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Metabolism of Puromycin Aminonucleoside in Transformed Human Lung Fibroblasts and the Mechanism of Its Inhibition of RNA Synthesis

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SUMMARY

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SV-40-transformed human lung fibroblasts (WI38–VA13 cells) were incubated for 4 hr with highly purified, tritium-labeled puromycin aminonucleoside (AMS), together with unlabeled AMS at a final concentration of 340 μM (100 μg/ml). Approximately 90% of AMS was found unchanged in the acid-soluble pool. Phosphorylated forms of the demethylated derivative of AMS, 3′-amino-3′-deoxyadenosine (3′-AmA) were also found; one form was shown to be the 5′-monophosphate, and the other a 5′-triphosphate. Tracer concentrations of AMS (0.066 μM) were converted to phosphorylated derivatives to a larger extent, and nonphosphorylated 3′-AmA was not found in the acid-soluble pool even at the higher AMS concentration, indicating that the demethylating step is slower than the phosphorylating reactions. Alkaline hydrolysis of the RNA from AMS-treated cells released only nonphosphorylated 3′-AmA. AMS or its derivatives were not detected in the DNA of treated cells. The results indicate that AMS is successively demethylated and phosphorylated, and that the resultant 3′-AmA triphosphate is incorporated into the terminal positions of nascent RNA chains. Further elongation of the growing RNA polynucleotide is prevented by the 3′-amino group of the analog, thus causing premature termination of RNA synthesis.

INTRODUCTION

Aminonucleoside of puromycin (AMS)[1] has a number of interesting biological effects. Administration of this compound to rats produces nephrotoxicity which mimics the nephrotic syndrome of man (1). In mice, AMS was reported to selectively reduce the RNA content of mammary tumor cells (2). The action of this inhibitor was more extensively studied in cultured mammalian cells. It was determined in several laboratories that AMS preferentially inhibits ribosomal RNA synthesis in transformed mammalian cells (3, 4), but is a rather nonselective inhibitor of nucleic acid synthesis in nontransformed, presumably normal cultured cells (5, 6). Curiously, it also induces aryl hydrocarbon hydroxylase activity in murine liver cell cultures by a process insensitive to actinomycin D (7).

The mechanisms through which this analog of adenosine produces the above effects have not been satisfactorily elucidated. The suggestions put forward to account for inhibition of RNA synthesis by AMS include false feedback inhibition of purine biosynthesis (8), the ability to induce a "stepdown" in nucleic acid synthesis through resemblance to uncharged transfer RNA (3), and interference with ATP formation or RNA polymerase action (9). However, premature termination of nascent RNA chains by addition of AMS, an adenosine analog lacking a 3′-OH group, was apparently excluded in work with murine L cells (9).

The differential effect of AMS on normal and transformed cells may offer valuable leads for cancer chemotherapy (10), but the analysis of this effect is hampered by the lack of understanding of the mechanism of AMS action. With this goal in mind we obtained a purer sample of radioactively labeled AMS than was previously available, and determined the fate of this inhibitor in transformed human fibroblasts. The results indicate that premature termination of growing RNA chains is the likely reason for inhibition of RNA synthesis by AMS.

MATERIALS AND METHODS

Materials. The enzymes, nucleotide pyrophosphatase (EC 3.6.1.9), alkaline phosphatase from *Escherichia coli*

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[1] The abbreviations used are: AMS, aminonucleoside of puromycin; PBS, phosphate-buffered saline; PCA, perchloric acid; 3′-AmA, 3′-amino-3′-deoxyadenosine.

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(EC 2.21.1), deoxyribonuclease (EC 3.14.5), snake venom phosphodiesterase (EC 3.1.4.1), and the purine derivatives AMS, ATP, ADP, AMP, adenosine, adenine and 6-*N*-monomethylaminopurine-9-riboside were purchased from Sigma Chemical Company, St. Louis, Missouri. 6-*N*-Dimethylaminopurine-9-riboside was purchased from Vega Biochemicals, Tucson, Arizona, and pancreatic RNase (EC 3.1.4.22) from Worthington, Freehold, New Jersey.

