Chemical and Morphological Studies of Bacterial Spore Formation

III. The Effect of 8-Azaguanine on Spore and Parasporal Protein Formation in *Bacillus cereus* var. *Alesti**‡

By I. ELIZABETH YOUNG,§ Ph.D., and PHILIP C. FITZ-JAMES,¶ Ph.D.

(*From the Department of Bacteriology and Immunology and the Department of Biochemistry, University of Western Ontario, London, Canada*)

PLATE 242

ABSTRACT

The purine analogue, 8-azaguanine, was added to cultures of the parasporal crystal-forming organism *Bacillus cereus* var. *alesti* at different times during growth and synchronous sporulation. The effect of its incorporation has been studied with particular reference to cell growth, nucleic acid composition, cytology, and the synthesis of the spore and crystal protein.

Additions of the analogue during any stage of growth prevented further cell proliferation and all spore and crystal formation. Since both nucleic acids continued to be formed, cells of an increased size developed, containing large masses of chromatin in the form of condensed balls or axial cords. Lipid-containing inclusions also appeared following these additions and were usually aggregated at the centre or poles of the cells. The analogue could be isolated as the ribonucleotide from both the acid soluble and RNA fractions of these inhibited cells.

Additions of the analogue following commencement of sporulation did not prevent either spore or crystal formation or affect the nucleic acid content of the sporulating cells. However, as before, the 8-azaguanine was incorporated into both the acid soluble and RNA of the cells, but not into these fractions of the spores ultimately formed.

The implications of these findings are discussed in relation to crystal protein synthesis.

INTRODUCTION

There is now much evidence linking the synthesis of protein with a concomitant synthesis of ribonucleic acid (RNA) (1, 10, 13, 14). The failure to detect any net synthesis of RNA during the formation of spores (15) or of spores and parasporal protein crystals (16) would at first seem incompatible with this concept. However, there is

suggestive evidence that the active synthesis of protein occurring during sporogenesis (and thus in non-dividing cells) was accompanied by a turnover of some of the RNA fraction (15, 16). Nevertheless, there was no information whether this metabolism was associated with or essential for the synthesis of these proteins.

It was known that the purine analogue, 8-azaguanine, could be specifically incorporated into the RNA of *Bacillus cereus* (6, 7) and that similar incorporations could prevent the induced synthesis of protein associated with adaptive enzyme formation (2, 3). Hence, it was reasoned that additions of 8-azaguanine to a culture during sporulation might render any RNA synthesized nonfunctional for protein formation and thus inhibit both spore and crystal development.

The chemical and cytological results presented

in this paper arose from studies of cultures of *Bacillus cereus* var. *alesti* to which 8-azaguanine had been added at various times during growth and sporulation.

Methods

The general techniques for the handling of growing and sporulating cultures of *B. cereus* var. *alesti* have been described previously (16).

A solution of 0.5 per cent 8-azaguanine (Nutritional Biochemicals Corporation, Cleveland, Ohio) dissolved in 0.5 per cent sodium carbonate was added at various times during growth and sporulation to identical lots of a culture. A final concentration of 100 μg. per ml. of culture was used throughout.

Samples for counting, cytology, electron microscopy, and nucleic acid analyses were removed and treated as described in the Methods of the preceding papers (15, 16). In addition, the cells from 80 ml. of culture were disrupted and the acid-soluble and RNA fractions separated and analysed for mononucleotides. The chromatographic and electrophoretic procedures already described (6, 8) were used for the separation of the nucleotides. The separated nucleotides were detected on the papers by their ultraviolet absorption or fluoroscence when examined in the light emitted from a Mineralite lamp (model SL, Ultraviolet Products, South Pasadena, California) to which was attached a quartz cell containing a solution of $CoSO_2 \cdot 7H_2O$ (10 per cent) and $NiSO_2 \cdot 7H_2O$ (35 per cent). The nucleotides which were eluted from the papers with 0.1 N HCl were estimated spectrophotometrically. The molecular extinction coefficient of guanylic acid was used to calculate the concentration of the 8-azaguanylic acid.

