Evidence for an Active Messenger Ribonucleic Acid Containing 8-Azaguanine*

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ABSTRACT: The exposure of *Bacillus cereus* to the guanine analog, 8-azaguanine, results in the incorporation of the analog into several ribonucleic acid (RNA) species by a replacement of guanine. Studies on the nature of azaguanine-containing messenger RNA reveal properties similar to those accepted as criteria for normal messenger RNA. These include an ability to complex strongly with deoxyribonucleic acid (DNA), a base composition complementary to DNA, and the migration of pulse-labeled RNA in the 4S–16S region of sucrose density gradients.

In addition azaguanine-containing messenger ribonucleic acid isolated from sucrose density gradients displays a high efficiency in stimulating polypeptide synthesis in *B. cereus* cell-free extracts.

The guanine analog, 8-azaguanine, is incorporated into several RNA fractions of *Bacillus cereus* by the substitution of guanine residues (Matthews and Smith, 1956; Mandel and Markham, 1958; Otaka *et al.*, 1961, 1962; Levin, 1963a,b; Grünberger *et al.*, 1964; Chantrenne, 1964; Grünberger, 1965). Based on studies with azaguanine-containing soluble ribonucleic acid [s-RNA(azaG)][1] the nucleotide sequences and composition are otherwise unchanged (Levin, 1963b). Prolonged exposure to the analog produces a two- to threefold increase in cellular RNA due primarily to the accumulation of s-RNA(azaG) and r-RNA(azaG) (Otaka *et al.*, 1961; Levin, 1963b; Grünberger *et al.*, 1964; Chantrenne, 1964). The increment in abnormal RNA is accompanied by a rapid decline in the rate of protein synthesis (Mandel, 1958; Grünberger and Sorm, 1963; Chantrenne, 1964). As a result of the replacement of G, a decrease in the s-RNA secondary structure occurs; the observed diminution is proportional to the extent of analog incorporation (Levin and Litt, 1965). In spite of these changes, an s-RNA(azaG) batch in which 20% of the G residues were replaced retained the ability to accept amino acids as well as to stimulate protein synthesis in bacterial cell-free systems (Levin, 1965; Weinstein and Grünberger, 1965). These findings imply that (1) in a given s-RNA chain, the acceptor function is not necessarily impaired by insertion of the analog; and (2) in those fractions where function is retained, biological specificity remains unaltered. The latter assertion is further supported by the report that azaG in synthetic copolymers codes like G (Grünberger *et al.*, 1966).

These observations have been extended as a result of studies on the functional nature of azaguanine-containing messenger RNA. Previous reports have described the separation of rapidly labeled RNA(azaG) components by density gradient centrifugation and by chromatography on MAK columns (Levin, 1963; Chantrenne, 1964; Grünberger, 1965). Recent evidence indicates that cell-free extracts prepared from azaguanine-treated *B. cereus* cultures carry out polypeptide synthesis using endogenous messenger RNA which contains azaguanine (Grünberger, 1965; D. H. Levin, unpublished data). In addition, isolated RNA(azaG) from *B. cereus* stimulates polypeptide formation in *Escherichia coli* extracts (Grünberger and Mandel, 1965).[2] This communication describes some properties of messengerlike RNA(azaG).

Methods and Materials

Growth of B. cereus. The growth medium was previously described (Matthews and Smith, 1956). Cultures were grown with vigorous aeration at 37° in a rotary shaker bath. Growth was measured turbidimetrically at 540 mμ in the Zeiss spectrophotometer. Where indicated, 8-azaguanine was added during early exponential growth at an OD_{540} of 0.2 (Mandel and Markham, 1958). Cultures were harvested in the cold and washed once in cold standard buffer containing 20 mM Tris–KCl (pH 7.9), 10 mM $MgCl_2$, 60 mM KCl,

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[1] Abbreviations: azaG, 8-azaguanosine 5′-P; azaGTP, 8-azaguanosine 5′-triphosphate; RNA(azaG), RNA containing 8-azaguanine and including m-RNA(azaG), s-RNA(azaG), and r-RNA(azaG); MAK, methylated serum albumin-kieselguhr; S30, 30,000*g* supernatant extract of *Bacillus cereus;* DNAase, deoxyribonuclease; PEP, phosphoenolpyruvate.

