Chemical and Morphological Studies of Bacterial Spore Formation

II. Spore and Parasporal Protein Formation in *Bacillus cereus* var. *Alesti**‡

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ABSTRACT

The development of both the spore and parasporal protein crystal of *Bacillus cereus* var. *alesti* was followed using chemical and cytological techniques. The changes which led to the formation of the fore-spore were similar to those already described for *Bacillus cereus*. However, adjacent to the developing fore-spore a small inclusion became discernible in phase contrast. This protein inclusion during its growth was differentiated from the chromatin and lipid-containing inclusions by sequential staining techniques.

During spore and crystal formation no net synthesis of either nucleic acid was detected. Tracer studies with radioactive phosphorus confirmed that the spore chromatin was derived from that in the vegetative cell. These same studies also indicated that a turnover of ribonucleic acid occurred during the sporulation process.

During their formation both the spore and crystal incorporated methionine-^{35}S from the medium and from cellular material into a bound form. Sequential extractions with alkali and with alkaline-thioglycollate reagent revealed that the solubility characteristics of the mature crystal were possibly related to the presence of intermolecular disulphide bonds which developed after the major synthesis of the crystal was complete.

The synthetic nature of sporogenesis and crystal formation is discussed with reference to the concept of "endotrophic" sporulation.

INTRODUCTION

During sporogenesis in certain organisms related to *Bacillus cereus* a parasporal body appears within the cell in the form of a diamond-shaped crystal (16). Although the protein nature of isolated mature crystals has been established, and some description given of their structure, solubility properties (17) and toxicity for larvae of Lepidoptera (1, 8), no critical information is available on their mode of formation. An understanding of sporulation in these organisms is hampered by the apparent variability of the process. It has been reported, for instance, that cells which contain four chromatin bodies form a spore and a crystal in a different fashion from those which contain two (16). However, such variability was apparently not unique to sporogenesis in crystal-forming organisms since similar deviations in the process have been described in organisms which do not form crystals (10). Since we recently had reason to believe that spore formation, at least, was a more

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intelligible process, we extended these studies to include a number of crystal-forming variants of *Bacillus cereus*.

In this paper we describe the mode of spore and crystal formation in *Bacillus cereus* var. *alesti* and present chemical evidence that the protein forming the crystal is actively synthesized from smaller units during its growth. Some studies of the nucleic acids during the synthesis of this specific protein will be presented in this and a subsequent paper (38).

Materials and Methods

Culturing Techniques:

The organism studied was originally isolated by Toumanoff and Vago (33) from the larvae of diseased silkworms. A culture of this isolate, *Bacillus cereus* var. *alesti*, was received in 1955 from Dr. C. Toumanoff of the Pasteur Institute. Colonies producing uniformly large crystals and spores were selected from blood agar plates and then maintained in the sporulated state on slopes of the agar medium (supplemented with 0.5 per cent casamino acids) of Howie and Cruickshank (20).

For the sporulation studies a fluid medium was required which would support adequate growth followed by reasonably synchronous sporulation. A nutrient stock containing 40 per cent beef papain-digest broth, 0.5 per cent proteose peptone 2 (Difco), 0.5 per cent proteose peptone 3 (Difco) in distilled water was diluted 1 in 4 with Grelet salts (15). A starter culture was grown and mixed with the total volume of medium required. Eighty ml. of this culture were then distributed to each litre flask and aeration continued at 29°–31°. A rapid growth was followed by sporulation in all cells. The degree of synchrony of the process was similar to that achieved in *B. cereus* (37); that is, all cells were within 15 to 20 minutes of each other in a process requiring some 6 hours.

Microscopy and Cytological Techniques:

These techniques including those for electron microscopy were the same as described in detail in the previous paper (37).

Chemical Fractionations and Analyses:

Nucleic Acids.—The preparation of samples, the fractionation procedures, and the methods of analyses have been described (37).

Amino Acids.—The free amino acid pools of vegetative and sporulating cells were extracted by a modification of the method of Gale (13). Ten to 20 mg. of cells were washed free of medium with cold Grelet salts, suspended in water, and boiled for 10 minutes in a water bath. The supernatants from this and a subsequent extraction contained the free amino acid pool of the cells.

The amino acids in these extracts were separated by the two-dimensional paper-chromatographic method of Redfield (31) as modified by Wyatt, Loughheed, and Wyatt (36) in which the first solvent system contained: *tert.*-butanol, methyl ethyl ketone, concentrated ammonium hydroxide, water (10:10:10:3:5, by volume) and the second: methanol, pyridine, water (20:1:5, by volume). The volume of the extract was adjusted so that approximately 4 μg. of each of the amino acids were applied to each paper (Schleicher and Schuell paper No. 507, blue ribbon grade). Following resolution, the amino acids were located with a ninhydrin spray, eluted from the paper with 70 per cent ethanol, and estimated quantitatively by the method of Kay, Harris, and Entenman (22).

The amino acid composition of proteins extracted from the cells was determined following hydrolysis of the samples in sealed tubes with 6 N HCl at 100°–110° for 24 hours. The HCl was removed by vacuum drying and the residues washed free of traces of the acid by at least two additions and re-evaporations of water.

Incorporation of Radioactive Compounds:

Phosphorus.—The transfer procedure described previously (37) for the incorporation of radioactive phosphorus into the nucleic acids of *B. cereus* was repeated with *B. cereus* var. *alesti*. The same methods of fractionation, analysis, and counting of radioactivity were also followed.

Methionine.—The degree of incorporation of radioactive methionine (methionine-^{35}S) into the crystals and spores of *B. cereus* var. *alesti* was determined in several experiments. The culturing procedures were essentially the same as those described for the incorporation of ^{32}P, although the salts solution of Grelet (15) with the full complement of phosphorus was used throughout.

In the *addition* experiments, the labelled methionine (as DL-methionine-^{35}S, Volk Radiochemical Company, Chicago) was added to separate flasks of the same culture at various times during spore and crystal formation. Sporulation was complete and the sporangia had lysed before the liberated spores and crystals were cleaned free of vegetative remnants and medium by 4 cycles of differential centrifugation. The crystal protein was then selectively extracted from a suspension of the spore-crystal mixture by the addition of an equal volume of sodium thioglycollate reagent (2 per cent thioglycollic acid in NaOH, pH 11.5). The unextracted spores were centrifuged and rewashed with the thioglycollate reagent; the pooled supernatants contained the crystal protein in solution. Upon addition of cold acetate buffer (5 M, pH 4.8) to the chilled supernatants the protein was recovered quantitatively as a white precipitate. The precipitate was twice washed in the cold with water before portions were transferred to hydrolysis tubes, dried, and then hydrolysed with 6 N

HCl as described above. The constituent amino acids in each sample were separated chromatographically and the methionine was estimated by the procedure described above.

The radioactivity of the methionine was determined on the ethanol eluate used for the absorbance measurements in the ninhydrin method. A portion (usually 500 μl.) was dried on aluminum planchets with 30 μl. of 2 per cent gelatin. These were placed in a windowless gas flow counter designed to measure soft radiation (Nuclear Instrument and Chemical Corporation, Chicago, Model D46A) and the counts were recorded on a Berkeley decimal scaler unit (Berkeley Scientific Company, Berkeley, California, model 2000). The amount and the activity of the methionine in each sample were estimated from the mean values derived from duplicate or triplicate chromatograms. Specific activities were expressed as counts per minute per μg. methionine. Autoradiograms of duplicate chromatograms were used to verify the discreteness of the methionine spot and to detect other active areas.

The spores which remained after the extraction of the crystal protein were washed with water, dilute HCl, and then water before they were dried and hydrolysed for the release of amino acids. The samples were then chromatographed and the specific activities of the methionine spots determined.

In the *transfer* procedure (37) cells labelled with methionine-^{35}S completed spore and crystal formation after transfer from the labelled to a non-labelled medium of the same characteristics. The specific activities of the methionine-^{35}S in the spores and crystals resulting from the transfer were determined as before.

The living cells were examined periodically in the phase contrast microscope to determine the stage of growth or sporulation. Impression smears were made at the times of sampling, of addition of a labelled compound to the culture, or of transfer of cells to a non-labelled medium. The stage of sporulation and the degree of synchrony of the process were then estimated from the appearance of these cells when stained for chromatin. The line drawings of the cells which have been appended to the figures are derived from the respective photomicrographs.

RESULTS

General Observations

Germination of the spore inoculum of *B. cereus* var. *alesti* was followed by approximately 9 hours of cell growth. During this interval the total cell count at any one time was directly related to both the optical density (650 mμ) of the culture and the deoxyribonucleic acid content of the cell fraction.

