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INHIBITION OF INITIATION OF TRANSLATION IN L1210 CELLS BY 8-AZAGUANINE

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Abstract—The inhibition of protein synthesis by 8-azaguanine (azaG) in L1210 cells in culture was investigated. AzaG selectively inhibited protein synthesis at concentrations where viability was decreased significantly. AzaG altered the polyribosome sedimentation profile, increasing the numbers of monosomes and smaller polysomes and decreasing the number of larger polysomes. The reversal by cycloheximide of the alterations in the polysome profile suggested that azaG inhibited the initiation of translation. This was confirmed by the demonstration of inhibition of the formation of the 43S and 80S initiation complexes.

Several purine analogs that inhibit tumor growth adversely affect the synthesis of proteins (reviewed in Ref. 1). 6-Thioguanine has a minor but still selective effect on the formation of specific proteins [2–8], but there is strong evidence for the incorporation of 6-thioguanine into DNA as the site of inhibition by this drug [9–11]. 3-Deazaguanine, a relatively new purine analog, inhibits protein and DNA syntheses but not RNA synthesis [12, 13]; however, its mechanism of action is not yet known. The selective effects of 8-azaguanine (azaG)‖ on the protein synthesizing machinery of the cell are now considered as a major cause of the action of azaG in the inhibition of growth of a variety of neoplastic tissues (reviewed in Refs. 14 and 15). Although the inhibition of protein synthesis by azaG has been studied for many years, the molecular basis for this action and its interrelationship with nucleic acid synthesis still has not been unequivocally established.

For this reason, we have investigated in greater detail the inhibition of protein synthesis by azaG in L1210 cells in culture. Alterations in the polyribosome sedimentation profiles from azaG-treated cells were reversed by cycloheximide, suggesting that azaG inhibited the initiation of translation. We then directly examined the inhibition of initiation by azaG by measuring the binding of [^{35}S]met-tRNA$_f$ to the ribosomal initiation complexes. The inhibition of initiation by azaG that we observed is discussed in light of the previous studies on the actions of azaG.

MATERIALS AND METHODS

Materials. AzaG was obtained from the Sigma Chemical Co., St. Louis, MO. Cell culture supplies (RPMI 1640 medium, fetal calf serum, streptomycin, penicillin G, and Hanks' Balanced Salt Solution) were received from Gibco, Grand Island, NY. RPMI 1630 medium without methionine was from the Media Unit, National Institutes of Health, Bethesda, MD. [Methyl-^{3}H]Thymidine (20 Ci/mmole), [5-^{3}H]uridine (28 Ci/mmole), L-[4,5-^{3}H]leucine (50 Ci/mmole), and Omnifluor were from the New England Nuclear Corp., Boston, MA. L-[^{35}S]Methionine (1200 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, England. Nonidet P-40 (NP-40) was from the Particle Data Laboratories Ltd., Elmhurst, IL.

Cell culture. The L1210 tumor line, obtained from Dr. Kurt Kohn, National Cancer Institute, Bethesda, MD, was maintained in RPMI 1640 medium supplemented with 10% dialyzed fetal calf serum. The medium contained 1×10^5 units penicillin G and 0.1 g streptomycin sulfate per liter of medium. Cells were maintained in suspension at 37° in a humidified incubator with a 5% CO_2 atmosphere.

Measurement of viability. Viability of L1210 cells was measured by the colony formation assay [16]. Cells were exposed for 5 hr to 0.1, 0.4, 0.5, 1.0, 2.0, 2.5, 3.0, 4.0, 10, 20, or 100 μM azaG dissolved in the medium. The cells were then washed with fresh medium and diluted in RPMI 1640 medium containing 20% dialyzed fetal calf serum. Three milliliters of RPMI 1640:20% dialyzed fetal calf serum containing 0.2% Noble agar at 44° was added to L1210 cells in 2 ml of RPMI 1640:20% dialyzed fetal calf serum; the suspension was mixed thoroughly in a 15-ml tube, placed on ice for 2–4 min, and then incubated upright at 37°. The spherical colonies (approximately 0.1 mm in diameter) were counted visually 10–12 days later.