RESULTS

Morphological Changes Following Additions of 8-Azaguanine During Logarithmic Growth:

Addition of 8-azaguanine to cells during late logarithmic growth inhibited all further increase in cell numbers as determined by direct cell counts. However, the cells continued to increase in size during the inhibition and were considerably longer following an hour in the presence of the analogue. Similarly, additions during early logarithmic growth caused a cessation of cell division and an increase in cell size but also caused the chains of cells to become twisted and tortuous.

A few minutes following the additions of the analogue, faintly phase-light areas similar to those shown in Fig. 3, appeared within the cells and could be shown by hydrolysis and staining to be aggregations of chromatin. For example, the two

nuclear bodies present in each cell at $5\frac{1}{2}$ hours of growth became aggregated and condensed into a single structure when incubated for a further 5 hours in the presence of the analogue (Fig. 6). Exposure of these cells to 8-azaguanine for longer periods provoked the appearance of phase-refractile inclusions which aggregated at the poles or in the centre of the cells. Normally, similar inclusions are not detectable by phase contrast illumination in growing cells (Fig. 1) but become prominent only upon the cessation of growth and the onset of sporulation. These inclusions stained with Sudan black B.

In cells to which 8-azaguanine was added later in logarithmic growth these phase-refractile inclusions appeared much sooner. Thus, they were apparent in the inhibited cells within 1 hour following addition of the analogue at $7\frac{1}{2}$ hours (Fig. 3) and appeared almost immediately after an addition at $8\frac{3}{4}$ hours (cf. Figs. 1 and 4). Subsequent nuclear stains of these cells following 2 hours of inhibition (Fig. 7) indicated, as before, that the chromatin had aggregated into large, densely staining masses. Addition of the analogue 15 minutes later, at 9 hours of growth, led to the formation of cells which 2 hours later possessed prominent axial cords of chromatin (Fig. 9).

In electron micrographs of thin sections, low density material similar in fine structure to that seen during normal spore formation occupied the sites of the chromatin (Figs. 10 and 11). The lipid-containing inclusions apparent in the phase contrast smears and in the stained preparations appeared in the electron micrographs as several small "vacuoles" within the cytoplasm and were usually clustered at the poles of the cell (Fig. 10). Similar vacuoles were occasionally seen skirting the longitudinal skein of chromatin (Fig. 11) much like those about the axial filament of spore formation (16). The curious rippling of the cell wall seen in these sections was always found with azaguanine inhibition.

In this typical experiment no spores had formed by 24 hours in those flasks of culture to which azaguanine was added prior to 9 hours (Fig. 5), although the control was 100 per cent sporulated at 15 hours. Cells inhibited prior to the onset of sporogenesis could accomplish the formation of spores only if they were transferred to fresh medium sufficiently soon following the addition of the analogue. The cells in Fig. 8 were placed in new medium at $9\frac{1}{2}$ hours; they had been in the presence

of the analogue for the previous 2½ hours. Some 40 per cent of these cells were able to form apparently normal, refractile spores and crystals. Shorter periods of exposure to the analogue increased the per cent sporulation while longer periods soon rendered the cells incapable of recovery.

Morphological Changes Following Additions of 8-Azaguanine During Sporulation:

In the control culture, the nuclear condensation associated with the first step into spore formation (15, 16) could be detected in all cells at 9⅔ hours of aeration. The lipid-containing inclusions characteristic of this stage appeared in close association with nuclear bodies (Fig. 2). Additions of 8-azaguanine now, or at any time after this period, did not prevent the subsequent formation of either spores or crystals although there was an increase of 30 to 60 minutes in the time required to complete the 6 hour process. Nor was there any alteration following these additions in the distribution of chromatin or in the sequence of chromatin patterns which have been associated with normal sporogenesis. For example, the cells shown in Fig. 12 contain chromatin bodies which are in an early stage of axial filament formation similar to that already seen in the control culture at this same time (Fig. 13); 8-azaguanine had been added to these cells 1 hour previously when they were in the stage of development shown in Fig. 2. Lysis of the cells and liberation of the spores and crystals, complete at 20 hours in the control culture, took until 30 to 40 hours in the presence of the analogue.