In the long slender chains of early log-phase cells, the cross-walls marking individual cell limits were difficult to distinguish in both dark-phase contrast mounts and nuclear-stained preparations, but, during later logarithmic growth the chains became shorter, the cells more plump, and the cross-walls readily discernible. The cessation of growth was accompanied by an aggregation of the cells, the evolution of a pungent and distinctive odour, and the cytological changes associated with the beginnings of spore formation. At this time there were approximately 2 mg. dry weight of cells per ml. of culture. In contrast to *B. cereus*, there was no clearing stage; that is, there was no marked fall in optical density of the culture nor any sudden alteration in the transparency of the cell cytoplasm as seen in phase contrast.

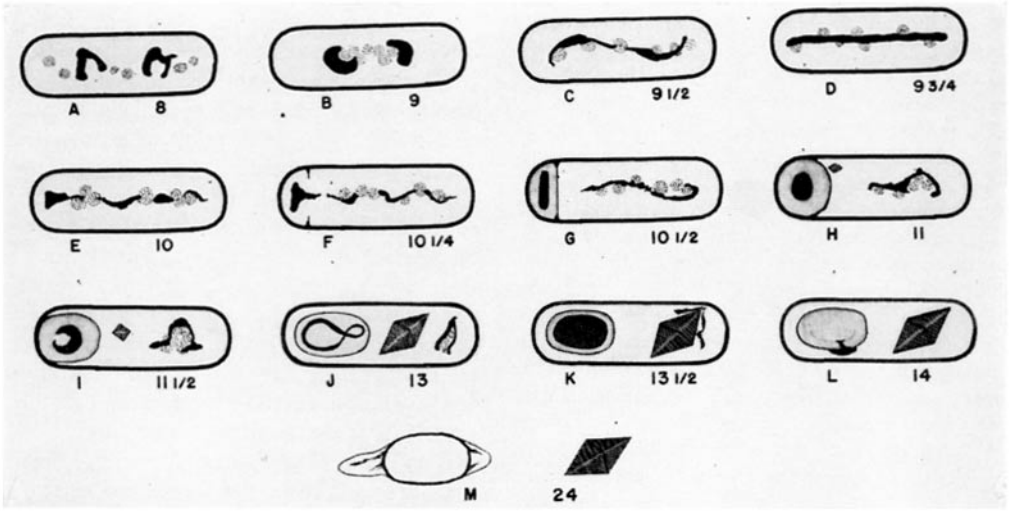
Fourteen to 15 hours from the time of the initial inoculation, a fully formed spore and crystal were present in each cell and, by 20 hours, as a result of the relatively rapid lysis of the sporangia, only free spores and crystals were present in the medium.

Cytology

The Early Stages of Spore Formation:

The phase-white granules which became apparent with the onset of sporulation differed from those observed in *B. cereus* by being larger, not as uniform in size and by having no apparent structural connection to each other (Figs. 1, 2). The movements of these beads and of the chromatin material with which they were closely associated were, however, very similar to those already observed in *B. cereus* (37). These early changes are summarized in Text-fig. 1, *A* to *E*. In electron micrographs of thin sections, lipid-containing inclusions again appeared as vacuole-like areas. In Fig. 12 three such inclusions frame the low density strand of chromatin while in Fig. 13 the chromatin appears to be constricted between two of them.

The developing spore wall already seen in thin sections of *B. cereus* and *B. cereus* var. *mycoides* (37) is shown in Figs. 14 and 15 in two characteristic steps of formation. As before, the spore chromatin can be recognized as the material of low electron-scattering power between the end of the cell and the developing spore wall. Despite the marked chromaticity and apparent condensation of this chromatin no alteration in fine-structural detail was noted. Since the sample of cells of which Fig. 14 is representative was removed from the culture only 30 minutes after that shown in Fig. 13, the rapidity with which the spore chromatin



TEXT-FIG. 1. Diagrammatic representation of the sequence of morphological changes as a cell of *B. cereus* var. *alesti* forms a spore and parasporal protein crystal; summarized from phase contrast, brightfield, and electron microscopy. The approximate number of hours of aeration at 30° at which these types of cells would be observed is indicated. The dark intracellular structures represent the chromatin, the dotted bodies lipid-containing inclusions, and the barred bodies, the protein crystals. *A*, divisional figures of chromatin. *B*, two condensed chromatin bodies about lipid-containing inclusions; this is the beginning of sporulation. *C*, extension and fusion of the chromatin accompanied by a dispersion of the lipid-containing bodies. *D*, axial filament of chromatin. *E*, apparent fragmentation of the filament. *F*, sequestration of the spore chromatin and growth of the spore septum. *G*, completed spore septum enclosing the spore chromatin in an end-pocket. *H*, growth of the spore septum around the cell end with cleavage from the cell wall. The primordial crystal appears in the angle made by the new spore wall and cell wall. *I*, continued growth of the spore septum encloses the fore-spore; the spore chromatin is expanding to an open ring; the crystal, increased in size, has moved to the centre of the cell and is now bipyramidal in shape. *J*, spore chromatin is a figure 8; crystal is moving to the opposite pole of the cell. *K*, the body of the prerefractile spore (phase-white with a dark outer rim) stains for chromatin. *L*, refractile spore and crystal within the sporangium; the spore chromatin has been extruded to the periphery by acid hydrolysis. *M*, free crystal and refractile spore with surrounding exosporium.

is segregated and completely enclosed by the wall becomes apparent. A diagrammatic summary of these stages is shown in Text-fig. 1, *F* to *J*.

Genesis of the Parasporal Crystal:

Of the several small phase-white or refractile granules present in the cell after the appearance of the fore-spore, one is unique. In the living cell it can be first distinguished from the lipid-containing beads only by its position in the sporangium, for it is usually first seen closely apposed to the outer rim of the fore-spore and often in the angle between the cell wall and the recently formed spore wall (Fig. 3; Text-fig. 1, *H*). To differentiate clearly this primordial crystal from the other inclusions in the cell and to follow its subsequent development, sequential examinations were made of fields of fixed cells throughout spore formation. First, they were examined in dark phase contrast; secondly, after hydrolysis and Azure A staining to locate the nuclear material; thirdly, after counterstaining with basic fuchsin to locate the crystal; and fourthly, in some cases, after staining with Sudan black B to locate the lipid-containing inclusions.

Thus in Fig. 8 the primordial crystal can be located as a small granule in phase contrast (Fig. 8 *a*) which subsequently does not stain for chromatin (Fig. 8 *b*), but does take up basic fuchsin on counterstaining (Fig. 8 *c*). No such inclusion could be detected in the cells fixed at earlier stages of sporulation (Figs. 7*a*, *b*, and *c*). Within ½ to ¾ of an hour following its initial appearance this granule had increased in size and could be recognized as having a definite bipyramidal shape.

Later Stages of Spore and Crystal Development:

The results of sequential staining of a field of sporulating cells when the crystals were half-grown are shown in Fig. 10 and again when three-

fourths grown after 13½ hours of aeration in Fig. 9.

In electron micrographs of thin sections the crystal inclusion can be recognized as an area near the fore-spore containing smooth textured material which is slightly less dense than the surrounding cytoplasm (Fig. 16, right arrow; Fig. 17). Within the fore-spore the chromatin lies in the homogeneous cytoplasm as an open ring (Fig. 16) and outside the surrounding wall other layers are beginning to form (Fig. 17). The lipid-containing inclusions are accumulating at the opposite pole of the cell.

In the final stage of development the fore-spore increased in density (Fig. 9 *a*) but the spore chromatin remained for some time as an open ring (Fig. 9 *b*) or as a figure 8 (Text-fig. 1, *I* to *J*). During this interval the crystal continued to increase in size and move to the opposite end of the cell where it crowded the non-spore chromatin from its position (Figs. 4, 5, 9 *a*, 9 *c*; Text-fig. 1, *J*).

As in *B. cereus*, the completion of sporulation was rapid once begun. The majority of the spores in Fig. 6 were at an early stage of becoming refractile, having a phase-white centre surrounded by a dark outer rim. The ultraviolet absorption spectra of extracts of disrupted cells at this stage of sporulation failed to show the presence of the characteristic spore compound, dipicolinic acid (39). Now, the spore chromatin was no longer in an open ring, but rather appeared to occupy the entire body of the spore (Fig. 11 *b*; Text-fig. 1, *K*). During these later stages of sporogenesis, the crystals had almost attained their full size, appeared slightly more refractile and stained more lightly with basic fuchsin (Fig. 11 *c*). They often appeared enveloped in strands and fragments of the vegetative chromatin (Fig. 11 *b*), a deposition probably brought about during the acid hydrolysis. The lipid-containing beads can no longer be detected (Fig. 11 *a*).

