The Depression of the Incorporation of Sulfur Amino Acids into Bacillus cereus by 8-Azaguanine*

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(Received for publication, October 6, 1959)

EXPERIMENTAL PROCEDURE

Growth of Microorganisms—The microorganism used for studying the effect of the analogue was Bacillus cereus, strain 569H. This strain is a constitutive penicillinase-producing mutant isolated from the inducible penicillinase-producing wild strain by Kogut et al. (12). The microorganism has been used in this laboratory for a number of investigations on 8-azaguanine (2, 5, 13). The bacteria were grown in a casein hydrolysate medium unless otherwise specified. The composition was KH₂PO₄ (3.5 g), MgSO₄·7H₂O (0.51 g), Fe(NH₄)₂(SO₄)·6H₂O (4.8 mg) and vitamin-free casamino acids (10 g) brought to 1 liter at pH 7.0. The addition of traces of the labeled amino acids produced no detectable change in the amino acid content of the medium. Microorganisms were grown during shaking in a Kahn-Aloe shaker mounted in a thermostated box and maintained at 37°. Bacterial suspensions in plastic wash bottles were sampled directly into cuvettes for turbidimetric assay at 540 μm (OD₅₄₀) in a Beckman spectrophotometer, model DU, as described previously (5). At OD₅₄₀ of 0.4, 1 ml of bacterial suspension contained 2 × 10⁶ cells or 0.3 mg dry weight. The bacterial suspension was then mixed with an equal volume of 10% trichloroacetic acid solution and centrifuged at 10,000 g. The supernatant was removed from the appropriate extracts by shaking with (a) the cold acid-soluble fraction; (b) the lipid fraction; and (c) 5% trichloroacetic acid at 100°. These extracts contained (a) the cold acid-soluble fraction; (b) the lipid fraction; and (c) the hot acid-soluble fraction, respectively. The bacterial suspension was then mixed with an equal volume of 10% trichloroacetic acid and the resulting precipitate was filtered through membrane filters (Schleicher and Schuell, Keene, N. H.) which removed the cells in an essentially quantitative and reproducible manner. This filtration procedure has been described by Britton et al. (14).

Materials—8-Azaguanine-4-C¹⁴ was prepared previously (15). L-Methionine-S⁳⁵ and L-cystine-S⁳⁵ were purchased from Schwarz Laboratories, Mount Vernon, New York. Carrier-free sulfate-S³⁵ was obtained from Oak Ridge National Laboratories, Tennessee. dl-Glutamic acid-2-C¹⁴ and acetate-1-C¹⁴ were bought from Tracerlab, Inc., Waltham, Massachusetts and Nuclear Instrument and Chemical Corp., Chicago, respectively.

Cellular Fractionation—The localization of isotopes in various cell fractions was carried out by a modification of the Schneider technique (16). Cells were harvested by centrifugation in a Sorvall refrigerated centrifuge at 15,000 × g, were washed with 0.9% NaCl solution and extracted with (a) 5% trichloroacetic acid at room temperature; (b) 70% aqueous ethanol at 70°, followed by ethanol-ether (1:1 volume per volume) at 40°; and (c) 5% trichloroacetic acid at 100°. These extracts contained (a) the cold acid-soluble fraction; (b) the lipid fraction; and (c) the hot acid-soluble fraction, respectively. Trichloroacetic acid was removed from the appropriate extracts by shaking with
ether several times and aspirating off the ether layer. The residue after the hot trichloroacetic acid extraction, herein referred to as the "total protein" fraction, was taken up in 6 N HCl, and protein was hydrolyzed in a pressure cooker at 8 pounds per sq. in. (112°) for 3 hours. The HCl was then removed in a vacuum and the hydrolysate was treated with water and evaporated repeatedly to remove HCl.

Aliquots of each fraction were plated for radioassay or were subjected to descending chromatography on Whatman No. 1 or 3MM paper, with the use of the following solvent systems: (1) 20% isopropanol, 30% water in an atmosphere of NH3 (17); (2) 170 ml of isopropanol, 44 ml of concentrated HCl and H2O to 250 ml (adapted from (18)). The latter system was found to be extremely useful for the rapid separation of amino acids. Although not all of the amino acids were dissociated from each other, most of the compounds were located in sharply defined bands. Radioautograms of chromatograms were made with single-coated blue-sensitive Kodak Medical X-ray film. Chromatograms developed in Solvent 2 (containing HCl) were carefully dried and the film exposed to ammonia fumes before radioautography. Counting was carried out in a windowless gas flow proportional counter, using natural gas, as has been previously described (13).