[2] The author wishes to thank Drs. D. Grünberger and H. G. Mandel for a copy of their manuscript prior to publication.

and 5 mM reduced glutathione. After centrifugation, the bacterial pellet was frozen at −20°.

Preparation of Alumina. Ca. 100 g of alumina powder (Alcoa, A-305) was stirred for several hours in 1 l. of 2 N HCl and then washed free of acid on a Büchner funnel with several liters of water followed by 95% ethanol. The powder was spread in large Pyrex Petri dishes, saturated with ethanol, and set on fire. The alumina was then stirred in about 600 ml of 10 mM EDTA for 60 min. After settling for 1 min, the supernatant fluid was withdrawn by aspiration to remove small quantities of debris. The suspension was transferred to a Büchner funnel and washed successively with water, ethanol, and acetone and then dried in air. This treatment resulted in the preparation of extracts with consistently higher and more reproducible activity.

Preparation of S30 Extracts. All steps were carried out at 3° except where otherwise stated. Frozen bacterial pellets of normal *B. cereus* cultures were ground in a mortar with alumina (1 g/g of wet weight of bacteria) for 5 min. For pellets of azaguanine cells, the amount of alumina and grinding time were increased threefold. The more intensive treatment was required due to the formation of heavy capsular mucopolysaccharide in azaguanine-treated cultures. The pastes were extracted by further grinding for 2 min in standard buffer (2 ml/g of wet weight of bacteria) containing 8 μg/ml of DNAase. The extract was then centrifuged successively at 2000*g* for 10 min, 10,000*g* for 20 min, and 30,000*g* for 25 min. The upper two-thirds of the final supernatant fluid (S30) was passed through a Sephadex G-25 column (0.9 cm × 29 cm) previously equilibrated with standard buffer. Normal S30 extracts were depleted of endogenous m-RNA by the method of Nirenberg and Matthaei (1961) and then passed through Sephadex G-25 as before. The extracts were stored in small aliquots at −60°.

Isolation of Nucleic Acid. RNA was isolated from DNAase-treated S30 extracts prepared from normal or azaguanine cells by the addition of recrystallized sodium dodecyl sulfate to a final concentration of 0.5% followed by shaking for 5 min at 40°. One volume of phenol was then added and shaking was continued for an additional 10 min. After centrifuging at 9000*g* for 20 min, the upper aqueous phase was collected and reextracted two more times with a half-volume of phenol for 5 min. RNA was precipitated from the combined aqueous phase with two volumes of ethanol containing 2% potassium acetate and the mixture was left at −20° for several hours. After centrifugation the RNA pellet was dissolved in H_2O and reprecipitated. The collected RNA was dissolved and then passed through an EDTA-washed Sephadex G-25 column (0.9 cm × 33 cm) in H_2O. The RNA effluent was precipitated as before, washed once in ethanol, dried, and stored *in vacuo*. Total nucleic acid for MAK fractionation was obtained in the same manner from S30 extracts which were prepared in the absence of DNAase.

Sucrose Density Gradient Centrifugation. RNA (1–5 mg) was dissolved in 0.25 ml of buffer containing 5 mM Tris–KCl (pH 7.4), 10 mM $MgCl_2$, and 30 mM KCl. The solution was layered on 4.6 ml of a linear sucrose gradient (4–20%) containing the same buffer and centrifuged in an SW-39 rotor in a Spinco Model L ultracentrifuge for 6 hr at −5°.

Fractionation of Nucleic Acid on MAK. Ca. 2 mg of nucleic acid from normal or azaguanine-treated cultures was added to MAK columns prepared according to the method of Mandell and Hershey (1960). Fractionation was carried out with a linear gradient of 150 ml of 0.3 M NaCl in the mixing chamber and 150 ml of 1.3 M NaCl in the reservoir, both solutions containing 50 mM potassium phosphate, pH 6.7. Fractions were collected at room temperature in 2-ml aliquots at a flow rate of 1 ml/min.

