The Differential Effect of 8-Azaguanine on Cell Wall and Protoplastic Protein Synthesis in Bacillus cereus*

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The previously reported studies of Mandel and Altman (1) with the sulfur-containing amino acids and the observations of Chantrenne and Devreux (2) with radioactive methionine, phenylalanine, valine, and leucine indicated that azaguanine inhibits the incorporation of amino acids into Bacillus cereus. However, the effect of the drug on the incorporation of acetate (3), glutamic acid (4), and aspartic acid (2) seemed to differ from the above protein precursors. Since it was thus apparent that not all the amino acids were responding in the same way to the drug, a survey has been made of the effects of 8-azaguanine on the incorporation of thirteen additional amino acids.

Most radioactive amino acids were found to be incorporated to a lesser extent into drug-treated than control cultures of B. cereus grown to the same turbidity. The incorporation of glycine, glutamic acid, and alanine, however, was greater in the inhibited cultures. In addition, growth in the presence of radioactive alanine, serine, or aspartic acid led to the appearance of far greater amounts of radioactivity in diaminopimelic acid of cells treated with 8-azaguanine than of control cells. Since diaminopimelic acid has been reported to be a specific component of bacterial cell walls (4-6) and since Chantrenne and Devreux (2) have observed that the synthesis of cell wall hexosamine was not inhibited by the drug, it became of interest to study in detail the possibility that 8-azaguanine produced different effects on cell wall synthesis and the synthesis of protoplasmic protein. A preliminary report of these investigations has been presented (7).

EXPERIMENTAL PROCEDURE

Materials and Methods

Bacterial Cultures—Bacillus cereus (penicillinase-constitutive strain 596H) was grown on a salts-casamino acid medium, as previously described (1). 8-Azaguanine was added to exponentially growing cultures to give a final concentration of 15 μg per ml in all the experiments. The drug was added simultaneously with the radioactive amino acid when the culture had grown to a turbidity of 0.2 measured as optical density at 540 nm (OD540) of 0.4 were extracted with cold trichloroacetic acid, described (1), and the residue after extraction with hot trichloroacetic acid (hereafter referred to as “total protein” fraction) was hydrolyzed in 6.0 N HCl (11). The hydrolysis products of the total protein fraction is shown in Fig. 1. Whatman No. 1 paper by two-dimensional ascending chromatography in Solvent 3 followed by Solvent 4, essentially as described by Roberts et al. (11). Chromatography was carried out in small museum jars (25 × 25 × 12 cm). The papers were freed of humin, was evaporated to dryness once and resuspended in 300 μl of water. Of the resulting solution, 100 μl were taken for radioactivity determinations or chemical assays and a similar aliquot was chromatographed on Whatman No. 1 paper by two-dimensional ascending chromatography in Solvent 3 followed by Solvent 4, essentially as described by Roberts et al. (11). Chromatography was carried out in small museum jars (25 × 25 × 12 cm). The papers were freed of phenol by several washes in acetone, dried at room temperature and exposed to blue-sensitive Kodak Medical X-ray film for a period of 4 days to several weeks. Radioactive spots were cut out and counted directly in a gas flow counter without eluting from the paper. They were then put back into position with a thin strip of adhesive tape and the paper dipped into a 0.25% solution of ninhydrin in acetone and heated for 3 minutes at 120° to locate the position of the amino acids. The identity of the radioactive spot was then established by the “fingerprinting” technique of Roberts et al. (11). The pattern of the major hydrolysis products of the total protein fraction is shown in Fig. 1.

Hexosamine Determinations—The method of Gardell (13),
adapted from the method of Elson and Morgan (14), was applied after precipitation of cells with cold trichloroacetic acid and hydrolysis of the residue in 6 N HCl in the pressure cooker.

Protein Determination—Alkali-soluble protein was estimated by the method of Lowry et al. (15) after extraction of the total protein fraction with 1.0 N NaOH at 70° for 30 minutes and filtration through a coarse collodion membrane. Quantitative ninhydrin analyses were done by the method of Harding and MacLean (16) with dL-leucine as standard.