Some chemical fractionations were carried out by the membrane filter fractionation technique currently developed in this laboratory. Aliquots of the radioactive bacterial suspension were filtered through membrane filters after the following treatments: (a) mixed with 0.9% NaCl and filtered directly to measure incorporation into intact bacteria; or (b) mixed with an equal volume of 10% trichloroacetic acid and filtered to measure the incorporation into the cold acid-insoluble material (difference between (a) and (b) being equal to cold acid-soluble fraction); or (c) heated with an equal volume of 10% trichloroacetic acid at 100° for 30 minutes and filtered to give material insoluble in hot trichloroacetic acid ("total protein" fraction), the difference between (b) and (c) being equal to the hot acid-soluble fraction; or (d) heated at 70° with 2 volumes of ethanol and filtered to measure the ethanol-soluble fraction (difference between (b) and (d)). The validity of this method and its agreement with conventional techniques is described in detail elsewhere (19).

Separation of Cysteic Acid—To determine the metabolic pathway of S4-azaguanine, cysteic acid was prepared from the cysteine derivatives of bacterial proteins by treating the unhydrolyzed protein with performic acid (20). A mixture of 1 ml of H2O2 and 9 ml of 70% formic acid was allowed to stand for 1 hour and was then added to the dried "total protein" fraction. After 15 minutes the mixture was taken to dryness and hydrolyzed in the usual manner. After the removal of HCl the amino acid mixture was subjected to paper electrophoresis in 0.2 M acetic acid at 500 volts for 90 minutes, with the Spino Durrum-type apparatus. The paper strips were dried and treated with 0.25% ninhydrin in acetone to locate the amino acids. Cysteic acid separated distinctly from the other amino acids because of its negative charge at this pH. The compound was eluted and measured by the method of Moore and Stein (21). A procedure similar to the above has recently appeared (22).

Bioassay for Methionine—To determine the content of methionine in bacterial proteins, only the method of bioassay proved satisfactory for measuring the small quantities of this amino acid. A methionine-requiring microorganism, Streptooccus sp. ATCC 8042 ("received as Leuconostoc mesenteroides P-60, ATCC 8042") was used in the methionine assay medium B 423 of Difco Laboratories, with the use of the procedure recommended (23).

For these determinations, Bacillus cultures were harvested and fractionated by the usual procedure. Protein hydrolysis in 6 N HCl was carried out at atmospheric pressure over reflux overnight to avoid possible destruction of methionine. The turbidity of the bioassay culture was measured in a Coleman junior spectrophotometer after 18 hours' growth at 37°. Standards of methionine were run simultaneously. The percentage of methionine in protein was calculated after hydrolysis of the total protein to amino acids and ninhydrin assay (24), with methionine serving as standard.

Protein Content—Two methods were used to measure total content of bacterial "total protein" fraction. The method of Lowry et al. (25) was applied after pretreatment with NaOH as recommended (25) to bring the microorganisms into solution. The ninhydrin method of Harding and MacLean (24) was carried out on hydrolysates of the "total protein" fraction.

RESULTS

Bacterial Uptake of Methionine—S-Azaguanine potently inhibits the growth of Bacillus cereus when the analogue is added to an exponentially growing bacterial culture (1, 5). In order to measure the effect that the analogue produced on the incorporation of a specific amino acid, L-methionine-S35 was added to bacteria in the logarithmic phase of growth. The suspension was immediately subdivided, one subculture receiving 15 μg of S-azaguanine dissolved in sodium carbonate per ml of medium and the other, receiving sodium carbonate only. The suspensions were then allowed to proliferate in the described manner. Periodically samples were removed to measure turbidity and incorporation into the trichloroacetic acid-insoluble bacterial residue by means of the membrane filter technique.

