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INHIBITION OF PROTEIN SYNTHESIS IN 5-AZACYTIDINE-TREATED HeLa CELLS*

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Abstract—Inhibition of protein and RNA synthesis was observed in HeLa cells treated with 5-azacytidine (5-aza-C). Intracellularly, the methylation of phenol-extractable RNA (4-5S, 18S and 28S) was rapidly diminished. The bulk tRNA isolated from 5-aza-C-treated cells differed from the tRNA isolated from untreated cells in its elution on DEAE-cellulose; the difference in the mobility of the tRNA species was dependent on the dose and length of 5-aza-C treatment. The reported reduction of tRNA acceptor activity in 5-aza-C-treated cells was also observed. A simultaneous addition of cytidine or uridine could partially reverse the inhibitory effects produced by 5-aza-C. It appears that the incorporation of the phosphorylated intermediates of 5-aza-C into nucleic acids is the major cytotoxic effect of this drug.

5-Azacytidine (5-aza-C), an analog of cytidine [1], has antimicrobial [2] and antineoplastic activity. Antitumor activity has been demonstrated against AKR and L1210 murine leukemias [2, 3], Ehrlich Ascites tumor [4], and more recently, acute myelogenous leukemia [5, 6]. 5-Aza-C has multiple effects on mammalian tissues [7–10]. It is phosphorylated [11] and incorporated into nucleic acids [12, 13]. The incorporation of 5-aza-C into RNA may result in loss of tRNA amino acid acceptor activity [14], disaggregation of polyribosomes [15, 16], and protein inhibition [12, 17]. However, the inhibitory effects of 5-aza-C treatment have not been adequately correlated with the mode of action of the drug [17].

In this paper, we study the effect of 5-aza-C treatment on RNA and protein synthesis. Intracellularly, the treatment results in loss of cellular RNA methylation, modification of tRNA in its elution behavior on DEAE-cellulose, and reduction of tRNA acceptor activity. The inhibitory effects produced by 5-aza-C can be partially reversed by cytidine addition. We believe that the incorporation of the phosphorylated intermediates of 5-aza-C into nucleic acids is the major cytotoxic effect of this drug.

MATERIALS AND METHODS

Materials. 5-Aza-C (NSC 102816 and [^{14}C]5-aza-C (46 mCi/m-mole) were supplied through the Chemical and Drug Procurement Section, Chemotherapy, National Cancer Institute. The radioactive nucleosides and amino acids were obtained from New England Nuclear, Boston, Mass. Yeast tRNA and oligouridylic acid (mol. wt 7500) were purchased from CalBiochem, San Diego, Calif.

Incorporation of [^{3}*H*]*uridine and* [^{14}*C*]*valine into RNA and protein.* HeLa cells were cultured in McCoy's 5A media (Flow Laboratories, Rockville, Md.) containing 10% calf serum (Grand Island Biological Co., Grand Island, N.Y.) and antibiotics. For macromolecular synthesis the cells in 30-mm petri dishes (10^5 cells/dish) were pulse-labeled with [^{3}H]uridine ([^{3}H]UR) or [^{14}C]valine for 30 min after drug treatment. The incorporation was stopped by rinsing the monolayer twice with cold saline. The cell mass was scraped off with a rubber policeman into cold saline and centrifuged at 800 *g* for 3 min at 4°. The soluble radioactivity of [^{3}H]UR was measured by suspending the cell pellet in 1 ml of cold 5% TCA and centrifuging at 3000 *g* for 5 min. Aliquots (0.5 ml) of the supernatant were added to 10 ml Aquasol (New England Nuclear) and assayed for radioactivity in a scintillation counter. The TCA precipitate was vortexed with 2 ml of cold 5% TCA, poured onto a Whatman GF/C filter, and washed with 5% TCA. The filters were dried and immersed in 10 ml of toluene scintillation fluid containing 5 g PPO§ and 100 mg POPOP in 1 liter toluene.

The soluble radioactivity of [^{14}C]valine was measured by suspending the pellet in 0.1 ml of 0.1 N KOH and incubating at 37° for 30 min. The mixture was acidified by adding 1 ml of cold 10% TCA, kept at 4° for 15 min, and centrifuged at 3000 *g* for 5 min. The soluble and insoluble radioactivity of [^{14}C]valine was measured using the same technique as described above for that of [^{3}H]UR.