Fractionation of Disrupted Bacteria—Since attempts to disrupt the cells by treatment with lysozyme or deoxycholate were unsuccessful, the following procedure was adopted. Suspensions of cells of B. cereus culture, 45 ml, were grown in the presence or absence of 8-azaguanine to a turbidimetric reading of OD660 of 0.4 and sedimented at 10,000 X g for 20 minutes in a Servall refrigerated centrifuge. The packed cells were washed once with growth medium, resuspended in 5.0 ml of 0.9% NaCl and disrupted in the Mickle apparatus (17) with 3 g of size 10 Ballotini beads (0.005 to 0.008 inch) for 1 hour at 4°. The disrupted bacteria and Ballotini beads were then filtered through a small pad of glass wool which was washed through with 10 ml of cold 0.4 N HCl at 88°. The HCl was removed by evaporation under reduced pressure and the residue taken up in water for assay or chromatography.

Radioactive Compounds—Uniformly C14-labeled L-arginine, L-histidine (ring-labeled)-2-C14, and uniformly C14-labeled L-threonine were obtained from Nuclear Chicago Corporation, Chicagou, Illinois; dL-alanine-1-C14, glycine-2-C14, uniformly C14-labeled glucose, dL-leucine-1-C14, dl-phenylalanine-3-C14, dL-serine-3-C14, and dL-valine-1-C14 from Isotopes Specialties Company, Inc., Burbank, California; and dL-aspartic acid-4-C14, dl-glutamic acid-2-C14, and dL-lysine-2-C14 from Tracerlab, Inc., Waltham, Massachusetts. Tritiated diaminopimelic acid was kindly furnished by Dr. J. L. Strominger, Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri. All radiocarbon amino acids were tested for radio-purity by two-dimensional chromatography (Solvents 3 and 4) before use. The radio-purity of the tritiated diaminopimelic acid was confirmed in Solvent 1. Between 2 and 8 μc of the C14 and 70 μc of the H3 compounds were added per 100 ml of culture. From the specific radioactivity of the tracer and the approximate constution of the casein hydrolysate, it was calculated that in all cases the amount of carbon-labeled amino acid added was less than 1% of that already in the medium. Counting was done in a proportional gas flow counter to a standard error of less than 1%.

RESULTS

Effect of 8-Azaguanine on Total Uptake of Radioactivity from Labeled Amino Acids—8-Azaguanine severely inhibits growth of B. cereus about 15 to 20 minutes after addition of the drug (18). As a result of this effect on growth, any biochemical property of the cell which proceeds at the same rate as total growth will appear to be inhibited when it is plotted against time. A good example of the use of time as a parameter giving misleading results is shown in Table 1. In these experiments, the incorporation of several radioactive amino acids was measured for 40 minutes in the presence and absence of 8-azaguanine. As might be
Effect of Azaguanine on Cell Wall and Protein Synthesis

Vol. 235, No. 7

2038 Effect of Azaguanine on Cell Wall and Protein Synthesis

Vol. 235, No. 7

The progressive effect of the drug on the incorporation of four different amino acids is shown in Fig. 2. It can be seen that a marked difference of behavior is observed when incorporation of radioactivity is plotted against increase in turbidity. As had been observed previously with methionine-S\(^{35}\) and cystine-S\(^{35}\) (1), the inhibitory effect by the drug on the incorporation of arginine and lysine did not begin until several minutes after the addition of 8-azaguanine. On the other hand, the stimulatory effect on the incorporation of alanine and glycine was immediate.

Chemical Fractionation of Bacteria Grown with C\(^{14}\)-labeled Amino Acids

As is evident from Table I, the analogue exerted a varied effect on the incorporation of the labeled amino acids. For a more detailed examination of this effect, the chemical fractions of the cell were removed in a stepwise fashion and examined for radioactivity. The results are presented as histograms in Fig. 3.

Extraction with Cold 5\% Trichloroacetic Acid—It was found, in general, that in control cultures, the cold acid-soluble fraction contained a small proportion (2 to 5\%) of the total radioactivity which was somewhat increased in inhibited organisms, although the effects observed were not very consistent. Of the various expected, in every case the uptake of radiocarbon by the drug-treated bacteria was inhibited. However, when the inhibited cultures were allowed to continue growing and were harvested when they had reached the same final turbidity as the controls, incorporation of radioactivity from labeled alanine, serine, and glycine was actually greater in the inhibited cultures than in the controls. On this more critical basis, the inhibited cells incorporated about the same amount of radioactivity from labeled aspartic acid and far less from valine and arginine than did the controls. It is felt by the authors that comparisons of cultures grown to the same extent rather than for the same time are more meaningful. Therefore, it was the general procedure in these experiments to add tracer and 8-azaguanine at OD\(_{540}\) of 0.2 and to grow both the control and inhibited cultures until the OD\(_{540}\) had doubled (about 50 minutes for the control, and 190 to 240 minutes for the inhibited culture).