Fig. 1 shows the results obtained when the uptake by the cells per ml of culture was plotted against either time or turbidimetric increase (AOD), indicative of cell mass increase or growth.

It is apparent that the uptake of methionine was sharply curtailed by the analogue, in comparison with the control culture, even when comparisons were made at the same turbidimetric readings. It was also observed that the drug effect did not commence immediately after the addition of the analogue, but required some time. There was thus a very close relationship between the time of onset of inhibition of growth and the effect on methionine uptake. This lag in the onset of inhibition of growth and of the uptake of S35-methionine was in sharp contrast to the incorporation of 8-azaguanine into the RNA, which commences immediately after the addition of the drug (5). The formation of S-azaguanine-containing polynucleotide and any associated intracellular anabolism of S-azaguanine to the active inhibitor, therefore preceded the other drug effects. The lag in onset may also be associated with the time required to reach a critical intracellular concentration of the active inhibitor.

The inhibition of methionine uptake in the inhibited culture started gradually and progressed until it reached 85% when compared at similar turbidimetric differences to a control culture. Thus the over-all depression of methionine uptake by S-azaguanine varied with the period of inhibition.

To localize the effect on S35-methionine, bacteria were grown to similar extents of cell mass and were harvested and fractionated as described. Table I shows that almost all of the radio-
sulfur was present in the protein fraction, where the drug produced its principal biochemical effect. Chromatography in Solvent 2 revealed that the radioactivity in this system was incorporated into protein as methionine, which was isolated as methionine and methionine sulfoxide or sulfinic. Again, the effect of azaguanine was to prevent almost entirely the uptake of radiosulfur into these products.

The radioactivity present in the acid-soluble fraction of the drug-treated cells usually appeared to be somewhat greater than that of the control bacteria. Thus the biochemical block does not appear to involve cellular permeability to exogenous methionine.

Reversal of Effect on Methionine Utilization—The addition of guanosine leads to recovery of the growth rate after inhibition by 8-azaguanine (5), as well as cessation of incorporation of 8-azaguanine into the nucleic acids and ejection of some of the analogue from the RNA into the bacterial medium (5). As shown in Fig. 2, the addition of guanosine resulted in the prompt return of the culture's ability to incorporate methionine. When plotted by increase in bacterial mass, the slope of the uptake curve for the drug reversal culture became similar to that of the control culture, implying that the effect on methionine utilization was due to some purine-antagonizing action of 8-azaguanine. It was noted that the reversal of the incorporation effect preceded the recovery of the growth rate, in agreement with a related observation by Chantrenne (26) that the reversal of the drug's effect on protein incorporation took place before the resumption of penicillinase formation.

Exclusion of Alternative Mechanisms—Other possible explanations for the observed effect of 8-azaguanine on the incorporation of methionine were examined. Since the following alternatives were considered unlikely, a direct effect by the drug on protein biosynthesis was postulated.

1. The biochemical effect observed in the drug-treated culture might be due to the longer time of incubation of the inhibited culture to reach the final turbidity of the control cells.

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**TABLE I**

**Characteristic distribution of \( ^{35} \)S from \( ^{35} \)S-methionine in cell fractions**

*B. cereus* grown with \( ^{35} \)S-methionine in the presence and absence of 8-azaguanine to identical turbidity values, harvested and fractionated with the use of 0.9% NaCl solution, cold and hot trichloroacetic acid treatments as described. Percentage of radioactivity in each fraction relative to radioactivity in cold acid-washed cells of control culture, arbitrarily set at (100).

<table>
<thead>
<tr>
<th>Radioactivity</th>
<th>Control cells</th>
<th>( ^{8} )-Azaguanine cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c.p.m.</td>
<td>%</td>
</tr>
<tr>
<td>Saline-insoluble</td>
<td>1298</td>
<td>103.4</td>
</tr>
<tr>
<td>Cold acid-soluble</td>
<td>48</td>
<td>3.4</td>
</tr>
<tr>
<td>Cold acid-insoluble</td>
<td>1255 (100.0)</td>
<td>671</td>
</tr>
<tr>
<td>Alcohol-soluble</td>
<td>40</td>
<td>3.2</td>
</tr>
<tr>
<td>Hot acid-soluble</td>
<td>55</td>
<td>4.4</td>
</tr>
<tr>
<td>Hot acid-insoluble</td>
<td>1200</td>
<td>95.6</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Effect of 8-azaguanine on growth and incorporation of exogenous methionine \( ^{35} \)S into cold trichloroacetic acid-washed cells of *Bacillus cereus*. Turbidimetric assay (recorded as OD at 540 rnp) described in text. 8-Azaguanine added at time 0. Top, effect of analogue on growth rate. Middle, uptake of radioactivity into cells per ml of bacterial suspension, as a function of time of incubation. Bottom, uptake of radioactivity into cells per ml of bacterial suspension as a function of turbidity (AOD). 8-Azaguanine added at AOD of 0. ○, control culture; ●, 8-azaguanine-treated culture.

