# Enhanced Messenger Activity of RNA from 8-Azaguanine-Treated *Bacillus cereus*

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SUMMARY

The messenger activity of total RNA from normal and 8-azaguanine-treated *Bacillus cereus* cultures was measured by the stimulation of amino acid incorporation into proteins in the preincubated S-30 *Escherichia coli* system. RNA from 8-azaguanine-treated cells enhanced the incorporation of various amino acids into proteins to a greater extent than did RNA from normal cells.

After sucrose gradient centrifugation, the messenger activity of each individual fraction was tested. Most of this activity in both RNA samples was found to be localized between the 16 S and 4 S fractions. In the RNA from analog-treated cells, however, an appreciably greater proportion of this activity was associated with the ribosomal RNA fraction and extended to particles of 23 S and greater.

The greater total messenger activity *in vitro* of analog-treated RNA is discussed in relation to the accumulation of precursors of ribosomal RNA with changed secondary structure and having template activity *in vitro* for amino acid incorporation.

## INTRODUCTION

It has been shown in a number of laboratories (1–4) that 8-azaguanine is incorporated into the RNA of exponentially growing *B. cereus* and that protein synthesis is inhibited soon after the addition of the analog. No 8-azaguanine was observed in the ribosomal particles (5, 6); instead, the analog was present in the sRNA (7)[2] fraction and also in the components which resembled pulse-labeled (messenger) RNA in normal cells (8). Since 8-azaguanine is incorporated into several RNA fractions, it is likely that this incorporation leads to alteration in biochemical functions, such as the interference with protein synthesis.

The function of sRNA containing 8-azaguanine was recently studied by Weinstein and Grünberger (9) in the poly U-directed phenylalanine incorporation. Their results indicate that *B. cereus* azaG-sRNA can participate in this reaction, and no miscoding was observed. Similarly Levin (10) has found that azaG-sRNA can accept leucine, lysine, alanine, and valine. During amino acid incorporation into the *B. cereus* extracts prepared from 8-azaguanine-treated cultures the distribution of radioactivity in the ribosomal and supernatant protein was found to be different from the normal system (11). Since in this system

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[2] Abbreviations used: mRNA, messenger ribonucleic acid; sRNA, transfer ribonucleic acid; CTA, cetyltrimethylammonium ion; ATP, adenosine triphosphate; GTP, guanosine triphosphate; PEP, phosphoenolpyruvate; PK, phosphoenolpyruvate kinase; PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis-2-(methyl-5-phenyloxazolyl)-benzene; poly U, polyuridylic acid.

the stable ribosomes were normal and were formed before the addition of the drug, the mRNA, which exhibits a rapid turnover and contained 8-azaguanine, might well be responsible for the observed effects. The messenger activity of RNA prepared from normal and azaG-treated *B. cereus* was therefore examined in the present work. Measurement of the stimulation of amino acid incorporation into proteins in an *in vitro* system from *Escherichia coli* in which the endogenous messenger activity had been largely destroyed by preincubation (12) served as assay for messenger activity of *B. cereus* RNA fractions. This composite system is more sensitive for amino acid incorporation studies than the reconstituted *B. cereus* system (13).

## METHODS

*E. coli* were grown in a medium containing 8 g of nutrient broth (Difco) and 5 g glucose per liter at 37° with aeration. Cells were harvested in log phase, washed with standard buffer (Tris-HCl 0.01 M, pH 7.8; KCl 0.06 M; magnesium acetate 0.01 M) and frozen at —20° until used.

*B. cereus* 569 H were grown at 37° with shaking in a medium containing 0.15 g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 0.005 g $(NH_4)\ Fe(SO_4)_2$, 1.5 g $K_2SO_4$, 2.0 g Tris, 0.5 mg $MnSO_4$, and 10 g Casamino acids (Difco) in 1 liter. The pH of the medium was adjusted to 7 with HCl. In the exponential phase of growth at a bacterial turbidity of 0.2 (O.D. at 540 mμ), 25 μg 8-azaguanine per 1 ml of medium was added to one cell culture (final concentration 0.16 mM), the other culture serving as control. Both cultures were then grown until an optical density of 0.4. The cells were then centrifuged and washed with standard buffer.