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amino acids tested, the highest relative radioactivity in this fraction was found after growth in the presence of serine-3-C\textsuperscript{14}, where 11\% of the total cellular radioactivity was extracted with trichloroacetic acid. This proportion usually was enhanced by the drug. Similar results were found with glutamic acid-2-C\textsuperscript{14}. The presence of measurable amounts of radioactivity extracted by cold acid from the inhibited organisms with most of the amino acids studied, as well as the evidence from the previous paper (1) indicate that passage of amino acid through the cell did not seem to be impaired by the drug. The possibility should not be overlooked, however, that failure on the part of one essential amino acid to be transported into the cell could lead to general inhibition of protein synthesis. All the amino acids required for optimal growth of B. cereus (1) have been examined during these investigations, however, and no such effect could be demonstrated.

**Extraction by 65% Ethanol for 30 Minutes at 70°**—Only small amounts of activity were extracted by ethanol and no significant drug effects could be noted. The ethanol extracted 14 and 10\% of the radioactivity from cells grown in the presence of alanine-1-C\textsuperscript{14} and serine-3-C\textsuperscript{14}, respectively. A greater amount was extracted from the inhibited than from the control cultures, in parallel with the increased total incorporation of radioactivity observed with these two amino acids in the presence of the analogue. In experiments with the other amino acids, less than 10\% and usually only 2 to 3\% of the total radioactivity was dissolved by the ethanol. This low value suggests that alcohol-soluble protein (as described by Roberts et al. (11) in *Escherichia coli*) was largely absent from *B. cereus*, and is also in agreement with the related observations that a group of bacterial species examined, gram-positive microorganisms contained less lipid-soluble material in their cell walls than gram-negative (19).

**Treatment with 5\% Trichloroacetic Acid at 100° for 30 Minutes (8)**—This reagent extracted little radioactivity (10\% of the total or less) from bacteria grown with labeled lysine, arginine, leucine, phenylalanine, and valine, as shown in Fig. 3. The radioactivity extracted by hot trichloroacetic acid was probably due more to release of small peptides from protein than to true incorporation into nucleic acids, and the amount was not greater in the inhibited cultures than in the controls.

In contrast to the above amino acids, however, experiments with radioactive alanine, serine, glycine, glutamic acid, aspartic acid, and threonine all showed considerable incorporation into the fractions extracted by hot trichloroacetic acid. It was interesting that in every case, the activity in these fractions was greater in the inhibited than in the control cultures. The increase in radioactivity in the fractions extracted by hot trichloroacetic acid in the case of the drug-inhibited culture compared to the control undoubtedly is related to the increase in polynucleotide formation occurring during inhibition by 8-azaguanine (20).

**Analysis of Hydrolysates of \"Total Protein\" Fractions**—The differences in the effect of the drug on the incorporation of radioactive amino acids are not due completely to differences in the extent of incorporation into nonprotein fractions. It is seen in Fig. 3 that there is still considerable variation in the effect of the drug on the incorporation of amino acids into \"total protein.\" For most of the amino acids examined (arginine, glutamic acid, glycine, histidine, leucine, lysine, phenylalanine, threonine, and valine), the addition of the labeled precursor led to the appearance of one major radioactive spot on the chromatogram which corresponded in its position to that of the original amino acid added. After the incorporation of alanine-1-C\textsuperscript{14}, serine-3-C\textsuperscript{14}, and aspartic acid-4-C\textsuperscript{14}, however, several radioactive spots appeared on the amino acid chromatograms of both control and inhibited cultures. The results of these investigations are shown in Table II. The identity of some of the spots was aided by chromatography in Solvent 1, whereas Solvent 2 was used for the separation of diaminopimelic acid.

