Inhibition of Protein Synthesis by 8-Azaguanine

I. Effects on Polyribosomes in HeLa Cells

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SUMMARY

In HeLa monolayer and suspension cultures azaguanine causes a preferential inhibition of protein synthesis; RNA and DNA synthesis are inhibited to a lesser extent and at later times. The effects of azaguanine were explored by measuring protein synthesis on the cytoplasmic polyribosomes that were isolated by sucrose gradient centrifugation. Azaguanine (3×10^{-4} M) after 24 hours pretreatment of HeLa, decreased protein synthesis per unit of polyribosome to 44% of control. The appearance of mRNA in polyribosomes of the drug-treated culture was not impaired. Synthesis of cytoplasmic ribosomes was suppressed by about 50–60%. Azaguanine was incorporated into both mRNA and ribosomal RNA molecules of the polyribosome structure.

Azaguanine (3×10^{-4} M) did not inhibit protein synthesis in rabbit reticulocytes even after 20 hours' exposure to the drug.

It was concluded that azaguanine inhibits protein synthesis not by blocking the formation of polyribosomes but rather by incorporation into mRNA of this structure.

INTRODUCTION

8-Azaguanine (azaguanine), an analog of guanine, has been shown to inhibit protein synthesis in *Bacillus cereus* (1, 2) while RNA synthesis was either stimulated, depressed, or not affected (3, 4) and DNA synthesis was depressed only at high doses. We chose to study the drug's action in the HeLa cell for the following reasons: (a) The cells have a generation time of 24 hr, proteins are synthesized in approximately 1 min (5), messenger RNA (mRNA) appears in polyribosomes in less than 30 min, and it takes about 60 min for ribosomal RNA to appear in completed ribosomes (6). Thus the time sequence for completion of macromolecules, especially proteins, is slow enough to be analyzed without difficulty, in contrast to the situation with bacteria. Also these mechanisms of synthesis are as well documented in animal cells as in bacteria. (b) Protein synthesis has been shown to occur on the polyribosome, a cluster of ribosomes held together by mRNA (6–8). The polyribosome is much more stable in animal cells than in bacteria, and at the time when this work was started, polyribosomes in bacteria could not be isolated. (c) RNA molecules are synthesized in the nucleus and have to pass into the cytoplasm to exert their biologic effects. This compartmentalization facilitates the analysis of the subsequent steps in the synthesis of polyribosomes; differential centrifugation of nucleus and cytoplasm separates function of ribosomes and mRNA from their sites of synthesis.

First we found suitable dose ranges of azaguanine in monolayer and suspension cultures. Then we showed preferential inhi-

bition of protein synthesis in HeLa. Analyses of protein synthesis on polyribosomes were undertaken next. We found that in azaguanine-treated cells less protein is synthesized per unit polyribosome than in control cells. mRNA transport into polyribosomes was not impaired, while synthesis of ribosomes which appear in the cytoplasm was inhibited by about 50%. Finally we showed that azaguanine was incorporated into both mRNA and ribosomes of the cytoplasmic polyribosomes.

METHODS

Cell Propagation

S3-1, a clone (isolated by N. P. Salzman) that grows exponentially in suspension (doubling time approximately 24 hr), was used in HeLa experiments. The medium in which the cells were grown was that of Eagle (9), containing glucose, essential amino acids, 4 mM glutamine, and 5% horse serum. The cells were grown in monolayer cultures fed every 5 days, or in suspension cultures fed daily.

Chemicals

Azaguanine was obtained from Nutritional Biochemicals Corporation and azaguanine-2-^{14}C from Volk Radiochemical Company; DL-Leucine-4,5-^{3}H, uridine-2-^{14}C, thymidine-2-^{14}C, and algal hydrolyzate-^{14}C were purchased from New England Nuclear Corporation.

Growth Inhibition Studies

In all experiments, azaguanine was dissolved in 0.5% Na_2CO_3 and added to cultures while control cultures had an equal volume 0.5% Na_2CO_3 added. pH of medium was made neutral with HCl or by gassing with 5% CO_2:95% air as needed.

Monolayer cultures. A suitable number of 8-oz. prescription bottles were inoculated with 20 ml of a cell suspension containing 1.5×10^5 cells. Cultures were incubated at 37° for 24 hr, at which time drug was added. Triplicate cultures for each drug concentration were removed at 0, 1, 2, 3, and 4 days. The medium was decanted, monolayer cells were resuspended in 0.9% saline, and cell numbers were determined in a Coulter counter.

Suspension cultures. 1000 ml of an exponentially growing culture at 1.5×10^5 cells/ml was divided into 4 equal parts. Azaguanine was added, and 24 hr later cell suspensions were fed with an equal volume of fresh medium containing the original drug concentration. At indicated times, three 1-ml samples were removed from each culture and cell counts were done twice, so that 900–2700 total cells were recorded. These counts were done with a hemacytometer at 80× magnification in a light microscope. Total cell concentrations were corrected for dilution with medium.

Total Protein, RNA and DNA Synthesis

Monolayer cultures. Incorporation of ^{3}H-leucine, ^{14}C-uridine, and ^{14}C-thymidine into cells was used as a measure of protein, RNA, and DNA synthesis, respectively. Replicate cultures containing 2×10^5 cells each were incubated for 24 hr at 37°, at which time drug was added; 24 hr later, 5 μC of ^{3}H-leucine (9.3 μC/μmole), 0.5 μC of ^{14}C-uridine (5 μC/μmole) and 2 μC of ^{14}C-thymidine (20 μC/μmole) were each added to triplicate bottles and incubated for 1 hr at 37°. Medium was removed, cells were scraped in 0.9% saline, two portions were removed for cell counting, and two portions were acidified with 5% trichloroacetic acid and assayed for radioactivity.

Suspension cultures. Protein and RNA synthesis were measured simultaneously by double labeling; 6×10^7 cells were centrifuged at room temperature (1000 *g*, 5 min) and resuspended in 200 ml of warmed Eagle's medium containing 1/20 normal concentration of leucine and 5% dialyzed horse serum. The suspension was incubated for 30 min at 37°, then 50 μC of ^{3}H-leucine (4700 μC/μmole) and 2.5 μC of ^{14}C-uridine (1.25 μC/μmole) were added, and the culture was divided into 4 equal parts. Azaguanine was added to give appropriate concentrations. Duplicate 2-ml samples from each culture were removed at specified times, pipetted into 2 ml of ice-cold medium, and centrifuged at 1500 *g*

for 5 min at 5°. The pellets were resuspended in 2 ml of 5% trichloroacetic acid and assayed for ^{3}H and ^{14}C radioactivity simultaneously.

