Effect of 8-Azaguanine on the Transition from Vegetative Growth to Presporulation in Bacillus cereus

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ABSTRACT

STAHL, D. P. (University of Illinois, Urbana), V. R. SRINIVASAN, AND H. ORIN HALVORSON. Effect of 8-azaguanine on the transition from vegetative growth to presporulation in Bacillus cereus. J. Bacteriol. 91:1875–1882. 1966.—The guanine analogue, 8-azaguanine (azaG), was found to inhibit sporulation of Bacillus cereus strain T when added to proliferating cells, but not to inhibit when added after the transition to presporulation. When azaG was added to vegetative cells, the growth rate was reduced, but no immediate bactericidal effect was demonstrated. Azaguanine was shown to be incorporated solely into ribonucleic acid (RNA). All of the natural purine bases and nucleosides were found to prevent azaG inhibition by blocking incorporation of the analogue into the RNA. Addition of a subinhibitory level of C14-azaG to proliferating cells resulted in an increase in incorporation paralleling the increase in number of cells. At the time of transition from growth to presporulation, a rapid removal of the azaG label from the cells occurred in the absence of net RNA breakdown. If differentiation was inhibited by increasing the concentration of azaG, then no expulsion took place. Instead, at the end of growth, net incorporation ceased, and a steady-state condition was established in which incorporation equaled breakdown. No azaG degradative enzymes are present in presporulating cells. The possibility is discussed that an increase in the ratio of natural purines to azaG occurred at the time of transition, and that the natural purine derivatives then were reincorporated into RNA preferentially to azaG. The data are consistent with the hypothesis that an increased rate of RNA turnover occurs at the time of transition from vegetative growth to presporulation. Addition of phosphate buffer (pH 7.0, 0.1 M) to azaG-inhibited vegetative cells caused reversal of inhibition, the reversal being accompanied by expulsion of the azaG. At least a partial explanation of this effect is that phosphate causes a decrease in the azaG intracellular pool size.

The structural changes accompanying bacterial spore formation are preceded by changes in the chemical composition and metabolism of the cells. As spore-forming bacilli reach the end of their growth phase, several significant metabolic changes occur which culminate in "commitment" to sporulation. The appearance of new enzymes during sporulation suggests that some shift in control mechanisms occurs during the transition from growth to sporulation.

Little is known concerning the control mechanisms for the transition from vegetative growth to presporulation. One method of gaining information concerning this stage of differentiation is to employ inhibitors which prevent sporulation when added prior to this transition, but do not inhibit sporulation when added thereafter. One inhibitor found to exert such a differential effect is the guanine analogue, 8-azaguainine (azaG). In other systems, the most significant inhibitory effect of azaG is on protein synthesis (2, 16, 20). It was thought by some that azaG triphos-
phate might be the active inhibitory form of the analogue, acting as a fraudulent coenzyme in the protein synthetic reaction. However, Roy et al. (22) found that azaG triphosphate produced no inhibition of C4-L-leucine incorporation in an in vitro protein-synthesizing system. The incorporation of azaG into the ribonucleic acid (RNA) of a wide variety of organisms and tissues has now been well established, and it is probable that "defective" RNA is the main cause of inhibition of growth and protein synthesis (2, 16). Testing the azaG-containing sRNA and mRNA in in vitro protein-synthesizing systems has thus far revealed no significant miscoding of amino acids (13, 19, 24). Although definitive evidence is still lacking, it has been suggested that perhaps the primary inhibitory effect of azaG may be on ribosome function (3, 7).

