methyl -^{14}C] tetrahydrofolic acid into Met, proteins and AdoMet in CCL 39 and Raji cells (Table II). The two types of cells metabolized Hcy and 5-

Table II: Metabolism of [5-methyl-^{14}C] tetrahydrofolic acid in CCL 39 and Raji cells

Metabolites	Cell lines	
	CCL 39	Raji
AdoMet in the acid soluble fraction	1,812 ± 153	715 ± 275
Met in the acid soluble fraction	3,766 ± 459	2,940 ± 307
Nucleic acids	14,849 ± 234	18,372 ± 375
Protein Met	101,737 ± 12,332	32,544 ± 3,520

Cells were incubated for 24 h at 37°C with [5-methyl-^{14}C] tetrahydrofolic acid (34 μM, 2 μCi/ml) in a Met-free medium supplemented with 8 μM vitamin B_{12} and 100 μM l-homocysteine thiolactone. Each point is the mean ± SD of triplicate determinations. Values are cpm.mg protein^{-1}.

Table III: Metabolism of [methyl-^{14}C] MTA in CCL 39, Raji and BHK cells

Metabolites	Cell lines		
	CCL 39	Raji	BHK
AdoMet in the acid soluble fraction	10,313 ± 2,970	29,231 ± 9,058	4,599 ± 2,247
Met in the acid soluble fraction	5,616 ± 2,761	6,613 ± 1,433	7,079 ± 4,493
Protein Met	377,407 ± 198,619	589,040 ± 137,405	185,548 ± 80,576

Cells were incubated for 6 h at 37°C with [methyl-^{14}C] MTA (25 µM, 0.62 µCi/ml) in a Met-free medium. Each point is the mean ± SD of five (CCL 39), four (Raji) and three (BHK) cell cultures. Values are cpm.mg protein^{-1}.

[methyl-^{14}C] tetrahydrofolic acid into Met and AdoMet in the acid-soluble fraction and incorporated endogenous Met into proteins.

Metabolism of [methyl-^{14}C] MTA in CCL 39, BHK and Raji cells

We similarly investigated the ability of the three cell lines to metabolize MTA. Table III shows that CCL 39, BHK and Raji cells metabolized MTA into Met and AdoMet in the acid-soluble fraction and incorporated Met synthesized from MTA into proteins.

Metabolism of exogenous and endogenous Met in CCL 39 and Raji cells

In order to determine why CCL 39 cannot proliferate when MTA replaces Met, we compared the metabolism of exogenous, preformed Met and of endogenous Met (newly synthesized from MTA) in these two cell lines

Table IV: Comparative study of the metabolism of preformed and endogenously synthesized Met in CCL 39 and Raji cells

Metabolites	Labeled precursor	Cell lines	
		CCL 39	Raji
AdoMet in the acid Soluble fraction	[methyl-^{14}C] MTA	0.246 ± 0.018	0.372 ± 0.126
	[methyl-^{14}C] Met	0.228 ± 0.102	0.516 ± 0.162
Protein Met	[methyl-^{14}C] MTA	6.852 ± 4.674	6.966 ± 1.386
	[methyl-^{14}C] Met	8.676 ± 2.022	9.492 ± 4.086

Cells were incubated for 6 h at 37°C in 25 µM MTA plus 25 µM Met with 0.62 µCi/ml labeled MTA or 0.62 µCi/ml labeled Met. Each point is the mean ± SD of five (CCL 39) or four (Raji) cell cultures incubated with [methyl-^{14}C] MTA, and two CCL 39 and Raji cell cultures incubated with [methyl-^{14}C] Met. Results are expressed as nmol.mg protein^{-1}.