Significant amounts of radioactivity and ninhydrin color appeared in the spot corresponding in position to diaminopimelic

### Table II

**Distribution of radioactivity in hydrolysates of total protein fraction of cells grown in the presence of labeled amino acids**

<p>| Isolated compound | Radioactivity (cpm per spot) in compounds after |</p>
<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diaminopimelic acid</td>
<td>56</td>
<td>308</td>
</tr>
<tr>
<td>Alanine</td>
<td>95</td>
<td>255</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>37</td>
<td>90</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>152</td>
<td>122</td>
</tr>
<tr>
<td>Serine</td>
<td>300</td>
<td>116</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>530</td>
<td>181</td>
</tr>
</tbody>
</table>

### Table III

**Variation in response of different amino acids to 8-azaguanine**

Control and inhibited bacteria were grown to same turbidity with labeled amino acids and corresponding fractions assayed for radioactivity.

<table>
<thead>
<tr>
<th>Exogenous amino acid</th>
<th>Ratios of radioactivity, inhibited culture</th>
<th>control culture</th>
<th>Radioactive spots detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original amino acid</td>
<td>Total protein fraction</td>
<td>Reconstituted amino acid</td>
<td>by chromatography</td>
</tr>
<tr>
<td>Alanine-1-C\textsuperscript{14}</td>
<td>2.40</td>
<td>2.59</td>
<td>Several*</td>
</tr>
<tr>
<td>Glutamic acid-2-C\textsuperscript{14}</td>
<td>1.21</td>
<td>1.28</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Glycine-2-C\textsuperscript{14}</td>
<td>1.22</td>
<td>1.17</td>
<td>Glycine</td>
</tr>
<tr>
<td>Phenylalanine-3-C\textsuperscript{14}</td>
<td>0.41</td>
<td>0.75</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>Histidine-(ring)-2-C\textsuperscript{14}</td>
<td>0.55</td>
<td>0.64</td>
<td>Histidine</td>
</tr>
<tr>
<td>Lysine-2-C\textsuperscript{14}</td>
<td>0.51</td>
<td>0.61</td>
<td>Lysine</td>
</tr>
<tr>
<td>Serine-3-C\textsuperscript{14}</td>
<td>1.56</td>
<td>1.28</td>
<td>Several*</td>
</tr>
<tr>
<td>Valine-1-C\textsuperscript{14}</td>
<td>0.31†</td>
<td>0.36</td>
<td>Valine</td>
</tr>
<tr>
<td>Leucine-1-C\textsuperscript{14}</td>
<td>0.37†</td>
<td>0.30</td>
<td>Leucine</td>
</tr>
<tr>
<td>Asparagine-1-C\textsuperscript{14}</td>
<td>0.96</td>
<td>0.34</td>
<td>Arginine</td>
</tr>
<tr>
<td>Aspartic acid-4-C\textsuperscript{14}</td>
<td>0.96</td>
<td>0.34</td>
<td>Several*</td>
</tr>
</tbody>
</table>

*See Table II.*

† A value of 1.0 was obtained for the first two experiments but four subsequent experiments resulted reproducibly in the value shown here. No such variability was observed for any other amino acid.

† Uniformly labeled.
acid, and both radioactivity and ninhydrin color in this spot were much greater in the sample from the culture inhibited by 8-aza-
guanine than in the control hydrolysates. Similar results were
indicated in the spot identified as glucosamine. The conversion
of radioactivity from alanine-1-C\(^{14}\) to diaminopimelic acid in the
inhibited culture appeared to be increased to a greater extent
than the direct incorporation of alanine. In addition, several
unidentified metabolites were found, representing 3 and 10% of
the activity of the known metabolites of the control and inhibit-
ed cells, respectively. The implications of these findings will
be discussed in detail below.

For purposes of comparison, information is also included on
results obtained from an experiment in which another purine
analogue, 6-thioguanine, was used as the growth inhibitory drug.
This analogue, which also inhibited the growth of \(B.\) \(cereus\), was
added to an exponentially growing culture of \(B.\) \(cereus\) to a con-
centration of 5 \(\mu\)g per ml of medium, and the usual procedure
was then followed. In contrast to 8-azaguanine, thioguanine
produced essentially no effect on the incorporation pattern of
alanine-C\(^{14}\) into the total protein fraction when results were
compared with a control culture at the same turbidity. On a
time basis, incorporation of alanine was inhibited to approxi-
mately the same extent as was growth.