*Preparation of RNA.* RNA was prepared from washed normal and 8-azaguanine-treated *B. cereus* cells using a modification of the method of Ralph and Bellamy (14). The cells, suspended in the standard buffer were disintegrated in the MSE model 60W ultrasonic disintegrator for 5 min at 0° in the presence of 0.5% sodium dodecylsulfate, extracted twice for 10 min with equal volumes of water-saturated phenol at 0°, and centrifuged at 12,000 *g* for 15 min. The aqueous upper phase was extracted three times with ether, and the ether was removed by blowing with nitrogen. To the clear supernatant 2 M sodium acetate was added to make the final concentration 0.1 M, and for each milliliter of solution 0.2 ml of a 1% cetyltrimethylammonium (CTA) bromide solution was slowly introduced. The resulting suspension containing the precipitated CTA-RNA was chilled at 0° for 10 min and centrifuged for 5 min at 5000 *g*. The precipitate was washed twice with 70% ethanol containing 0.1 M sodium acetate to convert CTA-RNA to Na-RNA. After centrifugation the precipitate was washed twice with 95% ethanol, acetone and dried over calcium sulfate in a vacuum desiccator.

*Sucrose gradient centrifugation.* Ten milligrams RNA was dissolved in 1 ml of 0.025 Tris-HCl buffer, pH 7.8, containing 0.05 M NaCl. Of this solution, 0.1 ml was placed on 5 ml of a linear sucrose gradient (20–5%) which was centrifuged in the SW39 rotor of the model L Spinco for 5 hr at 39,000 rpm. After this density gradient centrifugation, the bottom of the tube was pierced and successive 15 drops collected in tubes. One third of each fraction was diluted with water and the optical density at 260 mμ measured. The remaining two-thirds was used for assaying stimulation of amino acid incorporation into protein in the S-30 *E. coli* system.

*Incorporation of amino acids-$^{14}C$ into proteins.* S-30 preparations were made from frozen *E. coli* B cells, suspended in the standard buffer with 0.006 M mercaptoethanol, by disruption in the disintegrator for 5 min at 0°. The suspension was centrifuged twice for 20 min at 30,000 *g*, and the upper three-quarters of the final supernatant solution was stored in the frozen state until use. Preincubation of the S-30 fraction was carried out as described by Nirenberg and Matthaei (12).

Incorporation of amino acids was carried out in 0.25 ml reaction mixtures containing, in μmoles: Tris-HCl (pH 7.8) 25; KCl 15; Mg acetate 3.5; ATP 0.25; GTP 0.008;

PEP (Sigma) 1.88; PK (Sigma) 4 μg; 2-mercaptoethanol 1.5; a mixture of $^{12}$C-amino acids (excluding the $^{14}$C-amino acid), 0.025 of each; the indicated $^{14}$C-L-amino acid 0.025 μC (usually 0.1–0.3 mμmole); preincubated S-30 extract from *E. coli* and RNA from *B. cereus* as indicated. Reactions were carried out at 37° for 30 min and stopped by the addition of 4 ml 10% trichloroacetic acid. The suspension was boiled for 15 min, centrifuged, and the precipitate resuspended in 5% TCA, filtered through fiber filters (T-20A-60 Teflon-coated glass fiber filter 2.4 cm diameter) (15) and washed successively with 5% trichloroacetic acid containing 0.1% of the appropriate amino acid as carrier and then with ethanol. The filters were placed in a counting vial with 10 ml of scintillation fluid (containing 3 g PPO and 100 mg dimethyl PoPoP per liter of toluene) and counted in a Nuclear Chicago liquid scintillation spectrometer.

