RELATIONSHIP BETWEEN RNA SYNTHESIS, CELL DIVISION, AND MORPHOLOGY OF MAMMALIAN CELLS

I. Puromycin Aminonucleoside As An Inhibitor of RNA Synthesis and Division in HeLa Cells

GEORGE P. STUDZINSKI and KAY A. O. ELLEM

From the Department of Pathology, Jefferson Medical College, Philadelphia, Pennsylvania

ABSTRACT

Logarithmically growing HeLa cell monolayers were treated with a range of concentrations of puromycin aminonucleoside (AMS). The effects of AMS were studied by the following means: microscope examination of treated cells; enumeration of the cell number using an electronic particle counter; analyses for DNA, RNA, and protein content; incorporation of P^{32} and H^3-thymidine into nucleic acids; and fractionation of nucleic acids by column chromatography. Taking the rate of incorporation of the isotopic precursor as a measure of nucleic acid synthesis, it was found that concentrations of the inhibitor which had a rapid effect on the rate of cell division inhibited the synthesis of all types of nucleic acids and of protein, but depressed ribosomal RNA synthesis most markedly. Lower concentrations of AMS selectively inhibited ribosomal RNA and, to a lesser extent, transfer RNA synthesis. Partial inhibition of ribosomal RNA synthesis with low doses had no effect on the rate of cell division within the period studied (3 generation times). The cell content of RNA returned to normal when the inhibitor was removed.

INTRODUCTION

Hydrolytic cleavage of puromycin produces a nucleosidelike fragment structurally analogous to adenosine and known as aminonucleoside of puromycin (AMS) (Fig. 1). This compound shares some of the biological properties of puromycin, notably its antitrypanosomal and antiparasitic effects (17, 28), and is even more effective than the parent compound in the production of the nephrotic syndrome in the rat (14). The potential clinical importance of this finding stimulated intensive investigations of the action of AMS, but, although several plausible hypotheses of the mechanism of its action have been advanced (3, 4, 9, 20), there is, as yet, no general agreement on the biochemical basis for the observed biological effects of this antimetabolite. It is clear, however, that, unlike puromycin, AMS is not a specific inhibitor of protein biosynthesis (1, 18, 27, 31). Work with isolated mammalian mitochondria indicates that AMS may interfere with phosphorylation mechanisms (3, 4), and experiments with *E. coli* show that AMS inhibits nucleic acid synthesis in this or-

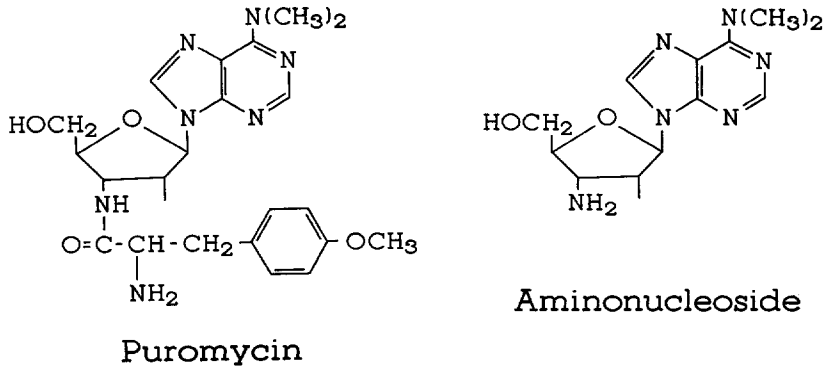

FIGURE 1 Comparison of the structural formulas of puromycin and aminonucleoside.

ganism (9), probably as a consequence of its inhibitory action on purine biosynthesis.

The experiments reported here were performed on HeLa cells, and it was found that, while high concentrations of AMS inhibited the synthesis of all types of nucleic acids and of protein, lower concentrations of AMS produced selective inhibition of RNA synthesis. In particular, ribosomal RNA (rRNA) synthesis was most markedly inhibited, with lesser inhibition of transfer RNA (sRNA), while DNA-like RNA (D-RNA) moieties were least affected. It was also shown that when AMS was used in concentrations which produced only partial depression of ribosomal RNA synthesis, HeLa cells continued to divide at the control rate, although their RNA content was decreased.

The biochemical and growth experiments to be presented here were performed together with cytochemical studies of the inhibited cells, and the next paper in this series will describe the morphological aspects of this work. These results have already been briefly reported (32, 34).

METHODS

Tissue Culture

A wild strain of HeLa cells originally obtained from Dr. J. J. Freed was grown in Petri dishes in a humidified CO_2 incubator as described previously (33). Some of the Petri dishes contained coverslips which were used for staining and microscope examination of the cell monolayer growing on them. For biochemical and growth studies the cells were harvested from Petri dishes, and the cells in each experimental group were pooled. The cell number was obtained by counting three aliquots from the pooled suspension in an electronic particle counter (Model B, Coulter Electronic Co., Hialeah, Florida). The observations reported here were made on cells in the logarithmic phase of culture growth. AMS and puromycin dihydrochloride (Nutritional Biochemical Co., Cleveland, Ohio) were added to the cultures dissolved in the medium.