Measurement of macromolecular synthesis. The effects of azaG on DNA, RNA, and protein syntheses were determined by the method of Mandel *et al.* [17]. L1210 cells were exposed to 0.5, 2, 10, or 50 μM azaG for 5 hr and then incubated with either [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine at 1 μCi/ml. An equal volume of ice-cold 0.9% NaCl

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‖ Abbreviations: azaG, 8-azaguanine; azaGTP, 8-azaguanosine-5′-triphosphate; and TCA, trichloroacetic acid.

was added to each sample to stop the reaction after 60 min, and the samples were put on ice. The cell suspensions were filtered directly on Whatman GF/C filters wetted with 0.9% NaCl and washed with 5 vol. of 0.9% NaCl, 10 vol. of 0.2 N perchloric acid, and 5 vol. of 0.9% NaCl. All of the filtering solutions were ice cold. The filters were dried and counted in 0.4% Omnifluor/toluene.

Polyribosome sedimentation profile analysis. L1210 cells were exposed to 10 μM azaG for 5 hr. The cells were chilled rapidly with ice-cold Hanks' Balanced Salt Solution, centrifuged, and lysed with gentle vortexing in isotonic buffer [150 mM NaCl, 10 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$] containing 0.5% NP-40, a nonionic detergent. The crude nuclei were pelleted by centrifugation at 1,000 *g* for 5 min, and the supernatant fraction was centrifuged at 10,000 *g* for 15 min to obtain a post-mitochondrial supernatant fraction. The post-mitochondrial supernatant fraction was layered over 35 ml 15–40% linear sucrose gradients made in lysing buffer without the NP-40. The gradients, centrifuged for 2.5 hr at 26,000 rpm in a SW 27 rotor, were pumped through a flow cell, and the absorbance at 260 nm was recorded continuously. The polysome fraction was calculated by expressing the absorption of the polysomes (dimers to bottom of gradient) as a percentage of the total ribosomal absorption (monomers to bottom of gradient).

The ability of cycloheximide to reverse the alterations in the polysome profile was determined by a modification of the method of Reichman and Penman [18]. L1210 cells were divided into four cultures of which two were control and two were exposed to 10 μM azaG for 5 hr. One control and one azaG culture were also exposed to 2 μg/ml cycloheximide for the last 30 min of the incubation. The cells were chilled rapidly with Hanks' Balanced Salt Solution, lysed, and fractionated. The polysomes were displayed on sucrose gradients as described above.

Initiation complex formation assay. The formation of the ribosomal initiation complexes in drug-treated cells was determined by a modification of the method of Henshaw [19, 20]. L1210 cells were labeled overnight with [^{3}H]uridine (1 μCi/ml) to uniformly label the RNA. The cells were resuspended in fresh medium and exposed to 10 μM azaG for 5 hr. The cells were centrifuged at 37°, and the pellet of 2×10^7 cells was resuspended in 0.5 ml of RPMI 1630 medium lacking methionine but containing azaG. The cells were preincubated at 37° for 2 min and then labeled with [^{35}S]methionine (200 μCi/ml) for 2 min. The reaction was stopped with 10 vol. of ice-cold Hanks' Balanced Salt Solution and an aliquot was removed to measure the incorporation of label into proteins by trichloroacetic acid (TCA) precipitation (see below). The rest of the sample of cells was lysed, and the post-mitochondrial supernatant fraction was isolated as described previously. The post-mitochondrial supernatant fraction was layered over 12 ml linear 20–30% sucrose gradients made in 100 mM KCl, 2 mM Mg acetate, 20 mM triethanolamine–HCl (pH 7.0), and 0.5 mM dithiothreitol. The gradients were centrifuged at 25,000 rpm for 16 hr at 4° in a SW 41 rotor. The absorbance of the gradients was monitored, and fractions containing the 40S to 43S ribosomal subunits and the 80S ribosomes were collected. Due to the poor resolving power of the sucrose gradients, 40S subunits and 80S ribosomes isolated from these gradients were contaminated by other ribonucleoprotein species and by ^{35}S-methionine-labeled proteins.

