EFFECT OF 8-AZAGUANINE ON GROWTH AND VIABILITY OF
BACILLUS MEGATERIUM

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ABSTRACT

MANGALO, R. (University of Illinois, Urbana) and J. T. WACHSMAN. Effect of 8-azaguanine on growth and viability of Bacillus megaterium. J. Bacteriol. 83:27–34. 1962.—The addition of 8-azaguanine to exponentially growing cells of Bacillus megaterium results in an inhibition of growth after a lag of approximately 30 min. However, 8-azaguanine-2-C14 is incorporated into the nucleic acids in a linear fashion without a detectable lag. The inhibitory action is reversed by purines and their derivatives, but not by uridine, thymidine, or cytidine. 8-Azaguanine is bactericidal, especially under conditions where growth (ribonucleic acid synthesis) is possible. Growth in the presence of a complete amino acid mixture, either before or during exposure to 8-azaguanine, increases the rate of killing. Chloramphenicol has little or no effect on the bactericidal action of the analogue.

During a study of the effect of various analogues on the growth of Bacillus megaterium, 8-azaguanine was found to be a powerful growth inhibitor. The present work shows that 8-azaguanine is incorporated into the nucleic acids of this organism and that it is a potent bactericidal agent, especially under conditions where growth is possible.

MATERIALS AND METHODS

B. megaterium strain KM was grown on a basal medium of the following composition: 1.0% glucose, 0.65% Na-l-glutamate, 0.3% K2HPO4, 0.1% KH2PO4, 0.1% Na2SO4, 0.01% MgSO4·7H2O, and 3 × 10^-4 M ferric citrate (final pH, 7.0). Mutant A, a tyrosine- or phenylalanine-requiring strain of this organism (isolated during the course of this investigation), was grown on the above basal medium supplemented with the complete amino acid mixture shown in Table 1. This is a modification of an equilibrated amino acid mixture (Roberts et al., 1957), containing L-amino acids in ratios corresponding to their occurrence in Escherichia coli proteins. The basal medium containing a complete amino acid mixture was also used in several experiments with strain KM. Vigorous aeration was provided by growing 10- to 40-ml quantities in 250-ml Erlenmeyer flasks, on a rotary shaker (30 or 37 C). Flasks with attached colorimeter tubes were used in experiments on growth rates. Optical densities were determined with a Klett-Summerson colorimeter, using filter no. 64.

Unless otherwise stated, 8-azaguanine and other compounds were added to exponentially growing cells at 50 Klett units (approximately 7 × 10^9 viable cells/ml).

The following procedure was used in most experiments on the bactericidal effect of 8-azaguanine: The inoculum consisted of cells growing exponentially at 30 C on basal medium supplemented with the complete amino acid mixture (Table 1). Cells were harvested by centrifugation, washed twice with basal medium, and resuspended in basal medium. Cells were incubated in growth flasks on a rotary shaker at 37 C, until approximately two generations had occurred (reading of 50 Klett units). A sample was then removed for a viable count and 8-azaguanine was added (final concentration, 100 μmoles/ml). After mixing, a 10.0-ml sample was transferred to a 200-ml Erlenmeyer flask, which was incubated with shaking at 37 C. Care was taken to avoid the adherence of small droplets of suspension to the sides of the flask. Samples of 1 ml were periodically removed, centrifuged, washed twice with basal medium, and finally diluted in the basal medium. Samples (0.1 ml) of appropriate dilutions were spread on the surface of plates containing the basal medium.
supplemented with 2% agar (basal agar). Colonies were counted after incubation for 48 to 72 hr at 30 C. In some experiments, the inoculum consisted of cells growing exponentially in the basal medium alone (absence of amino acid mixture) at 30 C. Cells were sometimes plated on basal agar containing guanosine (0.5 μmoles/ml) or on basal agar containing the amino acid mixture of Table 1.

For experiments on the uptake of the radioactive analogue, 8-azaguanine-2-C\(^{14}\) was added to exponentially growing cells at a final concentration of 100 μmoles/ml. The suspension was transferred to a fresh 200-ml Erlenmeyer flask, which was incubated with shaking. Samples (1 ml) were removed and mixed with 1.0 ml of 10% trichloroacetic acid (TCA) in an ice bath. After allowing 15 min for flocculation to occur, 8.0 ml of 5% TCA were added. The precipitate, obtained by centrifugation in the cold, was washed with 10 ml of cold water and resuspended in 2.0 ml of water. Samples (1 ml and 0.5 ml) were dried on plastic planchets and counted at infinite thinness in a gas flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) for a minimum of 640 counts.