RNA phenol extraction and column chromatography. The bulk RNA was extracted with phenol using the method of Karon *et al.* [18]. The RNA precipitated twice from cold ethanol, as dissolved in 1 ml of acetate buffer (0.01 N NaOAc, pH 6.7, 0.5 N NaCl containing 2 μg/ml of polyvinyl sulfate), layered onto a 5–20% sucrose density gradient in the same buffer, and centrifuged at 113,000 *g* in an SW 27.1 swinging bucket rotor for 15–17 hr. The gradient was fractionated using an ISCO gradient fractionator. The

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§ PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene.

4–5S RNA was precipitated from cold ethanol and dissolved in 0.01 N Tris–HCl, pH 7.5, containing 0·01 mM EDTA. It was applied to a column of Sephadex G-25 (90 cm 1 cm^2). Elution was carried out with the same Tris–HCl buffer. Yeast tRNA was eluted under identical conditions. The RNA which eluted at the same position as yeast tRNA was pooled. The radioactivity incorporated into RNA was measured after addition of 5% TCA. The precipitate was poured onto a Whatman GF/C filter, washed with 5% TCA, dried and immersed in toluene scintillation fluid for radioactivity counting. The tRNA purified from the Sephadex column was applied to a column of DEAE-cellulose (40 cm × 1 cm^2). The elution began with 100 ml of Tris–HCl buffer (20 mM Tris–HCl, pH 7.5, 1 mM EDTA containing 0.25 N NaCl) and was followed by a linear gradient of NaCl (0.55 to 0.75 N in 200 ml) in the Tris–HCl buffer, pH 7.5. Fractions of 5 ml were collected. The radioactivity was measured as described above.

Aminoacyl-tRNA synthetase assay. The assay mixture of 0.3 ml contained 20 μmoles Tris–HCl, pH 7.5; 20 μmoles MgOAc; 1 μmole KCl; 2 μmoles β-mercaptoethanol; 0.1 μmole CTP; 0.4 μmole ATP; 0.1 μCi of a [^{14}C]amino acid mixture containing 10 μCi/ml of each of the following amino acids: alanine aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine and valine, in 10 mCi/m-mole; 0.12 to 0.25 A_{260} of the hydrolyzed tRNA [19]; and 0.1 ml of enzyme (0.24 mg protein). The assay mixture was incubated at 37° for 15 min, during which time the incorporation of ^{14}C-labeled amino acid into tRNA reached a plateau. Cold 20% TCA (0.2 ml) was added to terminate the reaction. The precipitate was poured onto a Whatman GF/C filter, washed with 5% TCA, dried, and counted in toluene scintillation fluid.

The enzyme was obtained from the "pH 5" enzyme preparation of HeLa spinner culture [18]. The pH 5 enzyme precipitate was dissolved in 50 mM Tris–HCl, pH 7.5, buffer containing 5 mM $MgCl_2$ and 25 mM KCl in 0.25 M sucrose in a concentration of 3 mg protein/ml. The endogenous tRNA was removed by adding 0.1 g of washed DEAE-cellulose to each ml of the crude enzyme (2.4 mg protein), stirring gently at 4° for 30 min, and centrifuging at 12,000 *g* for 10 min at 4°. The supernatant was adjusted to 20 per cent by volume with glycerol containing 10 mM β-mercaptoethanol and stored at −20°.

RESULTS

Effect of 5-aza-C on RNA and protein synthesis. Inhibition of both RNA and protein synthesis was observed in 5-aza-C-treated HeLa cells (Fig. 1). The soluble radioactivity of [^{14}C]valine for protein synthesis fluctuated to a significantly lesser extent than that of [^{3}H]UR for RNA synthesis. The inhibition of protein synthesis appeared to be more pronounced than that of RNA synthesis. 5-Aza-C at concentrations of 1, 10 and 100 μg/ml inhibited protein synthesis by 20, 45 and 63 per cent, respectively, as compared to 11, 25 and 42 per cent inhibition of RNA synthesis after 5 hr of treatment.