Sucrose Gradient Analysis of Polyribosomes

Suspension cultures at 2×10^5 cells/ml were divided into equal parts usually containing 150–200 ml of suspension. Azaguanine was added (3×10^{-4} M) and suspension cultures were incubated at 37°. Radioactive precursors were added to cultures for the indicated periods of time. Synthesis of the macromolecules was stopped by pouring suspension over crushed frozen medium and centrifuging at 1500 *g* for 10 min. The following fractionation was carried out in the cold. Cells were washed with Earle's-saline and pellet resuspended in 2 ml of hypotonic buffer containing 0.01 M Tris (pH 7.5), 0.01 M KCl, and 0.001 M $MgCl_2$. After 5 min incubation, the swollen cells were lysed with 10 strokes of a tight fitting pestle in a Dounce homogenizer. The nuclei were removed from the lysate by centrifuging at 1500 *g* for 10 min. Sodium deoxycholate (0.5%) was added to the supernatant solution to free the polyribosomes from lipid material (7).

Sucrose gradient centrifugation was employed to separate the polyribosomes from the ribosomes. This was accomplished by layering 1.5 ml of the solution to be analyzed on 25 ml of a 5–30% (W/W) linear gradient of sucrose in the Tris-Mg buffer described above. The extracts were centrifuged for 90 min in a SW 25.1 rotor at 25,000 rpm at 0–5°. Appropriate volume fractions were collected for optical density (260 mμ) and radioactivity measurements.

Radioactivity Measurements

Nucleic acids and proteins were precipitated with cold 5% trichloroacetic acid. Albumin was added to coprecipitate the macromolecules when necessary. Precipitates were collected on Millipore filters and dried by infrared heat. These filters were placed in polyethylene vials, to which 10 ml of a toluene-PPO-POPOP scintillation mixture was added. Radioactivity was measured in a Packard scintillation counter.

RESULTS

Growth Inhibition Studies

Monolayer cultures. Initial studies on the growth-inhibitory effect of azaguanine were carried out in monolayer cultures. Azaguanine causes significant inhibition at a concentration of 4×10^{-5} M (6 μg/ml) (Fig. 1). At this concentration, monolayer cells seemed to recover from the effects of the drug. This may be due to the well documented metabolism of the drug to azaxanthine (10) after 1–4 days in the presence of the cells. At concentrations of 6 to 10×10^{-5} M, growth inhibition ranged from 75 to 83% after 4 days' exposure to the drug. A slight recovery of cells is apparent even at these higher concentrations of azaguanine.

Suspension cultures. Azaguanine was more effective in suspension cultures, although this varied with different experiments. A typical dose-response experiment is shown in Fig. 1, where 2, 3, and 4×10^{-5} M azaguanine was added during exponential growth. At 4×10^{-5} M azaguanine, cell growth was inhibited 65% in 48 hr. In contrast to monolayer cultures, the cells did not appear to recover. This can probably be explained by the fact that, at 24 hr, cell cultures were fed with medium containing azaguanine, replacing any metabolized drug.

Effect on Protein, RNA, and DNA Synthesis

Monolayer cultures. Metabolic studies in bacteria have shown that azaguanine, in low doses, inhibits protein synthesis before any inhibition of RNA or DNA synthesis is seen (1, 2). Therefore, a comparable study was carried out on HeLa. Azaguanine (6×10^{-5} M) caused a 75% inhibition of protein synthesis, a 36% inhibition of RNA synthesis, and a 6% inhibition of DNA synthesis after 24 hr of drug treatment in monolayer cultures, as determined by the incorporation for 1 hr of ^{14}C-leucine, ^{14}C-uridine, and ^{14}C-thymidine, respectively. During this time, growth, as measured by total cell number, was inhibited by 42%. These results are in accord with those observed in bacteria; namely, that azaguanine blocks