**Fig. 2.** Effect of guanosine on the depression of methionine-\( ^{35} \)S uptake into cold trichloroacetic acid-washed cells of *B. cereus*, resulting from 8-azaguanine treatment. 8-Azaguanine added at AOD 0, guanosine (250 \( \mu \)g per ml) at arrow. ○, control culture; ●, 8-azaguanine-treated culture.
example, if methionine were gradually depleted from the bacterial medium, the slower growing culture might not have available sufficient labeled methionine for protein biosynthesis, resulting in less than normal bacterial uptake of radioactivity. Chromatography and autoradiography of the growth media after the characteristic periods of incubation with and without 8-azaguanine showed identical chromatographic patterns of metabolites. Since both media still contained a large excess of S\textsuperscript{35}-methionine, this possible explanation was excluded.

2. Inhibition by 8-azaguanine might produce cell lysis, and the resulting decomposition products could dilute the radioactive precursor pool used in the synthesis of new cell protein. Alternatively, incorporation of methionine into protein of the control cells might represent the formation of protein for new cells plus exchange of labeled amino acid for the unlabeled constituents of previously formed cells. If 8-azaguanine should prevent this exchange reaction, the over-all effect of the analogue would appear as a decrease in incorporation of methionine into proteins. The possibility of "exchange" of methionine between the protein fraction and the medium was tested by labeling normal cells with methionine-S\textsuperscript{35}, and subculturing in S\textsuperscript{35}-free medium in the presence and absence of 8-azaguanine. As shown in Fig. 3, biochemical turnover under either condition led to only a very slight loss of radioactivity, in agreement with the work of Urbá (27) who calculated 1.4% replacement per hour in growing cells of B. cereus. For resting cells, the replacement of 7% per hour was reduced in the presence of the growth-inhibitory antibiotic, chloramphenicol, to 3.5% per hour (27).

3. Some of the methionine taken up by the control culture might become attached to protein molecules by the sulfur atom rather than as a peptide. The effect of the analogue might then be directed only against the former type of linkage. These structures may be differentiated by the use of mercaptoethanol which replaces methionine when linked through sulfur (28). Cells grown with methionine-S\textsuperscript{35} were fractionated and the proteins were allowed to stand overnight at room temperature with mercaptoethanol or water as control. Radioassays of the protein precipitated by trichloroacetic acid were identical, indicating that all of the methionine incorporated into the cells had been linked in the \( \alpha \) position characteristic of peptide formation, the only alternative linkage. Thus, the analogue must affect this pathway of methionine uptake.

4. The effect of 8-azaguanine may not have been specific on methionine uptake but was characteristic of a profound depression of the incorporation of all amino acids into the "total protein" fraction. At similar turbidity readings, cells harvested from control cultures and drug-treated bacteria showed none of the drastic reductions in total protein content that had been observed in the incorporation of methionine. When ninhydrin assays on hydrolyzed bacterial protein were expressed in terms of methionine as a standard, in two typical experiments control bacteria contained 0.185 and 0.173 mg of amino acid per ml of bacterial suspension at OD\textsubscript{560} of 0.40, whereas cells grown to this turbidity in the presence of 8-azaguanine contained 0.169 and 0.178 mg of amino acid per ml of suspension. Corresponding results were obtained with the Lowry method (29). Occasionally with cultures grown for long periods of time small but significant reductions in protein content were recorded after inhibition, compared to the corresponding control cells. In any case, the depression by the drug on methionine uptake was far greater than any concomitant decrease in total protein biosynthesis, even when allowing, in the latter calculations, for the dilution effect in the drug-treated culture by the normal cells formed before the addition of 8-azaguanine. A more detailed examination of the incorporation of additional protein precursors will be described in the following paper (9), in which it is concluded that 8-azaguanine inhibited the synthesis of proteoplastic protein, whereas the formation of bacterial cell wall (a component of the "total protein" fraction) continued almost unchanged.