Effect of 5-aza-C on RNA methylation. The transfer of the [^{3}H]CH_3 group from L-methionine-methyl[^{3}H] to phenol-extractable RNA was studied in 5-aza-C-treated cells (Fig. 2). The untreated culture which was used as the control showed a greater amount of methylation of 4–5S RNA at the 5-hr point than at the 2-hr point, which was again greater than at the 0.5-hr point. The increase in methylation of 18S and 28S RNA among the control culture was less pronounced. 5-Aza-C (25 μg/ml) treatment inhibited the methylation of RNA as early as 30 min. The inhibition of methylation of 4–5S, 18S and 28S RNA was greater than 50 per cent after 1.5 hr of drug treatment (Fig. 2). The methylation of 4–5S RNA was inhibited by 95 per cent or greater after 3.5 hr of drug exposure. Under the same conditions, the methylation of 18S and 28S RNA became undetectable in drug-treated cultures.

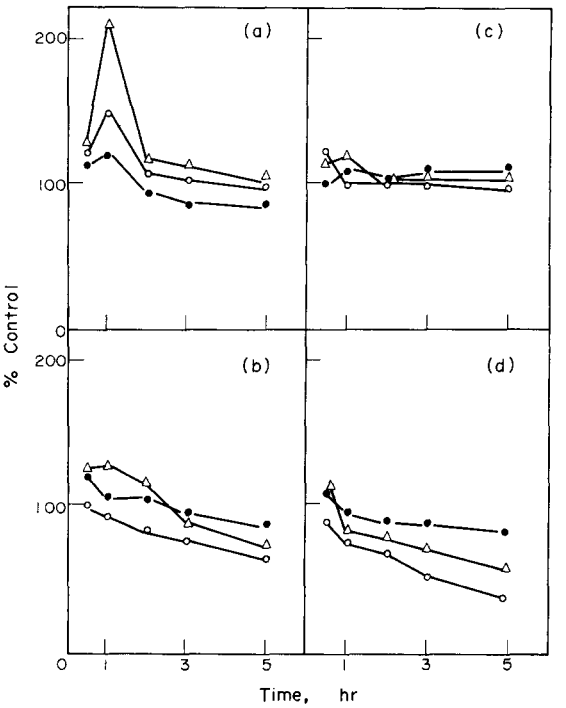

Fig. 1. Effect of 5-aza-C on cellular uptake of labeled uridine and valine. Twenty hr after the plating of 5 × 10^5 trypsinized cells, 5-aza-C was added to 60-mm petri dishes. At various intervals after the drug treatment, [^{3}H]UR (2 μCi/ml) or [^{14}C]valine (1 μCi/ml) was added to cells for 30 min. The assay for the acid-soluble radioactivity of [^{3}H]UR (A) and [^{14}C]valine (C), and the acid-insoluble radioactivity of [^{3}H]UR (B) and [^{14}C]valine (D) was described in Methods. Key: (●) 5-aza-C, 1 μg/ml; (△) 5-aza-C, 10 μg/ml; and (○) 5-aza-C, 100 μg/ml. The untreated cells were used as the control.

Effect of 5-aza-C on tRNA. On sucrose density gradient fractionation, the radioactivity of [^{14}C]5-aza-C was found to be incorporated into 4–5S, 18S and 28S RNA in HeLa cells pulse-labeled with [^{14}C]5-aza-C (data not shown). 4–5S RNA of ^{14}C-radioactivity chromatographed on a Sephadex column showed that greater than 85 per cent of ^{14}C-radioactivity was associated with the RNA species of the size of yeast tRNA (data not shown). tRNA isolated from drug-treated and untreated cultures and purified initially from a Sephedex column showed some difference in their elution on DEAE-cellulose. The tRNA species