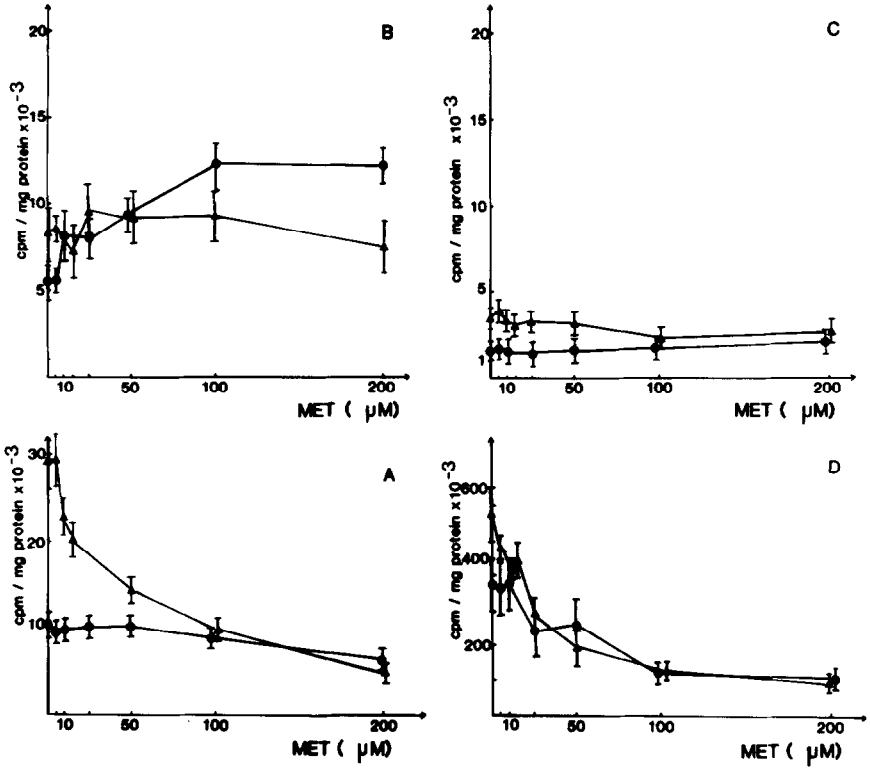

Fig. 2 Effect of Met on the metabolism of [methyl-^{14}C] MTA (25 μM) in CCL 39 (●) and in Raji cells (▲). The cells were incubated with labeled MTA (0.62 μCi/ml) for 6 hours at 37°C. Values are cpm/mg protein and represent means ± SD of five (CCL 39) and four (Raji) separate culture experiments.

A : AdoMet in the acid soluble fraction
B : Met in the acid soluble fraction
C : MTA in the acid soluble fraction
D : Protein Met

(Table IV) (Fig. 2). The cells were incubated with 25 μM [methyl-^{14}C] Met plus 25 μM MTA, or with 25 μM [methyl-^{14}C] MTA plus 25 μM Met. The 25 μM Met concentration chosen allows 80 and 100 percent of the growth in optimal Met concentrations for CCL 39 and Raji cells respectively. Table IV shows that the incorporation of [methyl-^{14}C] Met into proteins and AdoMet is the same in the two types of cells with the two labeled precursors. We also tested the effect of increasing concentrations of Met (1 to 200 μM) on the incorporation of [methyl-^{14}C] MTA. In both types of cells the [methyl-^{14}C] Met pools were not reduced by the addition of 200 μM Met (Fig. 2). Met newly synthesized from MTA was readily incorporated into proteins ; the addition of exogenous Met similarly inhibited this incorporation in the two types of cells. The ratio of the radioactivity incorpo-

rated into intracellular AdoMet in a Met-free medium to that incorporated in a medium supplemented with 200 µM Met were 6.20 and 1.58 for Raji and CCL 39 respectively. Exogenous Met thus exhibits an important ability to inhibit the synthesis of AdoMet from MTA in Raji cells, whereas it moderately affects it in CCL 39 cells.

DISCUSSION

Growth experiments revealed major differences between CCL 39, BHK and Raji cells : Raji cells, which are malignant human B lymphoid cells, can grow on MTA, as previously described (3), and also on Hcy, contrary to the findings of Kano et al. (6), who probably used unsufficient folic acid and vitamin B_{12} concentrations. BHK which are non malignant hamster fibroblasts can grow on Hcy, although less efficiently than on Met as described by Caboche (7), and do not grow on MTA. CCL 39, which are non-malignant hamster fibroblasts, do not grow either on MTA or on Hcy. Our findings that CCL 39 cells can, however, synthesize Met and AdoMet from Hcy, and incorporate the endogenously synthesized Met into proteins, are in accordance with the results obtained by Stern et al. in "Met-dependent"cells (8). The fact that Met-dependence is not, as initially suggested by Hoffman and Erbe (1), limited to malignant cells, has been also reported by Carson et al. (9). Our study of MTA metabolism in CCL 39 cells and BHK cells, showing that the inability of those cells to grow on MTA does not reflect their inability to synthesize Met and AdoMet from MTA and to incorporate Met, newly synthesized, into proteins, is the first demonstration that "Met-dependence" can be observed also with the other metabolic precursor of Met, MTA. However, the fact that BHK cells can be considered both as Met-dependent (referring to MTA) and Met-independent (referring to Hcy) indicates than the concept of Met dependence is questionable. The inability to grow on MTA was further studied in CCL 39 cells : in conditions of suboptimal cellular growth (25 µM MTA + 25 µM Met), there was not quantitative difference between the metabolism of MTA and that of exogenous Met, indicating that the metabolism of MTA to Met and its derivates is not a limiting factor in suboptimal growth conditions. On another hand, if we compare the incorporation of MTA into cellular AdoMet in CCL 39, BHK and Raji cells, it appears that in a Met-free medium (Table III and Fig. 2), the AdoMet pool of Raji cells is more than twice that of CCL 39 and BHK cells. Taken together, these results indicate that the inability of CCL 39 and BHK cells to grow on MTA alone is not due to defective synthesis of endogenous Met, but rather suggest a toxic effect of MTA in cells with low AdoMet synthesis (10).

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