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Effect of S-Adenosylhomocysteine and S-Tubercidinylhomocysteine on Transfer Ribonucleic Acid Methylation in Phytohemagglutinin-Stimulated Lymphocytes

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SUMMARY

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Methylation of tRNA in stimulated rat lymphocytes was studied by monitoring the incorporation of [*methyl*-^{3}H]methionine into tRNA. *S*-Adenosylhomocysteine and the 7-deaza analogue, *S*-tubercidinylhomocysteine, both known to inhibit tRNA methylases *in vitro*, were used to block the methylation of tRNA in stimulated lymphocytes. Both these drugs affected the synthesis and methylation of tRNA when added at various times during the cell cycle. However, *S*-adenosylhomocysteine stimulated DNA synthesis while *S*-tubercidinylhomocysteine inhibited it. These opposite effects are presumably due to metabolism of the drugs to homocysteine and either adenosine or tubercidin.

INTRODUCTION

Several studies have demonstrated differences in the activity of tRNA methylases between normal and malignant tissues (1). Measurement of the activity of these enzymes was used to monitor the onset of malignancies in liver even before any histological evidence of cancer was produced (2). In addition, recent data support the suggestion that increased tRNA methylase activity is associated with malignant but not benign tumors (3). Although many suggestions have been made as to the significance of methylated tRNA in cellular events (4), most workers in the field to date have studied the effect of various agents and/or events on the *extent* of tRNA methylation *in vitro* and *in vivo*. Relatively few investigations have attempted to ascertain a role for the methyl groups of methylated tRNA in the various stages of protein synthesis, and the cases when it has been done have all involved methionine-deficient mutants of *Escherichia coli* (5) and yeast (6). Such studies have not been carried out in mammalian cells, since it has not been possible to obtain hypomethylated tRNA from mammalian cells.

Specific inhibitors of tRNA methylation should be useful as pharmacological tools in the synthesis of hypomethylated mammalian tRNA and, if sufficiently specific, might be useful as potential chemotherapeutic agents in neoplastic diseases. Recent papers from this laboratory and others (7) have described the synthesis and activity of analogues of *S*-adenosylhomocysteine as inhibitors of several methylases *in vitro*. We have now evaluated the 7-deaza analogue of SAH[1] (*S*-tubercidinyl-

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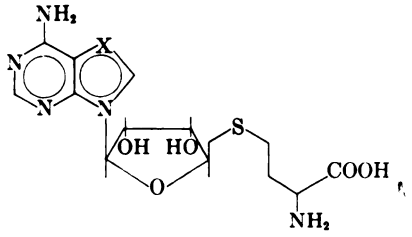
[1] The abbreviations used are: SAH, *S*-adenosylhomocysteine; STH, *S*-tubercidinylhomocysteine; PHA, phytohemagglutinin; [^{3}H]TdR, [5-methyl-^{3}H]-thymidine.

homocysteine) (8) and SAH as inhibitors of tRNA methylation in phytohemagglutinin-stimulated rat lymphocytes. The data presented in this paper support the idea that stable analogues of SAH can be used to inhibit biological methylation reactions known to occur in specific cells in culture (9, 10).



SAH, X = N
STH, X = C

MATERIALS AND METHODS

Male Lew/Mei rats (200–250 g) were obtained from Microbiological Associates, Bethesda, Md., and used as lymphocyte donors. Bacto-phytohemagglutinin P was obtained from Difco Laboratories. Fetal calf serum and Eagle's minimal essential medium (Spinner modification) for suspension cultures were purchased from Grand Island Biological Company. [*methyl*-^{3}H]Thymidine (6 Ci/mmole), L-[*methyl*-^{3}H]methionine (11 Ci/mmole), and [2-^{14}C]uridine (57 mCi/mmole) were purchased from New England Nuclear Corporation. *S*-Adenosyl-L-homocysteine was purchased from Boehringer/Mannheim, and *S*-tubercidinyl-DL-homocysteine was synthesized in our laboratory (8).