Changes in the Nucleic Acid Content of Cells Following Additions of 8-Azaguanine:

A young culture of B. cereus var. alesti was transferred in 80 ml. lots to 1 liter flasks and then aerated. At the times indicated in the text-figures, 8-azaguanine was added to separate lots of the culture. Samples were subsequently removed for cell count, optical density readings, and nucleic acid analyses as described previously (15).

Additions during the late logarithmic phase of growth retarded the rate of increase in optical density of the culture much as has been described in B. cereus (6). However, in these cultures of B. cereus var. alesti the continued increase in optical density (Text-fig. 1) was not due to an increase in cell numbers but was apparently due to the con-
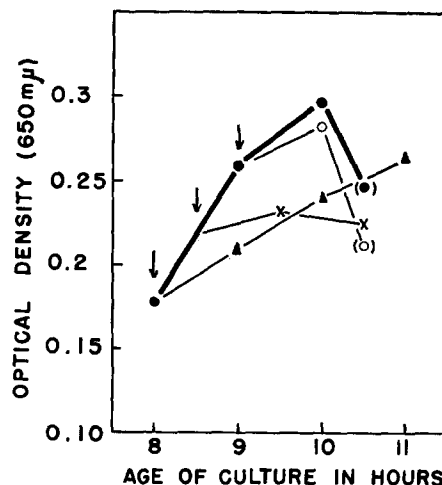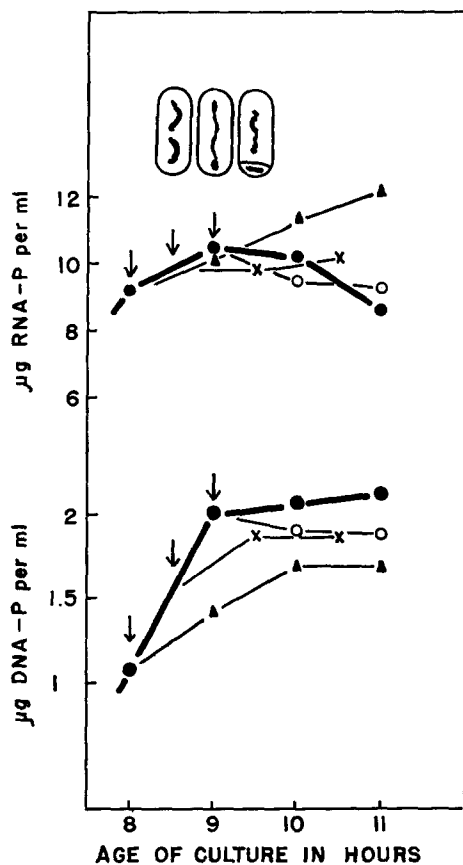

TEXT-FIG. 1. The effect of the time of addition of 8-azaguanine (100 µg. per ml.) on the optical density of a culture of B. cereus var. alesti. ●—●, control; ▲—▲, addition at 8 hours; ✕—✕, additiou at 8½ hours; ○—○, addition at 9 hours; (●), (○), cells aggregating.

siderable increase in cell mass (9) which was detected microscopically as an increase in cell length.

In spite of the absence of a further increase in the number of cells following the addition of the analogue, the synthesis of RNA continued at a linear, although reduced rate (Text-fig. 2). Even when the net synthesis of RNA ceased in the control culture due to the onset of sporulation, the synthesis of this compound continued unabated in the inhibited cultures (Text-figs. 2 and 4).

Synthesis of DNA also continued following addition of the analogue but at a rate considerably reduced from that of the control. Thus, cells developed during the inhibition of division and spore formation which contained nearly twice the normal content of DNA and a considerably increased content of RNA (Text-fig. 3). This accumulation of DNA gave direct evidence that the larger and well staining chromatin structures seen in the inhibited cells resulted from the continued synthesis of this compound as well as from an aggregation of the nuclear bodies.