Preliminary experiments were conducted using ^{3}H-AMS (15 Ci/mmole, general label), purchased from Amersham Corporation. The ^{3}H-AMS was found to be 90% pure, with the remainder of radioactivity being present in adenosine, as determined by mobility on high voltage paper electrophoresis. The ^{3}H-AMS was purified before use by elution after high-voltage paper electrophoresis. Definitive experiments were performed using a second batch of ^{3}H-AMS also purchased from Amersham (10.6 Ci/mmole, general label). It was found to be 98.4% pure as determined by high-voltage paper electrophoresis and paper chromatography in a solvent containing *N*-butanol, ethanol and H_2O (10.4:6.6:3). The remainder of the radioactivity was in adenosine (0.34%) and in adenine (1.2%).

Cell culture. SV-40-transformed human embryonic lung fibroblasts, WI38–VA13, were grown as monolayers in Corning 75-cm plastic flasks. Eagle's minimum essential medium was used, supplemented with 10% fetal calf serum (heat inactivated at 56° for 30 min) and with 1% glutamine. Antibiotics were not used. The cell line was originally obtained from Dr. V. J. Cristofalo of the Wistar Institute, Philadelphia, and has been maintained in this laboratory for the past 6 years. Periodic testing for mycoplasma contamination was performed by the autoradiographic screening method of Studzinski *et al.* (11).

Preparation of acid soluble pool extracts. In preliminary experiments six flasks of cells were exposed to 35 μCi of ^{3}H-AMS (15 Ci/mmole) each in 10 ml of medium containing unlabeled AMS at 100 μg/ml, which is 340 μM (final S.A. 10.2 mCi/mmole). When highly purified ^{3}H-AMS was obtained, five flasks were exposed to 100 μCi of this ^{3}H-AMS (10.6 Ci/mmole), each in 10 ml of medium containing unlabeled AMS at 100 μg/ml (final S.A. 10.2 mCi/mmole). With the exceptions noted, the cells were incubated for 4 hr at 37°. After exposure to AMS, the medium was poured off and the cell layers rinsed four times with ice-cold 0.1 M phosphate-buffered saline (PBS), (0.85% NaCl), pH 7.0. The cells were detached from the growth surface using a rubber spatula, suspended in PBS and centrifuged at 1800 rpm (800 × *g*) at 4° for 10 min in an IEC centrifuge (Model PR-6000). All subsequent centrifugations were done in this centrifuge. The cells were resuspended in PBS and centrifuged. The washed pellet was suspended in 2 ml of ice-cold distilled water and homogenized by 25 strokes in an 8-ml-capacity Potter–Elvehjem homogenizer (No. 22 Kontes). Seventy percent perchloric acid (PCA) was added to the homogenate to a final concentration of 5%. The mixture was vortexed and allowed to stand on ice for 15 min after which it was centrifuged for 10 min at 1800 rpm. The supernatant was saved and the pellet washed two times with 2 ml of 2.5% ice-cold PCA. The washes were combined with the original supernatant and the entire extract was considered to be the acid-soluble pool. This extract was neutralized with KOH and centrifuged at 3600 rpm (2,900 × *g*) for 10 min to remove the insoluble $KClO_4$. The supernatant was then lyophilized.

Incorporation into RNA. Five flasks of cells were exposed to 100 μCi of ^{3}H-AMS (10.6 Ci/mmole) each in 10 ml of medium containing unlabeled AMS at 100 μg/ml and incubated as described above. The same procedures were followed up to the final cell pellet resulting from extraction with ice-cold PCA. The pellet was washed two times with 10 ml of 2.5% PCA, dissolved in 7 ml of ice-cold 0.1 M NaOH, and reprecipitated with 14 ml of ice-cold 10% PCA, and then the pellet was washed and reprecipitated twice more. The final pellet was dissolved in 3 ml of 0.3 M KOH and incubated at 37° for 24 hr. The hydrolysate was neutralized with PCA, centrifuged at 3600 rpm, and the final supernatant lyophilized.

High voltage electrophoresis. The lyophilized samples were resuspended in 300 μl of distilled water just before electrophoresis and spotted across 5-cm-wide Whatman 3MM electrophoresis paper. Electrophoresis was carried out for 2.5 hr at 3500 V in a buffer containing pyridine, acetic acid, and water (1:10:89), pH 3.5. The apparatus was a Gilson Model D high-voltage electrophorator.