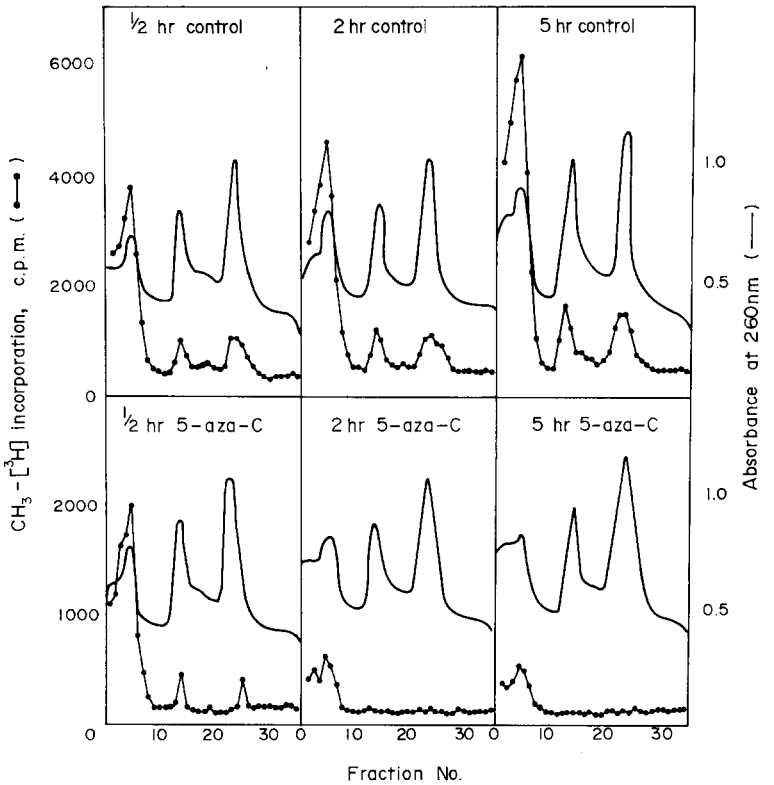

Fig. 2. Effect of 5-aza-C on the incorporation of $CH_3[^3H]$ into cellular RNA of HeLa cells. The monolayer cultures of HeLa cells, 2 to 3 × 10^7 cells in 250-ml plastic flasks, were treated with 5-aza-C (25 μg/ml) for the indicated time period. At each time interval, 20 μCi L-methionine-$CH_3[^3H]$ (1.2 Ci/m-mole) was added to each flask of culture (1.3 μCi/ml) for 30 min. The bulk RNA was extracted with phenol, layered onto a 5–20% sucrose density gradient in the acetate buffer, and centrifuged at 113,000 *g* in an SW 27.1 swinging bucket rotor for 15–17 hr. The gradient was fractionated using an ISCO gradient fractionator. The u.v. absorbance was recorded automatically. The incorporation of $CH_3[^3H]$ into cellular RNA (4-5S, 18S, 28S from left to right) was assayed as described in Methods.

pulse-labeled with [^{14}C]5-aza-C was eluted to a higher gradient concentration as compared to that of the bulk tRNA from drug-treated or untreated cells. The separation of tRNA synthesized during 5-aza-C treatment and pulsed with [^{3}H]UR from the bulk tRNA on DEAE-cellulose column chromatography appeared to be dependent on the dose (Fig. 3A) and length (Fig. 3B) of 5-aza-C addition.

The tRNA isolated from 5-aza-C-treated cells showed a loss in amino acid acceptor activity. A reduction of 43, 66 and 90 per cent of amino acid acceptor activity of tRNA (unfractionated) was observed in cells treated with 10, 25 and 50 μg/ml of 5-aza-C, respectively, for 3 hr (Table 1). 5-aza-C treatment at a concentration of 25 μg/ml resulted in a loss of 10, 66 and 88 per cent of amino acid acceptor activity of tRNA in cells exposed to the drug for 1, 3 and 6 hr respectively.

The tRNA isolated from 5-aza-C-treated cells (25 μg/ml, 3 hr) showed a varying degree in its loss of specific amino acid acceptor activity. The reduction of tRNA acceptor activity with respect to [^{14}C]aginine, [^{14}C]glycine, [^{14}C]leucine, [^{14}C]valine and [^{14}C]glutamic acid was 20, 53, 27, 75 and 25 per cent respectively (data not shown).