8-Azaguanine, adenine, guanine, guanosine, and uridine were obtained from Mann Research Laboratories, Inc., New York. Thymidine, inosine, and cytidine were products of Schwarz Laboratories, Inc., Mt. Vernon, N. Y. 4-Amino-5-imidazolecarboxamide was obtained from the Sigma Chemical Co., St. Louis, Mo., 2,6-diaminopurine from Nutritional Biochemicals Corp., and deoxyguanosine and 8-azaguanine-2-C\(^{14}\) from the California Corporation for Biochemical Research, Los Angeles.

8-Azaguanine and the complete amino acid mixture were sterilized by autoclaving; all other compounds were sterilized by filtration.

### RESULTS

The effect of the addition of different concentrations of 8-azaguanine on the growth rate of *B. megaterium* is shown in Fig. 1. There is a lag of approximately 30 min before the onset of growth inhibition, with the length of the lag independent of the analogue concentration. As the 8-azaguanine concentration is increased, there is a corresponding decrease in the growth rate (increase in generation time). This rate remains constant for at least 5 hr.

The inhibitory effect of the analogue can be completely reversed by the simultaneous addition of purines and their derivatives (Table 2). None of the pyrimidine derivatives, nor 4-amino-5-imidazolecarboxamide, has any effect. The effect of the addition of different concentrations of guanosine is shown in Fig. 2. It appears that the cells continue to grow at the same rate as the control, until the guanosine is exhausted. The greater the guanosine concentration, the longer the period of normal exponential growth prior to the onset of growth inhibition.

Although there is a 30-min lag in growth inhibition, there is no detectable lag in the uptake of 8-azaguanine-2-C\(^{14}\). The results of two experiments are presented in Fig. 3. There is a maximal linear uptake of the analogue for about 2 hr. Cells suspended in 5% TCA and heated at 90 C for 20 min, lose essentially all of their radioactivity. This suggests that 8-azaguanine-2-C\(^{14}\) has been incorporated into the nucleic acids. In *B. cereus*, this analogue has been found to replace guanine (Smith and Matthews, 1957). Based on the amount of ribonucleic acid (RNA) present after the 2-hr incubation (ribose determination) and assuming that the analogue is incorporated instead of guanine, there is a replacement of approximately 20% of the guanine by 8-azaguanine. This agrees well with the value of 18% reported by Jeener, Hamero-Casterman, and Mairesse (1959), for another strain of *B. megaterium*. Based on the amount of RNA synthesized in the presence of 8-azaguanine, the replacement is of the order of 40%.

### Table 1. Mixture of L-amino acids*

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Concentration (μmoles/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>1.50</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.63</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.60</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.60</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.20</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.63</td>
</tr>
<tr>
<td>Glutamine</td>
<td>0.63</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.30</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.13</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.55</td>
</tr>
</tbody>
</table>

* A tenfold concentrated amino acid mixture was adjusted to pH 6.6 and sterilized by autoclaving; 1 ml was added to 9.0 ml of basal medium to give the indicated concentrations of amino acids in μmoles per ml.
EFFECT OF 8-AZAGUANINE ON B. MEGATERIUM

FIG. 1. Effect of different concentrations of 8-azaguanine on the growth of strain KM in basal medium. The numbers refer to the final concentration of 8-azaguanine in µmole/ml. Temperature: 30°C.

TABLE 2. Effect of purines and pyrimidines on growth inhibition by 8-azaguanine*

<table>
<thead>
<tr>
<th>Complete reversal of inhibition</th>
<th>No effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>Cytidine</td>
</tr>
<tr>
<td>Guanine</td>
<td>Uridine</td>
</tr>
<tr>
<td>Guanosine</td>
<td>Thymidine</td>
</tr>
<tr>
<td>Deoxyguanosine</td>
<td>4-Amino-5-imidazolecarboxamide</td>
</tr>
<tr>
<td>Inosine</td>
<td></td>
</tr>
<tr>
<td>2,6-Diaminopurine</td>
<td></td>
</tr>
</tbody>
</table>

* Strain KM was grown in basal medium at 30°C. Both 8-azaguanine and the compound tested were added simultaneously to exponentially growing cells at a final concentration of 500 µmole/ml. Growth was followed turbidimetrically.