These species were further separated from each other using CsCl density gradients. To the fractions isolated from the sucrose gradients, 0.10 vol. of 0.11 M morpholinopropane sulfonic acid–KOH (pH 7.0) was added with mixing, followed by 0.11 vol. of 40% formaldehyde neutralized to pH 7 on the day of use. The solution was held on ice for a minimum of 30 min and was then layered over 9 ml preformed CsCl gradients, 1.35 to 1.60 g/ml. The gradients were centrifuged for 40 hr at 27,000 rpm at 4°. Fractions were collected on ice and one drop of 0.5% bovine serum albumin was added to each fraction as carrier, followed by 5 vol. of cold 20% TCA. The precipitate was collected on Whatman GF/C filters and was washed with 10% TCA, 5% TCA, water and 95% ethanol. The filters were dried and counted in 0.4% Omnifluor/toluene.

RESULTS

Macromolecular synthesis and viability. The effects of azaG on viability and macromolecular synthesis in L1210 cells are shown in Figs. 1 and 2 respectively. At 10 μM azaG, protein synthesis decreased to 17% of control and DNA and RNA syntheses decreased to 35 and 41% of control respectively. At this concentration, the viability of the cell population decreased to 24% of control. The selective decrease in protein synthesis that we observed from 0.5 to 50 μM azaG has been reported by various laboratories [21–23], and many studies have linked this selective effect of azaG on protein synthesis to the subsequent growth inhibition and decrease in cell viability (reviewed in Refs. 14 and 15).

Polyribosome sedimentation profile analysis. The polyribosome sedimentation profile from L1210 cells exposed to 10 μM azaG for 5 hr showed increased

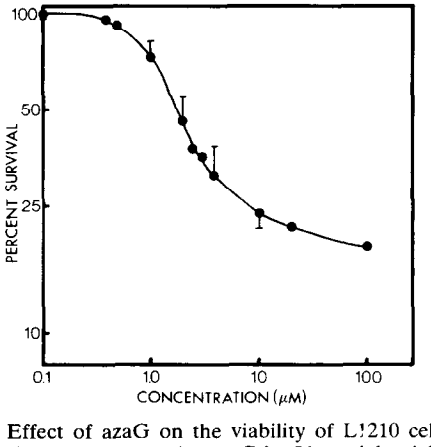

Fig. 1. Effect of azaG on the viability of L1210 cells in culture. Cells were exposed to azaG for 5 hr and the viability was measured by the colony formation assay. Values are means ± S.D.

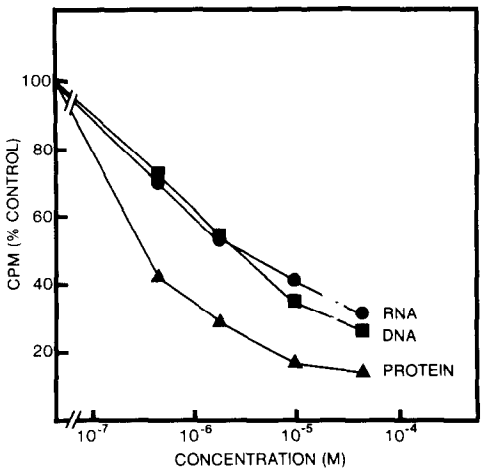

Fig. 2. Effect of azaG on macromolecular synthesis. L1210 cells were exposed to azaG for 5 hr and then labeled for 1 hr with [^{3}H]thymidine, [^{3}H]uridine, or [^{3}H]leucine. Incorporation of the isotopes into the acid-insoluble fraction was determined, and the values presented are the mean of triplicate samples. The standard deviations were less than 2% of mean values.