Preparation of cell suspensions. Rat lymphocytes were isolated by the method of Wilson (11). Pooled cell suspensions from cardiac blood and lymph tissues of a single animal were used in each culture experiment. The cell density was determined with a Coulter counter, and the cell viability was checked from time to time with trypan blue (12). The final cell preparation was adjusted to give a concentration of 2×10^6 cells/ml in culture medium consisting of 90% Eagle's minimal essential medium (Spinner modification), 10% fetal calf serum (inactivated for 30 min at 56° before use), 4 mM L-glutamine, 0.2% sodium bicarbonate, penicillin (100 units/ml), and streptomycin (100 μg/ml). Suspension cultures were incubated in loose-cap tubes in a 95% air–5% CO_2 humid atmosphere at 37°. All cultures were run in duplicate or triplicate, and cultures containing no PHA were routinely used as controls.

Estimation of DNA synthesis. Determination of the optimal PHA concentration required for transformation and DNA synthesis was carried out in 1-ml portions of the cell culture (2×10^6 cells). Incorporation of [^{3}H]thymidine was employed to monitor DNA synthesis as described previously (11), with slight modifications as follows. At 48 hr after initiation of the culture, 0.5 μCi of [^{3}H]TdR in phosphate-buffered saline (Grand Island Biological Company) was added. The tubes were returned to the incubator for 16 hr after the TdR addition. The cells were then centrifuged at $600 \times g$ for 10 min and washed with chilled isotonic NaCl. The cell pellets were suspended in 1 ml of 5% trichloracetic acid and disrupted by freezing and thawing three times. The precipitate was collected on a glass-fiber filter (Whatman GF/C), washed (5% trichloracetic acid, alcohol, and ether), dried, and counted with 10 ml of toluene-Liquifluor (New England Nuclear) in a Packard scintillation spectrometer. The optimal concentration of the rehydrated PHA-P for maximal TdR incorporation in stimulated rat lymphocytes was obtained with 3–4 μl/ml of culture fluid. The same optimal range of PHA concentration has been reported previously in mouse lymphocyte transformation (13). Therefore 3.3 μl of PHA per milliliter of cell suspension were used for all subsequent PHA-stimulated cultures.

Estimation of tRNA synthesis and methylation. At selected times after PHA stimulation, 5-ml portions of the cell suspension (10^7 cells) were labeled for 3 hr, under the standard incubation conditions described above, with 1.5 μCi of [^{14}C]uridine and 100 μCi of [*methyl*-^{3}H]methionine containing 0.01 M sodium formate to suppress ^{3}H incorporation into purines via synthesis *de novo* (14). When the inhibitory effects of SAH and STH were studied, the radioactive precursors were added simultaneously with the inhibitors. At the end of the 3-hr pulse,

cells were centrifuged at 600 × *g* for 10 min, washed twice with 10 ml of isotonic NaCl, and stored at −20° until isolation of the tRNA was carried out. Crude tRNA was isolated based on the methods of Atherton and Darby (15) and Parish (16) with some modifications. The cells were resuspended in pH 7.5 isotonic buffer (0.01 M Tris, 0.14 M NaCl, 0.5% sodium naphthalenedisulfonate, and 0.5 mM $MgCl_2$), and Triton X-100 (17) was added to a final concentration of 0.5%. The solution was extracted with an equal volume of phenol mixture (100 g of phenol, 11 ml of water, 14 ml of *m*-cresol, and 20 mg of 8-hydroxyquinoline). A mixture of NaCl and triisopropylnaphthalenesulfonate (6:4) was added to the aqueous phase to a final concentration of 1.2 M NaCl (18). The aqueous phase was deproteinized again with phenol mixture. *E. coli* tRNA (200 μg) was added as carrier, after which the crude tRNA was digested with DNase and Pronase, deproteinized with phenol, precipitated with ethanol, and stripped of endogenous amino acids as described by Sharma and Loeb (10).