Assay for Polypeptide Synthesis in Cell-Free Extracts. The messenger activity of RNA(azaG) fractions obtained from a sucrose density gradient (Figure 5) was determined in an incubation mixture containing the following components in a final volume of 0.25 ml: 50 mM Tris–KCl (pH 7.9), 19 mM $MgCl_2$, 92 mM KCl, 8 mM β-mercaptoethanol, 5 mM PEP, 1 mM ATP, 0.2 mM GTP, 20 μg of PEP kinase, 24 mμmoles each of 19 amino acids, 16 mμmoles (3 μc) of [^{3}H]L-lysine, and a 0.05-ml aliquot from each density gradient fraction containing from 30 to 90 μg of RNA. To this mixture was added *ca.* 0.8 mg of protein from an m-RNA-depleted S30 extract obtained from a normal *B. cereus* culture. Incubation was carried out at 37° for 20 min and was terminated by the addition of 0.50 ml of 7.5% cold trichloroacetic acid. After standing in ice for 10 min, the incubation tubes were heated at 90° for 15 min and cooled in ice. Radioactivity was determined as previously described (Levin, 1965).

Electrophoretically purified crystalline DNAase was obtained from Worthington Biochemical Corp. [2-^{14}C]8-Azaguanine (1.1 mc/mmole) was obtained from Baird Atomics. Uniformly labeled [^{3}H]L-lysine (210 mc/mmole) was obtained from Schwarz BioResearch.

Results

Sucrose Density Gradients of [^{14}C]Azaguanine Pulse-Labeled RNA. Upon pulse-labeling of an early exponential culture of *B. cereus* with [^{14}C]8-azaguanine (1 μg/ml) for 3 min, the radioactivity appeared in a fraction which migrated between the 4S and 16S RNA components in sucrose density gradients in a manner analogous to normal pulse labels (Figure 1). A 9-min pulse label in a separate culture displayed a similar RNA profile, but the radioactive peak had begun to separate into light and heavy regions of the density pattern. This probably was due to the degradation of the rapidly labeled RNA and the subsequent uptake of isotope into stable RNA species, a phenomenon characteristic of pulse-labeled RNA in normal bacterial systems (Gros *et al.*, 1961; Midgely and McCarthy, 1962; Attardi *et al.*, 1963; Woese *et al.*, 1963). The low concentrations of analog (1 μg/ml) had no effect on growth during the course of pulse labeling. By contrast, pulse labels of inhibitory concentrations of the

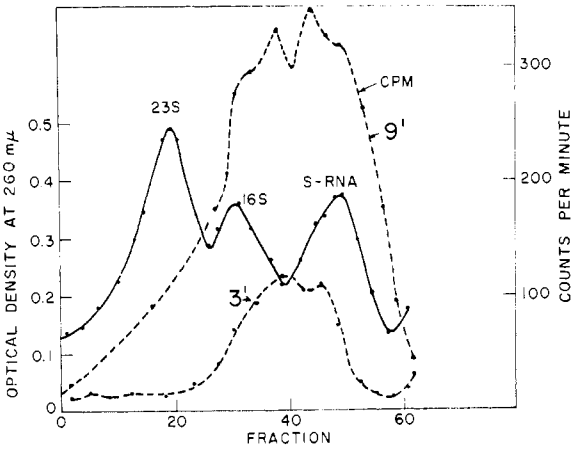

FIGURE 1: Sucrose density gradient of *B. cereus* RNA after pulse labeling with [2-^{14}C]8-azaguanine. To each of two 400-ml cultures (OD_{540} = 0.2) was added 0.4 mg of [^{14}C]azaguanine (2.92 μc). At 3 and 9 min, respectively, the cultures were poured over an equal volume of chopped ice and harvested. RNA was isolated and centrifuged in sucrose density gradients as described in the text. Fractions (0.075 ml) were collected from the bottom of the tube. Ultraviolet determinations at 260 mμ (solid line) measured with 0.05-ml aliquots were the same for both cultures. For radioactivity determinations (dashed lines) 0.02 ml was collected on millipore filters after precipitating with 1 ml of 5% trichloroacetic acid in the presence of added RNA carrier. Radioactivity was determined as previously described (Levin, 1965).