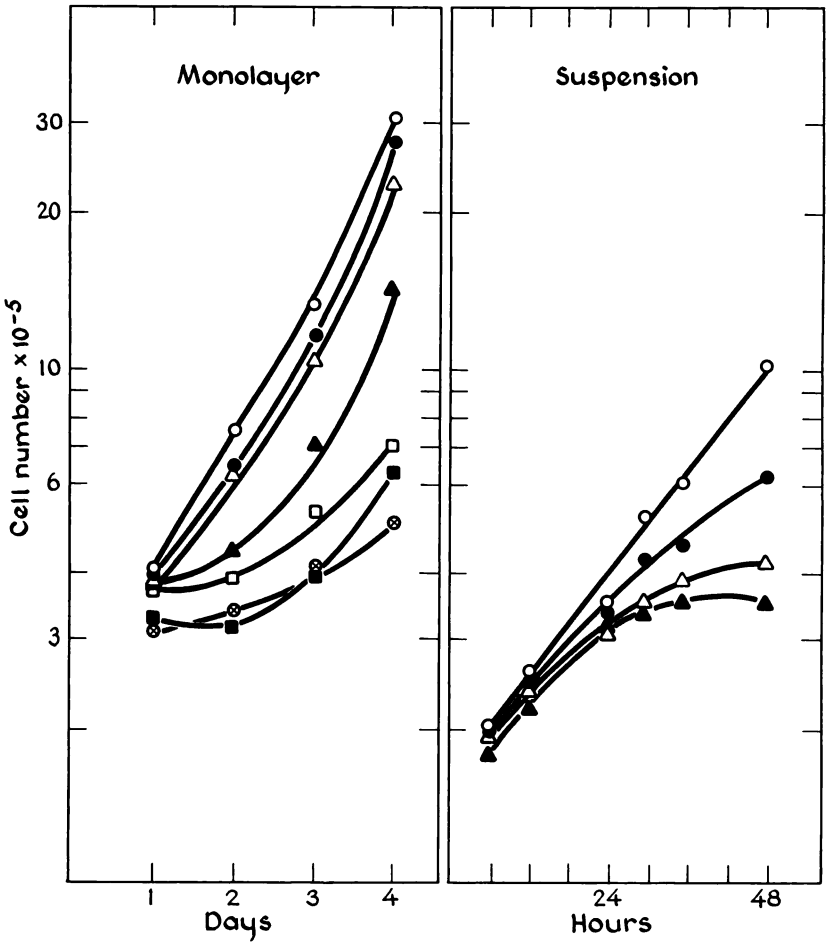

FIG. 1. *Effect of azaguanine on growth of HeLa with respect to time*

Left—Monolayer culture: control, ○; azaguanine: 1×10^{-5} M, ●; 2×10^{-5} M, △; 4×10^{-5} M, ▲; 6×10^{-5} M, □; 8×10^{-5} M, ■; 1×10^{-4} M, ⊗. *Right*—Suspension culture: control, ○; azaguanine: 2×10^{-5} M, ●; 3×10^{-5} M, △; 4×10^{-5} M, ▲.

cell growth by an early and preferential inhibition of protein biosynthesis.

Suspension cultures. Experiments were undertaken to look at protein synthesis on the polyribosomes of cells growing in suspension cultures. Since proteins are synthesized in animal cells on the order of 1 min, it was decided to pulse cells with radioactive leucine for 1 min so that newly synthesized proteins would still be located at their polyribosomal sites of synthesis (6, 7). However, when the culture was pretreated with 6×10^{-5} M azaguanine for one generation time or less and a 1-min pulse of radioactive amino acid was used to measure protein synthesis, inhibition was variable and low, e.g., 20–40%. It was therefore decided to use higher doses of azaguanine to suppress synthesis of protein. However, in order to ascertain that at these higher concentrations of azaguanine, the depression of protein synthesis was not due to a preferential block of RNA synthesis, this possibility was explored.

To a suspension culture was added ^{3}H-leucine and ^{14}C-uridine simultaneously. The culture was divided into 4 parts; and azaguanine, at concentrations of 3×10^{-5},

1×10^{-4}, and 3×10^{-4} M, was administered. Protein and RNA synthesis were measured in the same sample by differential analysis of ^{3}H and ^{14}C. The effect of azaguanine was observed for 9 hr (approximately one-third of a division cycle). Figure 2 shows that protein synthesis was depressed preferentially in comparison with RNA synthesis. Not until 9 hours did the highest concentration of azaguanine lower RNA synthesis to 91% of control. In other experiments, protein synthesis was suppressed to a much greater extent, e.g., see Fig. 4.

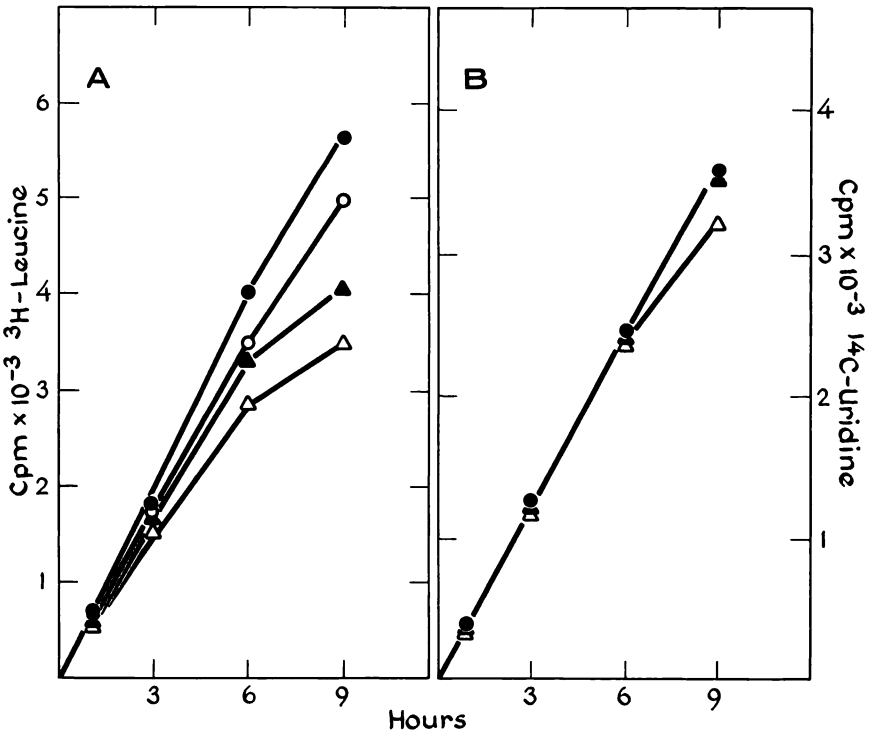

FIG. 2. *Effect of azaguanine on protein and RNA synthesis in suspension cultures*

(A) Protein synthesis measured by ^{3}H-leucine incorporation. (B) RNA synthesis measured by ^{14}C-uridine incorporation. Control, ●; azaguanine: 3×10^{-5} M, ○; 1×10^{-4} M, ▲; 3×10^{-4} M, △.

Protein Synthesis on Polyribosomes

The effects of azaguanine on synthesis of proteins that have not yet been released from polyribosomes were next investigated. This type of experiment was carried out by treating suspension cultures with 3×10^{-4} M azaguanine for varying periods of time. Then the cells were pulsed for 1 min with radioactive leucine. After rapid chilling of cell suspensions, the cells were broken, nuclei were removed from the cytoplasm, and the polyribosomes in the cytoplasm were analyzed by sucrose gradient centrifugation.