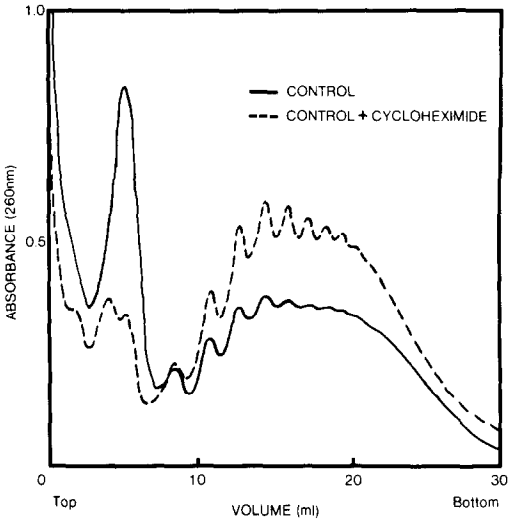

Fig. 4. Effect of cycloheximide on the polyribosome sedimentation profile of control cells. L1210 cells were exposed to 2 μg/ml cycloheximide for 30 min, and the polyribosomes were displayed on a 15–40% sucrose gradient.

numbers of monomers and small polysomes and a decrease in the number of large polysomes (Fig. 3). The polysome fraction, i.e. that fraction of total ribosomes engaged in translation, was decreased by azaG from 71 to 56% of control, whereas the percentage of free ribosomes or monosomes increased from 29 to 44% of control.

This shift in the polysome profile caused by azaG is similar to that resulting from impaired initiation of translation [18]. Cycloheximide at low concentrations is known to inhibit the elongation step of translation without significantly affecting initiation, whereas at high doses both steps are inhibited [24]. The slowing of elongation by low concentrations of cycloheximide would be expected to counteract the effect on the polysome profile of a drug which inhibits initiation, as was shown for 5-azacytidine [18]. The effect of a low concentration of cycloheximide on the polysome profile after azaG treatment was therefore examined. L1210 cells were divided into four cultures, two of which were untreated and two of which received 10 μM azaG for 5 hr. One control and one azaG-treated culture received a low concentration of cycloheximide (2 μg/ml) for the last 30 min of the incubation, and the polysome profile of each of these samples was determined.

In control cells exposed to cycloheximide, the polysome sedimentation profile shows a reduction in the monosome peak and an increase in all sizes

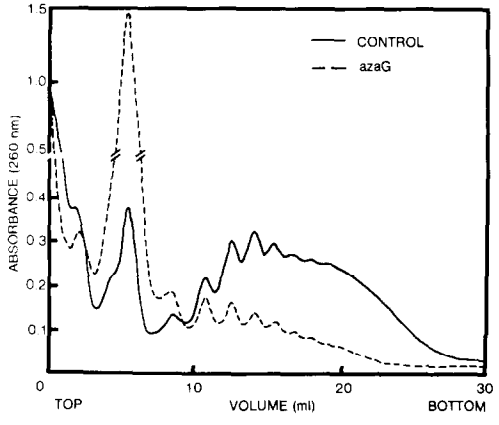

Fig. 3. Effect of azaG on the polyribosome sedimentation profile. L1210 cells were exposed to 10 μM azaG for 5 hr, and the polyribosomes were displayed on a 15–40% sucrose gradient.