Biochemical Procedures

Nucleic acids were estimated by a modified Schmidt-Thannhauser procedure (13), scaled down to require approximately a million cells per sample. Protein content was measured by the method of Lowry (25), using crystalline egg albumin as standard. Nuclei were obtained by treatment with cold citric acid, with the precautions described previously (33). For column chromatography of nucleic acids, the cells were exposed to P^{32}-labeled orthophosphate for different time periods as indicated in the results. After labeling, the cells were lysed into a 2% solution of sodium dodecyl sulphate and extracted twice with phenol (11), the interphase being removed with the aqueous phase after the first extraction, and left with the phenol phase after the second extraction. The nucleic acids were precipitated and chromatographed on methylated bovine serum albumin-Kieselguhr (MAK) columns, essentially as described previously (11). It was found, after exposure to AMS, that the P^{32}-labeled nucleic acid preparation extracted from the cells contained large amounts of low molecular weight phosphorylated materials. These caused unduly high backgrounds of acid-soluble isotope, thus contaminating the eluted nucleic acids. To eliminate this, the solutions of the nucleic acids from both controls and AMS-treated cells were passed through a G-25 Sephadex column equilibrated with 0.3 M Tris buffered sodium chloride (pH 6.7) containing 10^{-3} M EDTA, at 0°C, before application to the MAK column.

The eluted nucleic acids were located by continuous UV and scintillation-scanning devices recording on a two-pen chart recorder equipped with an event

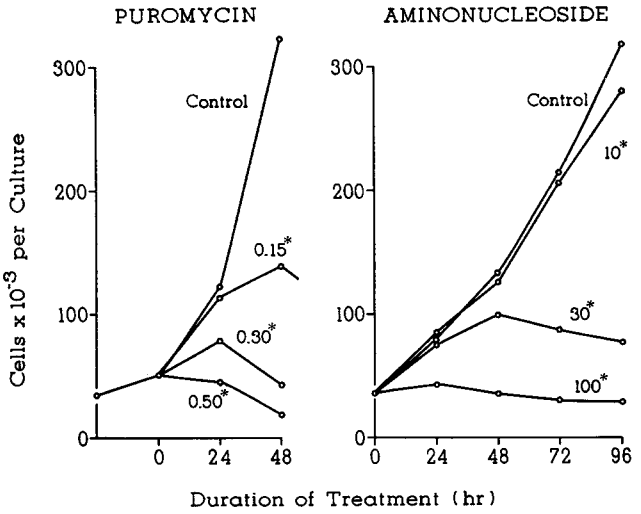

FIGURE 2 Inhibition of division of HeLa cells by aminonucleoside compared with the inhibitory effect of puromycin. On molar basis, aminonucleoside is approximately 200 times less powerful inhibitor of cell division than puromycin. Concentrations of aminonucleoside of 3 μg/ml or less had no discernible effect on the rate of cell division in this group of experiments, each performed in triplicate.

* μg/ml.

marker. The tubes containing the respective nucleic acids were pooled, the volumes measured, and the optical density at 260 mμ read. The P^{32} incorporated into each fraction was determined on aliquots in a low background end-window counter. For studies involving the use of H^3-thymidine, replicate plates were exposed to medium containing 16.5 μM thymidine and 4 μc of H^3-thymidine per ml for the same time as the parallel P^{32} exposure (60 min). The thymidine was used at this concentration to ensure "saturating concentration" conditions when the rate of thymidine incorporation is independent of its concentration (8, 16). The medium was removed and the cell monolayer washed with Hanks' balanced salt solution. The cells were harvested into a solution of EDTA in balanced salt (26), and then centrifuged at 3000 RPM for 5 min. The pellet was extracted five times with 5% trichloroacetic acid, digested with 0.5 N KOH for 20 hr at 37°C, and finally precipitated with 0.6 N perchloric acid. The DNA-protein pellet was extracted with 0.6 N perchloric acid at 90°C for 20 min, and the washings of the protein pellet were combined with the extract. Aliquots of all the solutions were then counted in Bray's solution (6) in a liquid scintillation spectrometer. The quenching of all the DNA samples was found to be the same, so that no correction was made for it. The DNA content was estimated by Burton's procedure (7).