*Radioactive materials.* Glycine-$^{14}$C with a specific activity 74.2 mC/mmole, leucine-$^{14}$C 237 mC/mmole, valine-$^{14}$C 200 mC/mmole were obtained from New England Nuclear Corporation; phenylalanine-$^{14}$C 302 mC/mmole, lysine-$^{14}$C 201 mC/mmole from Schwarz BioResearch, Inc.

## RESULTS

The effect of total RNA from normal and azaguanine-treated *B. cereus* cells on the incorporation of different amino acids into proteins was investigated in the preincubated S-30 fraction from *E. coli*. The incorporation of amino acids in this system is limited by messenger RNA content. Figure 1 shows that RNA from 8-azaguanine-treated *B. cereus* stimulated the incorporation of valine-$^{14}$C more than did the RNA from normal cells. Similarly in subsequent experiments the incorporation of glutamic acid, glycine, and an algal protein hydrolyzate was increased to a greater extent by the analog-treated RNA than by control RNA. Thus, RNA from 8-azaguanine-treated *B. cereus* had a higher messenger activity.

Figure 2 illustrates the time course of the incorporation of valine-$^{14}$C into proteins. The reaction started without apparent lag,

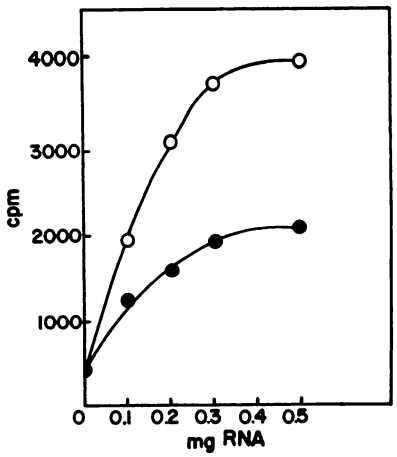


FIG. 1. *The effect of RNA isolated from normal and 8-azaguanine-treated Bacillus cereus cells on the incorporation of valine-$^{14}$C into proteins in the S-30 Escherichia coli system*

The experimental conditions of the assay are described under Methods. ●——●, control RNA; ○——○, 8-azaguanine RNA.

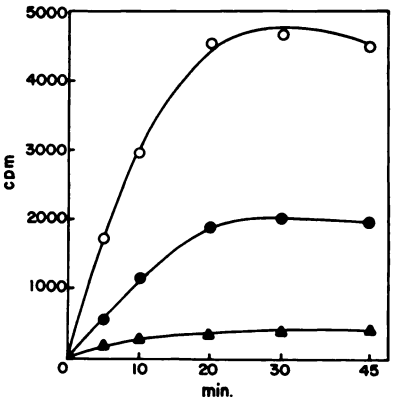


FIG. 2. *Time course of valine-$^{14}$C incorporation into proteins in the preincubated S-30 Escherichia coli system in the presence of 0.35 mg RNA from normal and 8-azaguanine-treated Bacillus cereus cells*

The experimental conditions are described under Methods. ●——●, control RNA; ○——○, 8-azaguanine RNA; ▲——▲ without RNA.

proceeded at an almost linear rate for approximately 20 min, and reached a plateau by 30 min. Although the kinetics of incorporation for both RNA samples were very similar, the higher messenger activity of RNA isolated from 8-azaguanine-treated cells is again apparent.

Since messenger RNA from inhibited cells differs from normal RNA partly by primary structure (guanine partly replaced by 8-azaguanine) and eventually by secondary structure, the possibility was considered that this drug-treated mRNA stimulated the incorporation of different amino acids to dissimilar extents. However, it is apparent from Table 1 that the relative increase in stimulation of incorporation of leucine, lysine, and phenylalanine by abnormal RNA compared to control RNA is essentially the same. These results are consistent with the recent observations that poly U-azaG stimulates the incorporation of amino acids into protein in a manner similar to poly UG, and no miscoding could be observed even when altering the pH or $Mg^{++}$ concentration (D. Grünberger, C. O'Neal, and M. W. Nirenberg, in preparation).