**Materials and Methods**

*Test organism. Bacillus cereus strain T was used as the test organism.*

*Cultural methods. The complex medium used in this study, G medium, was developed by Greenberg (Ph.D. Thesis, University of Illinois, Urbana, 1954). The constitution and methods of preparation of G medium were described by Gollakota and Halvorson (6). In some instances, the medium was buffered more strongly than normal by adding potassium phosphate buffer (0.1 M, pH 7.0). The "active culture" technique modified for the growth and sporulation of *B. cereus* T was employed (4, 8).*

*Measurement of optical absorbance and pH. Growth of cultures, as measured by optical absorbance, was determined by use of a Klett-Summerson photoelectric colorimeter with a no. 66 filter (640 to 700 mλ). The pH measurements were made with a Beckman Zeromatic pH meter.*

*Spor and viable-cell counts. Total viable-cell counts were determined by preparing pour plates of properly diluted samples with nutrient agar (Difco). Spore counts were obtained by heating the samples at 80 C for 15 min in a water bath prior to plating. All samples were plated in quintuplicate.*

*Cellular incorporation of radioactive isotopes. Cells were incubated in an appropriate medium with an amount of radioactive isotope suitable for obtaining a maximal incorporation of at least 2 × 10^5 counts per min per ml. At no time was more than 10% of the isotope incorporated into the cellular nucleic acids. Culture samples of 0.5 to 1 ml were treated in the desired manner (see fractionation procedure below) and filtered through Bact-T-flex 27-mm Schleicher & Schuell nitrocellulose membrane filters (Schleicher & Schuell Co., Keene, N.H.). The filters were washed with 50 ml of the appropriate solution, dried, and placed in scintillation vials. The scintillation fluid consisted of 4.02 g of 2,5-diphenyloxazole (PPO) and 0.1005 g of 1,4-bis-(5-phenyloxazolyl)benzene (POPOP) per liter of toluene. The radioactivity was determined in a Packard automatic Tri-Carb liquid scintillation spectrometer. When C4 and H3 were counted in a single sample, the H3 counts were corrected for channel crossover.*

*Cell fractionation. The fractionation procedure utilized was that of Roodyn and Mandel (21).*

**Determination of pool size. To determine the amount of a labeled substance present in the intracellular pool of *B. cereus* T, cells were grown with the labeled substance, and, at appropriate times, duplicate 1-ml samples of the culture were pipetted into two tubes containing 1 ml of 1 M NaCl; at the same time, duplicate 1-ml samples of the culture were pipetted into two tubes, each containing 1 ml of 10% trichloroacetic acid. The "NaCl cells" were filtered immediately, washed with 50 ml of 1 M NaCl, and the radioactivity was determined. This count represented the total cell radioactivity. The "trichloroacetic acid cells" were kept in an ice bath for 30 min, after which they were filtered, washed with 50 ml of 5% trichloroacetic acid, and assayed for radioactivity. The difference between the radioactivity in the cold trichloroacetic acid-preceptible cell fraction and the total cells gives an estimation of the pool size.*

*Cell-free extract preparation. Cells of the desired physiological age, determined by turbidity, morphology, and pH, were harvested by chilling the culture to 4 C, and were centrifuged. The cells were then washed with 0.05 M phosphate buffer (pH 7.0), suspended in the same buffer, and broken in a French pressure cell. The broken cell suspension was centrifuged at 15,000 X g for 10 min in a Servall SS-3 centrifuge at 4 C, and the supernatant fraction of the cell extracts was used for enzymatic assays.*

*Assay for azaG deaminase. Enzyme activity was assayed by microdetermination of the ammonia evolved. The incubation mixture described by Hirschberg, Kream, and Gelhorn (10) consisted of 5.0 ml of azaG (10 mg), 1.0 ml of cell-free extract, and 4.0 ml of the 0.05 M phosphate buffer (pH 7.0). Incubation was at 37 C for 2 hr. The reaction was stopped by the addition of 3 drops of 8 M H2SO4.*

*Colorimetric determination of ammonia. Ammonia was determined by the phenol hypochlorite method as adapted to the micro-Kjeldahl method of nitrogen analysis by Niss (Ph.D. Thesis, Purdue University, Lafayette, Ind., 1958).*

**Materials.** The following radioactive chemicals were used: 8-azaG-2-C^4 (1.1 mc/mmole), Nuclear Research Chemicals, Inc., Orlando, Fla.; H3-uracil (500 mc/mmole), Nuclear Chicago Corp., Chicago, Ill.; guanine-8-C^14-HCl (10 mc/mmole), New England Nuclear Corp., Boston, Mass. Unlabeled azaG was purchased from Mann Research Laboratories, New York, N.Y. The radioactive chemicals and the unlabeled azaG were found to be chromatographically pure.