In a preliminary study of [*methyl*-^{3}H] methionine incorporation, the tRNA isolated from duplicate 18-hr PHA-stimulated cultures was analyzed with 7.5% polyacrylamide gels by the method of Peacock and Dingman (19). The gels were scanned at 260 nm on a Gilford linear scanner and then were sliced into 2-mm fractions. Each slice was solubilized in 1 ml of Protosol (New England Nuclear) and counted in 10 ml of toluene-Omnifluor (New England Nuclear) scintillation fluid. The tRNA isolated by the above procedure was shown to migrate together with carrier *E. coli* tRNA almost exclusively in the 4 S region. Therefore tRNA synthesis and methylation were estimated by collecting the tRNA, isolated as described above, on a glass-fiber filter and counting the total macromolecular radioactivity trapped in the filter. The tRNA thus isolated has been degraded enzymatically to the component nucleosides and analyzed by high-pressure liquid chromatography. These experiments indicate that the methyl label is incorporated into appropriate base and ribose moieties of tRNA; their details will be published in a separate communication.

RESULTS AND DISCUSSION

In view of the possible variation of tRNA methylation during the cell cycle, the time course of tRNA synthesis and methylation in PHA-stimulated rat lymphocytes was studied. A preliminary study of the incorporation of the [^{3}H]methyl group from methionine into tRNA showed an immediate increase of methylation after the stimulation of the cultures with PHA. Figure 1 shows the measurement of tRNA synthesis and methylation rose gradually over the first 20 hr and then increased several fold after 24 hr of stimulation, to an elevated level which was maintained up to 54 hr after stimulation. Riddick and Gallo (20) have shown that the induction of tRNA methylases reaches a maximum level 38–42 hr after PHA stimulation of normal human lymphocytes. Their conclusion was based on the determination of tRNA methylase activity of extracts taken from the cell cultures at various times after PHA stimulation. The 5–7-fold in-

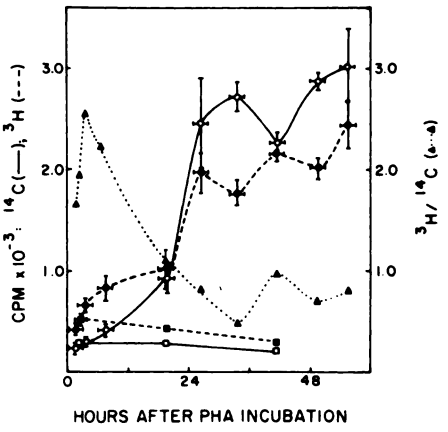


FIG. 1. *Time course of tRNA methylation in PHA-stimulated* (○, ●), *and control* (□, ■) *rat lymphocytes.*

Synthesis and methylation were measured by incorporation of [^{14}C]uridine and [*methyl*-^{3}H]methionine, followed by isolation of tRNA as described in the text. ——, tRNA synthesis (^{14}C incorporation); ---, tRNA methylation (^{3}H incorporation). Data points are in units of counts per minute per culture, and are average values obtained from triplicate determinations.

crease in methylation found in the present work 24 hr after PHA stimulation is comparable to the 5–6-fold stimulation of tRNA methylase activity in the S phase found by Raddick and Gallo (20). These workers have also shown that the tRNA methylases were not increased in activity until near late S phase and that the new tRNA methylases arising at the G_2 phase were qualitatively different from the early constitutive methylases. However, our observation of an increase of [^{3}H]methyl incorporation into tRNA may be simply a result of a simultaneous increase in tRNA synthesis. The fact that *both* methylation and synthesis are elevated from 24 to 60 hr after stimulation indicates that there are no large changes in the distribution of methyl groups in tRNA synthesized during the S phase. Interestingly, the ratio of tRNA methylation to synthesis (^{3}H:^{14}C) can be seen to increase sharply withing 3–6 hr after stimulation and then to decrease markedly after 24 hr. Together with the observation of Kay and Cooper (14) that an increase in mature tRNA occurs within 90 min of PHA addition, our data support the concept of pre-existing or constitutive tRNA methylases which methylate the early pre-tRNA. Maturation of pre-tRNA through methylation and size reduction has been demonstrated in malignant mammalian cells (21, 22). Tidwell and Stubblefield (23) have suggested that there is tight coupling between the changing methyl content of the tRNA "pool" and the rate of protein synthesis in mammalian systems. The "pool size" of methylated tRNA could exert a rate-limiting influence on protein synthesis and therefore on cellular reproduction. Our observation of this early increase of methyl content in the tRNA pool during initiation of lymphocyte-blast transformation by PHA is consistent with these proposals.