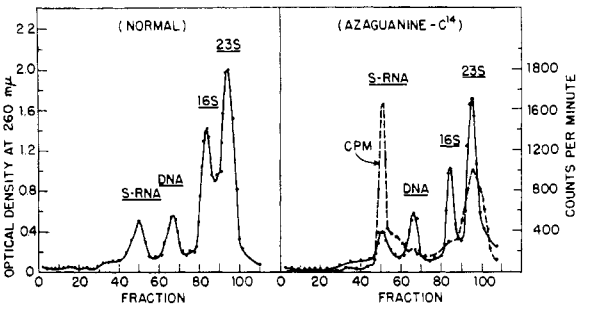

FIGURE 2: Fractionation on MAK of *B. cereus* nucleic acid isolated from normal and azaguanine-treated cultures. A 400-ml culture exposed to [2-^{14}C]8-azaguanine (2 μc, 25 μg/ml) was harvested after 3.5 hr. Nucleic acid was isolated and separated on MAK as described in the text (right graph). A separation of normal nucleic acid is shown in the left graph. For radioactivity determinations (dotted line, right graph) carrier RNA was added to 0.5 ml of each fraction followed by precipitation with 0.5 ml of 10% trichloroacetic acid. The suspensions were collected on millipore filters and counted as previously described (Levin, 1965). Solid lines represent absorption at 260 mμ.

analog (25 μg/ml) first appeared in high molecular weight RNA fractions similar to those previously reported by Grünberger *et al.* (1964).

Distribution of RNA(azaG) on MAK. In a culture exposed for 3.5 hr to [^{14}C]azaguanine (25 μg/ml), the analog was incorporated primarily into s-RNA and 23S r-RNA (Figure 2). Chantrenne (1964) and Grünberger *et al.* (1964) have shown that 1–2-hr azaguanine cultures accumulate an RNA(azaG) species heavier than 23 S which they tentatively identified as precursor ribosomal RNA. The results of this experiment suggest that longer incubation of the culture under highly efficient aeration produces a partial breakdown of the high molecular weight component which then appears on MAK as a broad peak composed primarily of 23S r-RNA(azaG). There is no explanation for the very limited amount of 16S r-RNA(azaG) which was found in this separation (Figure 2).

Base Ratios of Normal and Azaguanine-Containing Nucleic Acid. In order to examine the base ratios of the nucleic acid species of the treated and untreated *B. cereus*, cultures were grown in the presence of inorganic ^{32}P. Unlabeled azaguanine (25 μg/ml) was added to one culture 20 min prior to the addition of the isotope. Under these conditions, only RNA(azaG) was labeled. Fractionation of the isolated nucleic acid on MAK

TABLE 1: Nucleotide Composition of Normal and Azaguanine-Containing Nucleic Acid Fractions of *B. cereus*.[a]

		G	C	A	U(T)	Pyr/Pur	A+U(T)/G+C
S-RNA	(normal)	30.2	28.3	20.9	20.6	0.97	0.71
	(azaG)	21.1(G) 9.8(azaG)	29.3	20.5	19.3	0.95	0.66
16S	(normal)	28.9	21.7	26.8	22.6	0.80	0.98
	(azaG)	19.5(G) 9.1(azaG)	21.2	26.5	23.7	0.81	1.02
23S	(normal)	29.2	21.4	27.9	21.5	0.75	0.98
	(azaG)	19.6(G) 9.1(azaG)	20.9	28.1	22.3	0.76	0.98
DNA		19.6	19.5	29.9	(31.0)	0.98	(1.50)

[a] Normal and azaguanine cultures (400 ml) were grown with ^{32}P-orthophosphate (12 μc). In the abnormal cultures, the isotope was added 20 min after the addition of 8-azaguanine (25 μg/ml) and the culture was grown for an additional 3.5 hr. Nucleic acid was isolated as described in Methods except that the DNAase step was omitted. A separation of the total nucleic acid of each culture on MAK yielded the same ultraviolet absorption and radioactive profiles found in Figure 2. For base ratio determinations, only the peak tube of each fraction was analyzed. Determinations were carried out as described in the legend of Figure 3.