Reversal effect of cytidine and uridine. The inhibition of HeLa cell proliferation by 5-aza-C was par-

Table 1. tRNA acceptor activity in HeLa cells before and after 5-aza-C treatment*

5-aza-C treatment		[^{14}C]amino acid incorporation	
Duration (hr)	Dose (μg/ml)	(pmoles/15 min/mg tRNA)	Reduction (%)
0	0	1.37	0
1	25	1.24	10
3	10	0.78	43
3	25	0.46	66
3	50	0.14	90
3	100	0.04	97
6	25	0.16	88

* tRNA from 5-aza-C-treated or untreated cells was extracted with phenol, fractionated in sucrose density gradient, and purified by Sephadex column chromatography (see Methods). tRNA (5–10 A_{260}) was then applied to a small column of DEAE-cellulose (5 cm × 0.38 cm^2), washed with Tris–HCl, pH 7.5, buffer with 0.25 N NaCl, and eluted with Tris–HCl, pH 7.5, buffer with 0.75 N NaCl. tRNA was recovered by cold ethanol precipitation. A limiting concentration (0.12 to 0.25 A_{260}) of the hydrolyzed tRNA was used for the synthetase assay (see Methods). The ^{14}C-labeled amino acid mixture contained all the acidic and neutral amino acids of a specific activity of 10 mCi/m-mole. The counting efficiency for ^{14}C-radioactivity was 85 per cent.