Data on the kinetics of killing by 8-azaguanine are presented in Fig. 4. There is little change in the viable count for the first 30 min, in agreement with the lag in growth inhibition shown turbidimetrically (Fig. 1). This is followed by a period of rapid death, during which the viable count decreases by approximately 3 logarithms in 30 min. Subsequently, the rate of killing is much lower; there is a decrease in the viable count of approximately 2 logarithms in 4 hr. If the survivors are grown on basal medium in the absence of 8-azaguanine, and then exposed to the analogue under the conditions of the previous kinetic experiment, a curve similar to that of Fig. 4 is obtained. Therefore, the high and low death rates are not due to genetic heterogeneity of the population.

It was found that little or no killing occurs if the cultures are not shaken in the presence of 8-azaguanine. This is true even if as little as 4.0 ml of a cell suspension (7 × 10⁷ cells/ml) are left stationary in a 200-ml Erlenmeyer flask. This suggests that under conditions where little growth can occur, as in the case of a severe oxygen limitation, 8-azaguanine is not bactericidal. To test this hypothesis, we performed experiments with mutant A (tyrosine- or phenylalanine-requiring). As is shown in Table 3, 8-azaguanine is
FIG. 2. Effect of different concentrations of guanosine in reversing the action of 8-azaguanine. Strain KM was grown on basal medium at 30 C. 8-Azaguanine (500 μmoles/ml) and guanosine were added simultaneously. The numbers refer to the final concentration of guanosine in μmoles/ml.

FIG. 3. Uptake of radioactive 8-azaguanine. Strain KM was grown in basal medium at 30 C. 8-Azaguanine-8-C¹⁴ was added to exponentially growing cells at a final concentration of 100 μmoles/ml (71,000 count/min per μmole). Samples were removed and treated as described in Materials and Methods. The results of two different experiments are presented.
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FIG. 4. Kinetics of killing by 8-azaguanine. Strain KM was grown in basal medium supplemented with the amino acid mixture at 30 C. Exponentially growing cells were harvested, washed, and resuspended in basal medium at 37 C. After allowing two generations to occur, 8-azaguanine was added at a final concentration of 100 μmole/ml. Samples were removed and treated as described in Materials and Methods. Samples were plated on basal agar.

TABLE 3. Effect of 8-azaguanine on viability of mutant A*

<table>
<thead>
<tr>
<th>Amino acid mixture</th>
<th>8-Azaguanine</th>
<th>No. viable cells per ml</th>
<th>Klett units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
<td>2 hr</td>
<td>0 time</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>$8.0 \times 10^7$</td>
<td>$1.7 \times 10^6$</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>$4.0 \times 10^7$</td>
<td>$3.4 \times 10^7$</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>$4.0 \times 10^7$</td>
<td>$2.9 \times 10^7$</td>
</tr>
</tbody>
</table>

* Mutant A was grown on the basal medium supplemented with the complete amino acid mixture (AA). Exponentially growing cells were harvested, washed, and resuspended in basal medium with and without AA at 10 Klett units. Cells were shaken for 2 hr at 30 C, and 8-azaguanine was added at a final concentration of 500 μmole/ml. Samples were removed and treated as described in Materials and Methods. Samples were plated on basal agar containing guanosine. 8-Azaguanine was added at zero time.

bactericidal only under conditions where growth is possible, i.e., in the presence of the required amino acid (amino acid mixture). The turbidity of the cell suspension doubles, although the viable count decreases by almost 3 logarithms. In the absence of amino acids, the turbidity of the cell suspension increases slowly, and 8-azaguanine has no effect on viability (8-azaguanine added when cells depleted of residual amino acids). Also, amino acid starvation has little or

TABLE 4. Effect of amino acids in the culture medium on the bactericidal action of 8-azaguanine*

<table>
<thead>
<tr>
<th>Cultured on basal medium</th>
<th>No. viable cells per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
</tr>
<tr>
<td>+ AA</td>
<td>$7.7 \times 10^7$</td>
</tr>
<tr>
<td>- AA</td>
<td>$9.1 \times 10^7$</td>
</tr>
</tbody>
</table>

* Strain KM was grown either in basal medium alone or in basal medium supplemented with the amino acid mixture (AA) at 30 C. Exponentially growing cells were harvested, washed, and resuspended in basal medium at 37 C. After allowing two generations to occur, 8-azaguanine was added at a final concentration of 100 μmole/ml. Samples were removed and treated as described in Materials and Methods. Samples were plated on basal agar. 8-Azaguanine was added at zero time.
TABLE 5. Effect of amino acids on viability in the presence of 8-azaguanine*