Figure 3 shows a typical experiment in which the profile of protein synthesis on polyribosomes is seen in control cells and in cells treated for 24 hr with azaguanine. Absorption at 260 mμ identifies the various RNA components of the cytoplasm. The polyribosomes in the gradient are seen as the broad, heavy peak at tube 20, the monosomes (ribosome monomers) at tube 26 and the transfer RNA and soluble proteins at the top of the gradient. Treatment of cells with azaguanine inhibits protein synthesis markedly and the amount of protein synthesized per unit of ribosomal RNA in the polyribosome region is also markedly de-

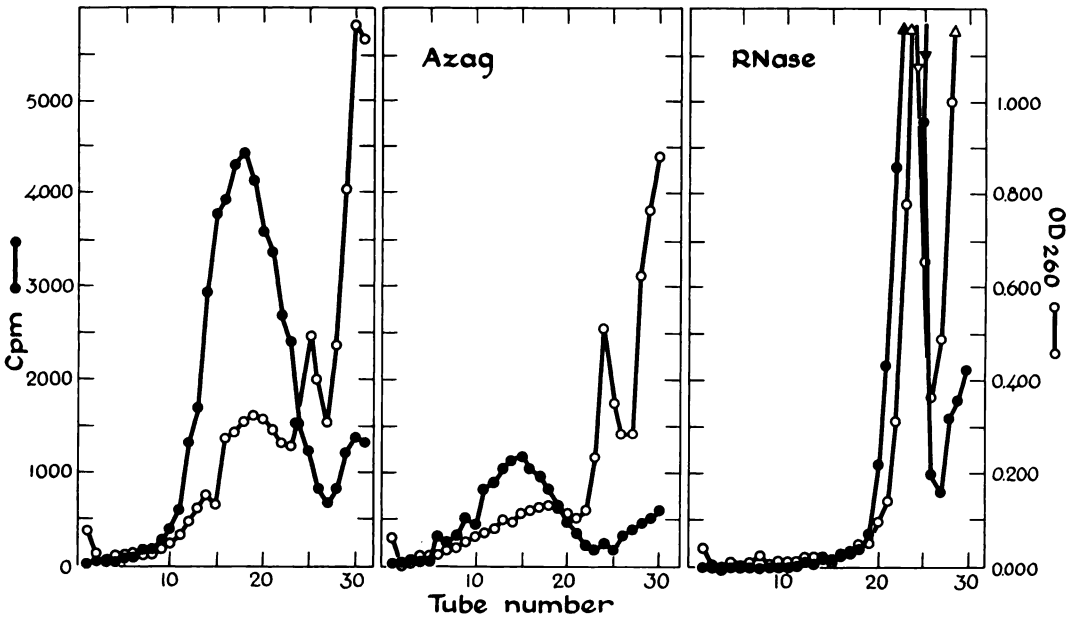

FIG. 3. *Sucrose gradient analysis of protein synthesis of HeLa after azaguanine pretreatment*

Cell suspension at 2×10^5/ml was subdivided: azaguanine (3×10^{-4} M) was added to 150 ml; two controls of same volume were also employed. After 24 hr incubation at 37°, cells were centrifuged at room temperature and resuspended in original volume of warmed medium containing 1/40 normal concentration of leucine, 5% dialyzed horse serum, and azaguanine (3×10^{-4} M), where indicated. Cultures were then incubated at 37° for 45 min, and exposed to 400 μC of ^{3}H-leucine (5500 μC/μmole) for 60 sec. The cells were fractionated and the 1500 *g* supernatant fraction was analyzed by sucrose gradient centrifugation as described in Methods. Ribonuclease (1 μg/ml) added to one control cytoplasmic extract at 0° for 10 min, then extract centrifuged.

pressed (60% of control).[1] The average specific activity seen in three such experiments is 44% of control. Another effect of azaguanine is to decrease the number of polyribosomes (Fig. 3).[2]

At shorter intervals of azaguanine treatment, similar results are seen (Fig. 4). After 4 hr azaguanine exposure, the cpm: OD_{260} ratio of the polyribosomes is 73% of control and at 12 hr the ratio is 47%. Thus we can see a progressive decrease in protein synthesis on the polyribosome. The other effect, namely a decrease in polyribosomes, is also seen, although it does not fall proportionately with time in this experiment.

In order to see whether the depression of protein synthesis is caused by a decrease in the number of polyribosomes, due to a depressed attachment of mRNA to ribosomes, data from many experiments were put together. Since actinomycin D completely blocks RNA synthesis in HeLa (11), the decay of mRNA can be calculated by measuring the depression of protein synthesis on polyribosomes after actinomycin treatment. Penman *et al.* (6) found by this technique that mRNA in HeLa has a half-life of 3 hr. In Fig. 5, the decay of mRNA

[1] Units of polyribosomes are the absorbance at 260 mμ of the broad peak heavier than the monosomes isolated by sucrose gradient centrifugation. All of this absorbance is polyribosomal because protein is synthesized in this region and the peak completely disappears upon prior mild RNase treatment (8) (Fig. 3). Hence a measure of polyribosome function is the total cpm/OD_{260} (specific activity).

[2] As a measure of the decrease in polyribosomes caused by azaguanine, that would reflect less mRNA attachment to ribosomes, the ratio of polyribosomes:monosomes is compared to a control. Absolute values of polyribosome number (OD_{260}) cannot be used since azaguanine inhibits the synthesis of ribosomes that appear in the polyribosome peak (Fig. 7B).

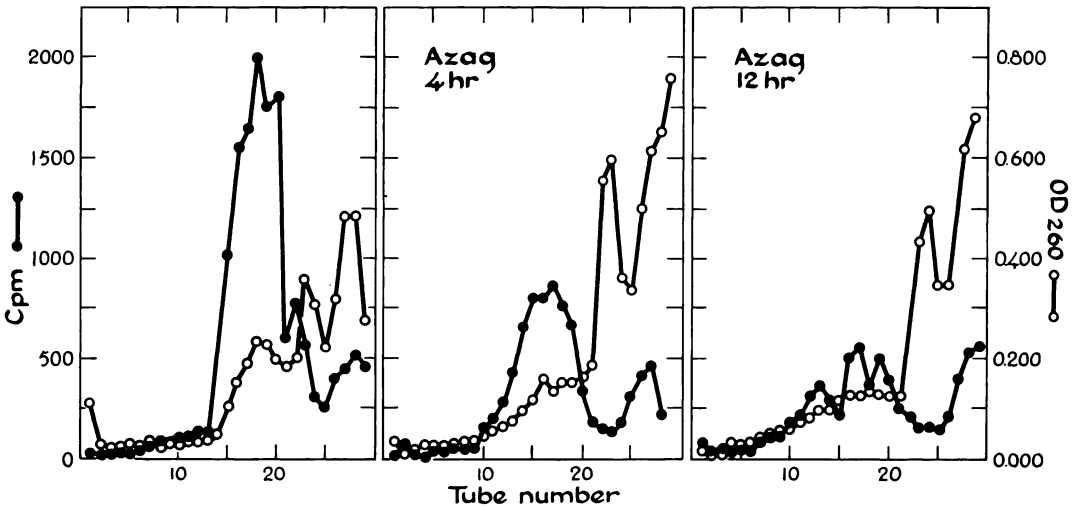

FIG. 4. *Effect of azaguanine (3×10^{-4} M) on protein synthesis with respect to time*

Cultures pretreated with azaguanine for indicated times before ^{3}H-leucine added. Procedure same as described in Fig. 3.