RESULTS

Inhibition of Cell Division by Aminonucleoside

Puromycin aminonucleoside was a less powerful inhibitor of HeLa cell multiplication than puromycin (Fig. 2). Concentrations of 3 μg/ml (1.02×10^{-5} M) or less did not inhibit the increase in the number of cells per culture within the time period studied (ca. 100 hr). Ten μg/ml allowed the normal growth rate to proceed for 72 hr, after which time there was partial inhibition of cell division. Concentrations of the inhibitor higher than 10 μg/ml led to retardation of cell division which was apparent at 24 hr of treatment. This was followed, after an interval, by a decrease in the cell number per culture, indicating that cell detachment and

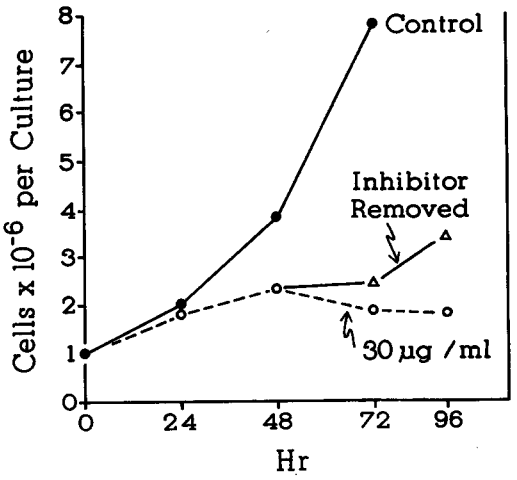

FIGURE 3 Reversibility of the growth inhibitory effect of aminonucleoside. If aminonucleoside is removed after 48 hr of treatment, HeLa cells resume growth after a lag period of approximately 24 hr.

TABLE I

Effect of Aminonucleoside on the Distribution of Mitotic Stages in Cultures of HeLa Cells

Treatment of the cultures	Total No. of mitotic figures per 1000 cells*	Distribution of mitotic stages‡			
		Prophase	Metaphase	Anaphase	Telophase
None	25.5	12	59	6	23
30 μg AMS for 24 hr	25.0	4	40	4	52
None	38.0	13	52	14	21
100 μg AMS for 24 hr	15.0	14	23	13	50

* Arithmetical means ot counts of 1000 mononucleated cells in three replicate cultures.
‡ % of the total number of mitotic figures.

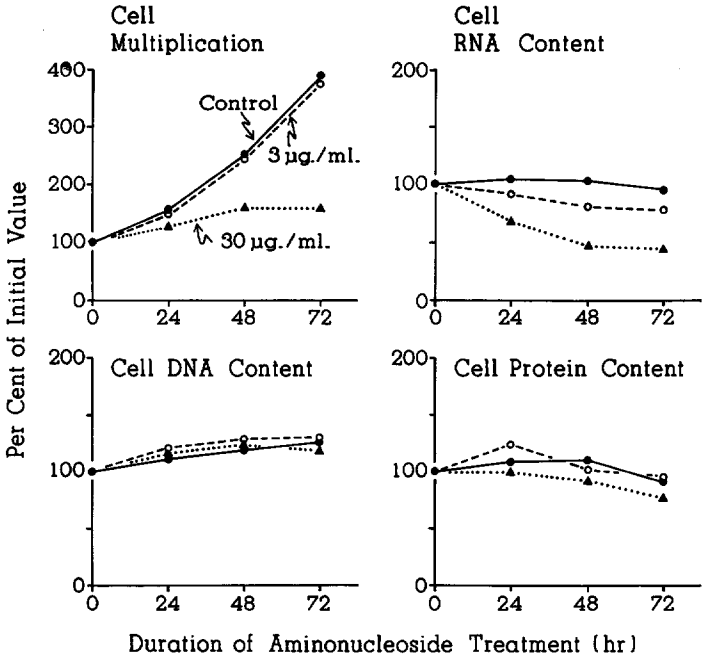

FIGURE 4 Effects of aminonucleoside on HeLa cell multiplication and macromolecular syntheses. Cell DNA content is not affected, protein content is lowered slightly by growth inhibitory concentrations of aminonucleoside, but cell RNA content is markedly affected, even at concentrations which have no effect on the rate of cell multiplication. The values are arithmetical means of four experiments which showed essentially the same pattern in each case.

degeneration exceeded the rate of cell multiplication.

The inhibition of cell division produced by AMS was not a result of processes immediately lethal to the cell, since established growth inhibition could be reversed by removal of the medium containing AMS and substitution of a normal medium (Fig. 3). The resumption of cell multiplication took place rather slowly at first, but attained the pretreatment rate 48 hr after the removal of the inhibitor.