The fortunate addition of 8-azaguanine just at the moment of commencement of sporulation in a rapidly growing and highly synchronous culture led to the remarkable changes in nucleic acid content shown in Text-fig. 4. Here, the synthesis of DNA continued linearly but at a slightly increased rate and led to the formation of cells with a six-
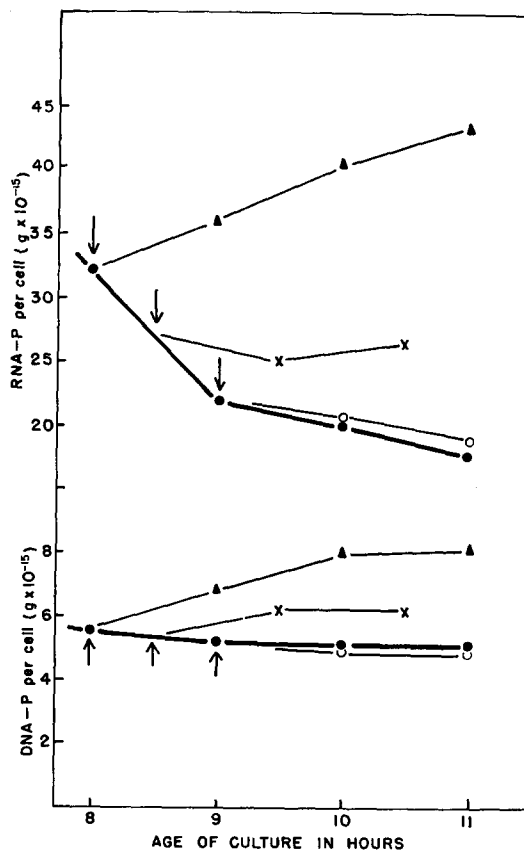
TEXT-FIG. 2. The effect of the time of addition of 8-azaguanine (100 μg. per ml.) on the content of nucleic acids in a culture of *B. cereus* var. *alesti*. ●—●, control; ▲—▲, addition at 8 hours; ×—×, addition at 8½ hours; ○—○, addition at 9 hours. Line drawings indicate that sporulation commenced between 8½ and 9 hours. Spore formation, inhibited following the first two additions, was at 15 hours complete in the control and 90 per cent in the 9 hour addition culture.

TEXT-FIG. 3. The effect of the time of addition of 8-azaguanine (100 μg. per ml.) on the content of nucleic acids per cell in a culture of *B. cereus* var. *alesti*, ●—●, control; ▲—▲, addition at 8 hours; ×—×. addition at 8½ hours; ○—○, addition at 9 hours. These values were derived from the same experiment pictured in Text-figs. 1 and 2.

fold increase in DNA. These cells are shown in Figs. 9 to 11. Similarly, RNA synthesis continued linearly at the rate underway prior to the time of addition.

Once the cells had commenced sporulation additions of 8-azaguanine to the cultures not only could not prevent the development of spores and crystals but also failed to alter the normal pattern of synthesis of either nucleic acid (Text-figs. 2 and 3).

The Incorporation of 8-Azaguanine into the Nucleotides of the Acid-Soluble and RNA Fractions:

The failure of 8-azaguanine to alter either the process of spore development or the pattern of nu-

cleic acid synthesis when added following the commencement of sporogenesis may be interpreted in three ways: (1) the analogue was not incorporated into the RNA because of either a sudden impermeability of the cells to the compound or, more probably, an absence of RNA synthesis; (2) although the analogue is incorporated into the cell and into the RNA fraction it does not alter the functional ability of this compound during sporogenesis; (3) the RNA plays no role in the formation of the spore or crystal proteins. To narrow these possibilities, studies were made to determine if 8-azaguanine were incorporated into the RNA of the sporulating cells.