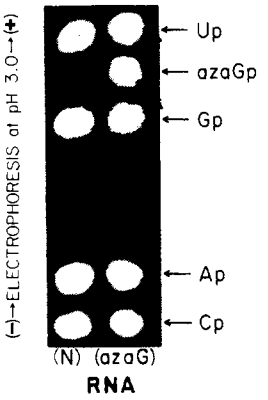

FIGURE 3: Separation of nucleotides by paper electrophoresis. Carrier RNA (100 μg) was added to aliquots of each ^{32}P-RNA(azaG) peak after separation on MAK (see Figure 2) of ^{32}P-labeled nucleic acid isolated from normal and azaguanine-treated cultures (see Table I). KOH hydrolysis was carried out in sealed capillary tubes for 18 hr at 37°. KOH was removed by adsorption on CM-50(H^+) paper (Ingram and Pierce, 1962). The nucleotides were separated by electrophoresis (2.5 kv for 150 min) on Whatman 3HR paper in 20% acetic acid brought to pH 3.0 with NH_4OH (Ingram and Sjöquist, 1963). In this procedure azaGMP-3′(2′) migrates between UMP-3′(2′) and GMP-3′(2′). The figure is a contact print of a typical separation. N = normal RNA; azaG = azaguanine-containing RNA. Deoxyribonucleotides were separated in a similar manner after treatment of isolated DNA with DNAase and venom diesterase.

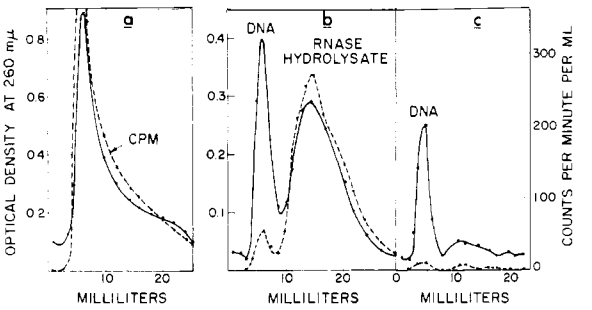

FIGURE 4: Fractionation of a DNA–RNA(^{14}C-azaG) complex on Sephadex G-25 after treatment with RNAase and alkali. The DNA peak of the MAK separation shown in Figure 2 was passed through Sephadex G-25 (0.9 cm × 27 cm) in H_2O to remove salt (Figure 4a). The first 10 fractions were volume reduced *in vacuo* to 0.5 ml; 10 μg of RNAase and 5 μmoles of Tris–KCl, pH 7.5, were added for 60 min at 37°. The mixture was passed again through Sephadex G-25 to separate small components (Figure 4b). The DNA peak was separately collected, dried *in vacuo*, and treated with 0.05 ml of 0.3 M KOH for 18 hr at 37° in a sealed capillary; the solution was then neutralized, diluted to 0.5 ml with H_2O, and passed through Sephadex as before (Figure 4c); ultraviolet absorption = (———); cpm = (----).

yielded the same profiles shown in Figure 2. Aliquots from the peak tube of each RNA species were hydrolyzed in alkali, and the nucleotides were separated by electrophoresis on paper (Figure 3). DNA was hydrolyzed enzymatically and the products similarly separated. As seen in Table I, if azaguanine is taken as guanine, the nucleotide compositions of the normal and abnormal RNA components were similar for s-RNA, 16S r-RNA, and 23S r-RNA, respectively. Similar values have been reported for normal r-RNA (Otaka *et al.*, 1962) and DNA (Belozersky and Spirin, 1960). *Ca.* 30% of the guanine in each RNA(azaG) component was replaced by azaguanine. Since only aliquots from the peak tubes of each MAK fraction were analyzed, it is possible that other RNA(azaG) species were present, particularly in the broad radioactive peak associated with 23S r-RNA.