**Results**

When *B. cereus* T cells are grown in G medium, the pH decreases and, after growth is complete, rises again. The drop in pH has been shown to be due to the production from glucose of several organic acids, principally acetic and
pyruvic acids (18). At the time when glucose is nearly exhausted, the enzymes of the tricarboxylic acid cycle are induced, thus resulting in the utilization of the acids and, hence, the pH rise (9). After acetate utilization has begun, the cells are spoken of as having been "committed" to sporulation. This term is derived from the observations that (i) washed cells at this stage can be transferred to a medium which does not support growth, and sporulation will still proceed (17, 23), and (ii) glucose can be added to the culture without repression of sporulation (H. M. Nakata, Ph.D. thesis, University of Illinois, Urbana, 1959).

Cells were grown in G medium, and azaG was added at two stages of development: (i) during the middle of exponential growth, when the pH had declined to 6.0 from an original value of 7.0, and (ii) after "commitment" to sporulation, when the pH had increased from a minimum of ca. 4.8 to 6.0. Sporulation was essentially completely inhibited when the analogue was added to the growing cells at concentrations of 10 μg/ml or more. However, concentrations up to and including 100 μg/ml were ineffective in blocking sporulation when added to "committed" cells.

Time of development of resistance to azaG. The results of Fig. 1 indicate that the cells became resistant to azaG by the time growth was complete and the pH minimum had been attained. These results are similar to those obtained by Young and Fitz-James (25) and Del Valle and Aronson (5).

Effect of azaG on growth. Addition of 100 μg/ml of azaG to a growing culture at zero-time resulted in a decrease in growth rate after about 1 hr, as determined by viable-cell count. The extent of growth in the inhibited culture was about 60% that of the control culture after 5 hr. In the culture containing analogue, the pH declined to a minimum value, but failed to subsequently rise, thus indicating repression of tricarboxylic acid cycle activity.

Reversal of azaG-induced inhibition of sporulation by purine derivatives. Azaguanine is incorporated solely into RNA as determined by cell fractionation after growth in the presence of the isotope. Treatment of cells labeled with C14-azaG with hot trichloroacetic acid or NaOH removed more than 99% of the radioactivity remaining after cold trichloroacetic acid precipitation.

Inhibition of sporulation by azaG was shown to be at least partially reversed by any of the normal purine bases or nucleosides (Table 1). It was subsequently found that these purines were capable of blocking the incorporation of C14-azaG into the RNA of growing cells. Since azaG is known to replace guanine in RNA (12), it is probable that the guanine and guanosine compete with azaG at one or more of the steps involved in conversion of the base to the nucleotide triphosphate and finally to the polynucleotide. The accumulation of an inhibitory level of azaG

![Fig. 1. Effect of time of addition of azaG (100 μg/ml) on sporulation. AzaG was added to cultures at different stages of development, and, after completion of sporulation, spore counts were made and compared with a control. The number of spores produced by the control culture is defined as 100% sporulation. The pH curve is that of the control culture.](http://jb.asm.org/)

**Table 1. Effect of natural purines, pyrimidines, and their derivatives on the inhibition of sporulation by azaG (100 μg/ml)***

<table>
<thead>
<tr>
<th>Reversal agent (250 μg/ml)</th>
<th>Per cent sporulation†</th>
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<tbody>
<tr>
<td>None</td>
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<tr>
<td>Adenine</td>
<td>78</td>
</tr>
<tr>
<td>Adenosine</td>
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</tr>
<tr>
<td>Guanine</td>
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* AzaG and reversal agents were added to cultures at zero-time.
† After completion of sporulation, spore counts were made and compared with that of a control culture without azaG. The number of spores produced by the control culture is defined as 100% sporulation.
in RNA would thus be prevented. The purines other than guanine are probably readily converted into guanine in *B. cereus* T. Such means of interconversion are known to occur in other bacteria (14).