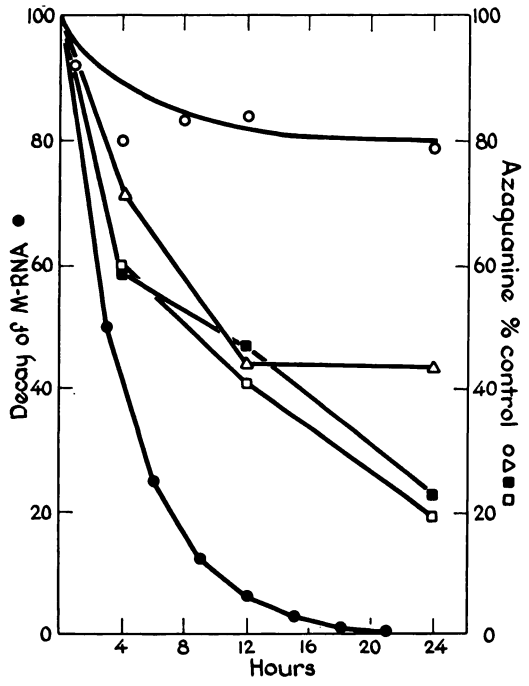

FIG. 5. *Comparison of decay of mRNA and effects of azaguanine treatment in HeLa*

●, Rate of disappearance of mRNA from polyribosomes (assuming a 3 hr half-life for mRNA (6); azaguanine effects—○, corrected polyribosome number; ■, protein synthesis in cytoplasm; □, protein synthesis on polyribosome; △, protein synthesis per unit of polyribosome.

is plotted using the above data. Also plotted are the effects of azaguanine on protein synthesis in the cytoplasm, protein synthesis on polyribosomes, protein synthesis per unit of polyribosome, and finally polyribosome number. This latter (polyribosome: ribosome ratio) has been corrected since any decrease in polyribosomes will cause a proportional increase in ribosomes. Thus, if the polyribosome:ribosome ratio of azaguanine-treated cells is 59% of control, after correction, the value is 79%.

Such a comparison reveals that the azaguanine-induced fall in polyribosomes is much smaller than either a decay of HeLa mRNA or the azaguanine-induced decrease in protein synthesis in the cytoplasm or on cytoplasmic polyribosomes or per unit polyribosome (specific activity). This makes it unlikely that azaguanine is depressing protein synthesis by completely preventing the attachment of mRNA into the polyribosomes. Possibly 25% of its depression of protein synthesis could be caused by this mechanism.

Messenger RNA Incorporation into Polyribosomes

Since a comparison of the rate of decrease of polyribosomes with the decay of mRNA is indirect, experiments were carried out to measure the rate of incorporation of mRNA