Elution of compounds from electrophoretic strips. In order to elute AMS and its metabolites from the electropherograms, the paper strips were cut into 1-cm sections. One end of each section was trimmed to a point. The pieces of paper were suspended in a beaker of distilled water, just touching the surface of the liquid. Air was blown over the beaker to facilitate the capillary movement of the water. After 4 to 5 hr of this treatment, the material was concentrated at the upper tip of the paper. The sections were secured to the top of a conical tube and centrifuged at 1000 rpm (300*g*) for 5 min. The water drawn out of the paper by the centrifugation washed the concentrated material from the tip of the paper into the tube. After elution all material was lyophilized.

Enzymatic digestion of material separated by paper electrophoresis. Procedures were those described by Germerhausen *et al.* (12). After paper electrophoresis, material from areas 2 and 3 (see Fig. 1) was eluted and lyophilized. Material from area 2 was resuspended in 0.2 ml of 0.1 M Tris (pH 8.0). Fifty micrograms of *E. coli* alkaline phosphatase was added to this suspension and it was incubated at 37° for 15 min. The incubation was repeated three times adding an additional 50 μg of enzyme with each 15 minute incubation. Area 3 material was dissolved in 0.2 ml of 0.02 M Tris pH 7.5 containing 1 mM $MgCl_2$; 0.01 unit (one unit will hydrolyze 1.0 μmole of β-NAD to NMN and AMP per minute at pH 7.4 at 37° in the presence of Mg^{2+} ions) of nucleotide pyrophosphatase was added to the sample and it was incubated at 37° for 15 min. After enzyme treatment the samples were frozen at −20° until electrophoresis could be performed.

Acid hydrolysis of nucleosides. Material eluted from the electrophoretic strips was lyophilized in 50 × 6-mm culture tubes, and dissolved in 50 μl of 1 N HCl. The culture tubes were then heat sealed and placed in a water bath at 100° for 1 hr. The hydrolysates were spotted on

Whatman 3 MM paper and chromatography carried out in a solvent system containing isopropanol, NH_4OH and H_2O (7:1:2) pH 11 (13). Adenine and hydrolysates of N^6-methyladenosine and N^6,N^6-dimethyladenosine were used as standards.

DNA isolation and digestion. Ten flasks of cells were exposed to 10 μCi of ^{3}H-AMS (15 Ci/mmole) each in 10 ml of medium (0.066 μM AMS). Five flasks were incubated at 37° for 4 hr and the other five incubated for 18 hr. After the incubation period, nuclei were isolated from the cells as previously described (14). DNA was extracted from the nuclei by the phenol, pH 9, RNase method of Saito and Miura (15). The purified DNA was enzymatically degraded by successive digestion with DNase I and snake venom phosphodiesterase (16).

Radioactivity measurements. Electrophoretic strips and chromatograms were cut into strips of equal size, and the strips were placed in glass scintillation vials. One milliliter of H_2O was added and the vials incubated at 60° for 2 hr. The vials were then vortexed for 30 sec. Biofluor was added, and the vials were vortexed for another 30 sec. The radioactivity of each sample was determined in a scintillation counter, Intertechnique Model 4000.

RESULTS

Metabolic fate of aminonucleoside. Three areas of radioactivity were found after high-voltage paper electrophoresis of the acid-soluble extracts of VA-13 cells exposed to tritiated AMS (Fig. 1). The major peak, containing approximately 90% of the total radioactivity, migrated toward the cathode together with the unlabeled AMS which was introduced as a marker. The two minor peaks were found close to the origin of the electropherogram, one toward the cathode, indicating a slight positive net charge, and the other toward the anode, with a slightly negative net charge.

TABLE 1

Effect of phosphatases on the electrophoretic mobility of ^{3}H AMS-containing compounds in the acid-soluble pool of VA-13 cells

Area eluted[a]	Enzyme treatment after elution	Percentage of radioactivity after electrophoresis		
		Area 1[a]	Area 2[a]	Area 3[a]
2	Alkaline phosphatase[b]	44	56	0
3	Pyrophosphatase	15[c]	78	7

[a] This designation refers to the areas of electropherograms shown in Fig. 1.

[b] The material in area 2 was a poor substrate for *E. coli* alkaline phosphatase. One possibility is that this area represents an approximately 50:50 mixture of mono- and diphosphate derivatives of 3′-AmA.