<table>
<thead>
<tr>
<th>Cells grown on basal me-</th>
<th>No. viable cells per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>dium + 8-aza-</td>
<td>0 time</td>
</tr>
<tr>
<td>guanine</td>
<td></td>
</tr>
<tr>
<td>+AA</td>
<td>$4.9 \times 10^7$</td>
</tr>
<tr>
<td>−AA</td>
<td>$4.7 \times 10^7$</td>
</tr>
</tbody>
</table>

* Strain KM was grown in basal medium at 30 C. Exponentially growing cells were then transferred to basal medium alone or basal medium supplemented with the amino acid mixture (AA). Cells were grown at 37 C, and, after allowing two generations to occur, 8-azaguanine was added (final concentration of 100 μmole/ml). Samples were removed and treated as described in Materials and Methods. Samples were plated on basal agar. 8-Azaguanine was added at zero time.

no effect on the viable count over a 2-hr period. Experiments on the uptake of 8-azaguanine-2-C14 by mutant A were in agreement with these findings. After 2 hr of incubation with 8-azaguanine (100 μmole/ml), the uptake per ml of cells in the presence of the complete amino acid mixture is approximately 200-fold greater than that of cells in the absence of amino acids. Therefore, death does not occur under conditions where the analogue is not incorporated into the nucleic acids.

Growth in the presence of the complete amino acid mixture was found to have a pronounced effect on the bactericidal action of 8-azaguanine. In the experiment described in Table 4, cells were cultured on basal medium plus amino acids, washed, and resuspended in basal medium at 37 C. 8-Azaguanine was added, after allowing two generations to occur. It is apparent that the viable count of cells cultured in the presence of amino acids is lower by a factor of 10 than those cultured in the absence of amino acids. In the experiment described in Table 5, cells were cultured on basal medium alone, and were then transferred to basal medium with and without amino acids. 8-Azaguanine was added after allowing two generations to occur at 37 C. It is clear that 8-azaguanine is a more effective bactericidal agent in the presence of the complete amino acid mixture. The viable count in the presence of amino acids is lower than the count in the absence of amino acids by a factor of 25 (5 hr) to 100 (2 hr). The effect of amino acids on the uptake of 8-azaguanine-2-C14 is shown in Fig. 5. Since amino acids have no effect on the growth rate at 37 C, it is clear that there is an increased uptake of the analogue in the presence of the amino acid mixture (factor of approximately 2).

An experiment on the effect of chloramphenicol on the bactericidal action of 8-azaguanine is shown in Table 6. The data show that chloramphenicol is primarily bacteriostatic. The concentration of the antibiotic used was five
TABLE 6. Effect of chloramphenicol on the bactericidal action of 8-azaguanine*

<table>
<thead>
<tr>
<th>Additions to basal medium</th>
<th>No. viable cells per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 time</td>
</tr>
<tr>
<td>8-Azaguanine</td>
<td>$9.6 \times 10^7$</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>$9.6 \times 10^7$</td>
</tr>
<tr>
<td>8-Azaguanine + chlorama-</td>
<td></td>
</tr>
<tr>
<td>phenicol</td>
<td>$9.6 \times 10^7$</td>
</tr>
</tbody>
</table>

* Strain KM was grown on basal medium at 30 C. 8-Azaguanine (final concentration of 500 m\(\mu\)moles/ml) and chloramphenicol (final concentration of 100 \(\mu\)g/ml) were added to exponentially growing cells, and incubation was continued at 30 C. Samples were removed and treated as described in Materials and Methods. Samples were plated on basal agar. 8-Azaguanine was added at zero time.

times the level needed to completely inhibit growth. It is apparent that chloramphenicol has little or no effect on the bactericidal action of 8-azaguanine. In addition, it was found that chloramphenicol (100 \(\mu\)g/ml) inhibits the uptake of 8-azaguanine-2-C\(^{14}\) only to the extent of about 25%.

The effect of different concentrations of 8-azaguanine was studied. The following results were obtained when the analogue was added to exponentially growing cells at a population density of $7 \times 10^7$ cells/ml and incubated for 5 hr: an increase in the viable count in the presence of 10 m\(\mu\)moles/ml, a slight decrease in the count in the presence of 50 m\(\mu\)moles/ml, and a maximal rate of death with 100, 500, or 1,000 m\(\mu\)moles/ml.