The development of complete refractility was the same here as described in *B. cereus* (Text-fig. 1, *L*, *M*). With phase contrast illumination liberated crystals appeared slightly more refractile than those which were fully formed but still confined within the cell.

Abnormal Forms:

Several of the photomicrographs were selected to demonstrate a type of cell which was representative of less than 5 per cent of the population. Both a spore and a crystal developed in these double-length cells (Figs. 5 and 6) but in such cells the crystals usually achieved a remarkable size. Electron micrographs of sections through these cells indicated that abortive cross-wall formation was associated with these longer forms.

Chemistry

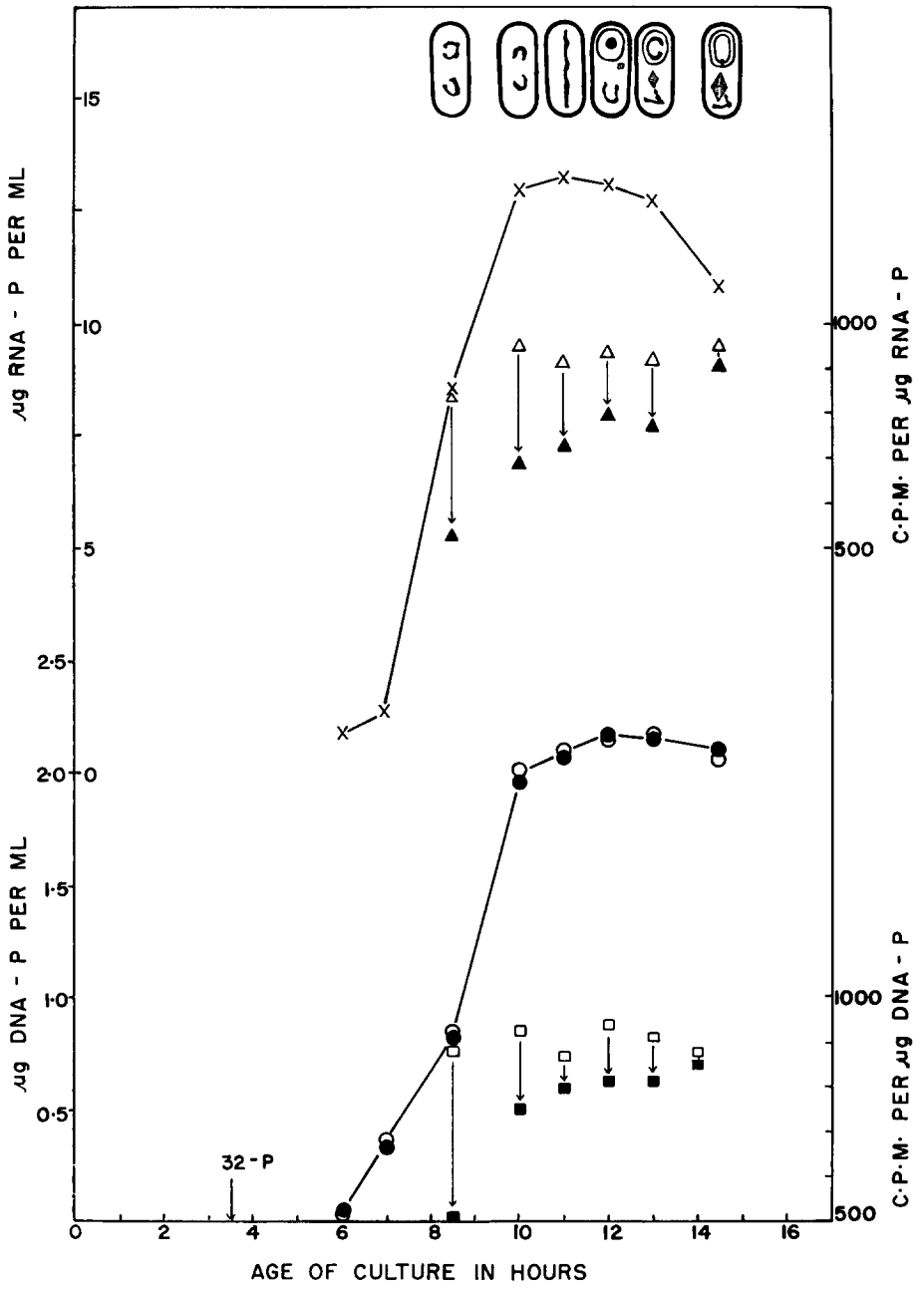
The Nucleic Acid Fractions During Growth and Sporulation:

A vegetative cell of *B. cereus* var. *alesti* contains an average of 3.95×10^{-9} μg. DNA-phosphorus (DNA-P) while the spore contains 2.08×10^{-9} μg. As the spore contains one chromatin body and the actively growing vegetative cell two chromatin bodies (in some stage of division), this increased amount of DNA in relation to that in *B. cereus* represents an increased amount of nucleic acid per chromatin body rather than an increased number of chromatin bodies per cell (9).

A representative experiment showing the changes in nucleic acid content of the cell fraction of a culture during growth and sporulation is graphed in Text-fig. 2. The rise in DNA-P during growth parallels the increase in the number of cells. At 10 hours the DNA-P content corresponded to a density of 0.51×10^9 cells per ml. of culture, a value equal to the number of spores eventually formed. Thus, the absence of lysis of cells prior to the ripening of the spores and of lysis or regrowth of the spores once formed indicate that the cell population remained constant throughout spore formation. Since at 10 hours the two chromatin bodies were either condensed or extending into an axial filament it is obvious that the events leading to spore formation were now underway.

In marked contrast to the pattern of synthesis of DNA during sporulation in *B. cereus* (37) no net synthesis of this compound occurred during spore and crystal development in var. *alesti*; rather the level of DNA-P throughout sporogenesis was invariably constant falling only upon lysis of the sporangia.

The changing distribution of DNA during these early stages of spore formation was followed as in *B. cereus* with ^{32}P. Five μc. of ^{32}P were added per ml. of culture to each of six flasks at 3½ hours of aeration. A transfer of these cells to non-labelled medium was effected at the times indicated. Although it is apparent that the DNA-P of the cells had reached a constant specific activity at each of the transfer times (Text-fig. 2), the specific activity of the DNA-P in the spores formed fol-



TEXT-FIG. 2. Changes in the DNA-P (●—●, by Dische reaction; ○—○, by total-P) and in the RNA-P (×—×) content of a culture of *B. cereus* var. *alesti* during growth and sporulation. The line drawings, derived from photomicrographs of the cells, indicate that sporulation began in this experiment at 10 hours. Radioactive phosphorus was added (↓) to lots of this same culture and the cells were transferred to non-labelled medium at 8½, 10, 11, 12, 13, and 14 hours. The specific activities of the DNA-P (□) and RNA-P (△) of the cells at the time of transfer were compared with the specific activities of the DNA-P (■) and RNA-P (▲) of the spores resulting from the transfer.

lowing the first transfer at 8½ hours was 40 per cent of that in the cells at the time of transfer. Such a marked fall can be accounted for by the synthesis of DNA associated with the continued vegetative growth in the non-labelled medium between 8½ and 10 hours. In transfers made following 10 hours the average drop was only 11 per cent. As the average difference between duplicate samples was 7 per cent, this is not significant and is probably due to contamination of the vegetative cell DNA-P by a small part of a more active phosphorus.

The absence of a significant difference between the specific activity of the DNA-P of the sporulating cell and that of the spore formed following the transfer, and the absence of net synthesis of DNA during sporogenesis lead to the conclusion that the spore chromatin here, as in *B. cereus* (37), is derived from the chromatin of the vegetative cell.

The striking absence of net synthesis of RNA previously observed in *B. cereus* (37) was encountered here during spore and crystal formation (Text-fig. 2). However, the sharp decrease in RNA detected in *B. cereus* prior to the onset of sporulation was not apparent in var. *alesti*. The considerable fall in the specific activity of the RNA-P, *i.e.*, RNA-P of the spores as compared to that of the vegetative cells at the time of transfer to unlabelled medium, suggested some turnover of this fraction occurred (Text-fig. 2). Indeed, electrophoretically separated ribonucleotides from cells exposed to ^{32}P for hourly periods during sporulation were actively labelled in spite of the absence of net RNA synthesis.