Microscope examination of treated cultures showed that AMS was not a spindle poison nor did it cause visible chromosome damage. Thus, metaphase arrest was not seen, abnormal mitoses were encountered with a frequency no higher than in

TABLE II

Comparison of the RNA Content of Isolated Nuclei and Unfractionated Cells Treated with Aminonucleoside for 48 hr

	Whole cells		Isolated nuclei	
	μg RNA/10 μg DNA*	% of control	μg RNA/ 10 μg DNA*	% of control
Control	33.6	100.0	8.2	100.0
AMS 3 μg/ml	25.0	74.5	6.2	75.6
AMS 30 μg/ml	18.1	53.9	4.1	50.0

* Arithmetical means of values obtained in three separate experiments.

the control cultures, and chromosome clumping or lagging chromosomes was rare. A few mitotic figures could be seen even after prolonged treatment with high doses of AMS, when the cell numbers per culture were decreasing e.g. 72 hr of treatment with AMS at 100 μg/ml. In some experiments, treatment with growth-inhibitory concentrations of AMS resulted in a proportion of mitotic figures greater than would be expected from the culture growth rate or the rate of DNA synthesis. Differential count of the various mitotic stages showed a higher proportion of telophases in such cultures (Table I), indicating that the relatively high number of mitotic figures noted was caused by slowing of the last stage of mitosis, or a partial telophase arrest.

Effects of Aminonucleoside on Nucleic Acid and Protein Metabolism Studied by Biochemical Methods

Effects on Cell Nucleic Acid and Protein Content

When HeLa cell monolayers were treated with concentrations of AMS ranging from 3μg/ml to 100 μg/ml (1.02×10^{-5} to 3.40×10^{-4} M) and the cultures were analyzed at intervals for their content of DNA, total RNA and total protein, it was found that the antimetabolite produced inhibition of synthesis of DNA and protein in proportion to the severity of inhibition of the rate of cell division. Consequently, the cell content of DNA and protein remained close to the value obtained for cells from untreated cultures (Fig. 4). In contrast, inhibition of RNA production was considerably more marked, being apparent after treatment with concentrations of AMS which produced no inhibition of the growth rate, and this resulted in a consistent decrease in RNA content per cell (Fig. 4). Higher concentrations of AMS lowered cell RNA content to approximately 50% of the control value (Table II).

The constancy of DNA content per cell during AMS treatment allowed the use of DNA as a reference in terms of which other cell constituents could be expressed. The ratio of the other cell values to DNA then becomes equivalent to expressing these values on a per cell basis. This feature was, therefore, utilized for presentation of subsequent results.

Unlike the DNA content, the protein content of AMS-treated cells was not invariant, but the decrease was only slight and observed only after the higher concentrations of AMS (Fig. 4). The results shown in Table III demonstrate that the total protein content of cultures could increase for a time under conditions in which there was no net RNA synthesis (AMS at 30 μg/ml). Furthermore, net protein synthesis could proceed at the control rate when the net synthesis of RNA was partially inhibited by AMS at 3 μg/ml. Cell samples from AMS-treated cultures gave a spuriously high color by the Lowry method if not extracted with TCA in the Schmidt-Thannhauser procedure, indicating

TABLE III

Effects of Aminonucleoside on Increases in Net RNA and Protein Contents of HeLa Cell Cultures

	μg RNA per culture		μg Protein per culture	
	Content*	Increase	Content*	Increase
		%		%
Content at the beginning of the experiment	60.6		454	
After 24 hr of treatment with:				
No treatment	111.2	83	806	78
AMS 3 μg/ml	102.8	69	820	81
AMS 10 μg/ml	91.7	51	795	75
AMS 30 μg/ml	60.7	0	625	38

* Arithmetical means of values obtained in four experiments.

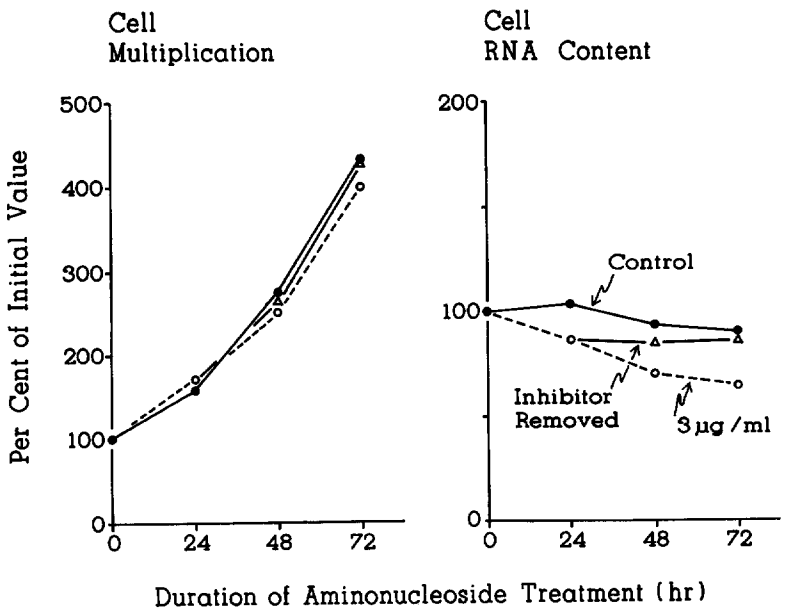

FIGURE 5 Readjustment of cell RNA content after removal of aminonucleoside. When the inhibitor is replaced by normal medium, the cell RNA content returns gradually to the control level, but these alterations in the rates of RNA synthesis do not affect the rate of cell division. Mean values of three separate experiments are shown in the charts.

an accumulation of an interfering substance as the result of AMS action.