Illustrative of the directness of the effect was the change in methionine content of "total protein." By bioassay with the methionine-requiring Streptococcus strain ATCC 5042, the contents of L-methionine in \( \mu \)g per 100 \( \mu \)g of total protein were determined for a control culture and a culture treated with 8-azaguanine. Duplicate measurements were made at five dilutions of each hydrolysate. Since both cultures had been grown with S\textsuperscript{35}-methionine, the ratio of uptake of S\textsuperscript{35}-methionine at the same turbidity was also calculated, as shown in Table II. It is evident that the methionine content of bacterial "total protein" had decreased, in accordance with the extent of depression of the incorporation of exogenous methionine. The control values are in good agreement with those of Urbá (27) who calculated a protein content of 1.4% methionine for a closely related strain of B. cereus with the use of a different bioassay.

Relationship of 8-Azaguanine Incorporation into Polynucleotides and Depression of Methionine Incorporation into Proteins—The participation of RNA and guanosine polyphosphates in protein synthesis (6, 7) prompted some comparative investigations correlating the role that 8-azaguanine played in antagonizing the normal incorporation of methionine during protein synthesis and the condensation of the analogue into polynucleotides.

The incorporation of 8-azaguanine into the RNA of B. cereus has already been described (5). Table III shows the extent of 8-azaguanine taken up into the nucleic acid fraction when cultures of microorganisms, exposed to varying concentrations of 8-azaguanine-C\textsuperscript{14}, were grown to identical increases in bacterial

![Fig. 3. Effect of subsequent growth on the bacterial content of radiosulfur](image-url)
cell mass. The depression of methionine-$S^{35}$ incorporation into the cells was also determined under identical conditions, and the extent of growth inhibition was recorded.

It is apparent that the biochemical effect on methionine uptake was related to the extent of incorporation of 8-azaguanine into nucleic acid. At concentrations of analogue (0.5 $\mu$g per ml) that were just slightly growth-inhibitory, the effects on nucleic acid and protein biosynthesis were both observed, and all effects increased with the concentration of the inhibitor until a maximal effect was reached near 20 $\mu$g per ml. Higher concentrations had little further effect on growth rate, 8-azaguanine incorporation into polyribonucleotides or the depression of methionine incorporation into proteins. It should be emphasized that these associations need not be cause and effect, as discussed below.

**Effect of 8-Azaguanine on the Utilization of Cystine-$S^{35}$**—The effect of 8-azaguanine on the utilization of labeled cystine provided results qualitatively and quantitatively comparable to those with methionine-$S^{35}$. The reduction in uptake of cystine again was localized in the protein fraction, as shown in Table IV.

Hydrolysis of the protein fraction followed by chromatography in Solvent 2 and radioautography indicated that most of the radioactivity was isolated as cystine (plus traces of cysteine and cysteic acid), and that the analogue had prevented the conversion of exogenous cystine into this constituent of proteins.

This observation was confirmed by the preparation and isolation of cysteic acid from bacterial protein. Although this experiment was difficult to quantitate accurately because of the minute amounts of cysteine or cystine present in the bacterial proteins, the specific activities of the isolated cysteic acid samples and the radioactivity ratios of the cysteic acid fraction from the control and drug-treated cultures (0.38) were of the order anticipated from the $S^{35}$ uptake studies on cold trichloroacetic acid-treated bacterial residues (0.40).