Additions of the analogue were made to lots of culture at the 8th, 9th, and 10th hours of aeration. The sequences of optical density and cytological
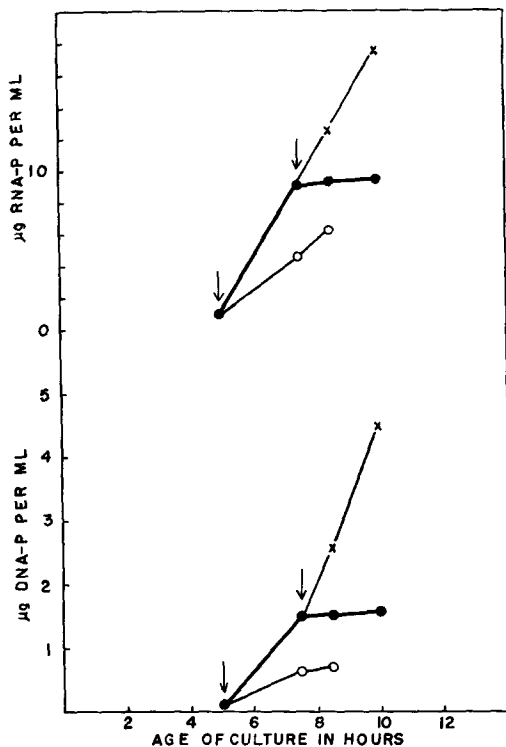
Text-Fig. 4. The effect of the time of addition of 8-azaguanine (100 μg. per ml.) on the nucleic acid content of a culture of *B. cereus* var. *alesti.* ●—●, control; O—O, addition at 5 hours; X—X, addition at 7½ hours. The latter addition was made just at the moment of commencement of sporulation. Both additions prevented spore and crystal formation.

changes of this experiment were identical to those recorded in Text-figs. 1 to 3. Typical blocked cells were present 2 hours after the 8th hour addition, while normal spore and crystal synthesis occurred in the cells following the 9th and 10th hour additions. Cells from 80 ml. of culture were analyzed for 8-azaguanine 2 hours after each of the additions. At 24 hours, a sample of the inhibited cells (8th hour addition) was again analyzed as well as samples of the free refractile spores and crystals which were formed following the later additions.

The crystals were removed from the cleaned spore-crystal mixture by two extractions with 1 per cent thioglycollate (pH 11.5) (16). The yield of crystal protein from cells sporulating in the presence of 8-azaguanine was similar to that from cells in the control culture as determined from the nitrogen content of these extracts.

The cold acid-soluble fractions freed of trichloroacetic acid by ether extraction were concentrated and then subjected to electrophoresis in borate buffer at pH 9.0 (8). The characteristic fluorescent band of 8-azaguanylic acid was readily observed in the cold acid fraction of cells removed 2 hours after the 8th hour addition, and also, to a lesser extent, in that of the sporulating cells following the 9th and 10th hour additions. It could not, however, be detected in the small acid-soluble fraction extracted from the spores formed in the presence of 8-azaguanine.

The RNA fractions treated with N alkali to liberate the nucleotides were subjected to paper electrophoresis in ammonium formate buffer at pH 3.0 following the techniques described by Mandel and Markham (6). In this system 8-azaguanylic acid can be detected as an ultraviolet-absorbing area between uridylic and guanylic acids. Such an area was present among the nucleotides released from the RNA of both inhibited cells and cells sporulating in the presence of the analogue but absent from the RNA of the spores formed in the presence of 8-azaguanine as well as from that of the untreated cells.

The 8-azaguanylic acid eluted in 0.1 N HCl showed an absorption maximum between 255 and 257 mμ and a minimum near 245 mμ. The ratio of the absorbency at 280 to 260 mμ varied from 0.53 to 0.57. On the other hand eluates of the guanylic acid spots and known guanylic acid in 0.1 N HCl possessed an absorption maximum near 255 mμ, a minimum near 228 mμ, and a ratio 280:260 which varied from 0.65 to 0.69.

These findings suggested that 8-azaguanine was incorporated into the RNA of cells to which the analogue had been added either prior to or after the commencement of sporulation. From the absorbencies it was calculated that 30 per cent of the guanylic acid had been replaced by 8-azaguanylic acid 2 hours after the 8th hour addition. By 24 hours in these blocked cells this value was 23 per cent. The cells sporulating 2 hours after the 9th hour addition showed 20 per cent replacement of guanylate by 8-azaguanylate and those analyzed 2 hours after the 10th hour addition showed a 12 per cent replacement.