Reversal of azaG-induced inhibition of sporulation by phosphate buffer. In these studies, it was felt that it would be desirable to buffer G medium to eliminate pH changes which might complicate interpretation of data. However, it was discovered that the differential effect caused by azaG was not observed when 0.1 M potassium phosphate buffer was included in the medium. Addition of phosphate buffer simultaneously with azaG (100 μg/ml) at zero-time or as late as 5 hr after addition of the analogue resulted in the formation of a substantial number of spores.

This led to the investigation of whether reversal of inhibition by phosphate buffer was due to the phosphate per se or to a pH effect. An attempt to answer this question was made by maintaining a vegetative culture containing 100 μg/ml of azaG at pH 7 by periodic additions of NaOH. After sporulation was complete, spore counts were made and compared with an inhibited and an uninhibited control. The results indicated that phosphate was only slightly better than NaOH as a reversal agent. Thus, the major effect of phosphate was probably a pH effect.

Figure 2 presents results of an experiment designed to determine what influence phosphate had on azaG incorporation into RNA. A subinhibitory concentration of C¹⁴-azaG (1.9 μg/ml) was added at zero-time to two vegetative cultures of *B. cereus* T, one in normal G medium and the other in phosphate-buffered medium. Tritiated uracil was included in both cultures as an internal control to determine the effect of phosphate on RNA synthesis in general.

The maximal incorporation of C¹⁴-azaG in the phosphate-buffered culture was ca. 65% less than that of the control culture. This decreased incorporation in the buffered culture was unique to azaG, as indicated by the fact that maximal H²-uracil incorporation was not greatly different in the presence of phosphate from that in its absence. Similarly, phosphate had no effect on guanine incorporation. The expulsion of C¹⁴-azaG from RNA at the time of "commitment" to sporulation was also shown to be unique to the analogue, as there was no decrease in the incorporated H²-uracil. In a separate experiment, it was also found that no decrease occurred in the level of C¹⁴-guanine incorporated into RNA after "commitment."

The effect of phosphate on relative azaG pool size during development of the organism in G medium supplemented with C¹⁴-azaG was determined, and it was found that, in the presence of phosphate, the pool size was 50 to 70% smaller than in unbuffered G medium. This effect correlates well with the phosphate-induced 65% inhibition of incorporation of azaG into RNA (Fig. 2). Therefore, it is probable that the ability of phosphate to decrease incorporation of azaG into RNA is related to the smaller azaG pool size evident in its presence.

The experiments involving C¹⁴-azaG incorporation that have been described thus far have all involved subinhibitory concentrations of azaG (1.9 μg/ml). The fact that the removal of azaG from the RNA occurred at the same time that "commitment" to sporulation normally occurs suggested that the two phenomena might be related. If they are related, then inhibition of differentiation by higher concentrations of azaG should inhibit the expulsion of azaG from the cells. Figure 3 shows results of an experiment in which C¹⁴-azaG incorporation was measured when the analogue was present at concentrations of 1.9 μg/ml (subinhibitory) and 13.5 μg/ml (inhibitory). Inhibition of differentiation did indeed result in a maintenance of incorporated azaG in the RNA. The equivalence of the maximal extents of incorporation in the two cases is of no significance, having arisen simply from use of a smaller proportion of labeled to nonlabeled...
Thus, it is probable that the mechanism of phosphate-induced removal of azaG from RNA during this steady-state period is the same as that described previously; i.e., by decreasing the azaG pool size so that incorporation is slowed down.