Examination of radioactivity in the acid-soluble fractions of 8-azaguanine-treated and control microorganisms revealed that the analogue did not prevent the penetration of $S^{35}$ into the cell. On the contrary, as is evident from Table IV and Fig. 4, there was more radioactivity in the acid-soluble fraction per unit of radioactivity in the protein fraction under conditions of inhibition. Fig. 4 also shows that the initially high ratios of $S^{35}$ in the acid-soluble fraction compared to the proteins gradually equilibrated at different values for the two cultures in two separate experiments, A and B. There was very little inhibition of total cellular uptake of cystine-$S^{35}$ for a period during which incorporation into the protein fraction was impaired by the drug. Thus, there was a relatively accumulation of intermediary metabolites related to cystine in the drug-inhibited cells, probably because of the failure to be utilized in protein biosynthesis.

Chromatography of the acid-soluble fraction in Solvent 1 followed by radioautography revealed a variety of $S^{35}$-labeled biochemical intermediates, not all of which were ninhydrin positive. The heaviest band could not be differentiated from glutathione. The chromatographic patterns of radioactive metabolites in the two cultures appeared to be identical, implying that 8-azaguanine did not affect specifically the biochemical pathways of cysteine before incorporation into proteins.

**Possible Compensatory Effects Involving Other Sources of Bacterial Sulfur**—The possibility existed that whereas 8-azaguanine inhibited the utilization of exogenous methionine and cystine, other sulfur-containing sources present in the medium might provide alternate biochemical schemes to compensate for the lack of direct incorporation of the two sulfur amino acids. Inorganic $S^{34}$-sulfate could not be used in conjunction with the filter membrane technique, apparently because a trace of radioactive im-
medium, where the only sulfur sources were the two amino acids, alanine, and the aromatic amino acids, were not necessary for growth, providing glucose was present. In this synthetic casein hydrolysate, as isoleucine, aspartic acid, proline, lysine, casein hydrolysate, a completely synthetic medium was devised. Table V shows the amino acids that were required for optimal growth of B. cereus. Other amino acids normally present in casein hydrolysate, as isoleucine, aspartic acid, proline, lysine, alanine, and the aromatic amino acids, were not necessary for growth, providing glucose was present. In this synthetic medium, where the only sulfur sources were the two amino acids, the incorporation of both methionine-S\(^{35}\) and cystine-S\(^{35}\) into B. cereus was inhibited by 8-azaguanine, as had been observed in the casein hydrolysate medium.

Since almost all of the cellular methionine is already in the protein fraction, redistribution of methionine from some reservoir in the cell can be excluded also as a possible mechanism to compensate for the effect observed. Such redistributions have been described for Escherichia coli growing during sulfur deficiency (29). The parallelism in the radioactivity distribution in the two cultures after labeled cystine, both in the acid-soluble fraction and the lack of extensive radioactivity in fractions other than proteins, implied that for cystine, also, little if any cellular redistribution of sulfur had occurred.

These studies confirmed the previous conclusions that the interference in the uptake of the exogenous methionine and cystine resulted in the formation of a "total protein" fraction relatively deficient in the sulfur-containing amino acids.

**Effect of 8-Azaguanine on Other Protein Precursors**—The decreased utilization of the sulfur amino acids for protein synthesis, not coupled with a corresponding drop in protein content prompted a preliminary examination of the drug's effect on other protein precursors, such as glutamic acid-2-C\(^{14}\) and acetate-1-C\(^{14}\). Results indicated that the drug did not depress the uptake of these compounds into the "total proteins" but actually increased their utilization, and it appeared at this point that 8-azaguanine altered the proportion of amino acids in the total protein fraction of B. cereus. In view of the formation of 8-azaguanine-containing RNA, and the possible antagonism of the participation of RNA and guanosine polyphosphates in protein synthesis, the differential effect of the analogue on the utilization of specific protein precursors became the next object for study, and will be described in the subsequent paper (9).

**DISCUSSION**

The depression in the incorporation of methionine and cystine into proteins was greater than that anticipated from the slower growth rate of the bacteria inhibited by the drug, since results have been expressed according to increase in cell mass rather than time. Such a criterion is essential in a description of a drug which is inhibitory to the growth process, for reduction in the proliferative rate must diminish the rate at which cellular mass is being synthesized. Comparisons made after equal increases in cell mass (related to cell number (5)) reveal biochemical responses differing from effects due to the mere slowing of the rate of cellular increase.