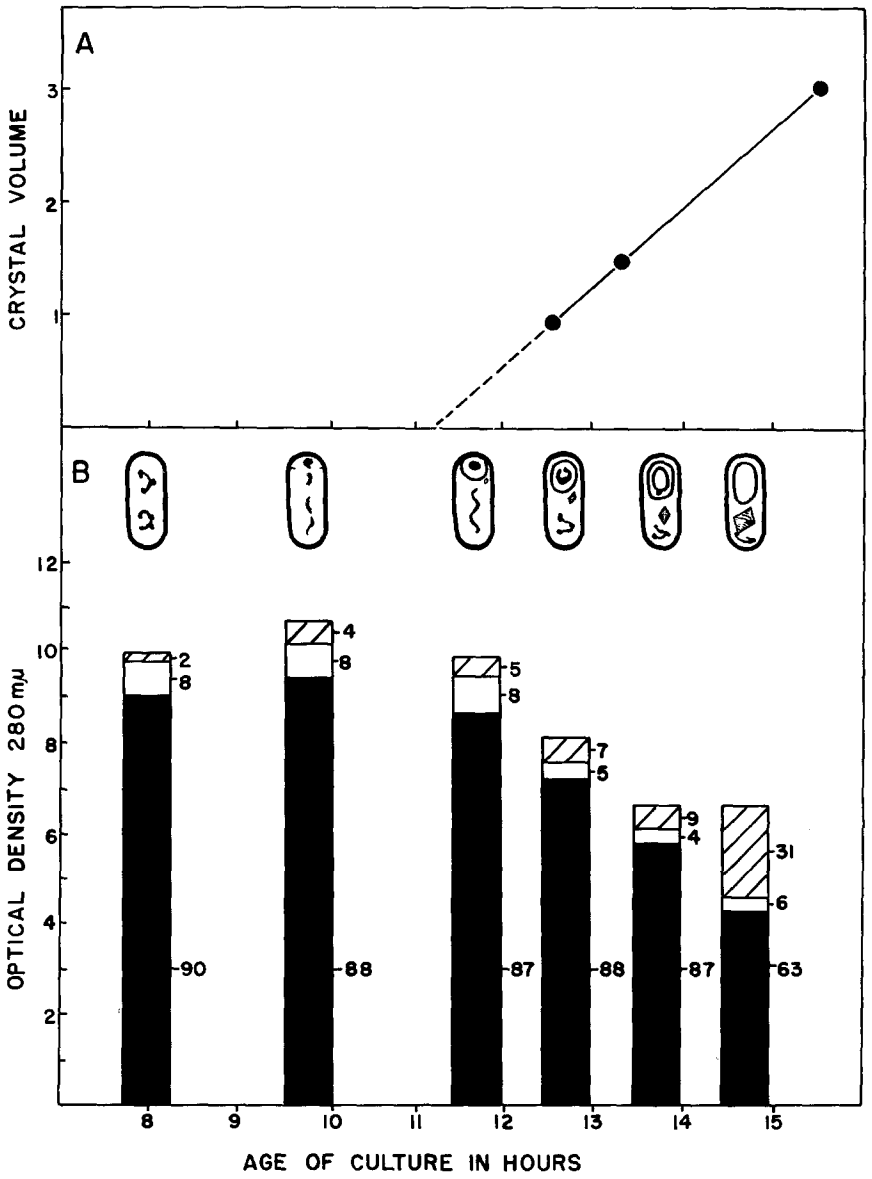
Extraction of Proteins from Cells During Spore and Crystal Formation:

At room temperature the mature crystals of *B. cereus* var. *alesti* become swollen by treatment with alkali between pH 10.5 and 11.8 but release no nitrogen into solution. They lose their structural outlines and go into solution only between pH 11.8 and 12.2 (8). However, subsequent studies have indicated that mature crystals in suspension can be completely dissolved at pH 11.5 by the addition of thioglycolic acid (to 1 per cent). On the other hand, vegetative cells of var. *alesti* which have been previously extracted to pH 11.5 retain a negligible amount of protein extractable by alkaline thioglycollate at the same pH. Thus, with such sequential extractions it was hoped to follow, during sporulation, the development of a protein with the solubility properties of the mature crystal.

In these experiments, vegetative and sporulating cells were disrupted at 4° by vibration with Ballotini glass beads (No. 10 size) (26) and were then extracted at room temperature with alkali by the addition of NaOH to pH 10.5. The residue which remained was then extracted at pH 11.5 and finally with 1 per cent sodium thioglycollate (pH 11.5). The residue was then extracted by incubation at 37° in N NaOH. The protein in the various extracts was precipitated in the cold by the addition of cold acetate buffer 5 M, pH 4.8). The relative amounts of protein in solution were estimated from the absorption at 280 mμ (corrected for non-specific light scattering) or, from a determination of the total nitrogen by a micro-Kjeldahl procedure.

In Text-fig. 3 *B*, it can be seen that 90 per cent of the total protein of cells not yet in the earliest stage of spore formation (8 hours) is soluble at pH 10.5 while 8 per cent is soluble at pH 11.5. From the residue an amount of protein equivalent to only 2 per cent of the total extractable protein is put into solution by alkaline thioglycollate. With the onset of sporulation, however, the amount in this latter fraction increases to 4 per cent. These ratios remained constant throughout the early stages of sporulation. With the appearance of the crystal, however, the amount soluble in alkaline-thioglycollate began to increase while that soluble in alkali fell. During the major growth of the crystal (from 13½ to 15 hours) the total extractable protein remained constant while the fraction soluble in alkaline thioglycollate increased markedly (Text-fig. 3 *B*).

The slight increase in protein soluble in thioglycollate with the onset of sporulation might suggest that protein with similar solubility characteristics to the mature crystal was present in the sporulating cell before the crystal became evident as a structure. However, the linear increase in the volume of the inclusion is not paralleled by a similar rate of increase in the amount of protein soluble in the thioglycollate (Text-fig. 3). Thus while the average volume of the crystals doubled between 12.7 and 13.7 hours (Text-fig. 3 *A*) there was only a 2 per cent increase in the amount of protein extracted by thioglycollate. However, between 13.7 and 14.7 hours when the crystal showed a further ⅔ increase in volume, the amount of



TEXT-FIG. 3. *A*, increase in crystal volume as calculated from measurements taken from projected photomicrographs ($V = \frac{2}{3}\ a^2b$, in arbitrary units). *B*, change in the amount of various alkali soluble proteins from *B. cereus* var. *alesti* during sporulation. Each amount is also expressed as a per cent of the total protein extracted. Sequential extraction at pH 10.5 (■); pH 11.5 (□); pH 11.5 + 1 per cent thioglycollic acid (▨). Cells from 10 ml. of culture were extracted at each time period. Concentration of protein was estimated by the optical density at 280 mμ. Sporulation began at 9 hours.

thioglycollate-extractable protein increased by some 230 per cent.

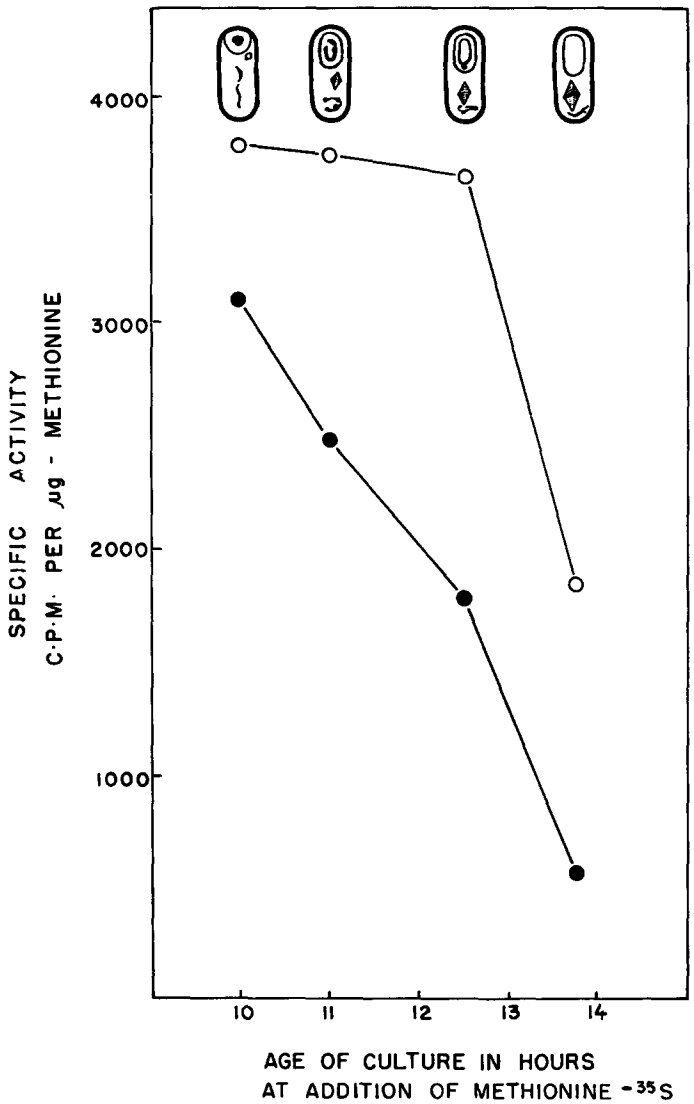
From these results it is inferred that, during development, the crystal protein remains in a form which can be extracted at a lower pH value or without the aid of a disulphide-bond reducing agent and that a "maturation" process confers on the crystal the solubility properties associated with the mature form. That such a process may occur is supported by two further observations: firstly, there is an increase in the refractility of the crystal as seen in both phase contrast and air-mounted nigrosin smears during late stages of sporulation, and secondly, there is a decrease at

these times in the affinity of the protein for basic fuchsin (after HCl hydrolysis). The finding that the toxic fraction for larvae can be extracted from sporulating cells at a pH value (pH 10.8) at which the toxicity of the mature crystal is largely insoluble is also consistent (8).