After exposure to 8-azaguanine, cells were usually plated on basal agar. A similar count was obtained when cells were plated on basal agar supplemented with the complete amino acid mixture. If the basal agar is supplemented with guanosine, however, there is a 10-fold increase in the viable count. This effect can be observed after a 2-hr incubation period in the presence of 8-azaguanine, but not after 5 hr. Therefore, there is some early reversal of the bactericidal effect of 8-azaguanine, if cells are exposed to guanosine.

DISCUSSION

When 8-azaguanine is added to exponentially growing cells of *B. megaterium*, there is a lag of approximately 30 min prior to the onset of growth inhibition. Similar results have been obtained with *B. cereus* (Mandel, 1957; Smith and Matthews, 1957). However, as reported for *B. cereus* (Mandel, 1957; Chantrenne and Devreux, 1958), 8-azaguanine-2-C\(^{14}\) is taken up in a linear fashion, without a detectable lag. The degree of growth inhibition varies directly with the 8-azaguanine concentration, and the growth rate remains constant for at least 5 hr. In part, the inhibition of growth may be explained in terms of a decreased intracellular concentration of an essential guanine derivative. Levin and Magasanik (1959) have shown that 8-azaguanine represses the formation of inosinic acid dehydrogenase in *Aerobacter aerogenes*, and Dewey and Kidder (1960) have evidence that this analogue inhibits the function of at least three enzymes involved in purine interconversions.

It is difficult to decide whether the inhibited growth is linear or exponential. A straight line is obtained with either a semilogarithmic or an arithmetic plot. 8-Azaguanine is reported to induce linear growth in *B. cereus* (Mandel, 1957) and in *B. subtilis* (Richmond, 1959). The reversal of 8-azaguanine inhibition by purines and their derivatives has been found in other biological systems (Dewey and Kidder, 1960; Mandel, 1957; Chantrenne and Devreux, 1960). In agreement with our experiments, neither thymine nor uracil has any effect on 8-azaguanine inhibition of *Staphylococcus aureus* (Creaser, 1966). In contrast to our experiments, 4-amino-5-imidazolecarboxamide induces a partial reversal of inhibition in *B. cereus* (Mandel, 1957).

The effect of the complete amino acid mixture on the bactericidal action of 8-azaguanine is currently under investigation. Cells grown in the presence of both the analogue and the amino acid mixture contain twice as much RNA as cells grown with the analogue alone. There is no effect on deoxyribonucleic acid synthesis. This is in agreement with the observed twofold increase in the uptake of 8-azaguanine-2-C\(^{14}\) in the presence of amino acids (Fig. 5). At present, it is uncertain why prior growth with amino acids has an effect on analogue action.

The bulk of the 8-azaguanine incorporated into the nucleic acids of *B. cereus* is present in RNA (Smith and Matthews, 1957), where it replaces guanine. The hot acid lability of the 8-azaguanine incorporated by *B. megaterium* is consistent with incorporation into nucleic acids.
In addition, chloramphenicol inhibits growth without affecting the bactericidal action of this analogue. Although we do not have rigorous proof, it is probable that 8-azaguanine is bactericidal only under conditions where RNA synthesis is possible. If incorporation of the analogue into RNA is essential for death, then our results suggest that the RNA synthesized in the presence of chloramphenicol is as functional as that synthesized in the absence of the antibiotic. This is in agreement with the experiments of Aronson and Spiegelman (1958) and in disagreement with those of Neidhart and Gros (1957) and Pardee, Paigen, and Prestidge (1957).

The results show that 8-azaguanine is bactericidal for B. megaterium, especially under conditions where growth is possible. This is in disagreement with the work of Mandel (1957) with B. cereus, in which no evidence for the bactericidal effect of this analogue was found. The reason for this discrepancy is not clear. The effect of 8-azaguanine on B. megaterium is similar to the effect of 5-fluorouracil on E. coli (Tomasz and Borek, 1960). 5-Fluorouracil was found to kill only growing cells. However, the observed decrease in the viable count was only 90 to 95%.

Since 8-azaguanine is bactericidal primarily with growing cultures, this analogue can be used instead of penicillin (Davis, 1948; Lederberg and Zinder, 1948) to obtain cell populations greatly enriched in auxotrophic mutants. This use of 8-azaguanine will be described in a following publication (Wachsmann and Mangalo, 1962).

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LITERATURE CITED