DISCUSSION

Extension of the two condensed chromatin bodies of a bacillus into an axial filament is the first reliable cytological indication of the transition of a vegetative cell into a sporulating cell. Associated with this change is the cessation of all further growth and of net synthesis of both nucleic acids. This decisive moment of commitment to sporo-

genesis is further reflected in the effects on the cells of additions of the purine analogue, 8-azaguanine. Prior to commitment such additions prevent further cell division and the onset of sporulation but do not prevent the continued net synthesis of either RNA or DNA. Subsequent to commitment such additions have no effect on sporogenesis or the normal pattern of nucleic acid synthesis but merely delay the terminal lysis of the sporangia. However, under both circumstances the analogue is found incorporated into both the acid-soluble and RNA fractions as a ribonucleotide, although the degree of displacement of guanylic by 8-azaguanylic acid is greater following the precommitment additions. No doubt this enhanced displacement of guanine is due to the continuing net synthesis of RNA following such early additions, although incorporation of the analogue into RNA during sporogenesis also indicates turnover of at least some of this fraction (16).

If protein synthesis depends on an active synthesis of a normal RNA (1, 10, 13, 14), then the continued formation of the crystal protein, despite the incorporation of the analogue, is an unexpected finding. There was no evidence of asynchrony which could account for the incorporation of the azaguanine; that is, inhibited cells were absent from the cultures sporulating in the presence of the analogue. Thus, one must conclude that following commitment 8-azaguanylic acid is incorporated into a position in, or a portion of, the RNA which is not involved in protein formation. In this regard, it has been shown by Mandel and Markham (6) that 8-azaguanine incorporated into *B. cereus* during growth is found at the ends of polynucleotide chains in the form of 8-azaguanosine-$2'$:$3'$-phosphate. Such positions probably play no role in the early steps, at least, of protein formation since activated amino acids appear to be linked to a specific polynucleotide chain with a terminal adenylyl-cytidylyl-cytidylyl sequence (5, 11).

No comparisons have yet been made of the biological activity, (*i.e.* of the toxicity to insect larvae) (4), of normal crystals and those formed following azaguanine incorporation. Some change in the specificity of the protein might be expected in view of the recent report of Rooden and Mandel (12) that 8-azaguanine markedly affects the relative incorporations of amino acids into the vegetative proteins of *B. cereus* without altering the total quantity of protein synthesized. In this re-

gard, the yield and appearance of the crystals formed in 8-azaguanine were indistinguishable from their controls.

Why 8-azaguanine should inhibit adaptive (3) as well as some constitutive enzyme formation (13) but not the synthesis of crystal protein remains completely obscure. A possible explanation may lie in the different cell states associated with these activities; *i.e.* the azaguanine-sensitive enzyme syntheses occur in growing cells while the insensitive crystal synthesis occurs only in non-growing, sporulating cells. In the former situation, nucleic acids continue to show an absolute increase; in the latter, only a turnover of RNA appears to occur.

REFERENCES

1. Ben-Ishai, R., Dependence of protein synthesis on ribonucleic acid synthesis. II. Nonparticipation of preformed ribonucleic acid in protein synthesis, *Biochim. et Biophysica Acta*, 1957, **26**, 477.
2. Creaser, E. H., Inhibition of induced enzyme formation by purine analogues, *Nature*, 1955, **175**, 899.
3. Creaser, E. H., Effect of 8-azaguanine on enzyme formation, *Nature*, 1955, **176**, 556.
4. Fitz-James, P. C., Toumanoff, C., and Young, I. E., Localization of a toxicity for silkworm larvae in the parasporal inclusion of *Bacillus cereus* var. *alesti*, *Canad. J. Microbiol.*, 1958, **4**, 385.
5. Hecht, L. I., Stephenson, M. L., and Zamecnik, P. C., Dependence of amino acid binding to soluble ribonucleic acid on cytidine triphosphate, *Biochim. et Biophysica Acta*, 1958, **29**, 460.
6. Mandel, H. G., and Markham, R., The effects of 8-azaguanine on the biosynthesis of ribonucleic acid in *Bacillus cereus*, *Biochem. J.*, 1958, **69**, 297.
7. Mandel, H. G., Sugarman, G. I., and Apter, R. A., Fractionation studies of *Bacillus cereus* containing 8-azaguanine, *J. Biol. Chem.*, 1957, **225**, 151.
8. Matthews, R. E. F., Effects of some purine analogues on Tobacco mosaic virus, *J. Gen. Microbiol.*, 1954, **10**, 521.
9. Monod, J., The growth of bacterial cultures, *Ann. Rev. Microbiol.*, 1949, **3**, 371.
10. Pardee, A. B., Nucleic acid precursors and protein synthesis, *Proc. Nat. Acad. Sc.*, 1954, **40**, 263.
11. Preiss, J., Berg, P., Ofengand, E. J., Bergmann, F. H., and Dieckmann, M., The chemical nature of the RNA-amino acid compound formed by amino acid-activating enzymes, *Proc. Nat. Acad. Sc.*, 1959, **45**, 319.
12. Rooden, D. B., and Mandel, H. G., Disruption by