Studies of the Incorporation of Radioactive Methionine:

Although the mode of formation of the crystal protein could not be decided from these cytological observations, the extraction and toxicity studies suggested that a new protein is formed during crystal development. If so, the protein could be derived by synthesis either from components of the vegetative cell, nutrients in the medium, or both sources. The possibility that the crystal formed by "crystallization" of a pre-existent vegetative protein due to a change in the environment had not been entirely eliminated.

To answer this question the experimental procedures of transfer and addition used in the studies of incorporation of ^{32}P were extended to a study of the incorporation of methionine-^{35}S into protein



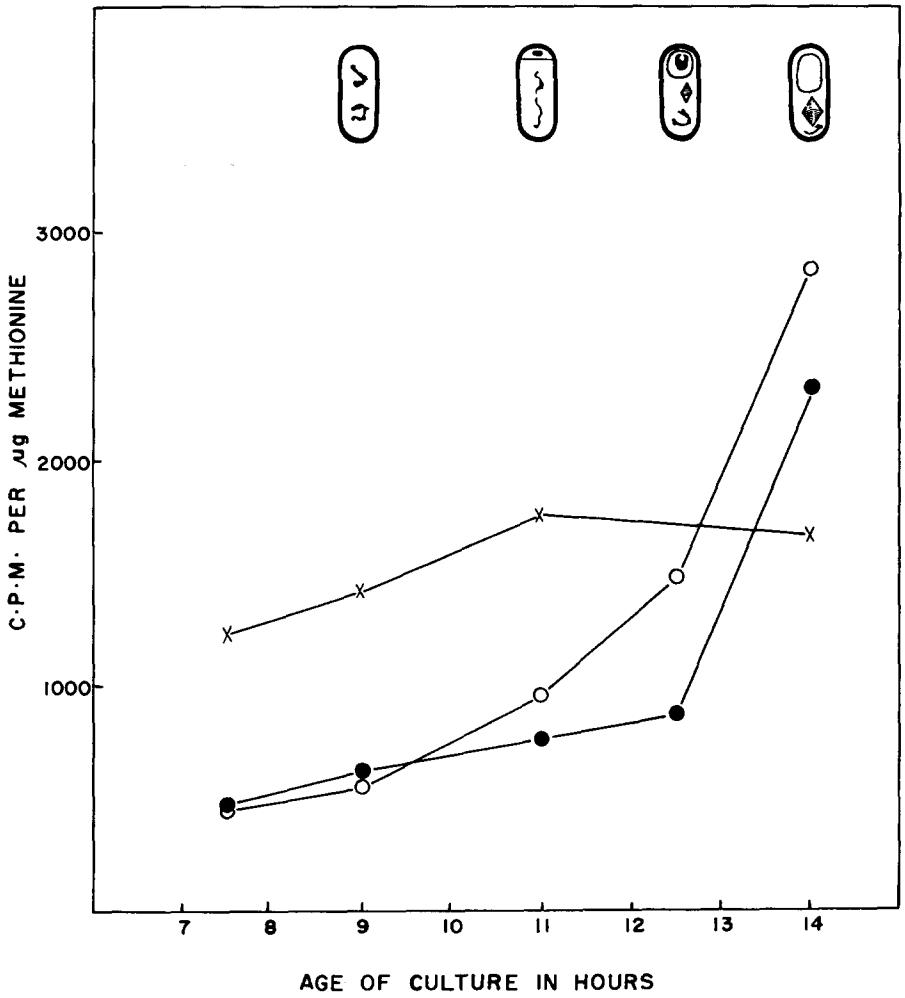
TEST-FIG. 4. Specific activity of methionine in ripe spores (●—●) and crystals (○—○) of *B. cereus* var. *alesti* formed in lots of the same culture to which methionine-^{35}S was added (0.62 µc. per ml. of culture) at 10, 11, 12½, and 13¾ hours. The attendant stage of sporulation is indicated by the line drawings.

of the vegetative cell, spore, and crystal. (Previous analyses of the constituent amino acids of the crystal had indicated that methionine was present.)

In Text-fig. 4 the specific activity of the methionine in the total spore proteins and in the crystal protein is plotted against the time of addition of the labelled amino acid to the culture. The nearly constant specific activity of the methionine in the crystal protein formed following additions at 10, 11, and 12½ hours suggest two possibilities: either a constant specific activity is reached regardless of the time of addition due to a continued high rate of incorporation, or a progressive increase in the rate of incorporation occurs between 10 and 12½ hours. The sharp fall in specific activity upon addition of the labelled methionine at 13¾ hours indicates a greatly decreased incorporation following this time.

The specific activity of the methionine in the spore proteins showed a near linear rate of decline with the successively later additions of the label to the culture. At all times the specific activity of the methionine in the spores was less than that measured in the crystal protein.

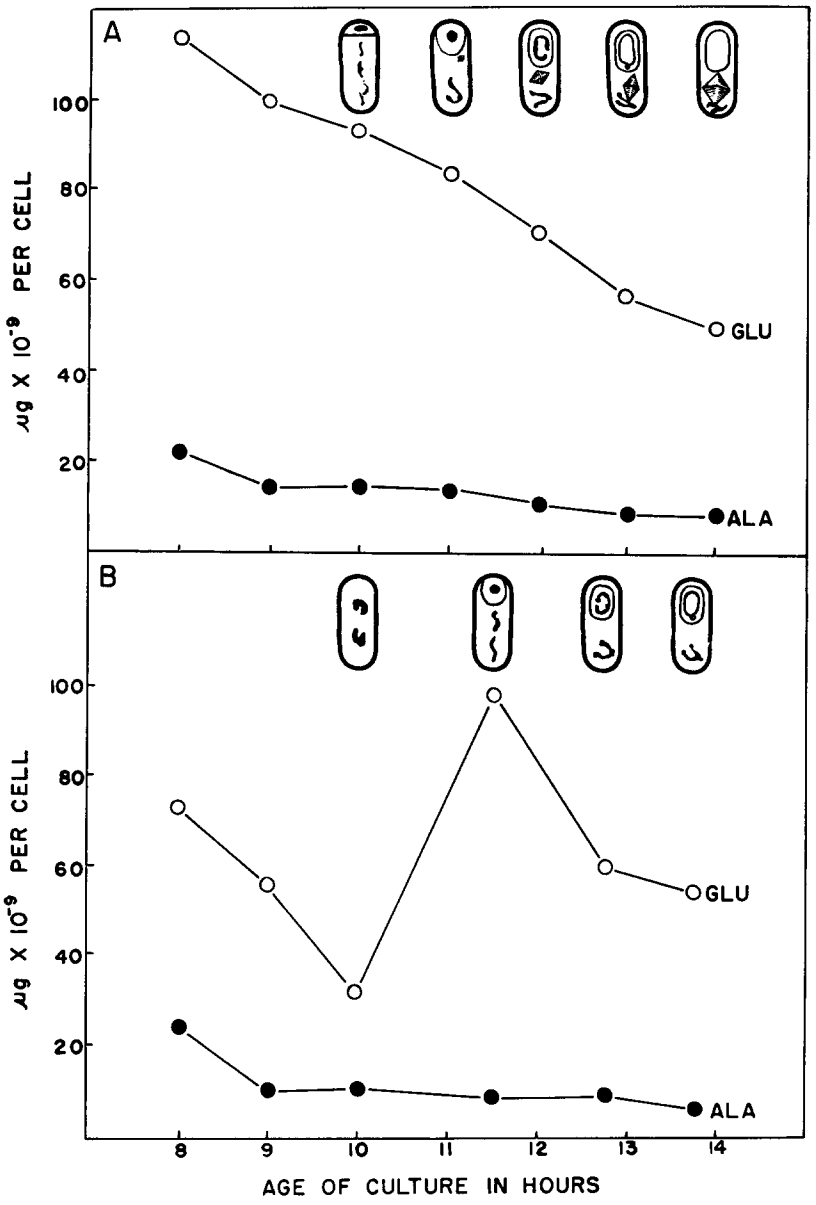
The results of a typical transfer experiment are presented in Text-fig. 5. The labelled methionine was added (5 μc. per ml.) to the culture during



TEXT-FIG. 5. Specific activity of methionine in ripe spores (●—●) and crystals (○—○) of *B. cereus* var. *alesti* formed following transfer of cells from labelled to non-labelled medium at 7½, 9, 11, 12½, and 14 hours; (×—×) specific activity of methionine in pH 10.5 soluble proteins of cells at the time of transfer. The stages of sporulation at the times of transfer are indicated by the line drawings.

early logarithmic growth; the transfer of the labelled cells to the non-labelled medium was effected at the times indicated. The pH 10.5 soluble proteins were extracted from a duplicate lot of cells at the times of transfer and these as well as the spore and crystal proteins were precipitated, washed, and hydrolyzed and their amino acids separated by chromatography.