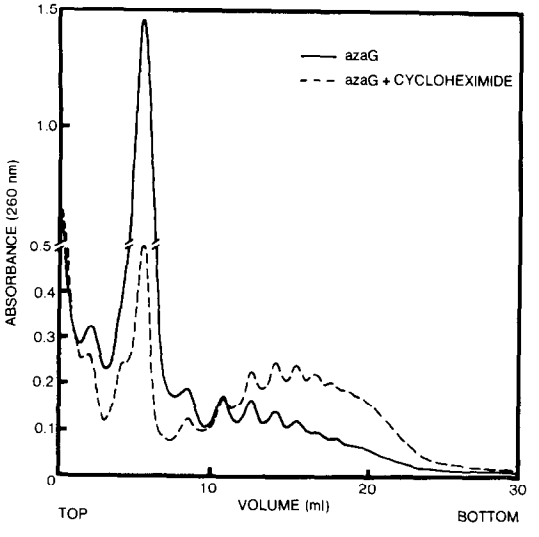

Fig. 5. Effect of cycloheximide on polyribosome sedimentation profile alterations in azaG-treated cells. Cells were exposed to 10 μM azaG for 5 hr, and for the last 30 min were also exposed to 2 μg/ml cycloheximide. Polyribosomes were displayed on a 15–40% sucrose gradient.

of the polysomes (Fig. 4). Cycloheximide, which inhibited [^{3}H]leucine incorporation into proteins by 80% [13], prevented the analog-induced shift towards monosomes and returned the polysome fraction of azaG-treated cells back towards that of control cells (Fig. 5). In addition, the relative size distribution of polysomes was not altered by cycloheximide in control cells, whereas in azaG-treated cells cycloheximide reversed the greater preponderance of smaller polysomes and increased the fraction of larger polysomes. The reversal by cycloheximide of the shift in the polysome profile from azaG-treated cells suggested that azaG inhibited the initiation of translation.

Initiation complex formation. The results obtained by the use of cycloheximide suggested, but did not directly prove, that azaG inhibited the initiation of translation. In the subsequent experiments, formation of radiolabeled met-tRNA$_f$ (the initiator aminoacyl tRNA species) and incorporation into the 43S and 80S initiation complexes served as the basis for the direct assay of initiating activity in azaG-treated cells. L1210 cells, therefore, were grown overnight with [^{3}H]uridine to uniformly label the RNA, were treated with 10 μM azaG for 5 hr, and then were exposed for 2 min to [^{35}S]methionine. The 43S and 80S initiation complexes thus contained the [^{35}S]methionine which had condensed with the initiator methionyl tRNA. The 40S subunit and 80S ribosome fractions, isolated from sucrose gradients, were then further displayed on CsCl gradients.

The results of the effects of azaG on the formation of the 43S and 80S initiation complexes are shown in Figs. 6 and 7 respectively. The absolute values of the radiolabels on the ordinates of each of the figures are different but a constant ratio of ^{35}S cpm to ^{3}H cpm has been maintained. The various ribonucleoprotein species, shown in Fig. 6, separated on the CsCl gradients are, from left to right, the 60S ribosomal subunit, the 80S ribosome and initiation complex, the 40S ribosomal subunit, and the 43S ribosomal subunit and preinitiation complex. The ^{35}S