Decrease in incorporated azaG during the transition to presporulation. It has been indicated that the decrease in incorporated azaG in RNA during the normal transition from vegetative growth to presporulation is peculiar to the analogue; i.e., C4'-guanine and H2-uracil are not expelled at this time.

One possible mechanism whereby azaG could be removed from RNA after "commitment" to sporulation is by the induction at this time of an enzyme capable of degrading the analogue. The azaG released during normal "turnover" would be degraded and hence would be unavailable for reutilization. Azaguanine deaminase, an enzyme which catalyzes the conversion of azaG to azaxanthine, has been reported to be the means of resistance to azaG in certain mam-

azaG in the case of the culture with the higher concentration of analogue.

When phosphate was added to a culture containing an inhibitory amount of azaG either during the stage of increasing incorporation or after maximal incorporation had been attained, the RNA C4'-azaG content rapidly decreased (Fig. 4). It has been suggested that the ability of phosphate to remove azaG from RNA has been related to its ability to decrease the azaG intracellular pool size and, hence, the extent of its incorporation into RNA. This mechanism can be tentatively invoked in the case of phosphate addition after attainment of maximal azaG incorporation only if it is demonstrated that "turnover" of RNA is occurring during this stage. If "turnover" of RNA is occurring during the state of no net change in RNA-C4'-azaG content, then the label should be effectively "chased" from the RNA by addition of a large excess of unlabeled azaG. Figure 5 shows the effect of adding 250 μg/ml of unlabeled azaG to an azaG-inhibited culture during growth and after attainment of the stage referred to above. The label was readily "chased" from the cells in both cases, indicating that "turnover" was indeed occurring.

**Fig. 3.** Incorporation of C4'-azaG into cells when the analogue is present at a subinhibitory concentration (1.9 μg/ml) and at an inhibitory concentration (13.5 μg/ml). The azaG was added in both instances at zero-time. Samples were taken periodically during development, and the radioactivity of the cold trichloroacetic acid-precipitable material was determined.

**Fig. 4.** Effect of time of addition of phosphate buffer on C4'-azaG incorporation into RNA and expulsion from RNA. An inhibitory level of C4'-azaG (13.5 μg/ml) was added to a culture growing in G medium at zero-time, and phosphate buffer (0.1 M, pH 7.0) was added to portions of this culture at various times thereafter. The arrows indicate time of addition of the buffer. Samples of each culture were taken periodically, and the radioactivity of the cold trichloroacetic acid-precipitable material was determined.
malian tissues (10). If this reaction was carried out by “committed” B. cereus cells but not by vegetative cells, then the release of azaG from the former cells would be easily explainable.

The product azaxanthine, when added to vegetative cells or presporulating cells in G medium at a concentration of 500 μg/ml, produced no inhibition of sporulation. All attempts to detect azaG deaminase in cells “committed” to sporulation have been unsuccessful, however. Further evidence for the absence of azaG deaminase activity lies in the fact that 4-amino-5-imidazolecarboxamide, a potent inhibitor of azaG deaminase (1), did not render “committed” cells susceptible to azaG inhibition.

Another possible mechanism whereby azaG might be expelled from RNA at the time of “commitment” is by an increase at this time in the ratio of natural purines to azaG in the intracellular pool. A hypothesis as to how this change in ratio might occur is included in the discussion section. Guanine and guanosine are effective as reversal agents of azaG inhibition (Table 1). It is possible that guanine and derivatives present in the pool after “commitment” to sporulation are utilized preferentially to the azaG released by “turnover.” Evidence for preferential incorporation of guanine over azaG was provided by the following experiment, the results of which are presented in Fig. 6. An inhibitory concentration of C^4-azaG was added to a vegetative culture, and guanine was added to samples of the culture at concentrations of 5, 20, and 100 μg/ml (Fig. 6A). Unlabeled azaG was added to similar samples at the same time (Fig. 6B). The results clearly demonstrate that guanine was much more effective in “chasing” than azaG, guanine at a
concentration of 5 µg/ml being as effective as 100 µg/ml of azaG.