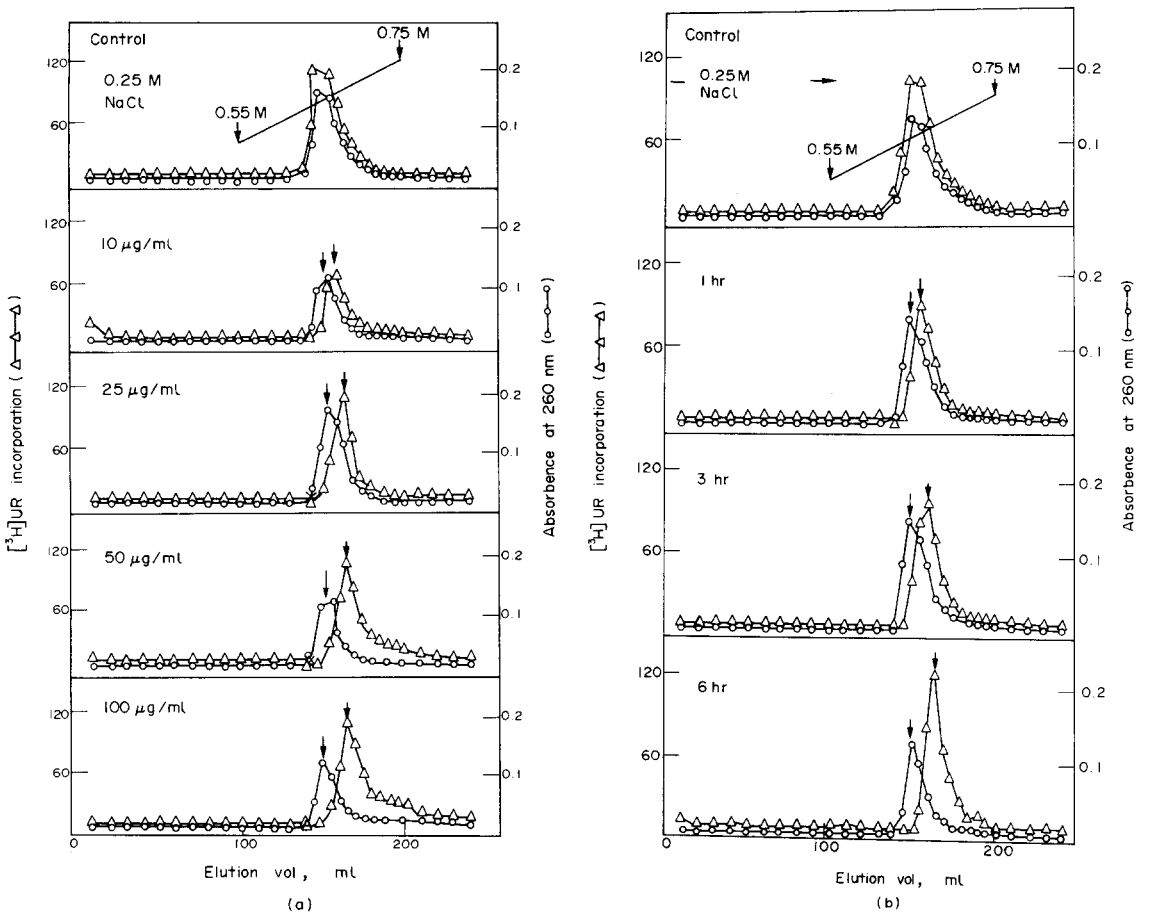

Fig. 3. Effect of dose and length of 5-aza-C treatment on the chromatographic elution of tRNA on DEAE-cellulose. The monolayer HeLa cells, 2 to 3 × 10^7 cells in 250-ml plastic flasks, were treated with 0, 10, 25, 50 or 100 μg/ml of 5-aza-C for 3 hr and followed by a pulse-labelling of 10 μCi [^{3}H]UR for 60 min (A). To some HeLa cells the treatment with 25 μg/ml of 5-aza-C continued for 0, 1, 3 and 6 hr and was followed by a pulse-labeling of 10 μCi [^{3}H]UR for 60 min (B). The chromatographic elution of tRNA isolated from drug-treated and untreated cells was described in Methods.

tially reversed by a simultaneous addition of uridine or cytidine (Fig. 4). The addition of 10 μg/ml of uridine or cytidine reduced the inhibitory effect of 5-aza-C (10 μg/ml) on cell number by 35–60 per cent after 5 days of treatment. The cells recovering from 5-aza-C treatment showed about the same size as untreated cultures (data not shown).

The methylation of tRNA was diminished rapidly in 5-aza-C-treated cells. The transfer of the [^{3}H]CH_3 group from radioactive L-methionine to tRNA was

Table 2. tRNA methylation in HeLa cells before and after 5-aza-C treatment*

5-aza-C treatment		Cytidine addition (μg/ml)	[^{3}H]CH_3 group incorporation	
Duration (hr)	Dose (μg/ml)		(pmoles/mg tRNA)	(% Control)
0	0	0	11.1	100.
1	25	0	3.4	30.6
1	25	25	12.7	114.4
3	25	0	2.9	26.1
3	25	25	11.6	104.5
5	25	0	1.1	10.0

* An HeLa monolayer culture of 2 to 3 × 10^7 cells in a 250-ml plastic flask was pulse-labeled with 50 μCi L-methionine-CH_3[^{3}H] (1.2 Ci/m-mole) for 60 min after the indicated period of 5-aza-C treatment. Cytidine was added simultaneously with the radioactive methionine. tRNA was purified as described in Table 1. The radioactivity was measured using aliquots ($2A_{260}$) of purified tRNA in the assay as described in Methods.

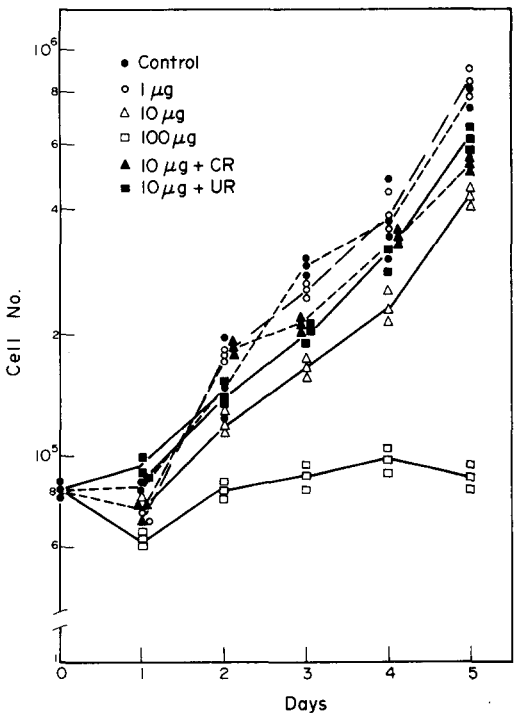

Fig. 4. Changes in the number of cells/plate after exposure of HeLa cells to 5-aza-C. Twenty hr after the plating of 1.3×10^5 trypsinized cells, 5-aza-C was added to 60-mm petri dishes. When cytidine (CR) or uridine (UR) was added simultaneously with 5-aza-C, their concentration was 10 μg/ml. Cell number was determined after trypsinization.

reduced by 70.0, 73.9 and 90.0 per cent in cells treated with 5-aza-C (25 μg/ml) for 1, 3 and 5 hr respectively. The cells treated with 5-aza-C (25 μg/ml) for 1 and 3 hr by cytidine addition for 1 hr appeared to fully recover their capacity to methylate tRNA (Table 2). The incorporation of the $[^3H]CH_3$ group from L-methionine $CH_3[^3H]$ into tRNA was 114 and 105 per cent of the control value in those cells treated with 25 μg/ml of 5-aza-C for 1 and 3 hr, respectively, and followed by cytidine addition.