Following even the earliest transfer (at least 2 hours prior to the beginning of spore formation) there was still some incorporation of labelled methionine into both the spores and crystals. This indicated that methionine incorporated into vegetative cell components must contribute to the formation of these bodies. (It will be shown that methionine is not found in the free amino acid pool



TEXT-FIG. 6. Quantitative changes in the free amino acid pool from cells of *B. cereus* var. *alesti* (*A*) and *B. cereus* (*B*) during late growth and sporulation; (○—○, glutamic acid; ●—●, alanine). The line drawings indicate the stage of sporulation at the time of sampling.

during late growth and sporulation.) However, following all but the final exchange, the lower specific activity of the methionine in the spores and crystals as compared to that in the pH 10.5 soluble proteins from the progenitor cells suggests that these two bodies are not formed from vegetative cell constituents alone. This confirms the results of the addition experiments.

From preliminary electrophoretic and ultracentrifugal analyses, the mature crystal in solution appears to be composed of a single species of protein molecule. Hence, the further evidence of two sources of amino acid for crystal development is a strong indication that the protein is synthesized during crystal growth. Similarly, amino acids from both the medium and vegetative cell contribute to the formation of spore proteins. However, it is impossible at this time to determine whether all of the spore proteins are synthesized anew, or if some are incorporated directly into the spore from the vegetative cell. Most probably some spore proteins are synthesized *de novo* for it is known that bacterial spores contain antigens not detectable in the vegetative cell (7, 20).

The Free Amino Acid Pool of Sporulating Cells:

The considerable synthesis which occurs during sporogenesis in var. *alesti* was apparently associated with degradation of some existing cell proteins. During this period the free amino acid pools of var. *alesti* and for comparison *B. cereus*, were extracted and analyzed quantitatively for component amino acids. Glutamic acid and alanine were the only two which appeared in significant amounts in the free pool of either organism (Text-fig. 6). In var. *alesti* the glutamic acid concentration per cell fell from the end of vegetative growth to the completion of crystal formation, whereas, in *B. cereus* a sharp increase in its concentration at the beginning of sporulation preceded a decline throughout the remainder of the process. No marked change occurred in the pool concentration of alanine in either organism.

DISCUSSION

From the results presented in this and the preceding communication, the bacterial spore may now be considered the product of a peculiar division of a genetically competent cell. Differing from all previous divisions in the growth cycle (40), this one occurs when there is no continuing synthesis of DNA and at a time when each cell contains just twice the spore level of this compound. Upon confinement of half of this chromatin at one pole, the cell becomes irrevocably committed to either the formation of a spore, or alternatively to death. In other words, the cell cannot now revert to vegetative growth without first forming a spore. Thus, the period extending from the end of vegetative division until commitment (usually some 30 to 40 minutes) marks the transition from a vegetative to a sporulative type of metabolism.

Events characteristic of this precommitment period are the sequestration of the select half of chromatin to the cell end, the absence of net nucleic acid synthesis, a decreased oxygen consumption, and an altered response to incorporated purine analogues (38). The events of the post-commitment period are associated with pre-spore development and the laying down of those peripheral coats and layers characteristic of the mature spores. From three lines of evidence these latter processes appear to be synthetic in nature: first, an amino acid free in the sporulation medium can be found in a bound form within the resulting spores; second, phosphorus is incorporated from the medium into a bound organic form in the spore coat fraction (37), and third, the development of the cortex about the spore body can be directly related to the initial appearance and accumulation of the spore compound, dipicolinic acid (39).

Sporulation as an "Endotrophic" Process:

The above description of spore formation is at variance with the recently proposed and somewhat widely accepted concept that sporulation is a strictly endogenous process. This concept of "endotrophic" sporulation maintains that the exogenous nutrition of the cell is completed before sporogenesis begins; thus, the reserve of intracellular energy supports the conversion of cell substances into the specific spore components. As a corollary to this concept, it is stated that washed vegetative cells, regardless of age, will begin and complete normal sporulation when aerated in distilled water (11, 18).

Although it is true that spores are found following 12 to 14 hours of aeration of vegetative cells in distilled water, it is still a point in controversy whether there is a direct conversion of each original cell to a spore (28) or whether there is some lysis and regrowth with eventual sporulation of

only a portion of the cells (29). In this connection the incorporation of nutrients from the medium into the spore, the increase in cell dry weight during sporogenesis (37), and the depletion of carbon and nitrogen containing compounds from the sporulation medium (30) are all direct evidences that during normal sporulation exogenous nutrition is utilized if not required for the formation of refractile spores. Furthermore, normal spores have a considerably enhanced content over their progenitor cells of at least two components, calcium (30) and cyst(e)ine (34), neither of which could be derived in such magnitude entirely from intracellular constituents.

Thus, the results presented in this series of papers have shown that sporogenesis involves the confinement of vegetative material in the forespore as well as the *de novo* synthesis of spore compounds. Materials for this synthesis are derived from the medium as well as from intracellular sources.

It is difficult to reconcile further our results on sporogenesis with those derived from "endotrophic" systems (11, 12, 18) particularly since no cytological evidence of the process in such systems has been provided. Nevertheless, it seems reasonable that during the first 8 hours of aeration following transfer to distilled water, the cells maintain essentially a vegetative metabolism not associated with pre-spore synthesis. In the first instance, it is curious that 16 hour old cells, purported to contain all the intracellular reserves needed for sporulation, require a further 8 to 10 hours aeration in water before commitment to the process occurs, since, if left in the growth medium, these same cells would have sporulated in 4 hours (18). In our studies, the interval between the cessation of growth and the commitment to sporulation is less than one hour.

More direct evidence for believing that the "endotrophic" precommitment period is vegetative in nature was derived from studies of purine analogues (38). 8-azaguanine and 2:6-diaminopurine both prevent spore formation if added to cells at any time during vegetative growth. Additions of these during and following commitment have no inhibitory effect. On the other hand, Hardwick and Foster (18) and Foster and Perry (12) observed that 2:6-diaminopurine did inhibit "endotrophic" sporulation. However, the analogue was added at the time of transfer of the cells to distilled water. Since sporulation occurred some 12 hours later, it seems reasonable that the analogue exerted its effect on a vegetative metabolism rather than specifically on the spore-forming process. This metabolism could be associated with lysis and regrowth (29) or with intracellular turnover of ribonucleoprotein (*cf.* reference 6).

Sporogenesis and Adaptive Enzyme Synthesis:

As a consequence of these studies of "endotrophic" sporulation, Hardwick and Foster (18) envisaged sporulation as a process akin to adaptive enzyme synthesis. Evidence for the synthetic nature of the process was based primarily on the observation that methionine-^{35}S added to distilled water suspensions of cells was incorporated into an insoluble cellular fraction. Of the two maxima in rate of incorporation found, the first between the 7th and 8th hours of aeration, was considered evidence for pre-spore synthesis. Aside from the obvious criticism that the system was no longer "endotrophic" the data do not indicate whether this incorporation was into the vegetative or pre-spore portion of the cell. Thus, in harmony with our earlier suggestions, the first maximum in rate of incorporation may be interpreted as due to vegetative synthesis and the second to spore synthesis. The release of insoluble sulphur to a soluble form can also be interpreted to support this view.