Analyses performed in parallel on isolated nuclei and on unfractionated cells showed that the loss of RNA per cell or per nucleus was proportionately the same (Table II). This showed by inference that the decreases in RNA content in the cytoplasm and in the nucleus of the cell were equal. 50% appeared to be the maximal value for the decrease in RNA without incurring the loss of structural integrity, since increasing the concentration of AMS or the duration of treatment failed to lower the RNA content of the surviving cells below this level.

When AMS was removed from the cells' environment, the RNA content returned to the control value. For instance, cultures treated with AMS at 3 µg/ml continued to grow at the control rate, although the RNA content became lowered; removal of AMS from such cultures resulted in the return of cell RNA content to the control value (Fig. 5).

SELECTIVITY OF AMS EFFECT ON THE NUCLEIC ACIDS

The amount of radioactively labeled precursor incorporated into the different types of nucleic acid in a given time was used to estimate the rate of synthesis of the different nucleic acids. Different precursors were used to check the possible effect of AMS on pool size. The nucleic acids were separated in order of elution from the column into sRNA, DNA, ribosomal and ribosomal precursor (Q_1) RNA, and two types of DNAlike RNA—one eluted by salt (Q_2-RNA) (11, 36), and the second major part tenaciously bound to the MAK and in need of vigorous elution methods (D-RNA) (11). D-RNA was eluted with 2% sodium dodecylsulphate in 0.05 M Tris-HCl pH 6.7 in two steps (at 35°C and the remainder at 70°C) rather than with hot saline as was previously used (11).

Fig. 6 illustrates the patterns of incorporation of P^{32}, during a 1-hr period, into control cells and cells treated for 48 hr with a relatively low concentration of AMS. The most notable effect was the relative depression of incorporation into ribosomal and ribosomal precursor (Q1) RNA. When the incorporation of P^{32} was measured (Table IV), it was found that, relative to that of DNA, the labeling of the ribosomal-Q1 complex had decreased some 2- to 4-fold in different experiments as a result of AMS treatment, while sRNA was less affected, and the DNAlike RNA fractions were