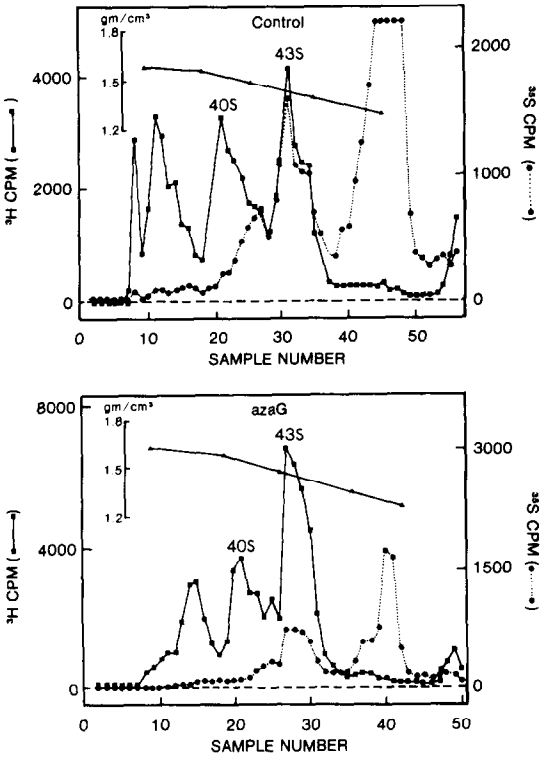

Fig. 6. Formation of 43S met-tRNA$_f$ initiation complex in azaG-treated and control cells. L1210 cells, labeled over night with [^{3}H]uridine, were treated with 10 μM azaG for 5 hr and then were exposed for 2 min to [^{35}S]methionine. The 40S subunits, isolated on sucrose gradients, were displayed on CsCl gradients. Fractions were treated as described in Materials and Methods. The different absolute values of the ordinates reflect differences in the quantities of 40S subunits used for the CsCl gradients, but the ratio of ^{35}S to ^{3}H cpm was maintained constant. The major tritium peaks represent, from left to right, the 60S, 80S, 40S (ρ = 1.52), and 43S (ρ = 1.44) ribonucleoprotein species, and the ^{35}S activity was distributed mainly in two peaks, representing the 43S preinitiation complex (ρ = 1.44) and proteins (ρ = 1.30).

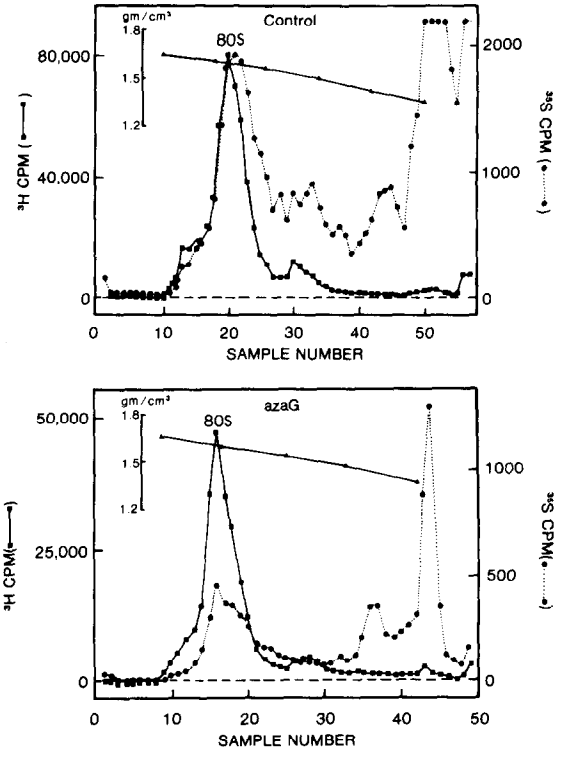

Fig. 7. Formation of the 80S met-tRNA$_f$ initiation complex in azaG-treated and control cells. L1210 cells, labeled overnight with [^{3}H]uridine, were treated with 10 μM azaG for 5 hr and then were exposed for 2 min to [^{35}S]methionine. The 80S ribosomes, isolated on sucrose gradients, were displayed on CsCl gradients. Fractions were treated as described in Materials and Methods. The different absolute values of the ordinates reflect differences in the quantities of 80S ribosomes used for the CsCl gradient, but the ratio of ^{35}S to ^{3}H cpm was maintained constant. The major tritium peaks represent the 80S ribosome and initiation complex (ρ = 1.58) and the 40S subunit (ρ = 1.52). The ^{35}S is distributed mainly in the 80S peak and to a lesser extent in the 40S peak. The two ^{35}S peaks not associated with any tritium are an unidentified peak (ρ = 1.40) and a protein peak (ρ = 1.30).