[c] The small conversion of material from area 3 to area 1 was probably due to a slight phosphomonoesterase activity in the nucleotide pyrophosphatase preparation, reported by the supplier, which splits off the phosphate from the product of the pyrophosphatase reaction. This is supported by the finding that increasing the incubation time with this enzyme produced increasing conversion to material migrating in area 1.

The constituents of the two minor peaks were shown to be phosphorylated. Areas of the electropherogram designated 2 and 3 in Fig. 1 were eluted, the eluates treated with phosphatases, and reaction products were subjected to further electrophoresis (Table 1). In each case phosphatase treatment resulted in conversion to compounds with enhanced mobility toward the cathode, and after treatment with appropriate enzymes each compound could be converted into a form which migrated with AMS on electrophoresis.

AMS and its demethylated derivatives, N^6-methyl-3′-amino-3′-deoxyadenosine and 3′-amino-3′deoxyaden-

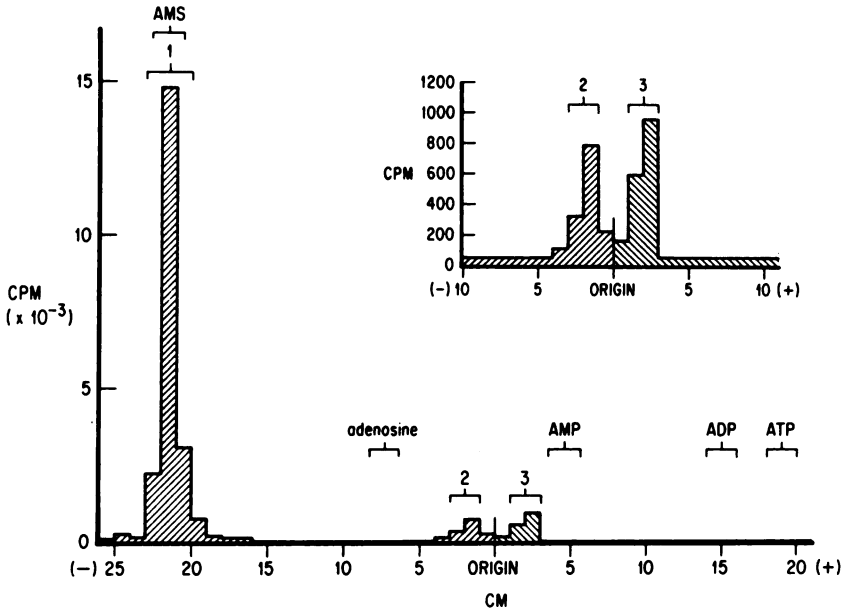

FIG. 1. *Electropherogram of the radioactive compounds present in the acid-soluble pool of VA-13 cells after a 4-hr exposure to 10 μCi/ml ^{3}H-AMS at the final concentration of 340 μM*

Unlabeled AMS, adenosine, AMP, ADP, and ATP were coelectrophoresed with the radioactive sample as markers and were visualized under UV light. Areas 1, 2, and 3 were eluted from the paper and further characterized. The inset is an enlargement of areas 2 and 3.

osine, are not easily separated by paper electrophoresis. The level of adenine methylation was, therefore, determined by paper chromatography in a system that easily distinguished between adenine, N^6-methyladenine and N^6,N^6-dimethyladenine. Areas 1, 2, and 3 resulting from paper electrophoresis of the acid-soluble pool of ^{3}H-AMS-treated cells were eluted, exposed to acid hydrolysis to liberate the bases, and then subjected to paper chromatography as described under Materials and Methods (Fig. 2). The component of the acid-soluble pool that migrated with AMS marker (area 1) in experiments shown in Fig. 1 was shown to contain only N^6,N^6-dimethyladenine (Fig. 2A), indicating that the principal radioactive component of the acid-soluble pool was unchanged AMS. On the other hand, the bases released from areas 2 and 3 of the paper electropherogram proved to be adenine in each case (Figs. 2B and C). Thus, the phosphorylated derivatives of AMS found in the acid-soluble cell extract are mono- and triphosphates of 3′-amino-3′-deoxyadenosine, with possibly an admixture of the diphosphate derivative.

The chromatography system used in these studies separates hypoxanthine from adenine and its methylated derivatives. Since hypoxanthine was not detected on chromatography after acid hydrolysis as described above, deamination of 3′-AmA did not appear to take place in these cells to any significant extent.