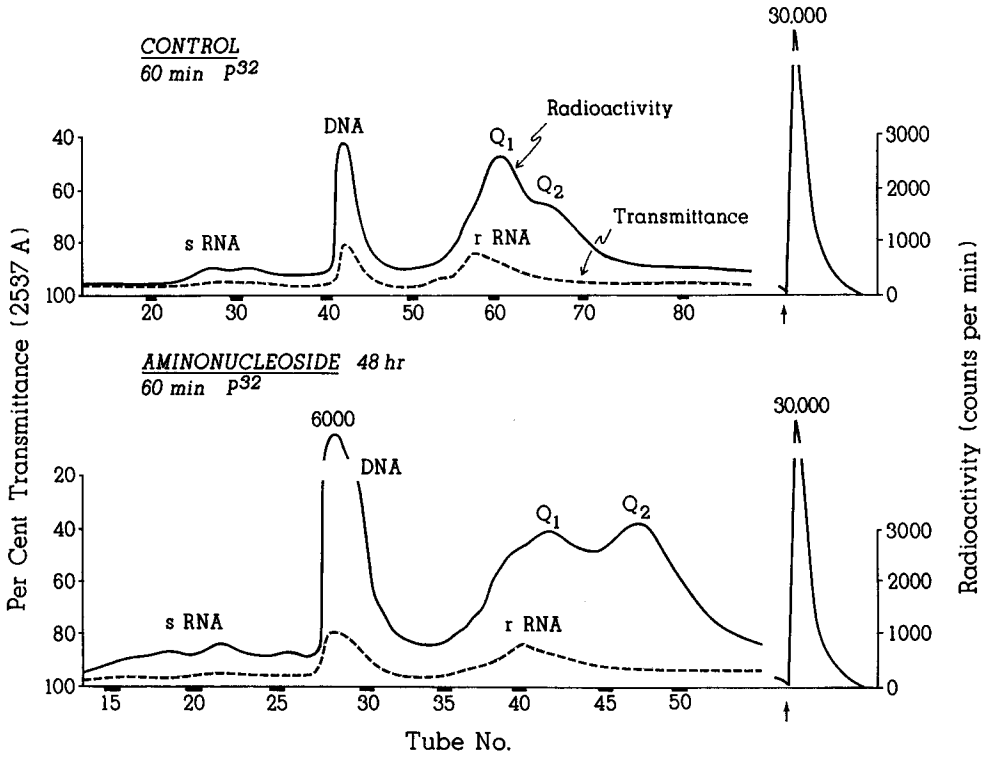

FIGURE 6 Actual tracings of the chart records of the nucleic acids eluted from MAK columns with a linear gradient of NaCl from 0.3 M to 1.6 M. The linear gradient was terminated at the points indicated by arrows, and tenaciously bound D-RNA was eluted in two steps, only the first of which is shown in the figure. Since different amounts of nucleic acid were chromatographed and different volumes of salt used to elute them, the magnitudes of the values on the chart are not comparable. The important feature is the relative depression of P^{32} incorporation into the ribosomal-Q_1 complex, relative to DNA and Q_2, of the nucleic acids from the cells treated with AMS (6 μg/ml).

least affected. However, it was found that the amount of incorporation of P^{32} into DNA and D-RNA during short exposures to the radioactive precursor (1 hr, which is approximately $\frac{1}{36}$th of the cell-doubling time) was actually increased relative to the control cells, despite the independently demonstrable decrease in net synthesis of RNA (Table III).

As an additional measure of nucleic acid synthesis, it was decided to observe the incorporation of H^3-thymidine into DNA in replicate dishes, in parallel with those used for P^{32} incorporation. It is clear from Table V that AMS alters the relative incorporation rates of P^{32} and H^3-thymidine into DNA. AMS at a moderate concentration, which caused the gradual inhibition of cell multiplication, resulted in progressive decrease in the amount of incorporation, in 1 hr, of the H^3-thymidine into DNA (Table V, DNA values in columns headed "Corrected"). The effect was small at 24 hr (15.4% inhibition) but marked after 72 hr (49.0% inhibition). P^{32} incorporation, while depressed at 24 hr, actually increased above control levels at 48 and 72 hr, as was also seen in Table IV. Since the cell growth at this concentration was depressed after 24 hr, H^3-thymidine incorporation indicated the state of DNA synthesis, while the the P^{32} incorporation was altered by changes either in the purine pool size or, perhaps, in the uptake of P^{32} into the cells in the presence of AMS, such as has been seen with 3-'deoxyadenosine (22). By assuming that the incorporated P^{32} was present in ribotides with the same activity as in deoxyribotides, though it must be admitted that support for this assumption is not at present available, the percentage incorporation of

TABLE IV

Effects of Aminonucleoside on P^{32} Incorporation‡ into HeLa Cell Nucleic Acids*

Nucleic acid	Control		AMS 24 hr		AMS 48 hr	
	P^{32} Incorp. CPM/OD DNA§	Incorp. rel. to DNA Incorp.	P^{32} Incorp. CPM/OD DNA	Incorp. rel. to DNA Incorp.	P^{32} Incorp. CPM/OD DNA	Incorp. rel. to DNA Incorp.
sRNA	5,137	0.82	5,840	0.28	6,486	0.47
DNA	6,279	1.00	20,734	1.00	13,741	1.00
r + Q_1RNA‖	21,670	3.45	16,222	0.78	12,085	0.88
D-RNA**	36,243	5.77	75,874	3.66	63,561	4.63

* Concentration of AMS was 10 μg/ml.

‡ Incorporation during 70-min exposure to isotope at 160 μc/ml.

§ The values for the isotope incorporation were normalized by dividing the measured values by the total DNA recovered from the MAK column; they are thus expressed as counts per minute of P^{32} per optical density unit (260 mμ) of DNA.

‖ Ribosomal + ribosomal precursor RNA.

** DNA like RNA is the sum of Q_2 and tenaciously bound RNA.

TABLE V

Effects of Aminonucleoside on Rates of Synthesis of Nucleic Acids of HeLa Cells*

Nucleic acid‡	Control P^{32} Incorp. CPM/OD DNA	Incorporation of P^{32} as % of control incorporation					
		AMS 24 hr		AMS 48 hr		AMS 72 hr	
		Observed§	Corrected‖	Observed	Corrected	Observed	Corrected
sRNA	3,087	49.5	68.3	124.1	47.9	106.4	33.6
DNA	6,765	61.4	84.6	194.1	74.9	161.3	51.0
r + Q_1RNA	13,811	29.9	41.3	83.9	32.4	78.2	24.7
D-RNA	39,616	69.8	96.2	142.5	55.0	135.0	42.7

* Duration of isotope incorporation was 60 min. P^{32} concentration was 160 μc/ml; AMS concentration was 6 μg/ml. The batch of AMS used in this experiment proved to have markedly higher potency than the batches of AMS purchased previously and used in other experiments reported here.