**Discussion**

The guanine analogue azaG has been shown to inhibit sporulation of *B. cereus* T only when added to the cells prior to the time of transition from vegetative growth to presporulation. When a subinhibitory concentration of C¹⁴-azaG was added to vegetative cells, an increase in incorporation occurred which paralleled the increase in number of cells. Coincident with the time of transition from vegetative growth to presporulation, an expulsion of 80 to 85% of the azaG from the RNA occurred. This expulsion took place in the absence of net RNA breakdown as shown by the maintenance within the cells of incorporated H³-uracil and C¹⁴-guanine. If an inhibitory concentration of azaG was added to a vegetative culture, no such C¹⁴-azaG expulsion was observed, the incorporation reaching a maximum and no net change occurring thereafter. Most of the azaG was expelled before the pH of the medium began to rise (Fig. 2), so simple neutralization by utilization of the acids in the medium is not the explanation.

One possible mechanism whereby azaG could be removed from RNA after the transition to presporulation is by the induction of an enzyme(s) capable of degrading the analogue. None was found, however.

Another possible mechanism whereby azaG might be expelled from RNA at the time of transition to presporulation is by some process causing an increase in the ratio of natural purines to azaG in the intracellular pool. Guanine and guanosine were shown to be effective in reversal of azaG-induced inhibition of sporulation (Table 1). Guanine was also shown to be more effective than azaG in chasing the C¹⁴-azaG label from inhibited cells (Fig. 6). The latter finding constituted indirect evidence for preferential incorporation of the normal base over the analogue. More direct evidence for preferential incorporation of guanine over azaG was provided by Mandel (15). This author reported that, in *B. cereus* 569H, C¹⁴-guanine incorporation into nucleic acid was unaffected by azaG. Inhibition of growth and incorporation of azaG into RNA began only when the guanine was completely taken up. The same phenomenon occurred when adenine was used in place of guanine. Therefore, it seems possible that if the ratio of natural purines to azaG increased sufficiently at the time of transition to presporulation, then the azaG released by normal RNA "turnover" would be discriminated against, the guanine being incorporated preferentially.

Intracellular pool size may be determined in part by extracellular concentration of the substance in question and in part by the intracellular release of the material from a polymerized form. Since azaG is present in the medium at a high concentration (1.9 µg/ml), of which no more than 10% is taken into the cells, it is probable that the extracellular concentration of azaG is the main factor determining its pool size. On the other hand, intracellular breakdown of RNA releasing natural purines may contribute significantly to their pool size. Thus, if a greater rate of RNA turnover occurs at the time of transition from vegetative growth to sporulation than during growth, the ratio of normal purines to azaG in the pool may well increase. Whether such an increased turnover occurs in *B. cereus* T remains to be determined. Leitzmann and Bernlohr (11) found that in *B. licheniformis* an increase in nucleotide pool size did occur after "commitment" to sporulation, thus lending credibility to this hypothesis.

Addition of potassium phosphate buffer (pH 7.0; 0.1 M) simultaneously with azaG (100 µg/ml) at zero-time or as late as 5 hr after the addition of the analogue resulted in reversal of inhibition of sporulation. This reversal was shown to be accompanied by a 90% removal of azaG from the cellular RNA. Probably, the explanation for the ability of phosphate to remove azaG from the RNA is its effect on azaG intracellular pool size. In the presence of phosphate, the azaG pool size was 50 to 70% smaller than in unbuffered G medium. The phosphate effect on azaG pool size could possibly be due solely to the increase in pH. It is known that azaG has a pKₐ of 7.0 as compared with a pKₐ of 9.3 for guanine. Therefore, at pH 7.0, the azaG would be more anionic than guanine. If the more negatively charged molecule was less reactive with the permease than the normal molecule, then phosphate would be expected to reduce azaG permeation while not affecting guanine permeation.

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