Table 1. Inhibition by azaG of formation of 43S and 80S initiation complexes and of total protein synthesis*

Expt.	Protein synthesis† (% of control)	43S Initiation complex formation‡ (% of control)	80S Initiation complex formation‡ (% of control)
1	38	35	59
2	30	29	44
Mean	34	32	52

* L1210 cells, labeled overnight with [^{3}H]uridine, were treated with 10 μM azaG for 5 hr and then were exposed for 2 min to [^{35}S]methionine. Aliquots were removed to measure the [^{35}S]methionine incorporation into proteins. The 80S ribosomes and 40S subunits, isolated on sucrose gradients, were displayed on CsCl gradients. The ratios of ^{35}S to ^{3}H cpm in the 43S and 80S initiation complex regions of the CsCl gradients were calculated.

† [^{35}S]Methionine incorporation into TCA-insoluble material.

‡ ^{35}S cpm/^{3}H cpm; TCA precipitable material.

label was not associated with the first three species but was recovered around sample 30, which represents the 43S preinitiation complex, and around fraction 45, which represents the proteins. The ratio of ^{35}S to ^{3}H counts associated with the 43S peak decreased after azaG treatment, indicating that azaG inhibited the formation of the 43S preinitiation complex.

Similarly, in Fig. 7, the various ribonucleoprotein species that separated on the CsCl gradient were, from left to right, the 60S subunit, the 80S ribosome and initiation complex, and the 40S subunit. The largest peak, composed of the 80S ribosome and the 80S initiation complex, had ^{35}S activity associated with it which indicated the formation of the 80S initiation complex. Treatment with azaG also inhibited the formation of this 80S initiation complex since the ratio of ^{35}S to ^{3}H radioactivity associated with the 80S peak was decreased in azaG-treated cells.

These results, summarized in Table 1, show the agreement between the decrease in the incorporation of [^{35}S]methionine into total proteins and the diminished formation of the 43S preinitiation complex. Protein synthesis decreased to 34% of control and the formation of the 43S preinitiation complex decreased to 32% of control after treatment with 10 μM azaG. The inhibition of the formation of the 80S initiation complex did not appear to be as great. However, the actual inhibition of the formation of the 80S initiation complex is difficult to quantify since the specific activity of the 80S initiation complex was diluted by the presence of preexisting inactive 80S ribosomes sedimenting at approximately the same density on the CsCl gradient.

DISCUSSION

8-Azaguanine selectively inhibited protein synthesis in L1210 cells in culture at concentrations of azaG where cell viability was decreased significantly. The shift in the polysome profile caused by azaG, noted before in other systems [23, 25], was similar to that resulting from impaired initiation of translation or fragmentation of active mRNA. However, this latter possibility had been eliminated since labeling of the nascent polypeptides with [^{14}C]leucine resulted in incorporation of isotope into polysomes but not into monosomes [25]. This indicated that the monomers had not been derived directly from the degradation of active polysomes.

The slowing of elongation by low concentrations of cycloheximide would be expected to counteract the effect on the polysome profile of a drug which inhibits initiation, as was shown for 5-azacytidine [18]. Treatment of control cells with cycloheximide indicated an increase in the loading of all mRNAs with ribosomes due to the decreased rate of elongation; once on the mRNA, each ribosome took a longer time to travel down the message before it dissociated, leading to a uniform increase in the amount of rRNA associated with each size of mRNA. For azaG-treated cells later exposed to cycloheximide, the shift from lighter to heavier polysomes suggested that mRNAs had not been fully loaded with ribosomes in the presence of azaG. When ribosomes attached at substantially lower rates than in controls because of a block in intiation, the slowing of elongation by cycloheximide then allowed more time for messages to become fully loaded with ribosomes.

These results, which suggested that azaG inhibited the initiation of translation, were confirmed by demonstration of inhibition of the formation of the 43S and 80S initiation complexes. There are several steps in the initiation process that azaG might inhibit, and the sequence of events comprising the initiation process is depicted in Fig. 8. First, a large initiation factor (IF-3) binds to the 40S ribosomal subunit, changing the sedimentation coefficient to 43S. The ternary complex (met-tRNA$_f$·GTP·IF-2) then forms and binds to the 43S ribosomal subunit resulting in the 43S preinitiation complex. Finally, the mRNA binds to this preinitiation complex which is then joined to a 60S ribosomal subunit to complete the formation of an 80S initiation complex.