‡ Designation of nucleic acid fractions as in Table IV.

§ Incorporation of P^{32}.

‖ Correction based on H^3-thymidine uptake. The DNA % incorporation was the actual incorporation of H^3-thymidine observed, and the RNA % incorporation values were adjusted by the factor of difference between the P^{32} and H^3-thymidine incorporation values for DNA.

P^{32} into RNA was corrected by making the same fractional adjustment to the observed values as was necessary to correct the P^{32} incorporation figures for DNA to those observed with H^3-thymidine. The corrected RNA synthesis rates show that the inhibition of the different species was greatest in ribosomal RNA and least in DNAlike RNA. At 24 hr of AMS treatment, ribosomal RNA synthesis was decreased more than twofold, while DNAlike RNA was still being synthesized at the control rate.

The effect of AMS was not immediate. After 1 hr of exposure to 10 μg/ml, the P^{32} incorporated during a further 40 min showed the values for all nucleic acids to be within 5% of the control values, with the exception of the ribosomal complex, which exhibited a scarcely significant 8% depression.

Correlation of the Observed Net Synthesis of RNA with Isotopically Determined Rates of Synthesis

A calculation was made to check whether the proposed reduction in the rate of rRNA synthesis was commensurate with the observed net reduction of

TABLE VI

Correlation of Net RNA Synthesis with the Rate of Incorporation of Isotope into RNA

	sRNA	Ribosomal RNA	DNA-like RNA
Proportion of the species of RNA as percentage of total RNA*	8.8	81.0	10.2
Expected increase in 24 hr in untreated culture‡	$8.8 \times \frac{83}{100} = 7.3$	$81 \times \frac{83}{100} = 67.1$	$10.2 \times \frac{83}{100} = 8.5$
Rate of incorporation of P^{32}§ as a percentage of control incorporation‖	$68.3 \times \frac{6.03}{6.40} = 64.4$	$41.3 \times \frac{6.03}{6.40} = 38.9$	$96.2 \times \frac{6.03}{6.40} = 90.6$
i.e. increase in 24 hr in AMS-treated culture	$7.3 \times \frac{64.4}{100} = 4.7$	$67.1 \times \frac{38.9}{100} = 26.2$	$8.5 \times \frac{90.6}{100} = 7.8$
i.e. increase in total culture RNA calculated from the rate of incorporation of P^{32}	$4.7 + 26.2 + 7.8 = 38.7\%$		
Increase in culture RNA found by Schmidt-Thannhauser analysis		44.0%	

* Calculated from data presented reference 10.

‡ It was found by direct analysis that untreated HeLa cells of this strain synthesize 83% of their RNA in 24 hr (*See* Table III). The values are expressed at this stage as % increase, taking the value for total RNA of untreated cells at 0 time as 100.

§ From Table V.

‖ The rates are corrected for the fact that treated cultures in this experiment contained 6.03×10^6 cells, while controls contained 6.40×10^6 cells.

cell RNA. The value for RNA content of cells treated with AMS obtained by direct analysis was close to that calculated from the rate of incorporation of the isotope (Table VI). This makes it unlikely that there was an increase in the breakdown of ribosomal RNA present in the cells prior to the AMS treatment. It does not, however, eliminate the possibility that there was an increase in the rate of degradation specifically of the newly synthesized ribosomal RNA, producing the greater depression of isotope accumulation observed in this fraction, as well as the depressed net synthesis. Nevertheless, the simplest interpretation of the depressed isotope incorporation is that the synthesis of the various RNA types has been inhibited, and that ribosomal RNA has been more inhibited than the other nucleic acids.

DISCUSSION

The studies reported here demonstrate that AMS acts as an inhibitor of RNA synthesis in the HeLa cell. When the concentration of the compound is relatively low, the inhibition of RNA synthesis is selective, affecting principally the synthesis of ribosomal types of RNA. Higher concentrations (> 10 μg/ml) inhibit, in addition, the synthesis of DNA and other types of RNA, and subsequently a decrease in protein synthesis is noted. These observations on cultured cells derived from a human carcinoma are similar to the finding of van Meter et al., in 1956, that the RNA content of mammary tumors obtained from mice treated with AMS is considerably lower than that of tumors from untreated mice (35). Quite recently, Farnham found that AMS inhibited incorporation of uridine by cultured L cells under conditions in which uptake of thymidine and leucine into macromolecules was not inhibited (12). This provides an independent line of evidence that AMS selectively inhibits RNA synthesis in established lines of cultured mammalian cells.