It is apparent from Table III that, for most of the amino acids
investigated, 8-azaguanine produced a striking decrease in the
radioactivity of the amino acids isolated from the hydrolysates
of the total protein fraction, and the ratio of radioactivities of
the isolated compounds agreed well with that of the total protein
fractions. The discrepancy for serine and aspartic acid was re-
lated to the labeling of other constituents, as described in Table
II, and these two precursors were also incorporated into the
corresponding amino acids of the "total protein" fraction to a
lesser extent in inhibited than control cells. Thus, only the first
three of the listed amino acids, alanine, glutamic acid, and

glycine differed appreciably from the others in that their incor-
poration directly into the "total protein" fraction was increased
by the drug.

Effect of 8-Azaguanine on Cell Wall Constituents

Since alanine, glutamic acid, hexosamine, and diaminopimelic
acid have been shown to be characteristic components of the cell
wall of strains of \(B.\) \(cereus\) \((21)\), and the evidence obtained thus
far has consistently shown an increase in the incorporation of, or
conversion to, these compounds, experiments were carried out to
measure the effect of 8-azaguanine on the bacterial content of
hexosamine and the incorporation of diaminopimelic acid.

Hexosamine Determinations—Cultures were grown to the same
turbidity in the presence or absence of 8-azaguanine, and the
hexosamine content measured. The values at turbidity of OD\(_{540}\)
of 0.4 were 0.0275 and 0.0682 \(\mu\)moles for the cells in 1 ml of sus-
cension for the control and inhibited cultures, respectively (mean
of four experiments). These results indicated that more hexos-
amine was synthesized for the same quantity of new cellular
matter in the presence of the inhibitor. The hexosamine con-
tent was also followed during the course of growth, as shown in
Fig. 4, and it was confirmed that much greater amounts of hexos-
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amine were synthesized per unit increase in cell mass (as meas-
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of 0.4 were 0.0275 and 0.0682 \(\mu\)moles for the cells in 1 ml of sus-
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TABLE IV
Effect of 8-azaguanine on incorporation of radioactivity into bacterial diaminopimelic acid

Equal volumes of control and inhibited cultures, grown to the same turbidity, were extracted and the “total protein” fraction was hydrolyzed. Radioactivity was measured after chromatography of corresponding samples of diaminopimelic acid. Solvents described under Methods.

<table>
<thead>
<tr>
<th>Exogenous amino acid</th>
<th>Chromatographic solvents</th>
<th>Radioactivity in diaminopimelic acid, inhibited culture</th>
<th>Radioactivity in diaminopimelic acid, control culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid-4-C¹⁴</td>
<td>3, followed by 4</td>
<td>3.2</td>
<td>6.2</td>
</tr>
<tr>
<td>Glucose-4-C¹⁴</td>
<td>3, followed by 4</td>
<td>6.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Serine-3-C¹⁴</td>
<td>3, followed by 4</td>
<td>5.6</td>
<td>3.2</td>
</tr>
<tr>
<td>Alanine-1-C¹⁴</td>
<td>3, followed by 4</td>
<td>6.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Alanine-1-C¹⁴</td>
<td>2</td>
<td>4.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Diaminopimelic acid-H²</td>
<td>1</td>
<td>4.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* Uniformly labeled.

than in cultures grown to same turbidity, as may be seen in Fig. 5 (bottom). On the other hand, where comparisons were made between cultures at identical turbidities, the incorporation of diaminopimelic acid by the drug-treated cultures was appreciably greater than that of the control (Fig. 5, top), the curve resembling that obtained with the hexosamine assays. Chromatographic analysis in Solvent 1 after growth in the presence of tritiated diaminopimelic acid revealed that the radioactivity was found only in the spot corresponding to diaminopimelic acid.

Cultures were also grown in the presence of uniformly C¹⁴-labeled glucose in the synthetic medium described (1), and the activity found in diaminopimelic acid isolated from the cells was measured. The results of these experiments, together with results obtained for the conversion of exogenous alanine-1-C¹⁴, serine-3-C¹⁴, and aspartic acid-4-C¹⁴ into diaminopimelic acid isolated from the “total protein” fraction are summarized in Table IV.