Because of the different tRNA methylase activities in early and late phases of the cell cycle, the inhibitors SAH and STH were added at peak tRNA methylation, 40 hr after PHA incubation. Attempts to study the effects of SAH and STH on the early "burst" methylation (3 hr) were unsuccessful because of the low incorporation of radioactivity into tRNA at this point of the cell cycle. Preliminary dose experiments, using final drug concentrations of 20 and 50 μM, indicated that the latter concentration led to a more significant and reproducible inhibition of tRNA methylation. At this concentration (50 μM) SAH produced 28%, and STH, 49%, inhibition of PHA-induced methyl incorporation into tRNA (Table 1). However, the observed effects might be partially due to the inhibition of tRNA synthesis, since both drugs inhibited [^{14}C]uridine incorporation to an extent of 25%. In our previous study *in vitro* (8) SAH and STH were shown to inhibit rat liver tRNA methylases with K_i values of 25 and 20 μM, respectively.

The same preparation of cells (1-ml portions) was used to measure DNA synthesis. The data in Table 2 show that SAH and STH had opposite effects on DNA synthesis at a concentration of 50 μM. SAH produced an increase of 20–40% in PHA-induced DNA synthesis, whereas STH inhibited TdR incorporation into DNA by 20–70%. The opposite effects of STH and SAH on DNA synthesis cannot be correlated with their inhibitory effects on tRNA methylation. DNA synthesis in PHA stimulated lymphocytes begins as early as 24 hr after stimulation, and enzymes associated with DNA synthesis, such as DNA polymerase, are increased in activity during the S phase (24). Any effect of SAH and STH on the synthesis of these enzymes via inhibition of tRNA methylation might be more pronounced when the drug is given early in the cell cycle. Although the inhibitory effect of STH (Table 2) is in accord with this hypothesis, the opposite effect of SAH cannot be so reconciled. The stimulatory effect of SAH is unexpected and of considerable interest, since SAH is a naturally occurring nucleoside which is produced in numerous transmethylation reactions and is present in tissues at a level of approximately 0.01–0.1 μmole/g (25). The stimulation by SAH decreases slightly as the time of drug incubation is increased, whereas STH inhibition of DNA synthesis increased 3-fold (22% vs. 67%) as the time of drug incubation is increased. These data suggest that some sort of metabolic break-

TABLE 1

Inhibition of tRNA synthesis and methylation in PHA-stimulated rat lymphocytes

Inhibitor was added 40 hr after stimulation by PHA. A 3-hr pulse of [^{14}C]uridine and [^{3}H] methionine was initiated at the time of addition of the inhibitor (final concentration, 50 μM), and the tRNA was then isolated and analyzed as described in the text. ^{14}C was counted at 12% gain, and ^{3}H, at 100% gain. All counts are averages of triplicate assays. Appropriate controls without PHA were run, and the counts indicated were corrected for the radioactivity incorporated in each control run (approximately 210 cpm for ^{14}C, 300 cpm for ^{3}H).

Label and inhibitor	Incorporation	Inhibition
	cpm/culture	%
^{14}C (tRNA synthesis)		
No inhibitor	2070 ± 90	
SAH	1635 ± 300	24
STH	1590 ± 200	26
Adenosine	1635 ± 400	24
Homocysteine	1605 ± 210	25
^{3}H (tRNA methylation)		
No inhibitor	1860 ± 60	
SAH	1340 ± 130	28
STH	945 ± 120	49
Adenosine	1400 ± 400	25
Homocysteine	1540 ± 250	17

down of the drug causes the observed effects on DNA synthesis. It is known that tubercidin is very cytotoxic nucleoside because of its activation to the 5'-phosphate (26); it has been shown to inhibit the growth of KB cells with I_{50} = 30 nM (27).