The effect of 8-azaguanine on the uptake of the sulfur-containing amino acids needed to be examined carefully to evaluate its mechanism. Before it was concluded that the incorporation of the amino acids into protein was impeded directly, a series of alternatives was examined and eliminated. For example, changes in cellular permeability and possible alterations in the composition of bacterial medium or cells due to the longer incubation period of the inhibited culture were found not to play a role in the observed effect. Since total protein synthesis continued while methionine and cystine incorporation virtually stopped, a change in the amino acid proportion of the total bacterial protein was expected and was observed. Thus, it appeared that the action of 8-azaguanine involved one or both of the following alternatives. Protein with improper amino acid composition could be synthesized during growth inhibition, or the drug produced a selective effect on the formation of certain amino acids.
acid containing compounds in the total protein fraction. The action of 8-azaguanine on protein formation was studied further, therefore, by examining the incorporation of additional protein precursors. In the subsequent paper (9) it has been concluded that the drug’s effect on methionine and cystine incorporation is characteristic of the depressed uptake of most other amino acids tested. In a few cases, however, the uptake into the inhibited culture was found to be greater than that into the control, and the final conclusion has been drawn that the utilization of amino acids for bacterial protoplastic protein synthesis is curtailed by 8-azaguanine in conformity with the decreased formation of this cellular constituent, whereas bacterial cell wall synthesis continued almost unchanged.

Reports that 8-azaguanine affected protein biosynthesis have appeared in the literature and have been compiled in a recent review (30). Among the most pertinent observations is that of Creaser (31), who noted that the analogue inhibited the biosynthesis of the inducible enzymes, β-galactosidase and catalase in Staphylococcus aureus Dunn. The synthesis of induced and constitutive penicillinase was inhibited in B. cereus (11) and the formation of extracellular lytic enzyme was prevented in Bacillus subtilis by the analogue (32). In the chick embryo the cellular degeneration resulting from 8-azaguanine treatment was most severe in those regions which normally accumulated methionine (33). Simultaneous with the preliminary report on the present findings (8), Chantrenne and Devreux (10) observed that 8-azaguanine reversed the utilization of methionine in B. cereus.

It was not the purpose of these investigations to elucidate the metabolic pathway of 8-azaguanine responsible for the effect on protein biosynthesis. Previously it was postulated (2) that the analogue owed its effect to the formation of a derivative, as azaguanosine diphosphate, which interfered with the coenzymes responsible for various metabolic reactions. Another possibility involved the formation of a particular type of 8-azaguanine-containing RNA (5) which interfered with the function of normal RNA. These mechanisms have been discussed recently in greater detail (30). The incorporation of 8-azaguanine into RNA and the drug’s effect on protein biosynthesis as compared in Table III, need not be related directly and etiologically, since undoubtedly other factors are also involved. As a matter of fact, considerable evidence has been provided that such a simple relationship does not exist (5). These correlations are still under investigation.

**SUMMARY**

1. Cultures of Bacillus cereus proliferating in a defined amino acid medium incorporated far less exogenous Sα-methionine or Sα-cystine per unit of bacterial mass synthesized, when growth was partially inhibited by 8-azaguanine. These effects could be reversed by the addition of guanosine.

2. The observed inhibitory effect of the drug was localized at the step of incorporation into cellular proteins. In contrast the acid-soluble fraction of the drug-treated cells contained relatively large accumulations of the radioactive intermediates present in control bacteria.

3. The effect did not commence instantly and was associated with the delay characteristic of the drug’s effect on the growth rate. The incorporation of 8-azaguanine into cellular polynucleotides thus preceded the observed effect on the amino acids.

4. “Total protein” content of drug-treated cells was nearly that of the control cells after similar increases in cell mass, and a deficiency of sulfur amino acids in the protein residue was demonstrated by direct measurement and exclusion of compensatory biochemical pathways. At the same time, certain nonsulfur-containing protein precursors were incorporated to a greater extent by the inhibited cells.

5. Since the overall composition of the protein residue from bacterial cells grown in the presence of 8-azaguanine differed sharply from that of control microorganisms, inhibition by 8-azaguanine apparently involved the synthesis of protein molecules with altered composition, and/or produced a selective inhibitory effect on the formation of certain amino acid-containing compounds. Evidence for the latter effect will be described in the subsequent paper.

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