In every case, whether the radioactivity was added directly as tritiated diaminopimelic acid or whether it was derived from other precursors, there was much more radioactivity in diaminopimelic acid of inhibited cultures than in control cultures grown to the same turbidity. Since the radioisotopes had been added at the same time as the inhibitor, and the turbidity had been allowed to double after this addition, the change in total cellular content would only be approximately one-half of the isotope effect. On this basis, the inhibited cells had about three times the content of diaminopimelic acid of the control.

Fractionation of Disrupted Bacteria

In order to dissociate cell wall from protoplasmic protein effects, bacteria grown to the same turbidity under various specified conditions in the presence and absence of 8-azaguanine were disrupted in the Mickle apparatus and subjected to a simple centrifugal fractionation, as described. The distribution within the cell fractions of hexosamine, protein, and of radioactivity after growth in the presence of radioactive alanine and lysine were examined. In addition, the distribution of radioactive diaminopimelic acid derived from alanine-1-C¹⁴ was studied by the use of chromatography in Solvent 2. The results are presented in Table V.

The hexosamine and diaminopimelic acid were recovered quantitatively in the sediment after centrifuging at 10,300 × 𝑔 for 20 minutes. Microscopic examination of this first fraction revealed cell wall material in addition to some unbroken cells and debris. The ratio of values from inhibited and control cultures (B/A, Table V) in this first sediment, in the case of radioactivity derived from exogenous alanine-1-C¹⁴, was far greater than that after growth with lysine-2-C¹⁴, and it also exceeded the ratio of alkali-soluble proteins. It is important to observe that the stimulations previously observed in the content of inhibited cells of C¹⁴-alanine (Table II), hexosamine (Fig. 4) and diaminopimelic acid (Fig. 5) were found only in this sediment, whereas the contents of radioactivity for the alanine-1-C¹⁴ culture in the remaining fractions were actually less in the drug-treated culture than in Control and inhibited cultures grown to same turbidity. Equal volumes taken for fractionation. Results expressed as per cent of amount in unfractionated disrupted bacteria from control culture.

A = control cells; B = 8-azaguanine-treated cells. Ratios of values B/A from corresponding fraction printed in roman type.
The synthesis of new protein was observed throughout the period of partial inhibition of growth. In three consecutive experiments with the Lowry technique (15), measurement of drug-treated cultures harvested at identical turbidities as controls showed depressions of 16, 15, and 2%. Similarly, in seven experiments with the ninhydrin method of Harding and MacLean (16), depressions of 0, 1, 1.5, 2.5, 17, 10, and 20% were observed. There was thus some variability in the "protein" content in inhibited cultures grown to the same turbidity as controls, and the average depression for all the experiments was 10%. This figure is considerably less than the amount one would expect from the inhibition in uptake observed with the majority of the radioactive amino acids (1).

Since cell wall constituents give a color with the ninhydrin assay, their contribution in the measurement of "true" protein will increase as inhibition proceeds, and the final results will depend on the exact point at which the cultures were harvested. The 10% cell depression in protein content could also be partly due to the effect of changes in cell size and shape in the turbidimetric assay. Such cellular alterations have been described during 8-azaguanine inhibition (18).

**Effect of Azaguanine on Total Protein Content**

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**Summary of Drug Effects on Cell Wall and Cytoplasmic Protein**

Fig. 6 is a simplified representation of the action of 8-azaguanine on cell wall and cytoplasmic protein synthesis. It has been assumed: (a) that the inhibited cell had approximately three times the cell wall material of the control at OD$_{540}$ of 0.4 (Tables IV and V, Fig. 4); and (b) after 20 minutes, the production of new cytoplasmic protein per unit increase in turbidity was reduced by 85% (1). The ratio of cell wall to cytoplasmic protein expressed in arbitrary units, has been set at 10 to 100. An exact description of the drug's action would require accurate analyses of the relative and absolute amounts of protein, cell wall, and nucleic acids during the entire course of the experiment.

It will be noticed that when both cultures had grown to OD$_{540}$ of 0.4, the total quantities of cellular "total protein" material were approximately equal, but the relative content of cell wall and cytoplasmic protein was drastically altered.