8-azaguanine of the incorporation of amino acid into *Bacillus cereus*, *Fed. Proc.*, 1959, **18**, 1735.

13. Spiegelman, S., Nucleic acids and the synthesis of proteins, *in* Chemical Basis of Heredity, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 232.

14. Spiegelman, S., Halvorson, H. O., and Ben-Ishai, R., Free amino acids and the enzyme-forming mechanism, *in* Amino Acid Metabolism, (W. D. McElroy and B. Glass, editors), Baltimore, The Johns Hopkins Press, 1955, 124.

15. Young, I. E., and Fitz-James, P. C., Chemical and morphological studies of bacterial spore formation. I. The formation of spores in *Bacillus cereus*, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 467.

16. Young, I. E., and Fitz-James, P. C., Chemical and morphological studies of bacterial spore formation. II. Spore and parasporal protein formation in *Bacillus cereus* var. *alesti*, *J. Biophysic. and Biochem. Cytol.*, 1959, **6**, 483.

<div style="text-align:center">

EXPLANATION OF PLATE 242

</div>

FIGS. 1 to 5. Dark phase contrast photomicrographs. $\times$ 3880.

FIG. 1. Two control cells of *B. cereus* var. *alesti* at $8\frac{3}{4}$ hours of aeration.

FIG. 2. Control cells at $9\frac{3}{4}$ hours of aeration. Growth has ceased and the inclusions heralding the onset of spore formation are detectable.

FIG. 3. 8-Azaguanine (100 μg. per ml.) was added at $7\frac{1}{2}$ hours of aeration; cells were photographed at $8\frac{3}{4}$ hours.

FIG. 4. 8-Azaguanine (100 μg. per ml.) was added at $8\frac{3}{4}$ hours and photographed an hour later.

FIG. 5. Cells of the same culture shown in Fig. 4 photographed at 23 hours.

FIG. 6. 8-Azaguanine (100 μg. per ml.) was added to the culture at $5\frac{1}{2}$ hours and the cells were fixed and stained after a further 5 hours of aeration; HCl-azure A. $\times$ 3600.

FIG. 7. The same culture and stage as shown in Fig. 4; HCl-azure A. $\times$ 3600.

FIG. 8. Dark phase contrast of cells at 26 hours showing partial recovery from 8-azaguanine inhibition of sporulation. $\times$ 3880.

FIG. 9. These cells, inhibited by 8-azaguanine just before commitment to sporulation, show the extensive axial cord of chromatin; HCl-azure A. $\times$ 3600.

FIGS. 10 and 11. Electron micrographs of thin sections of cells from the same culture shown in Fig. 9. Osmium-fixed, cut onto water. The longitudinally arranged chromatin bodies contain low density strands interspersed with a more dense background material. The lipid-containing inclusions appear as vacuoles clustered at the cell ends in Fig. 10 ($\times$ 36000), or along the chromatin strand in Fig. 11 ($\times$ 52000).

FIG. 12. Cells of *B. cereus* var. *alesti* to which 8-azaguanine was added after sporulation had commenced (at $9\frac{3}{4}$ hours of aeration, the stage shown in Fig. 2). This stained smear made at $10\frac{2}{3}$ hours shows the normal condensation of the chromatin into an axial filament of spore formation; HCl-azure A. $\times$ 3600.