Furthermore, our results do not support the analogy drawn by Hardwick and Foster (18) that spore synthesis is adaptive in nature. Although most data indicate that adaptive enzyme syntheses are inhibited by the additions of purine analogues (4, 5, 32), we have been unable to prevent either spore or crystal development if such analogues are added following the cessation of growth and with the onset of sporulation (38). The observation by Hardwick and Foster (18) that such could be done has been discussed above. In addition, both spore and crystal protein syntheses normally proceed simultaneously without obvious competition for intracellular precursors (*cf.* reference 18). Even in cells transferred to distilled water both bodies may be formed. However, as in non-crystal-forming organisms, to avoid massive cell lysis such transfers must be effected following the cessation of growth. In spite of such a late transfer, sporogenesis is delayed, the crystals are smaller, and 50 per cent of the resulting spores autolyze within 5 days.

RNA Metabolism and Sporogenesis:

Since the continued synthesis of some RNA is reported to be associated with the synthesis of both induced enzymes (3, 27, 32) and general proteins (3), the absence of net RNA synthesis during sporulation was an unexpected finding. Yet, during this part of its life cycle, *B. cereus* var. *alesti* and other similar organisms synthesize not only spore material but also a considerable quantity of parasporal protein. Two pieces of evidence, however, indicated that the RNA of the sporulating cell, although declining in quantity was actively turning over: firstly, the apparent metabolism of RNA as judged from the incorporation of ^{32}P and, secondly, the continued incorporation of 8-azaguanine into this fraction of sporulating cells (38).

Previous studies had indicated, however, that turnover of RNA did not occur in bacteria (19, 23) except perhaps under abnormal conditions; for example, 2 to 3 per cent of the RNA appears to turn over in *E. coli* during phage infection (35). However, there was no evidence associating this metabolically active fraction with the specific synthesis of proteins.

Accordingly, in our studies of sporulation, two conclusions seemed possible: either the formation of specific proteins associated with the formation of the spore (and in var. *alesti* the protein crystal as well) did not require net co-synthesis of RNA, or, the synthesis of RNA associated with turnover functioned during the synthesis of these proteins. The recent definitive experiments of Barner and Cohen (2) on a pyrimidine-requiring mutant of *E. coli* gave the first evidence in support of the latter alternative. From their experiments it now seems apparent that the synthesis of both general protein as well as specific protein (directed by induction) can be effected during periods of no net synthesis of RNA; but such syntheses are accompanied by a marked turnover of a fraction of the RNA.

The Parasporal Crystal:

In this scheme of sporulation the formation of the parasporal crystal appears to be associated entirely with the postcommitment phase of sporulation. Prior to this time the crystal cannot be demonstrated cytologically, nor is there present any protein fraction possessing toxicity for insect larvae (8). Further, there is now some serological evidence (in *B. cereus* var. *thuringiensis*) which suggests that "the crystal antigen is absent in vegetative cells but arises during sporulation. The appearance of the antigen can be correlated with the formation and growth of the crystal."[1]

Although the crystal appears to result from the synthesis of protein from small molecular weight compounds, its final form, associated with characteristic solubility properties, is achieved at the end of its growth. The insolubility of the mature crystal in alkali at pH 11.5, but its solubility at the same pH upon addition of thioglycollic acid, suggests that reduction of S—S bonds (or bonds involving the oxidized form of sulphur) aids the splitting of secondary linkages by the alkali. Although similar solubility properties have been reported for the isolated mitotic apparatus from dividing sea urchin eggs (24), unlike the mitotic apparatus and like keratins (14) the crystal, although swollen, is not rendered soluble by concentrated urea.[2] Such solubility phenomena suggest that S—S bonds, as in keratins, form strong intermolecular linkages. In this connection, autoradiograms of the constituent amino acids of crystals formed in the presence of methionine-^{35}S possess a highly active spot in the cyst(e)ine region.

It is thus proposed that there is a process of "maturation" of the crystal which involves the formation of S—S cross-linkages much as may occur during gel formation (21) or in the formation of the organized fiber system of the mitotic apparatus (24, 25).

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[2] Unpublished data.

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Explanation of Plates

Plate 238

Figs. 1 to 6. Dark phase contrast photomicrographs of living cells from the same culture of *B. cereus* var. *alesti* at various stages of sporulation. × 3880.

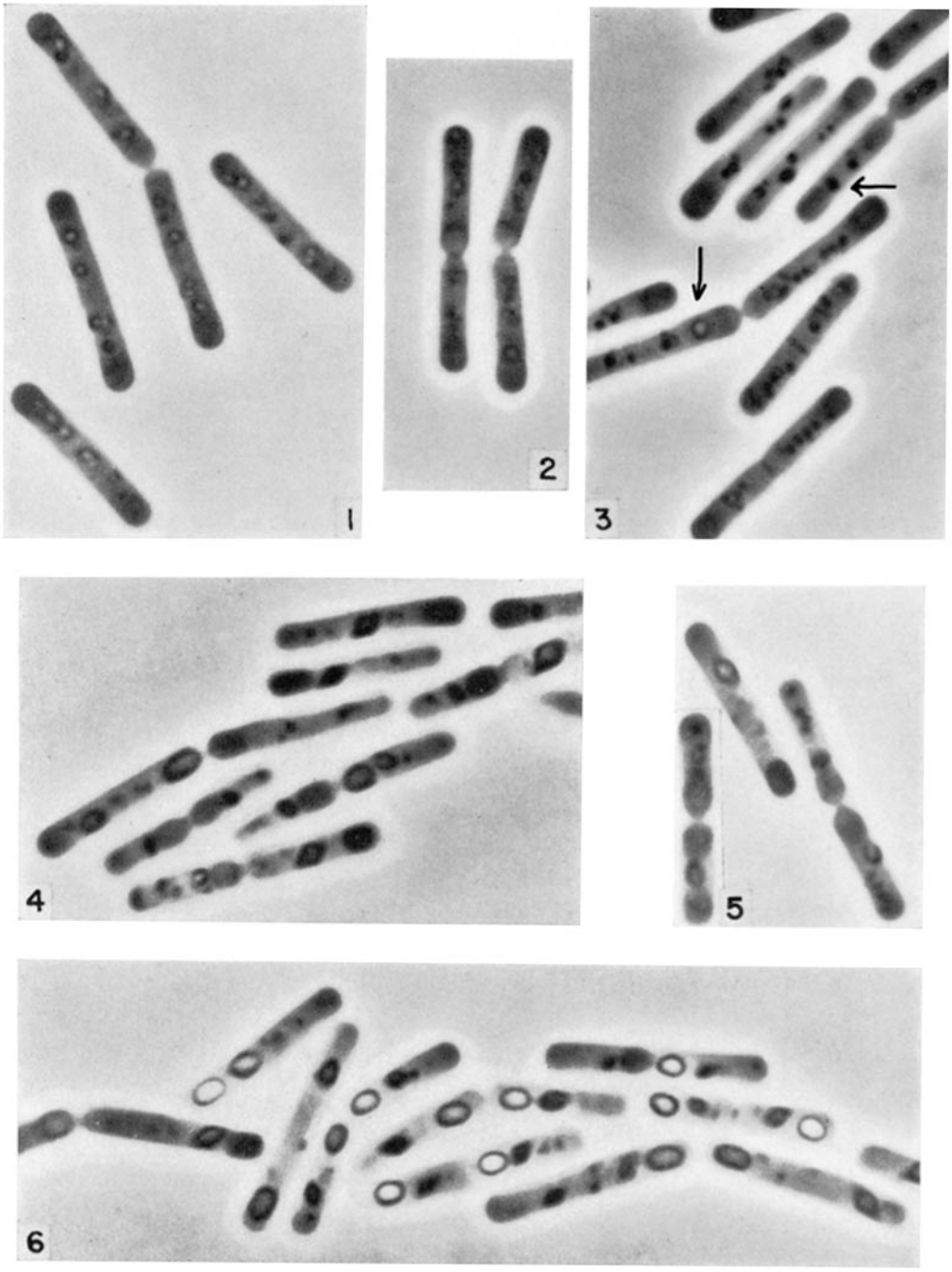
Fig. 1. Cells at 8¾ hours of aeration showing the lipid-containing inclusions which become prominent at the onset of sporulation.

Fig. 2. Development of the fore-spore (9½ hours).

Fig. 3. At 10¼ hours the fore-spore is enlarging and the crystal just discernible (arrows).

Figs. 4 and 5. By 12¾ hours the majority of the spores are becoming dense and the growing crystals show their bipyramidal form.

Fig. 6. At 13¼ hours the spores are phase-white with a dark outer rim. Crystal growth is as yet incomplete.