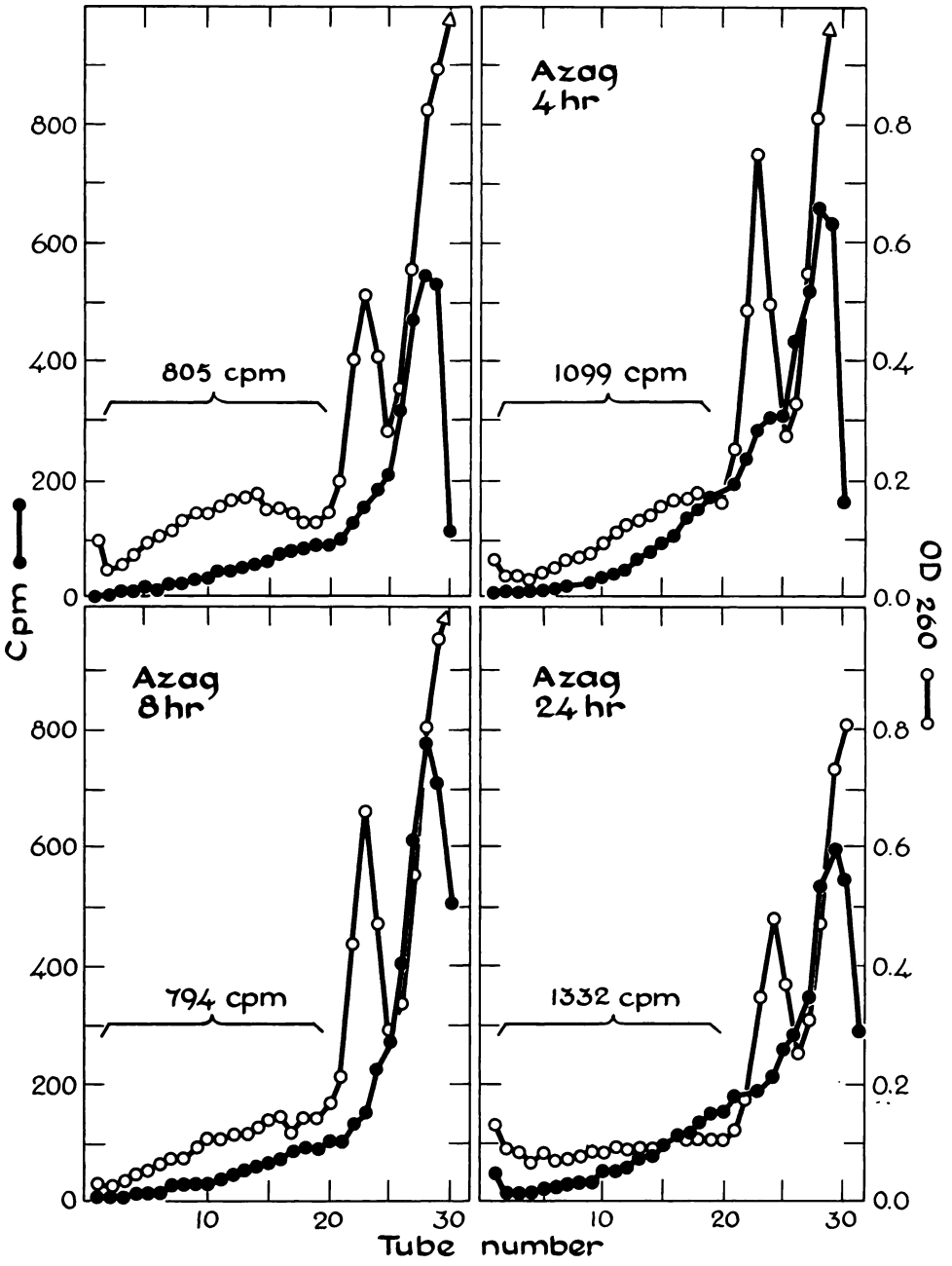

FIG. 6. *Sucrose gradient analysis of mRNA incorporation into polyribosomes of HeLa after various times of pretreatment with azaguanine*

2.4×10^5 cells were divided into 4 equal parts and allowed to grow exponentially. Azaguanine (3×10^{-4} M) was added to appropriate cultures at times that would allow the indicated period of pretreatment to end simultaneously. Cells were exposed to 5 μC of ^{14}C-uridine (30 μC/μmole, 8.4×10^{-7} M) for 30 min. Analysis by sucrose gradient centrifugation as described in Methods.

into polyribosomes directly. Girard *et al.* (12) showed that a 30 min pulse of radioactive uridine was incorporated into RNA of the cytoplasmic polyribosomes of HeLa that was messenger-like (10 *S*) rather than ribosomal (28 *S* and 18 *S*).

Preliminary experiments confirmed that a 30-min pulse of uridine labeled the mRNA in preference to ribosomal RNA in the polyribosomes. Thereupon suspension cultures of HeLa were pretreated for 4, 8, and 24 hr with azaguanine (3×10^{-4} M), pulsed for 30 min with ^{14}C-uridine, and the polyribosomes were assayed for uridine incorporation. The results are presented in Fig. 6. It can be seen that the incorporation of radioactivity into the polyribosomes, representing predominantly mRNA, is not depressed after azaguanine treatment. The slight increases at 4 and 24 hr are not considered significant. It is concluded that the inhibition of protein synthesis by azaguanine is not due to a depressed rate of transfer of mRNA into polyribosomes.

Ribosomal RNA Incorporation into Ribosomes and Polyribosomes

Comparison of the OD_{260} profiles from sucrose gradients of extracts from azaguanine (24 hr treatment) and control cells indicated that azaguanine inhibited the synthesis of ribosomes, both monosomes and polyribosomes. After suitable subtraction of the ribosomes present before addition of drug, it was found that azaguanine caused a 61% depression of synthesis of cytoplasmic ribosomes (6 experiments). The effects of azaguanine on ribosome synthesis were also measured directly with radioactive uridine.

Suspension cultures were treated with azaguanine for 15.5 hr, pulsed with radioactive uridine for 30 min, and chased with an excess of nonradioactive uridine for an additional 4 hr; comparisons were made with a control culture similarly treated. Portions of the cell suspensions were removed every hour to follow the passage of uridine into stable RNA (Fig. 7A). It can

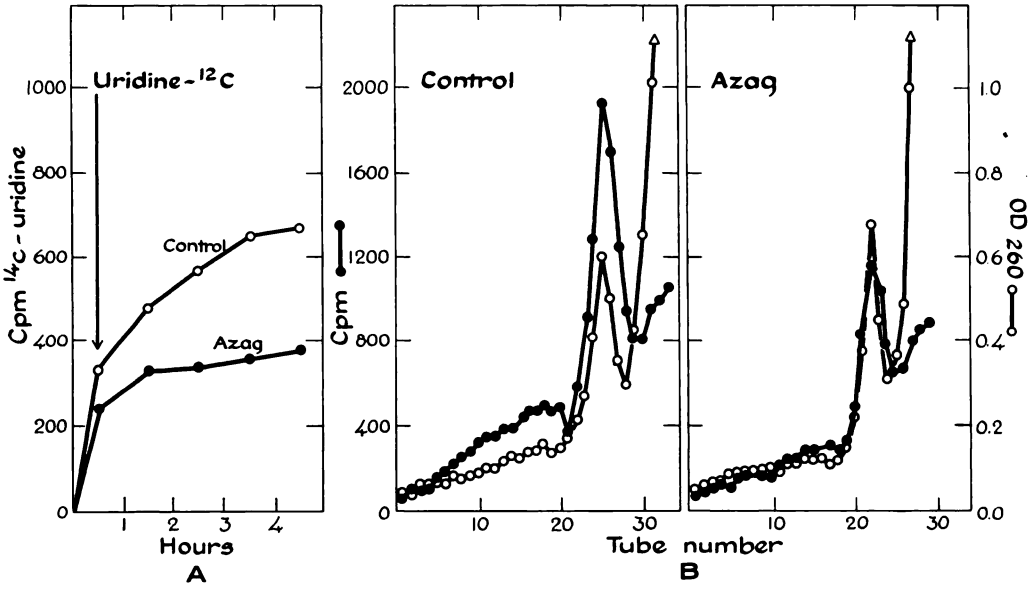

FIG. 7. *Effect of azaguanine on a 30-min ^{14}C-uridine pulse chased into stable RNA with ^{12}C-uridine*

A. Control, ○; azaguanine (3×10^{-4} M), ●. Azaguanine was added to one of two 250 ml cultures at 2×10^5 cells/ml; 15.5 hr later 5 μC of ^{14}C-uridine (30 μC/μmole, 6.7×10^{-7} M) was added to each culture, and 30 min later ^{12}C-uridine (6.7×10^{-5} M) was added. Duplicate 1 ml portions were removed at indicated times, treated with trichloroacetic acid, and assayed for radioactivity as described in Methods.