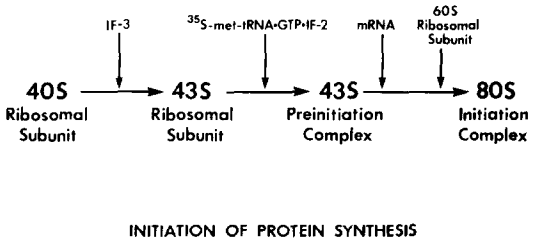

Fig. 8. Diagram of the initiation of protein synthesis. The four basic steps in the formation of the 80S initiation complex are shown.

Alterations in GTP levels or formation of 8-azaguanosine-5′-triphosphate (azaGTP) could lead to a decrease in the formation or function of the ternary complex, met-tRNA$_f$·GTP·IF-2. However, azaGTP did not inhibit the incorporation of leucine into microsomal protein of rat liver and actually partially substituted for GTP [26]. In addition, although azaGTP failed to stimulate amino acid incorporation in 30S fractions from *Bacillus cereus*, levels of GTP and ATP remained sufficiently high after azaG treatment to support amino acid incorporation [27]. Since azaG did not inhibit protein synthesis in a rabbit reticulocyte lysate system [23], which does not synthesize RNA but which does form azaGTP [28], the site of action of the analog undoubtedly is more closely related to a macromolecular effect than an action at the level of nucleotide triphosphates. The ability of 6-azauridine, which inhibits RNA synthesis, to protect against azaG toxicity [29] also confirms that the major site of action is at the level of RNA.

Incorporation of azaG into tRNA or rRNA could lead to an inhibition of several steps in the initiation process. The analog blocked the processing of rRNA [30] even though it was not incorporated into rRNA to any great extent [31–33]. Although there is extensive incorporation of azaG into tRNA [34, 35], many studies have indicated that these species of RNA were not responsible for the inhibitory actions of azaG [36–40].

AzaG may have altered the function of mRNA by incorporation either internally into the message or into the 5′-7-methyl-G cap structure of mRNA, thus inhibiting mRNA binding to the 43S preinitiation complex. There is strong evidence in bacteria and eukaryotic cells of incorporation of azaG into mRNA and of altered mRNA function in protein biosynthesis as a result [23, 38–43]. Although azaG inhibited the incorporation of [^{14}C]phenylalanine into proteins in extracts from *B. cereus*, HeLa cells, and regenerating rat liver, polyphenylalanine synthesis was restored in the presence of the synthetic messenger poly (U) [38–40]. Analog-containing polynucleotides were less efficient in stimulating polypeptide synthesis than were the corresponding guanine-containing polynucleotides [44]. The inhibition was particularly pronounced when two guanines were replaced by azaG in a triplet codon [45], even though no evidence of miscoding was found [44].

These experiments bear similarity to those of a totally different drug which also inhibits protein synthesis. Aurintricarboxylic acid, which has been shown to inhibit initiation by preventing the binding of mRNA [46–48], caused a decrease in the relative amount of [^{35}S]met-tRNA$_f$ radioactivity cosedimenting with the 40S subunits in a sucrose gradient [49]. This is similar to the results we obtained with azaG, i.e. azaG caused a decrease in the [^{35}S]met-tRNA$_f$ radioactivity as a percentage of the ^{3}H-labeled RNA banding in a CsCl gradient with the 43S ribonucleoprotein species. Thus, it is possible that azaG incorporation into mRNA reduces the efficiency of binding of mRNA to the 43S preinitiation complex.

Many studies have shown that the effect of azaG on protein synthesis is mediated by its incorporation into mRNA. In addition, the selective effect of azaG on protein synthesis is a major site of action of azaG in tumor cells. Therefore, the incorporation of azaG into mRNA and the subsequent reduced binding of mRNA to the initiation complex may be the mechanism of action of azaG.

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