FIG. 13. Control cells at $10\frac{2}{3}$ hours of aeration; HCl-azure A. $\times$ 3600.
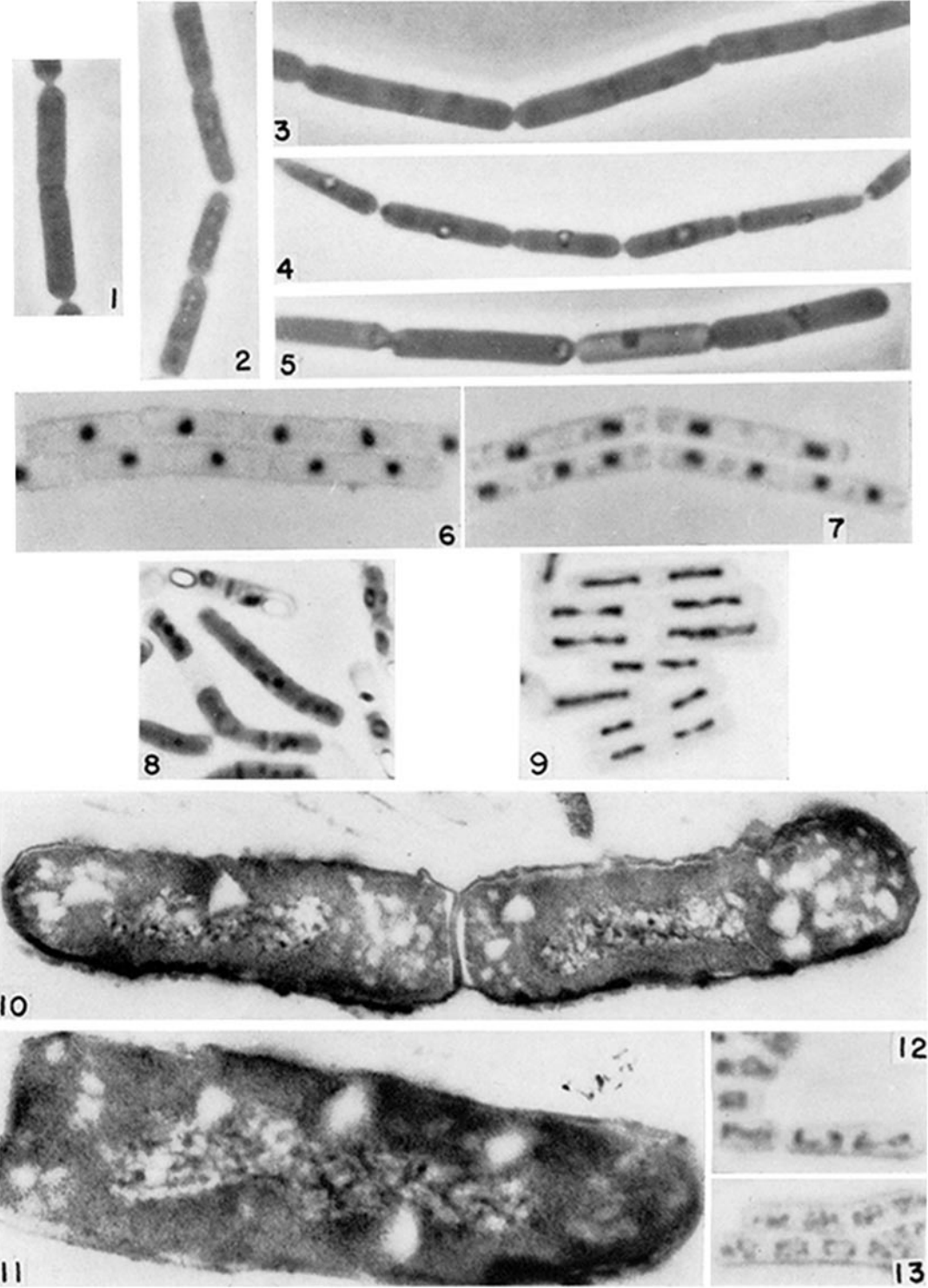
(Young and Fitz-James: Bacterial spore formation. III)