**DISCUSSION**

Expression of Results—Parameters such as dry weight and protein content suffer from the fact that 8-azaguanine produces independent changes in the constitution of the cell. The present investigations have relied on the comparisons of drug-treated and control cells grown to the same turbidity as a convenient measure of the drug's action. Thus, cell wall synthesis appeared to be stimulated when considered on an equal growth basis for the two cultures, but on a time basis the effect was a slight inhibition. This latter basis of comparison, however, masks the fact that the over-all composition of the cell was drastically altered by the drug, the inhibited cell having a much higher content of RNA (20) and cell wall and a lower content of cytoplasmic protein than the control.

**Differential Effect of 8-Azaguanine on Amino Acid Incorporation**

Part of the variation in behavior of the different amino acids resulted from the fact that some labeled compounds, but not all, contributed considerable radioactivity to nonprotein fractions of the cell, and that these fractions responded differently to the drug's action than did the protein fraction. For example, a large percentage of the total radioactivity in cells grown in the presence of glycine-$C^{14}$ was found to be extracted by hot tri-
chloroacetic acid under conditions which removed nucleic acids (8). Since glycine is known to be incorporated into the purine ring, and since azaguanine raises the cellular content of RNA (20), it was not surprising, therefore, to find greater radioactivity in the extract obtained after hot trichloroacetic acid treatment of inhibited cells than in that obtained from control cells.

Studies on the effect of the analogue on the incorporation of amino acids into the "total protein" fraction (i.e. that material not extracted by lipid solvents and hot trichloroacetic acid) still showed variation in response, however. In the case of several radioactive amino acids (alanine, serine, and aspartic acid), part of this difference was due to the formation of several radioactive products, and the analogue affected the rates of formation of these compounds to different extents. The results with aspartic acid-4-C\(^14\) were of particular interest, since its greater utilization for dianaminopimelic acid synthesis in the inhibited cells masked the depression in the direct incorporation of the amino acid. Similar results were obtained with serine-3-C\(^14\). Chantrenne and Devreux (2) have mentioned that aspartic acid incorporation was inhibited less on a time basis than that of other amino acids, an effect they ascribed to the presence of aspartic acid in the bacterial cell wall. In all likelihood, however, their results were due to the partial conversion to dianaminopimelic acid. The synthesis of dianaminopimelic acid from aspartic acid and pyruvate by cell-free extracts of Escherichia coli has been reported by Givarg (22), and it is probable that conversion of radioactive alanine into dianaminopimelic acid observed in the above work occurs after deamination to pyruvic acid.

In addition to the above effects, analysis of the radioactivity of individual components of protein hydrolysates after growth in the presence of various labeled amino acids (alanine, glutamic acid, and to a lesser extent, glycine) still showed differences in response to the drug, even though for most exogenous amino acids, an inhibition in uptake was observed.

**Cell Wall Synthesis**—For equal growth of inhibited and control cultures, (a) the incorporation of radioactivity into both glucosamine and dianaminopimelic acid from added radioactive alanine, serine, aspartic acid, and glucose was stimulated in drug-treated cells; (b) the list of those bacterial components which were present in greater quantity in inhibited than in control cells (dianaminopimelic acid, glucosamine, alanine, glutamic acid, and glycine) was strikingly similar to the reported major components of the cell wall of strains of *B. cereus* (dianaminopimelic acid, glucosamine, alanine, and glutamic acid) (21); and (c) the stimulatory effects with hexosamine, alanine, and dianaminopimelic acid were restricted to a fraction isolated from disrupted bacteria containing cell walls. On a time basis, the incorporation of labeled dianaminopimelic acid proceeded at almost the same rate in inhibited and control organisms. Thus, whereas 8-azaguanine produced a marked depression of protoplasmic protein synthesis, the inhibited cells still had the ability to synthesize the major components of the cell wall quite independently of any effect on growth, and the formation of this substance per cell was greatly increased.

At the moment, there is no direct information that the structure of the inhibited cell wall is completely normal. Not all the reported cell wall constituents, e.g. muramic acid (23) or teichoic acid (24), have been studied, but several unidentified labeled compounds were uncovered after growth with radioactive alanine that had greater activity in the inhibited than in the control cultures, characteristic of cell wall constituents.