TABLE 1
*Incorporation of various amino acids into proteins using S-30 extract from E. coli and RNA from normal and 8-azaguanine-treated B. cereus*[a]

| Amino acid | Incorporation: μμmoles per mg RNA | Incorporation: μμmoles per mg AzaG-RNA | Relative incorporation AzaG: Control |
|---|---|---|---|
| Leu-$^{14}$C | 125 | 195 | 1.56 |
| Phe-$^{14}$C | 44 | 67 | 1.52 |
| Lys-$^{14}$C | 27 | 41 | 1.52 |

[a] Results are expressed as μμmoles of incorporated amino acids per milligram added RNA.

Since in these experiments total RNA preparations were used, the possibility existed that the stimulation of the incorporation of amino acids was due to the presence of sRNA. Therefore, sRNA was separated from total RNA by treatment with 1.5 M NaCl at 0° (16) and its effect on incorporation of phenylalanine into proteins was determined. Table 2 shows that considerable stimulation of the incorporation of phenylalanine into proteins of the preincubated S-30 fraction takes place only in the presence of total RNA or poly U. In the presence of sRNA, on the other hand, the incorporation is only slightly higher than in the absence of added RNA. These results confirm the role of mRNA as the limiting component of the RNA preparations in the incorporation of amino acids into proteins.

TABLE 2
*The effect of various RNA preparations on the incorporation of phenylalanine-$^{14}$C into proteins in S-30 E. coli system*[a]

| Added | μμmoles phe-$^{14}$C per mg protein | % Incorporation |
|---|---|---|
| — | 2.04 | 100 |
| 0.30 mg RNA | 7.20 | 340 |
| 0.30 mg AzaG-RNA | 9.48 | 458 |
| 0.07 mg sRNA | 2.34 | 118 |
| 0.015 mg poly U | 60.40 | 2900 |

[a] The results are expressed in μμmoles of incorporated phenylalanine per milligram extract protein.

Various investigators have reported the sedimentation coefficient of mRNA to be about 14 S; by linear sucrose gradient centrifugation of total RNA, mRNA is positioned between the 4 S and 16 S fractions (17). In the present experiments messenger

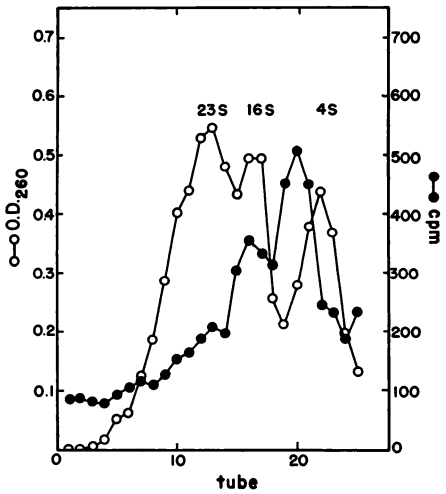


FIG. 3. *Stimulation of $^{14}$C-valine incorporation into proteins by fractions isolated from RNA of normal Bacillus cereus obtained from sucrose gradient analysis*

Details are presented under Methods. ●——●, O.D. at 260 mμ; ○——○, radioactivity (cpm).

activity was characterized after linear sucrose gradient ultracentrifugation of total RNA of *B. cereus*. The individual fractions obtained were tested for their ability to stimulate incorporation of valine-$^{14}$C into proteins in the preincubated S-30 system from *E. coli*. Figure 3 is characteristic of the results normally obtained and shows that the template activity of total RNA from normal *B. cereus* cells is located partly in the 16 S component but mainly between the 16 S and 4 S fractions, as was expected for mRNA.