The acceptor activity of tRNA in cells treated with 25 μg/ml of 5-aza-C for 3 hr was increased after cytidine addition. The tRNA acceptor activity was 25.9, 65.5, 75.3 and 76.9 per cent of that of untreated cells when 25 μg/ml of cytidine was added to 5-aza-C-treated cells for 0, 1, 2 and 3 hr respectively (Table 3). A high dose of cytidine (50 μg/ml) increased the acceptor activity of tRNA in 5-aza-C-treated cells to about 80.0 per cent of the control value in 1 hr (Table 3).

DISCUSSION

The inhibition of RNA and protein synthesis in drug-treated HeLa cells, which is dependent on the dose and length of 5-aza-C treatment (Fig. 1), can be explained by the incorporation of the phosphorylated intermediates [11] of 5-aza-C into nucleic acids [12, 13]. Since inhibition of protein synthesis precedes that of RNA synthesis, the interference with the activity of a particular enzyme to block *de novo* pyrimidine synthesis [20] appears less important in cytotoxic effects produced by 5-aza-C. Nonetheless, the inhibition of orotidylic acid decarboxylase by 5-aza-cytidine-5′-monophosphate [20] may result in an early rise of soluble radioactivity of $[^3H]$UR in drug-treated cells (Fig. 1).

The increase of methylation of bulk RNA in untreated cells with time (Fig. 2) may be partially caused by the media change. Fresh serum has a growth-stimulating effect on HeLa cells. The cells treated with 5-aza-C, however, show a rapid loss of methylation in both 4–5S RNA and 18S and 28S RNA (Fig. 2). Under a similar condition of drug treatment, the radioactivity of $[^{14}C]$5-aza-C is found to be incorporated in all three RNA species and more than 85 per cent of ^{14}C-radioactivity of 4–5S RNA is associated with tRNA or with RNA of the size of yeast tRNA (see Results). This suggests that diminution of RNA methylation is a result of 5-aza-C incorporation.

Further study of the effect of 5-aza-C treatment on RNA modification is carried out with tRNA molecule. On DEAE-cellulose elution, the tRNA isolated from 5-aza-C-treated cells is different in comparison to the tRNA isolated from untreated cells. When cells are pulse-labeled with $[^{14}C]$5-aza-C and then treated with cold 5-aza-C, the tRNA synthesized during this period of drug treatment is also different from the bulk unlabeled tRNA (data not shown). The reason for the difference is largely unknown. One of the conditions to induce a greater alteration of the elution behavior of tRNA is to increase the dose and length of 5-aza-C treatment (Fig. 3, panels A and B).

The tRNA isolated from drug-treated cells shows a loss in its amino acid acceptor activity. The degree

Table 3. Recovery of tRNA acceptor activity by cytidine addition*

5-aza-C treatment		Cytidine addition			
Duration (hr)	Dose (μg/ml)	Duration (hr)	Dose (μg/ml)	$[^{14}C]$amino acid incorporation (pmoles/15 min/mg tRNA)	% Control
0	0	0	0	1.42	100
3	25	0	0	0.41	28.9
3	25	1	25	0.93	65.5
3	25	1	50	1.14	80.0
3	25	2	25	1.07	75.3
3	25	3	25	1.13	79.6

* Cytidine was added at the end of 5-aza-C treatment. tRNA was purified and assayed as described in Table 1.