A rather unexpected finding is the ability of

HeLa cells to continue their multiplication at an unimpaired rate while the syntheses of ribosomal RNA and, to a lesser extent, transfer RNA are being depressed by AMS. This indicates that there is some reserve potential in the machinery for protein synthesis of the HeLa cells, since the protein content of cells treated in this manner is not depressed. A degree of stability of the apparatus for protein synthesis is shown by treatment with higher concentrations of AMS, which result in complete inhibition of net RNA synthesis: the protein content of such cultures continues to increase for a time (Table III).

While the continuation of the normal growth rate in the face of diminished RNA content of HeLa cells may appear to contrast with the situation in bacteria, in which the total RNA content per cell closely parallels the growth rate (21, 30), this may be due to the big difference in time parameters between the microbial and metazoan cell growth cycles. Thus, in the bacterial systems the growth rates used in the studies represent the steady-state situation, several generations after the growth-controlling environment has been established (29). In the present system, growth is observed for approximately 2 generations after the modulating conditions have been introduced. Cells grown as monolayers cannot be maintained in uniform logarithmic growth for more than 3 or 4 generations, so that steady-state conditions with regard to growth rate and RNA content are not achieved. On the other hand, our results are closely parallel to the finding of Lederberg and Mazia (23) that, in media deficient in uracil but otherwise complete, *Tetrahymena pyriformis* continues to make protein, divide, and form normal cells during 20 hr of uracil deprivation, although its net RNA content does not increase. Another analogy, with respect to the case of the HeLa cell dividing normally while its production of ribosomal RNA is depressed, is afforded by the sea urchin oocyte, which upon fertilization forms new proteins and divides to the four-cell stage without any significant synthesis of RNA, and grows to the 32-cell stage with only the production of "messenger" RNA and additions of terminal nucleotides to transfer RNA (15). These instances and the observations reported here show that the cell content of ribosomal RNA does not necessarily determine the rate of growth or the rate of division of at least some metazoan cells.

There does seem to be a limit on the reduction in RNA content that can be produced in HeLa cells, and this limit is close to 50% of the RNA content of untreated cells. In contrast with the findings obtained after treatment with puromycin, in which case marked decrease of cytoplasmic RNA content is accompanied by only a minimal change in nuclear RNA (33), the depression in RNA content after treatment with AMS is proportionately just as marked in the nucleus as in the cytoplasm. This conclusion is subject to the reservation that losses of RNA from the nucleus during isolation are greater in nuclei from the AMS-treated cultures than in nuclei from controls, although the different effects noted with the related inhibitor puromycin makes this unlikely.

The present observations on the different directions taken by the change of rate of incorporation of two different nucleic acid precursors as a result of AMS treatment emphasize the need for coordinated net synthesis and multiple precursor studies for the determination of rates of synthesis. Although the synthesis of DNA was found to be decreased, as indicated by both H^3-thymidine incorporation and the measurement of net synthesis, the rate of incorporation of P^{32} was actually increased. The increased P^{32} incorporation could be the result of at least two possible changes: the rate of P^{32} entry into the cell may have been markedly increased, and fluctuations in the nucleotide precursor pools may have facilitated P^{32} entry into the nucleic acids. Klenow and Overgaard-Hansen (22) have observed the stimulation of P^{32} incorporation into the DNA of Ehrlich ascites tumor cells incubated with low concentrations of a similar inhibitor, 3′-deoxyadenosine (cordycepin). In this case, they attributed the more rapid labeling to a more rapid equilibration of the phosphorus of the nucleotide pool with that of the isotope in the suspending fluid (four times faster in the 3′-deoxyadenosine–treated cells). This inhibitor, like AMS, also decreases the size of the pool of adenine derivatives in the cell, and diminishing supplies of precursors could be expected to result in a slowing down of the macromolecular synthesis dependent upon them. The actual rate of isotope incorporation will thus be the algebraic sum of these two opposed factors. A third complexity in the interpretation of these results may arise from intracellular compartmentation of acid-soluble pools, for which there is increasing evidence (2, 5, 19, 24). In particular, Behki and Schneider's (5) demonstration that the acid-soluble deoxyribosidic compounds are present in a nuclear compartment and Letnansky's find-

ings (24), which suggest that there are separate ATP pools which equilibrate with exogenous phosphate at different rates, raise the possibility that depletion of the intranuclear pools by AMS may lead to more rapid supply of high specific activity phosphate from the cytoplasmic sources of ATP.

While the isotopic data do not completely rule out the possibility of rapid degradation of the newly synthesized ribosomal RNA, we have interpreted them to indicate the selective inhibition of ribosomal RNA synthesis.

We are grateful to Miss Anna Lisa Dopirak, Miss Jeanne Schweitzer, and Miss Agnes T. Masse for enthusiastic technical assistance. This work was supported in full by United States Public Health Service Grants CA-05402-06 VR and GM-813-04 from the National Institutes of Health.

Received for publication 11 November 1965.

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