S-Adenosylhomocysteinase (EC 3.3.1.1) catalyzes the hydrolysis of SAH to adenosine and L-homocysteine, although the equilibrium of this reversible reaction is very much in favor of synthesis of SAH (28). In experiments described in Tables 1 and 2, the addition of adenosine and homocysteine at a final concentration of 50 μM caused 17–25% inhibition of tRNA synthesis or methylation at 40 hr, and marked stimulation in the synthesis of DNA throughout the cell cycle. In contrast, addition of tubercidin (50 μM) caused a severe inhibition of both tRNA and DNA synthesis to below control (without PHA) values. The net effects of SAH and STH on tRNA synthesis, methylation, and DNA synthesis might all be interpreted in terms of inhibition or stimulation of these reactions by adenosine, tubercidin, and homocysteine arising from the degradation of the drugs. Adenosine is only a weak inhibitor of tRNA methylases *in vitro* (24) and therefore should not have inhibited tRNA methylation at the concentration of 50 μM used (Table 1). Since sodium formate (0.01 M) was used in our cultures to suppress incorporation of tritium into tRNA via purine synthesis *de novo* (14), adenosine (50 μM) should not have affected this means of tRNA labeling in the present work. This is in contrast to reported effects in the absence of sodium formate (22). The observed inhibition by adenosine and homocysteine of ^{3}H incorporation into tRNA appears to be the result of a quantitatively similar inhibition of tRNA synthesis (Table 1). Although the permeability of SAH analogues and the effect of STH on methionine pool size were not studied in the present work, the consistent inhibition (approximately 50%) of tRNA methylation by STH (50 μM) cannot be attributed to the metabolite, tu-

TABLE 2

Effects of SAH and STH on DNA synthesis in PHA-stimulated rat lymphocytes

Inhibitor was added at the times indicated after stimulation by PHA. A 16-hr pulse of [*methyl*-^{3}H]thymidine was initiated 48 hr after stimulation, and the DNA was analyzed as described in the text. All inhibitors were added at a final concentration of 50 μM. All counts are averages of duplicate assays. Appropriate controls without PHA were run, and the counts indicated were corrected for the radioactivity incorporated in each control run (approximately 4600 cpm).

Time	Inhibitor	Incorporation	Inhibition[a]
hr		*cpm/culture*	%
	None	47,000 ± 6,600	
0	SAH	56,850 ± 1,500	−21
	STH	15,550 ± 1,500	67
30	SAH	63,850 ± 2,000	−36
	STH	23,900 ± 1,200	49
40	SAH	64,250 ± 1,500	−37
	STH	36,700 ± 4,200	22

[a] Negative numbers indicate stimulation of DNA synthesis.

bercidin, since the latter is only a weak inhibitor of tRNA methylation *in vitro* (29). It should be noted that STH does not appear to be cytotoxic in short-term rat lymphocyte cultures, as determined by the loss of cell viability measured by the trypan blue exclusion method. More than 85% of the cells containing STH were viable by this analysis at the termination of TdR incorporation studies (64 hr), and few of the cells were blast-transformed. In contrast, nearly 50% of the cells were nonviable in the presence of 50 μM tubercidin, whereas mostly viable cells, most of which were transformed, were observed in 64-hr cultures containing SAH or its metabolites adenosine and homocysteine.

Early work on the metabolism of SAH in bacteria indicated that the primary metabolic pathway involved cleavage of the purine-ribose bond (30, 31). Only recently have similar data become available on the metabolism of SAH in higher organisms (32). Rather than cleavage of the purine-ribose bond, these data indicate that the major hydrolytic pathway in vertebrates involves cleavage of the 5′-thioether bond. Hydrolysis of the 5′-thioether is also suggested by studies of sulfur amino acid metabolism in patients with clearly defined genetic disorders (33). The present work tends to support these concepts of SAH metabolism in mammalian cells. Further studies to investigate the enzymatic degradation of STH and to obtain SAH analogues which have a stable 5′-linkage to homocysteine, in addition to the stable glycosyl-purine bond of STH, are in progress.