Characterization of Radioactivity Associated with DNA. The small radioactive peak associated with the DNA fraction of the MAK separation of Figure 2 was further characterized. Passage of the isolated DNA through Sephadex G-25 did not alter the radioactivity profile (Figure 4a). However, after treatment with RNAase *ca.* 78% of the radioactivity was hydrolyzed to smaller components which were retarded on Sephadex G-25 (Figure 4b). These components were collected and identified as RNA fragments. An RNAase-resistant radioactive fraction remained bound to the DNA. Treatment of this fraction with alkali resulted in the separation from DNA of a second RNA hydrolysate fraction containing an additional 19% of the radioactivity (Figure 4c). The residual radioactivity in the DNA after both treatments represented <0.1% of the total nucleic acid radioactivity recovered in the separation on MAK. A base analysis of the treated DNA revealed no azaguanine in agreement with results obtained by other investigators (Lasnitzki *et al.*, 1955; Matthews and Smith, 1956; Grünberger *et al.*, 1964). The residual isotope in the DNA, therefore, may have been contamination although a limited incorporation of analog into DNA beyond the method of detection could not be entirely ruled out. In this respect, Mandel (1961) reported a small incorporation of azaguanine into DNA. The discrepancy in the two results is not yet resolved.

The DNA itself was not degraded by the RNAase and alkali treatment described above. Table II summarizes the amount of radioactivity remaining in the DNA peak after each step in the dissociation of the RNA fractions. The s-RNA and 23S r-RNA peaks isolated in the same fractionation on MAK are included for comparison.

Base Ratios of Two RNA(azaG) Species Bound to DNA. The two DNA-bound RNA(azaG) fractions obtained by treatment of isolated DNA successively with RNAase and alkali were analyzed for their respective base compositions. As shown in Table III,

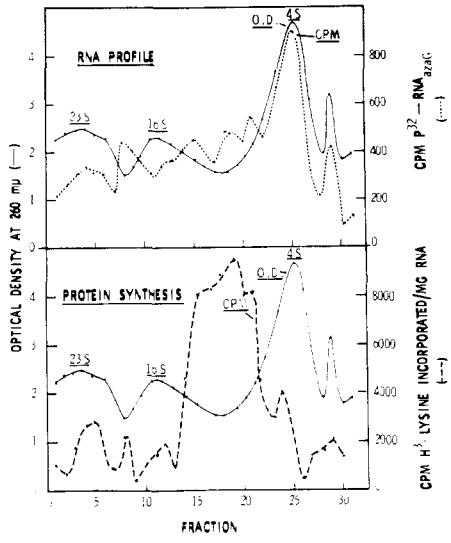

FIGURE 5: Messenger activity of RNA(azaG) fractions obtained by sucrose density gradient centrifugation. ^{32}P-Orthophosphate (3.2 μc) was added to a 1-l. culture 30 min after the addition of azaguanine (25 μg/ml) and the culture was further incubated 60 min. RNA(azaG)-^{32}P was isolated and exposed to density gradient centrifugation as described in the text. Upper figure: RNA profile. Fractions (0.15 ml) were collected after piercing the bottom of the tube; 0.03-ml aliquots were used for measurements at 260 mμ (solid line); 0.05-ml aliquots were used for radioactivity assays (dotted line). Lower figure: protein synthesis. Ultraviolet absorption profile from upper figure is repeated (solid line); messsenger activity (dashed line) of each RNA fraction in the gradient was assayed as described in the text. The ^{32}P-counts in the RNA fractions of the density gradient were solubilized during the heating step of the assay procedure and passed through the millipore filters without interfering with the counting of the tritium-labeled polypeptide which was retained on the filter membranes.