B. After 20 hr of azaguanine treatment, the above cells were harvested and analyzed by sucrose gradient centrifugation as described in Methods.

be seen that azaguanine depressed the amount of uridine incorporated after the 30 min pulse by only 28%. The RNA synthesized at ths time is mostly 45 *S* and 35 *S* ribosomal precursor (14).[3] However, after 4 hr it has been incorporated into stable ribosomes. After a total of 20 hr incubation with the drug, azaguanine depressed the increase of stable RNA by 44%. This greater inhibition of formation of total stable RNA is due to a partial blockage of synthesis of ribosomes which are later transported into the cytoplasm (Fig. 7B). Integration of polyribosome and monosome peaks in Fig. 7B revealed that incorporation of ribosomal RNA into these structures was depressed by 46% in the azaguanine-treated culture.

[3] Although most of the RNA that is made by the cell is ribosomal precursor, nevertheless, since it takes more than 60 min to complete the ribosomes, the RNA that appears in cytoplasmic polyribosomes at this time is mRNA (6). In this experiment, the chase depressed ^{14}C-uridine incorporation into total nucleic acid by 80%, and since most of the RNA synthesized is ribosomal RNA, it is evident that the amount of mRNA synthesized after the chase is negligible. Hence the amount of ^{14}C-uridine incorporated into cytoplasmic ribosomes 4 hr after the chase is essentially ribosomal RNA.

Incorporation of Azaguanine into Cytoplasmic Ribosomes and Polyribosomes

The previous experiments revealed that 40–50% of cytoplasmic ribosomes are still synthesized after 20 hr of treatment with azaguanine, in comparison with a control. Therefore, the incorporation of the analog into cytoplasmic structures was measured, since such incorporation by azaguanine into ribosomes or mRNA of polyribosomes could explain the inhibition of cytoplasmic protein synthesis. 3×10^{-4} M of ^{14}C-azaguanine was used for incorporation into a suspension culture for 24 hr. The uptake of ^{14}C-uridine in a comparable culture served as a measure of RNA synthesis. As Fig. 8 indicates, radioactive azaguanine was incorporated into polyribosomes and monosomes of the cytoplasm, as was the incorporation of ^{14}C-uridine in the control. After suitable corrections for differences in specific activities of ^{14}C-uridine and ^{14}C-azaguanine, the depression of ribosome synthesis by azaguanine and varying uracil

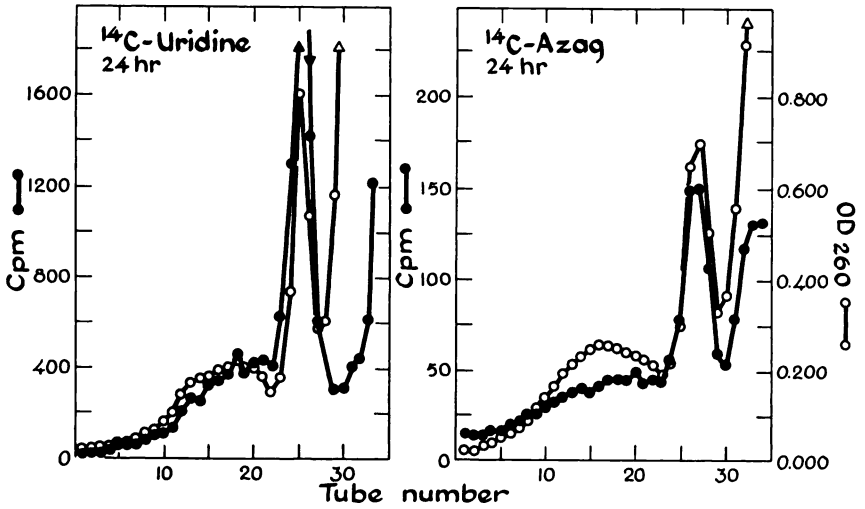

FIG. 8. *Sucrose gradient analysis of cytoplasm of suspension cultures exposed to ^{14}C-azaguanine and ^{14}C-uridine for 24 hr.* To each 1×10^7 exponentially growing cells in a volume of 50 ml was added ^{14}C-azaguanine (3×10^{-4} M, 1.3 μC/μmole) and ^{14}C-uridine (2×10^{-5} M, 0.2 μC/μmole), respectively. At 24 hr each culture was harvested and sufficient nonradioactive control cells were added to yield a total of 8×10^7 cells. Analysis of sucrose gradient centrifugation as described in Methods.

and guanine proportions in ribosomal RNA, it is estimated that 3% of total ribosomal RNA contains azaguanine. It is also seen that azaguanine is incorporated into sRNA, in accord with the results reported for *B. cereus* (15, 16). mRNA should comprise only about 2% of the RNA in a polyribosome, assuming 8 ribosomes in a polyribosome and molecular weights of 2.5×10^5 for mRNA and 2×10^6 (28 *S* and 18 *S*) for ribosomal RNA. Since the relative distribution of the incorporation of labeled azaguanine into polyribosomes is not less than in monosomes in comparison with the ^{14}C-uridine-incorporated control culture, the ribosomes in the polyribosomes as well as the monosomes have incorporated azaguanine.

On the other hand, from these data it is not certain that azaguanine is incorporated into the mRNA of the polyribosome. To test this possibility, cultures were labeled with ^{14}C-azaguanine and ^{14}C-uridine; the polyribosomes and ribosomes were isolated by sucrose gradient centrifugation, and treated with high and low concentrations of RNase. Table 1 presents the results of this experiment. Treating monosomes and polyribosomes labeled with ^{14}C-azaguanine or ^{14}C-uridine for 24 hr or 1 hr with 1000 μg/ml of RNase at 37° for 30 min results in essentially complete hydrolysis of the RNA in these particles. However, if low concentrations of RNase (0.1 μg/ml, 0°, 10 min) are incubated with these particles only mRNA should be hydrolyzed and ribosomal RNA, encapsulated by the protein coat in the ribosome, should be protected (see Fig. 3 in which 1 μg/ml RNase at 0° was employed). When polyribosomes and monosomes from cells labeled with ^{14}C-azaguanine for 24 hr are incubated with 0.1 μg/ml RNase, 4 and 0% hydrolysis occur, respectively. This 4% difference which is attributable to mRNA agrees with the 2% mRNA calculated to be present in polyribosomes. Similarly, ^{14}C-uridine-labeled particles (24 hr) also show a 4% difference between polyribosome and monosome hydrolysis.