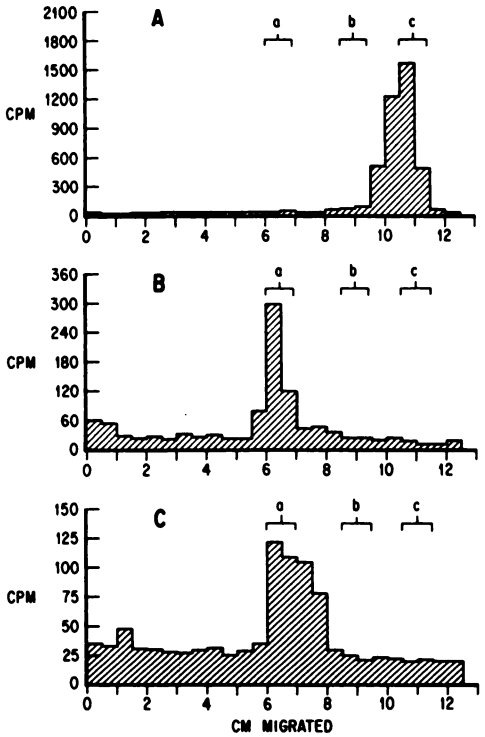

FIG. 2. *Chromatograms of the products of acid hydrolysis of the radioactive compounds eluted from the electropherogram represented in Fig. 1*

(A) Hydrolysate of the compound eluted from area 1 in Fig. 1. (B) Hydrolysate of compound eluted from area 2. (C) Hydrolysate of compound eluted from area 3. Adenine and acid hydrolysates of mono- and di-N^6-methyl derivatives of adenosine were cochromatographed as standards and visualized under UV light. These are identified in the figure as a, b, and c, respectively.

TABLE 2

Distribution of radioactive compounds in paper electrophoregrams of the acid-soluble pool of VA-13 cells treated with AMS

Concentration of AMS (μM)	Time of exposure to AMS (hr)	Percentage of total radioactivity recovered		
		Area 1	Area 2	Area 3
0.066[a]	4	49	29	22
0.066[a]	18	32	48	20
340[b]	4	89	4.4	6.6
340[b]	18	85	8.0	7.0

[a] Cells were exposed to ^{3}H-AMS as described under Materials and Methods for isolation of DNA. Acid-soluble extracts were prepared.

[b] Cells were incubated as described for the preparation of the acid-soluble pool except that one group was incubated with the ^{3}H-AMS for 18 hr. Acid-soluble extracts were prepared as described under Materials and Methods.

The bulk of the experiments reported here were performed in the presence of ^{3}H-AMS added to cold AMS in high concentrations as described in previous work (14, 17). It was also of interest to determine the cellular handling of tracer amounts of the inhibitor. Table 2 shows that when the concentration of AMS in the medium was 0.066 μM, a large proportion (approximately 70% at 18 hr of treatment) was converted to phosphorylated forms. This indicates that demethylation is the principal rate-limiting step in the metabolic conversion of AMS in transformed human fibroblasts.

Incorporation of ^{3}H-AMS into RNA. Incorporation of ^{3}H-AMS into RNA was detected by the alkaline hydrolysis of the PCA pellet. Following repeated washings and reprecipitations to remove noncovalently bound AMS, the pellet was hydrolysed in 0.3 M KOH. The hydrolysis liberated a compound which migrated with marker AMS (Fig. 3). The monophosphate peak, which would be released by alkaline hydrolysis if the labeled compound was incorporated into internal positions in the polynucleotide, was not found. The compound migrating with AMS was eluted from the electrophoretic strips, digested with 1 N HCl at 100° for 1 hr and examined by paper chromatography (Fig. 4). The cleavage product migrated with adenine. This shows that the demethylated derivative of AMS is capable of being phosphorylated and incorporated into RNA. Alkaline hydrolysis releases its nucleoside form from the 3′-terminal position of the polyribonucleotide.

Incorporation into DNA. When DNA isolated from cells incubated with ^{3}H-AMS was digested with DNase I and snake venom phosphodiesterase, radioactive compounds were not detected on high voltage paper electrophoresis. Thus, AMS derivatives do not appear to be incorporated into the DNA of transformed fibroblasts.