TABLE II: Distribution of Radioactivity in Nucleic Acid Fractions Obtained from [^{14}C-]Azaguanine-Treated Cultures.[a]

Fraction from CH_3-BSA kieselguhr column	CPM per OD_{260} unit of peak tube
sRNA	4150
23S rRNA	560
DNA	408
a) RNase	131
b) RNase + Alkali	18

[a] The nucleic acid fractions were obtained by separation on MAK of nucleic acid from [^{14}C-]azaguanine-treated *B. cereus* and are the same as those shown in Figure 2. Radioactivity was determined as described in Figure 2. The DNA–RNA(^{14}C-aza) complex was treated successively with RNAase and alkali as described in Figure 4. The counts remaining in the DNA after the two treatments are given in the table.

TABLE III: Nucleotide Composition of Two DNA-Bound RNA(azaG) Fractions.[a]

	G	azaG	C	A	U(T)
DNA	19.6	-	19.5	29.9	(31.0)
DNA-bound RNA					
a) RNase-labile	15.2	12.0	23.4	25.6	23.8
b) Alkali-labile	11.2	9.8	20.4	28.6	30.0

[a] A DNA–RNA(azaG) complex labeled with inorganic ^{32}P (see legend of Table I) was isolated by chromatography on MAK as described in Figure 2. The complex was treated successively with RNAase and alkali to obtain two RNA(azaG) hydrolysate fractions as described in Figure 4. The fractions were separately collected and their nucleotide composition determined as described in Figure 3.

the nucleotide composition of the RNAase-sensitive component (Figure 4b) reflected a mixture of r-RNA and s-RNA. A similar observation was reported by Otaka *et al.* (1961, 1962) who isolated a DNA–RNA-(azaG) complex by chromatography of crude extracts on DEAE-cellulose; they tentatively identified an RNAase-sensitive component of the complex as precursor ribosomal RNA. The base ratios of the RNAase-resistant fraction (Figure 4c) were unrelated to s-RNA or r-RNA but were similar to DNA suggesting the presence of a DNA–RNA hybrid. This RNA(azaG) fraction represented <1% of the total radioactivity. Both of the DNA-bound RNA fractions had analog substitution values of 45% compared to an average of 30% for the total RNA(azaG). The increasing incorporation of analog reflected a continuous *de novo* synthesis of s-RNA(azaG), m-RNA(azaG), and r-RNA-(azaG).

Messenger Activity of RNA(azaG). The ability of RNA(azaG) to serve as messenger for polypeptide synthesis was examined in the following way. A *B. cereus* culture was exposed for 2 hr to azaguanine and inorganic ^{32}P so that only RNA(azaG) chains were labeled. A sucrose density gradient of the isolated RNA(azaG)-^{32}P revealed the pattern shown in Figure 5. The highest incorporation of $^{32}P_i$ occurred in s-RNA; a lower radioactivity was observed in the ribosomal RNA fractions. A heterogeneous distribution was found in the 4S–16S region (Figure 5). Messenger activity was assayed by adding aliquots from each fraction in the density gradient to m-RNA-depleted S30 extracts prepared from normal *B. cereus*. The highest stimulation of protein synthesis occurred in the presence of the RNA(azaG) fractions located in the 4S–16S region (Figure 5). This is in agreement with previous observa-

tions that free m-RNA migrates primarily in this region in density gradients (Gros *et al.*, 1961; Attardi *et al.*, 1963).

Discussion

Under certain conditions *B. cereus* produces a number of azaguanine-containing RNA species which display properties generally accepted as criteria for messenger RNA. These include an ability to complex strongly to DNA, a base composition similar to DNA, the migration of pulse-labeled RNA in the 4S–16S region of sucrose density gradients, and a high efficiency in stimulating polypeptide synthesis in bacterial cell-free systems. In addition, the appearance of azaguanine pulse labels in rapidly labeled RNA and the subsequent incorporation into s-RNA and r-RNA represent a distribution of isotope similar to that observed in normal systems. These criteria are consistent with the hypothesis that azaGTP is utilized in the RNA polymerase reactions in competition with endogenous GTP, a mechanism which would account for the appearance of analog in all of the intracellular RNA species.