Since this difference is so small, cells were exposed to each radioactive precursor for 1 hr. In this situation, the polyribosomes should have a higher proportion of labeled mRNA since the synthesis of mRNA is faster than rRNA. Treatment of these particles with 0.1 μg/ml RNase show a 13% net difference between polyribosomes and monosomes in azaguanine-treated cells and a 16% net difference in control cells. There-

TABLE 1

Differential hydrolysis of polyribosomes and monosomes labeled with ^{14}C-azaguanine and ^{14}C-uridine

HeLa cells labeled: 400 ml of cells at 2×10^5/ml exposed to ^{14}C-azaguanine (3×10^{-4} M, 0.42 μC/μmole) and 400 ml of cells at 2×10^5/ml exposed to ^{14}C-uridine (5×10^{-5} M, 0.1 μC/μmole) for 24 hr; 20 ml of cells at 6×10^6/ml exposed to ^{14}C-azaguanine (1.7×10^{-4} M, 6 μC/μmole) and 10 ml of cells at 6×10^6/ml exposed to ^{14}C-uridine (1.7×10^{-5} M, 30 μC/μmole) for 1 hr. Particles separated by sucrose gradient centrifugation and incubated with RNase: 0.1 μg/ml, 0° for 10 min and 1000 μg/ml, 37° for 30 min. Reaction stopped with trichloroacetic acid and assayed for radioactivity as described in Methods.

Isotopic precursor	Pulse	Particles	Per cent decrease in radioactivity concentration RNase	
			0.1 μg/ml	1000 μg/ml
^{14}C-Azaguanine	24 hr	Polyribosomes	4	97
		Monosomes	0	97
^{14}C-Uridine	24 hr	Polyribosomes	6	98
		Monosomes	2	98
^{14}C-Azaguanine	1 hr	Polyribosomes	15	93
		Monosomes	2	86
^{14}C-Uridine	1 hr	Polyribosomes	20	96
		Monosomes	4	92

fore, it is concluded that azaguanine is incorporated into the mRNA molecules entering the polyribosome structure involved in protein synthesis.

Failure of Azaguanine to Inhibit Protein Synthesis in Rabbit Reticulocytes

If azaguanine inhibited protein synthesis in a system that does not synthesize any new RNA, it would be clear that the incorporation of azaguanine into RNA was not necessary for its inhibition of protein synthesis. Rabbit reticulocytes were used for this purpose, using the methods outlined by Warner *et al.* (8). Samples were removed from a suspension of reticulocytes 1–20 hr after the addition of azaguanine and ^{14}C-algal hydrolyzate. Incorporation into hot trichloroacetic acid-precipitable material was used as a measure of protein synthesis. Azaguanine, at 3×10^{-4} M, which caused more than 75% inhibition of protein synthesis in the HeLa cell after 20 hr, did not inhibit protein synthesis at all in the rabbit reticulocyte.

DISCUSSION

Although the inhibition of protein synthesis by azaguanine has been studied for many years, the precise mechanism by which this guanine analog functions has not been elucidated. It appears that the incorporation of azaguanine into RNA is necessary for the inhibition of protein synthesis. The substitution of azaguanine for guanine into the cofactor GTP, which is necessary for protein synthesis, does not appear to be harmful to the cell. Parks has shown that azaGTP does not block protein synthesis in a cell-free system from rat liver (17), while azaguanine depresses induction of three enzymes *in vivo* (18). We have shown that rabbit reticulocytes (cells that cannot synthesize new RNA) do not have their ability to synthesize protein impaired by high concentrations of azaguanine (3×10^{-4}). This result has recently also been reported by Marbaix (19).

Since it has been shown that RNA accumulates in the nucleus of HeLa (20) and the "nuclear fraction" of *Bacillus megaterium* (21) after treatment with azaguanine, the amount of newly synthesized mRNA that appears in the cytoplasm was measured after HeLa was exposed to azaguanine. The results indicate that azaguanine does not inhibit attachment of mRNA in the polyribosomes, thus precluding this possibility as a mechanism of inhibition of protein synthesis. Ribosome synthesis, on the other hand, was inhibited by about 50–60% by azaguanine. Not surprisingly, the mRNA that appeared in the polyribosomes and the ribosomal RNA that appeared in both polyribosomes and monosomes contained azaguanine residues. Azaguanine was also incorporated into sRNA, as seen by others (13, 16). However, it is not likely that such incorporation in sRNA could be responsible for the inhibition of protein synthesis since in *Bacillus cereus* the base composition of the sRNA was unaltered (16) and the sRNA functioned in protein synthesis (15); Zimmerman, unpublished observations).

In *B. cereus* the possibility existed that azaguanine was functioning by its almost complete inhibition of ribosome synthesis (13). However, since ribosome synthesis was inhibited much less in HeLa and since the amount of synthesis of proteins per unit polyribosome was depressed, this indicates that azaguanine functions not by inhibition of synthesis of polyribosomes, but more likely by incorporation into them. Furthermore since ribosomes are stable and about 40–50% of the newly synthesized ribosomes are made in the presence of azaguanine during one generation time, then less than 1/3 of the ribosomes could contain azaguanine. Even if each ribosome that incorporated azaguanine were completely nonfunctional in protein synthesis, then depression of protein synthesis per unit polyribosome would be less than 33%. In 3 experiments, azaguanine inhibited protein synthesis per unit polyribosome by 56% (an average of 44% of control). Therefore, it seems more likely that substitution of azaguanine for guanine into mRNA than into ribosomal RNA would be responsible for the inhibition of cell growth. This mRNA containing azaguanine could lead to miscoding in protein synthesis. In fact

Barnett and Brockman (22) have shown that azaguanine causes phenotypic suppression in mutants of neurospora, implicating misreading of the mRNA. Others have reported altered rates of incorporation of radioactive amino acids into protein (23, 24) and altered rates of enzyme synthesis (4) after treatment with azaguanine. However, these studies do not prove synthesis of miscoded proteins since the relative pool sizes of each amino acid may be differentially altered. Furthermore, Chantrenne (25) has been unsuccessful in finding cross-reacting material related to penicillinase that was depressed by azaguanine. Thus, if azaguanine functions through its incorporation into informational RNA, the precise mechanism by which protein synthesis is suppressed is still not clarified.

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