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REFERENCES

1. Borek, E. & Kerr, S. J. (1972) *Adv. Cancer Res.*, 15, 163–190.
2. Waalkes, T. P., Adamson, R. H., O'Gara, R. W. & Gallo, R. C. (1971) *Cancer Res.*, 31, 1069–1073.
3. Sheid, B., Lu, T. & Nelson, J. H., Jr. (1974) *Cancer Res.*, 34, 2416–2418.
4. Kerr, S. J. & Borek, E. (1973) in *The Enzymes* (Boyer, P., ed.), Ed. 3, Vol. 9, Pt. B, pp. 167–195, Academic Press, New York.
5. Shugart, L. & Stulberg, M. P. (1976) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., and Borek, E., eds.), Columbia University Press, New York, in press.
6. Björk, G. R., Kjellin-Straby, K., & Neidhardt, F. C. (1976) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., and Borek, E., eds.), Columbia University Press, New York, in press.
7. Borchardt, R. T. (1976) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., and Borek, E., eds.), Columbia University Press, New York, in press.
8. Coward, J. K., Bussolotti, D. L. & Chang, C.-D. (1974) *J. Med. Chem.*, 17, 1286–1289.
9. Imashuku, S., Tnui, A., Nakamura, T., Tanaka, J. & Miyake, S. (1973) *J. Clin. Endocrinol. Metab.*, 36, 931–936.
10. Sharma, O. K. & Loeb, L. A. (1973) *Biochem. Biophys. Res. Commun.*, 50, 172–178.
11. Wilson, D. B. (1967) *J. Exp. Med.*, 126, 625–654.
12. Phillips, H. J. (1973) in *Tissue Culture* (Kruse, P. F., Jr. & Patterson, M. K., Jr., eds.), pp. 406–408, Academic Press, New York.
13. Bryan, J. H. D. & Hybertson, R. L. (1972) *Cytogenetics*, 11, 25–34.
14. Kay, E. J. & Cooper, H. L. (1969) *Biochim. Biophys. Acta*, 186, 62–84.
15. Atherton, K. T. & Darby, G. (1974) *Anal. Biochem.*, 57, 403–412.
16. Parish, J. H. (1972) in *Subcellular Components—Preparation and Fractionation* (Birnie, G. D., ed.), pp. 251–278, University Park Press, Baltimore.
17. Hymer, W. C. & Kuff, E. L. (1964) *J. Histochem. Cytochem.*, 12, 359–363.
18. Munn, T. W. & Katzman, P. A. (1973) *Biochem. Biophys. Res. Commun.*, 53, 119–125.
19. Peacock, A. C. & Dingman, C. W. (1967) *Biochemistry*, 6, 1818–1827.
20. Riddick, D. H. & Gallo, R. C. (1971) *Blood*, 37, 282–292.
21. Mowshowitz, D. B. (1970) *J. Mol. Biol.*, 50, 143–151.
22. Stevens, R. H. & Amos, H. (1972) *J. Cell Biol.*, 54, 1–7.
23. Tidwell, T. & Stubblefield, E. C. (1971) *Exp. Cell Res.*, 64, 350–354.
24. Loeb, L. A., Agarwal, S. S. & Woodside, A. M. (1968) *Proc. Natl. Acad. Sci. U. S. A.*, 61, 827–834.
25. Salvatore, F., Utili, R., Zappia, V. & Shapiro, S. K. (1971) *Anal. Biochem.*, 41, 16–28.
26. Smith, C. G., Gray, G. D., Carlson, R. G. & Hanze, A. K. (1967) *Anal. Biochem.*, 5, 121–151.
27. Owen, S. P. & Smith, C. G. (1964) *Cancer Chemother. Rep.*, 36, 19–22.

28. De la Haba, G. & Cantoni, G. (1959) *J. Biol. Chem.*, **234**, 603–608.
29. Wainfan, E. & Borek, E. (1967) *Mol. Pharmacol.*, **2**, 595–598.
30. Duerre, J. A. (1962) *J. Biol. Chem.*, **237**, 3737–3741.
31. Chung, A. E. & Law, J. H. (1964) *Biochemistry*, **3**, 1989–1993.
32. Walker, R. D. & Duerre, J. A. (1975) *Can. J. Biochem.*, **53**, 312–319.
33. Finkelstein, J. D. (1974) *Metabolism (Clin. Exp.)*, **23**, 387–398.