The high azaguanine content found in cultures exposed to the analog for several hours mitigates strongly against the biosynthesis of normal RNA *in vivo*. This is supported in part by an analysis of two DNA-bound RNA(azaG) fractions, both of which have high analog concentrations indicative of late RNA(azaG) synthesis. One component is RNAase sensitive and appears to be a mixture of s-RNA(azaG) and r-RNA(azaG). The other component is RNAase resistant with a base composition characteristic of m-RNA, which suggests the presence of a hybrid of DNA and nascent m-RNA chains. Such a hybrid would be of particular interest since most evidence indicates that only a single DNA strand is copied by RNA polymerase *in vivo* (Elson, 1965). In the azaguanine-treated organisms, both DNA strands appear to be copied. This could give rise to several types of hybrid intermediates, such as two double-stranded DNA–RNA(azaG) helices or a multiple-stranded complex. Further studies are now in progress to determine the nature of the complex. The high rate of RNA synthesis is in contrast to the limited DNA turnover. It is convenient to speculate that both phenomena may be due to some distortion in the DNA helix *in vivo* which inhibits DNA polymerase but enables RNA polymerase to copy both DNA strands.

Although normal s-RNA and r-RNA, present at the time of azaguanine addition, are retained in a 2-hr azaguanine culture it is unlikely that normal m-RNA is present. This estimate is based on studies which indicate that the half-life of normal m-RNA is 1–4 min and that no stable m-RNA can be demonstrated in bacteria (Midgely and McCarthy, 1962; Attardi *et al.*, 1963; Woese *et al.*, 1963; Gros *et al.*, 1963; Levinthal *et al.*, 1962; Nakada and Magasanik, 1962). It is known, however, that certain agents such as chloramphenicol, which interfere with protein synthesis but not with RNA synthesis, produce an apparent accumulation of m-RNA by inducing conditions which tend to protect m-RNA *vs.* degradation (Midgely and McCarthy, 1962; Nakada and Magasanik, 1962; Levinthal *et al.*, 1963). AzaG induces similar physiological changes, but no evidence exists for the accumulation of normal m-RNA in this system. On the other hand, a rapidly labeled high-molecular weight RNA(azaG) component which has messenger activity does form in this system; but this fraction is rich in azaguanine (Grünberger, 1965; Chantrenne, 1964). It seems probable, therefore, that the high messenger efficiency displayed by the 6S–14S density gradient fractions of the RNA(azaG) profile is due entirely to azaguanine-containing m-RNA. The relatively poor messenger activity of the s-RNA(azaG) and r-RNA(azaG) regions supports this contention.

In an independent investigation, Grünberger and Mandel (1965)[2] surveyed the messenger activity of fractions of the same RNA(azaG) profile using messenger-dependent *E. coli* extracts and reported similar results. Other studies show that endogenous m-RNA(azaG) bound to ribosomes can direct the synthesis of polypeptides (Grünberger, 1965; D. H. Levin, unpublished data). A preliminary analysis of the products formed has revealed the presence of long chain polypeptides which indicates that azaG residues in m-RNA(azaG) are coding (D. H. Levin, unpublished data).

In addition to the results cited here other evidence supports the conclusion that the transcription process from DNA to m-RNA(azaG) catalyzed by RNA polymerase is only partially affected by the incorporation of azaguanine and that the product formed is essentially a complementary copy of DNA (Kahan and Hurwitz, 1962). It is clear, however, that the translation from m-RNA(azaG) into protein *in vivo* is inhibited. This need not imply that m-RNA(azaG) is nonfunctional since other factors in the inhibited cells could interfere with protein synthesis. The possibility that azaG residues in endogenous m-RNA(azaG) permit the synthesis of functional protein is now being investigated.

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