DISCUSSION

The sequence of metabolic conversions of AMS in transformed human fibroblasts appears clear (Fig. 5). This adenosine analog enters the cell, perhaps by facilitated diffusion as do other purine nucleosides (18), and becomes N^6-demethylated. The enzyme system for this demethylation, or whether the intermediate N^6-monomethyl derivative is formed, is not known, but microsomal methylating systems perform this function in liver

cells (19). The demethylating system in the fibroblasts appears to be easily saturated and when its capacity is exceeded, unchanged AMS remains in the cell and is not otherwise processed, thus not having any effect on RNA synthesis.

The demethylated derivative of AMS, 3′-AmA, is efficiently phosphorylated to 5′-monophosphate and the corresponding 5′-triphosphate, so that only phosphorylated forms of 3′-AmA can be detected in the cell. It has been previously shown that in the presence of ATP, myokinase and adenosine kinase, 3′-AmA is converted to its 5′-triphosphate (20), and a similar system may be operating in the transformed fibroblasts. This triphosphate then becomes an acceptable substrate for the RNA polymerases (Figs. 3 and 5), but not for the DNA polymerase. Inhibition of RNA synthesis is thus perceived as a direct

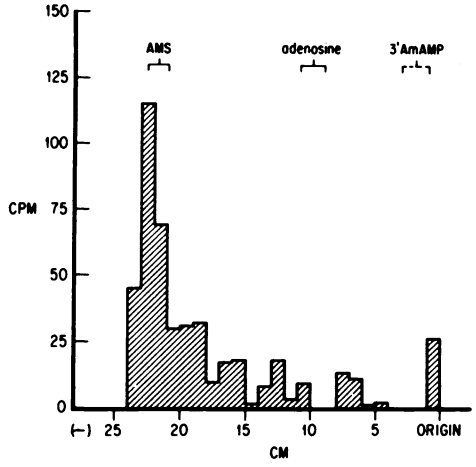

FIG. 3. *Electropherogram of the alkaline hydrolysate of the PCA pellet obtained from cells exposed to 10 μCi/ml of ^{3}H-AMS (together with 340 μM unlabeled AMS) for 4 hr*

Unlabeled AMS and adenosine were coelectrophoresed with the radioactive sample as markers and visualized under UV light. 3′-AmAMP denotes the area to which the monophosphate of 3′-AmA, in the acid-soluble pool, migrated in other electropherograms. No significant radioactivity was found in this area in this case.

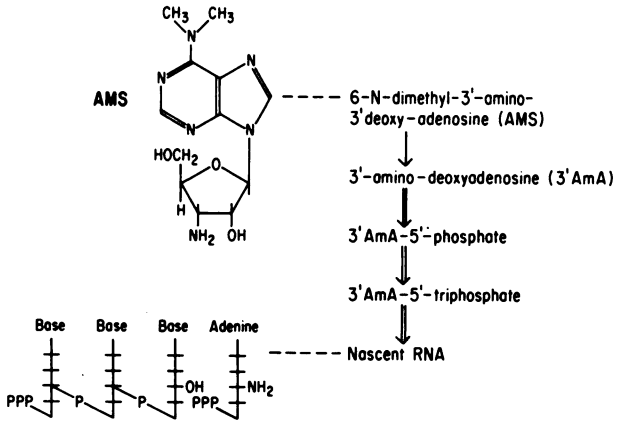

FIG. 5. *Structural formula of AMS and its postulated conversions in transformed fibroblasts, together with a representation of the growing (3′-OH) end of the nascent RNA chain and the incoming amino sugar-containing analog*

effect of the incorporated AMS derivative, and the effect on DNA replication, observed after prolonged treatment of transformed cells with high concentrations of AMS, seems likely to be a consequence of impaired transcription of RNA. However, inhibition of the DNA polymerase reaction cannot be excluded at this stage.

The above scheme for the inhibitory effects of AMS on RNA synthesis is based on data presented in this report, but is to a large extent also supported by results of previous work in other laboratories. However, the approach utilized by us and the availability of radioactive AMS uncontaminated by adenosine permitted unequivocal demonstration of incorporation of the demethylated derivative of AMS in 3′-terminal positions of RNA.