Other investigators have also studied the differential effect of 8-azaguanine on cell wall and protoplasmic protein. Chantrenne and Devreux (2) have already observed that hexosamine synthesis in control and treated cells continued at equal rates, and concluded that cell wall synthesis was unaffected by the drug. The present experiments supply the additional evidence needed to validate the hypothesis. Similar conclusions have recently been suggested by Richmond (25) using *Bacillus subtilis*.

Disassociative effects between cell wall synthesis and protoplasmic protein synthesis have been recognized previously. Chloramphenicol has been shown to inhibit protein synthesis in *Staphylococcus aureus* without affecting cell wall synthesis (26, 27). Park and Strominger (28) have postulated that penicillin inhibited the growth of *S. aureus* by interfering specifically with cell wall synthesis.

**Protoplasmic Protein Synthesis**—With the exception of precursors for cell wall, all of the amino acids were incorporated into the protein fraction of inhibited cells to a lesser extent than into that of control cells, comparisons being made on a growth basis. On the basis that the incorporation of these amino acids was a measure of true protein synthesis, about 85% inhibition was observed once the drug's action had been initiated.

It cannot be said whether the uptakes of the various amino acids into protoplasmic protein were inhibited by 8-azaguanine to precisely the same extent. The small variations that have been observed in Tables III and V may be due to slight differences in the various experiments in extent of inhibition produced. The presence in cell walls of small amounts of amino acids other than the major ones described by Salton and Ghuysen (21) may raise the relative values in "total protein." Another possibility to account for the observed variation might be that the amino acid proportion of the small amount of protoplasmic protein that is synthesized under conditions of inhibition by 8-azaguanine differs from that of the combined protoplasmic protein. This effect could be due to the disproportionate synthesis of normal proteins richer in certain amino acids than the average, or the synthesis of abnormal proteins.

It is surprising that many biochemical reactions continue in spite of the profound depression of protein synthesis produced by azaguanine. The increased formation of polynucleotides (20) and cell wall implied that the entire complex of enzymes necessary for their synthesis from the amino acids of the medium, the only source of carbon, still functions actively. One would assume, thus, that either the enzymes required for these reactions are not among those components of protoplasmic protein whose synthesis was curtailed, or that intra- or extracellular enzyme stores, available in the culture at the time of addition of the inhibitor, were adequate to allow synthetic reactions to continue for several hours. The recent observations (29) that the "cytoplasmic membrane" (30) can still incorporate amino acids after separation from the cell suggests that this membrane and cell wall may act as an independent replicating system, even when most protoplasmic protein synthesis is inhibited.

**SUMMARY**

1. Control cultures of *Bacillus cereus* and cultures partially inhibited by 8-azaguanine were grown to the same turbidity in a casein-hydrolysate medium in the presence of radioactive protein precursors, and the distribution of radioactivity in the various chemical fractions was analyzed.
2. The radioactivity in the total protein fraction (i.e. material
insoluble in lipid solvents and hot trichloroacetic acid) appearing in aspartic acid, arginine, threonine, valine, phenylalanine, leucine, serine, lysine, and histidine was considerably less in inhibited than in control cultures, in parallel with the previously observed decrease in the incorporation of methionine and cystine. The formation of protoplasmic protein was sharply depressed by 8-azaguanine. 3. However, the radioactivity appearing in the total protein fraction in alanine, glutamic acid, glycine, diaminopimelic acid, or glucosamine, either directly after growth in the presence of the appropriate amino acid, or indirectly by metabolic conversion from added C14-labeled aspartic acid, alanine, or serine was considerably greater in inhibited than in control cultures.

4. The list of substances found to have greater radioactivity in inhibited than in control cells agreed strikingly with the reported constitution of the bacterial cell wall. Differential centrifugation of disrupted bacteria revealed that the stimulatory effects were restricted to the fraction containing cell walls, whereas the incorporation of these precursors into the protoplasmic protein fraction was depressed by the analogue.

5. It was concluded that cells grown during partial inhibition by 8-azaguanine had a higher content of cell wall material and a lower content of protoplasmic protein than the controls grown to the same turbidity.

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Donald B. Roodyn and H. George Mandel


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