of reduction of tRNA acceptor activity, which is non-specific with respect to amino acids (see Results), appears to lag initially behind the rate of diminution of tRNA methylation. After exposure to 25 μg/ml of 5-aza-C for 1 hr, the reduction of tRNA acceptor activity is only 10 per cent (Table 1) as compared to 70 per cent of loss in tRNA methylation (Table 2). On the other hand, tRNA methylation can be restored immediately after cytidine addition (Table 2), whereas the acceptor activity of tRNA in 5-aza-C-treated cells is only partially recovered (Table 3). Apparently, the loss of tRNA methylation may not entirely stem from the same mechanism responsible for the reduction of tRNA acceptor activity. The relatively fast turnover of the 3'-CCA terminal end of tRNA [14] may explain the loss of tRNA methylation and its quick recovery after cytidine addition in 5-aza-C-treated cells. Meanwhile, the incorporation of 5-aza-C into the internal structure as compared to the 3'-terminal end of tRNA, no matter how limited, may produce an inhibitor of amino acyl-tRNA synthesis which would explain the partial recovery of tRNA acceptor activity (Table 3). Recently the tRNA isolated from 5-aza-C-treated A(T_1)CL-6 hamster fibrosarcoma cells is shown to inhibit protein synthesis in the cell-free reticulocyte system *in vitro* [21]. The inhibition is partially explained by the irreversible binding of 5-aza-C tRNA to the polyribosome complex [21].

The partial recovery of tRNA acceptor activity in 5-aza-C-treated cells after cytidine addition may explain why the proliferation of HeLa cells is recovered only by 35–60 per cent (Fig. 4). We believe the incorporation of the phosphorylated intermediates into nucleic acids to be the major cytotoxic effect of this drug.

REFERENCES

1. A. Piskala and F. Sorm, *Colln Czech. chem. Commun.* **29**, 2060 (1964).
2. L. J. Hanka, J. S. Evans, D. J. Mason and A. Diety, *Antimicrobial Agents in Chemotherapy*, pp. 619–24. American Society of Microbiologists, Ann Arbor, Mich. (1967).
3. F. Sorm and J. Vesely, *Neoplasm* **11**, 123 (1964).
4. F. Sorm, A. Piskala, A. Cihak and J. Vesely, Jr., *Experientia* **20**, 202 (1964).
5. M. Karon, L. Sieger, S. Leimbrock, J. Z. Finkelstein, M. E. Nesbit and J. J. Swaney, *Blood* **42**, 359 (1973).
6. K. B. McCredie, G. P. Bodey, M. A. Burgess, J. U. Gutherman, V. Rodriquez, M. P. Sullivan and E. J. Freireich, *Cancer Chemother. Rep.* **57**, 319 (1973).
7. A. Cihak and J. Vesely, **34**, 910 (1969).
8. A. Cihak, J. Vesely and F. Sorm, *Colln Czech. chem. Commun.* **32**, 3427 (1967).
9. K. Raska, M. Jurovcik, V. Fucik and F. Sorm, *Colln Czech. chem. Commun.* **31**, 2809 (1966).
10. K. Raska, M. Jurovcik, Z. Sormova and F. Sorm, *Colln Czech. chem. Commun.* **31**, 2803 (1966).
11. T. Lee, M. Karon and R. L. Momparler, *Cancer Res.* **34**, 2482 (1974).
12. M. Jurovcik, K. Raska, Z. Sormova and F. Sorm, *Colln Czech. chem. Commun.* **30**, 3370 (1965).
13. V. Paces, J. Porkocil and F. Sorm, *Biochim. biophys. Acta* **161**, 352 (1968).
14. J. Kalousek, K. Raska, M. Jurovcik and F. Sorm, *Colln Czech. chem. Commun.* **31**, 1421 (1966).
15. I. B. Levitan and T. E. Webb, *Biochim. biophys. Acta* **182**, 491 (1969).
16. A. Cihak, L. M. Narurka and H. C. Pitot, *Colln Czech. chem. Commun.* **34**, 948 (1973).
17. Z. B. Sayeida, R. L. P. Adams and R. C. Imric, *Cancer Res.* **33**, 40 (1973).
18. M. Karon, S. Weissman, C. Meyer and P. Henry, *Cancer Res.* **25**, 185 (1965).
19. T. Tidwell, B. J. Bruce and A. C. Griffin, *Cancer Res.* **32**, 1002 (1972).
20. A. Cihak and J. Broucek, *Biochem. Pharmac.* **21**, 2497 (1972).
21. R. L. Momparler, S. Siegel, F. Avila, T. Lee and M. Karon, *Biochem. Pharmac.* **25**, 389 (1976).