Farnham and Dubin investigated the incorporation of ^{14}C-AMS into the RNA of mouse fibroblasts (9). They found a small amount of 3′-AmA in the RNA of AMS-treated cells, but 95% of the radioactivity released by alkaline hydrolysis of this RNA was due to radioactive AMS. They concluded that since the AMS was most probably not covalently linked to the RNA but bound by ionic forces, the 3′-AmA was probably also a contami-

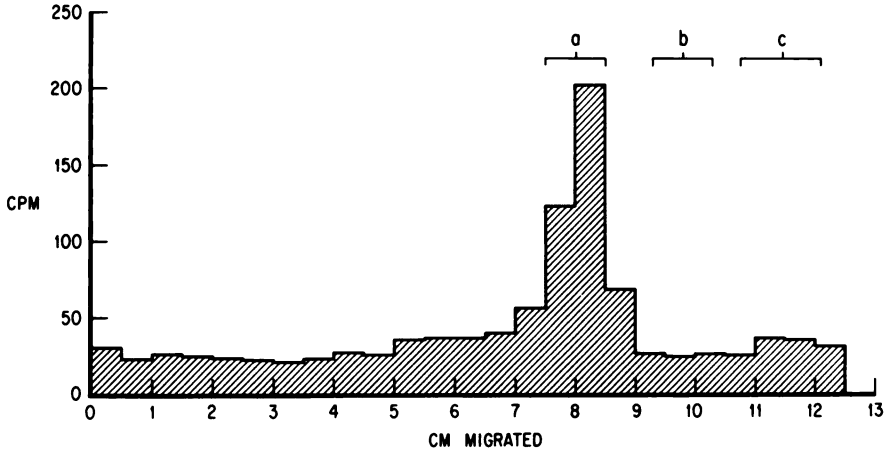

FIG. 4. *Chromatogram of the products of the acid hydrolysis of the radioactive compound eluted from the electropherogram of the alkaline hydrolysate of the PCA pellet of VA-13 cells shown in Fig. 3*

Adenine and acid hydrolysates of mono- and di-N^6-methyl derivatives of adenosine were cochromatographed as markers and visualized by UV light. These are identified in the figure as a, b, and c, respectively.

nant. We were able to separate the RNA from any such ionically bound AMS by repeated PCA precipitation of the RNA dissolved in cold NaOH. Since no AMS was detected in our preparations after alkaline hydrolysis of the RNA, the 3′-AmA recovered should represent material incorporated into RNA.

Methylation level of purine derivatives has been shown to be important for inhibition of RNA synthesis by these analogs in L cells. A marked inhibition of RNA synthesis was observed after treatment with AMS, which appeared to be demethylated in the cell, since phosphorylated 3′-AmA derivatives were tentatively identified in the acid-soluble pool of the treated cells (9). On the other hand, Ehrlich ascites cells have been reported to be relatively insensitive to inhibition of RNA synthesis by AMS and not to demethylate AMS, but when these cells were treated directly with 3′-AmA RNA synthesis was markedly inhibited (20). Similarly, Ehrlich ascites cells were found to be incapable of demethylating 6-methylaminopurine-3′-deoxyriboside and this compound produced only a minimal inhibitory effect on RNA synthesis (21), while KB cells, sensitive to the drug, demethylated it to the potent inhibitor of RNA synthesis, 3′-deoxyadenosine (22). It appears that N^6-methylated adenosine analogs such as AMS are effective RNA inhibitors only if the treated cells have the ability to remove the methyl groups of these compounds. The demethylated analogs can then be phosphorylated and incorporated into RNA.

We found no evidence of direct phosphorylation of AMS. This is in line with the finding that AMS was not a substrate for the adenosine kinase from rabbit liver, while 3′-AmA was found to be readily phosphorylated (23) and a potent inhibitor of RNA synthesis, by virtue of its 3′-amino group which is thought to prevent further addition of nucleotides to the growing RNA chain.

Several previously observed features of the inhibitory action of AMS can now be understood. For instance, the relatively slow onset of its action (4), the cytostatic rather than cytotoxic effect of high concentrations of AMS (17), and the persistence of the inhibitory effect in nuclei or nucleoli isolated from AMS-treated cells (14), all now become logical consequences of the fact that AMS has to be converted into a form capable of inhibiting RNA transcription, that this process has a limited capacity (especially the demethylation step), and that the stable attachment of the AMS derivative to nascent RNA chains may cause difficulty in reutilization of many of the units of transcription, particularly in the nucleolus. Hopefully, this knowledge will permit us to analyze the differential effect of AMS on normal, as contrasted with transformed, cultured cells. Such studies are in progress.

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