The distribution of messenger activity of RNA from 8-azaguanine-treated cells differed from that of control cells. As can be seen in Fig. 4, the spectrum of amino acid-

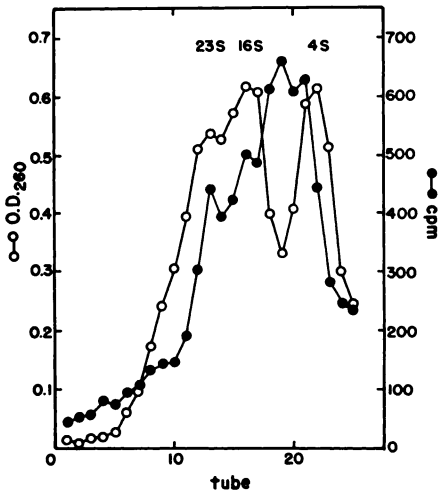


FIG. 4. *Stimulation of $^{14}$C-valine incorporation into proteins by fractions isolated from RNA of 8-azaguanine-treated Bacillus cereus obtained from sucrose gradient analysis*

Details are presented under Methods. ●——●, O.D. at 260 mμ; ○——○, radioactivity (cpm).

incorporating activity of RNA from analog-treated cells is much broader than that of normal RNA. Most of the template activity was associated with the peak at 16 S and the area between the 16 S and 4 S fractions, but activity was also recovered in fractions of 23 S, or even higher. The relative ultraviolet absorption profile for RNA from analog-treated cells at 16 S was greater than that of 23 S, in contrast to normal RNA, probably because of accumulation of large quantities of ribosomal RNA in the 16 S area.

## DISCUSSION

Several possibilities have been considered to explain the mechanism of inhibition of protein synthesis by azaguanine observed in *B. cereus* cells. Inhibition of amino acid incorporation by the anabolite azaguanosine triphosphate, by interference with the GTP requirements during protein synthesis, has been ruled out (18). In the *in vitro* model system the incorporation of amino acids not only continues (11) but per milligram of RNA is actually enhanced by treatment of cells with the analog. Incorporation of the analog into sRNA apparently does not lead to inhibition of amino acid incorporation or miscoding (9, 10). There is little or no incorporation of the analog into ribosomes (5, 6), and instead a part of the analog after brief exposure to cells is recovered in RNA fractions which sediment on sucrose gradients in a manner similar to that of mRNA and may be ribosomal precursor (6, 8). Thus it is conceivable that such 8-azaguanine-containing RNA may only serve as an artificial messenger *in vitro* for the incorporation of amino acids into polypeptide chains which, however, would lack the proper biological functions *in vivo*.

Kono *et al.* (19) have shown that the mature ribosomal particles are formed in several intermediate steps starting from a set of free ribosomal RNA's with nearly the same sedimentation coefficients as the mature RNA. These "premature" ribosomal RNA's are accumulated in the *E. coli* cells in the presence of chloramphenicol, 5-fluorouracil (19) or in the early phase of recovery from magnesium starvation (20), during which the synthesis of new ribosomes is blocked. In contrast to the mature ribosomal RNA, these particles possessed "messenger" activity (21), possibly due to the changed secondary structure which was observed in the "premature" RNA's (20, 22).

In previous work using methylated albumin columns, the accumulation of an abnormal RNA peak containing 8-azaguanine

was described (8) and has been confirmed independently by Chantrenne (6). Since the half-life of this fraction was much longer than is characteristic for mRNA (6), and since the analog prevents the formation of mature ribosomal particles, it is likely that this fraction contains a precursor of ribosomal RNA, similar to the results obtained under the conditions described previously (19, 20). Thus, the observed enhancement of messenger activity of RNA from 8-azaguanine-treated cells may then be due to the accumulation of premature ribosomal RNA containing the analog and exhibiting messenger activity, as has been observed by Otaka *et al.* (21) for chloramphenicol-treated cells. The possibility cannot be ruled out, however, that RNA from 8-azaguanine-treated cells contains messenger that is less degradable than in normal cells, and consequently displays a higher average sedimentation rate.

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