(Young and Fitz-James: Bacterial spore formation. II)

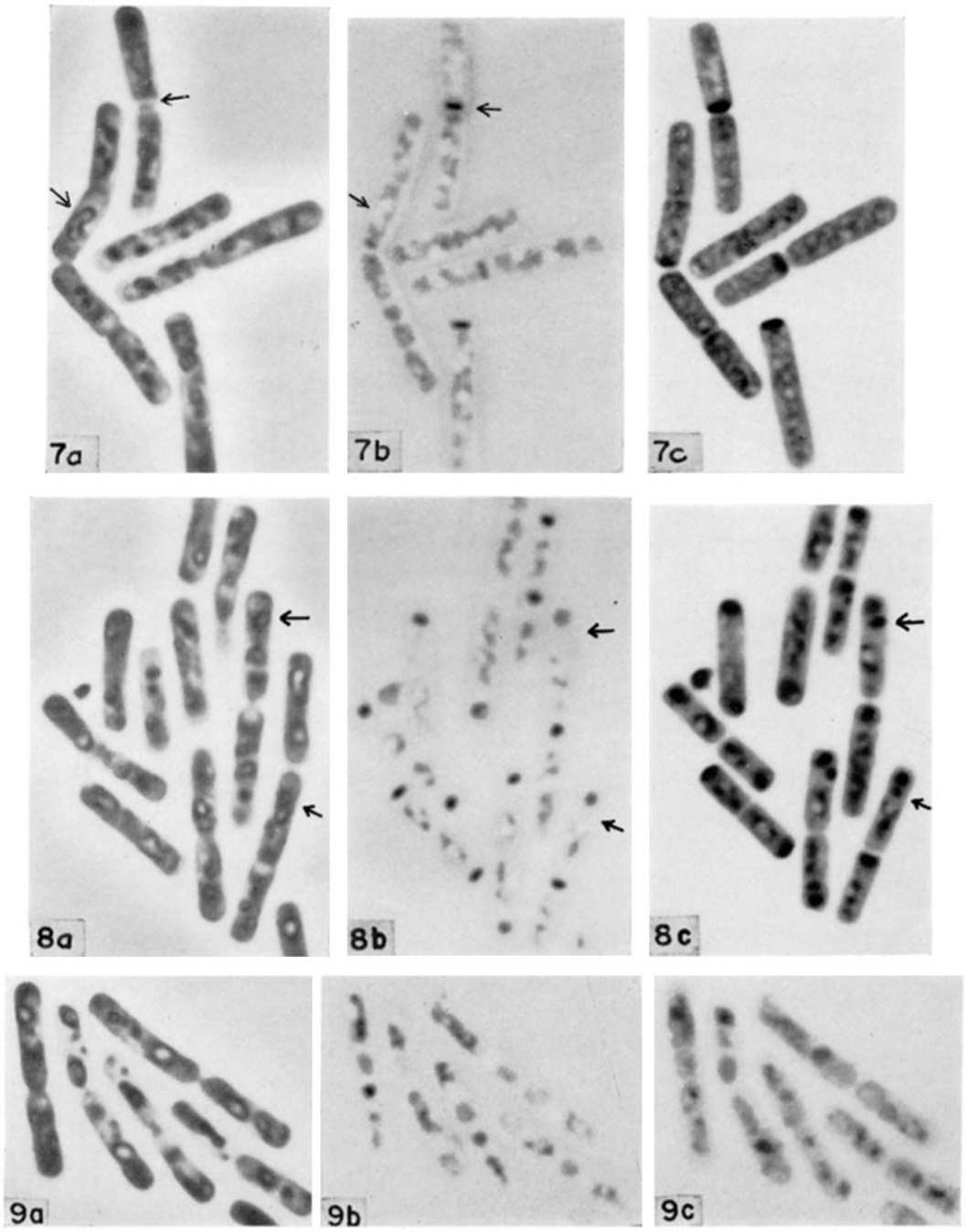
PLATE 239

FIGS. 7, 8, and 9. Sequential examination of fixed cells of *B. cereus* var. *alesti* during three stages of sporulation; *a*, dark phase contrast of fixed cells × 3880; *b*, chromatin stained with SO_2-azure A; *c*, SO_2-azure A–stained preparation counterstained with basic fuchsin to reveal the crystal. × 3600.

FIG. 7. At 10¼ hours the localization of the spore chromatin has occurred in most cells (upper arrow). The prominent lipid-containing inclusions can be seen in both phase contrast and brightfield illumination (lower arrows); no definite crystal is yet visible on counterstaining.

FIG. 8. By 11 hours the chromatin body of the developing spore is rounding out and a definite crystal is seen in each cell close to the spore (arrows).

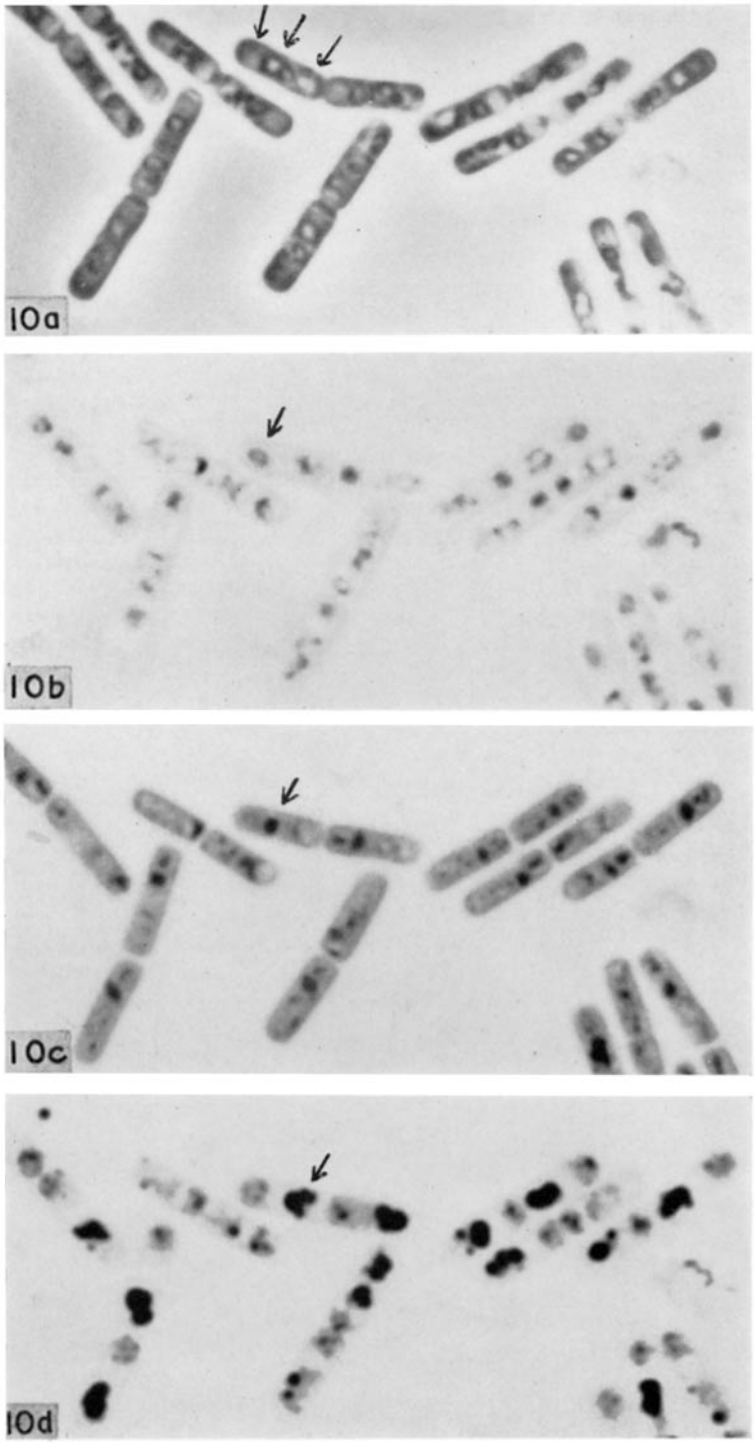
FIG. 9. The nearly fully formed crystal at 13¼ hours is in most instances between the spore and the remaining chromatin of the cell. The ring-shaped chromatin pattern of the developing spores is characteristic of this stage of development.



(Young and Fitz-James: Bacterial spore formation. II)

PLATE 240

FIG. 10. Sequential staining of a field of fixed cells of *B. cereus* var. *alesti* after 11¾ hours of aeration (crystal size 50 per cent of maximum). *a*, dark phase contrast following osmium fixation. Arrows mark from left to right the developing spore, protein crystal, non-spore chromatin partly encircling phase-refractile inclusions. × 3880. *b*, after hydrolysis and staining of the chromatin (azure A); the arrow marks the chromatin of the spore. *c*, counterstaining with basic fuchsin reveals the crystal (arrow). *d*, Sudan black B stains the lipid-containing phase-refractile inclusions (arrow). Figs. 10 *b* to *d*, × 3600.



(Young and Fitz-James: Bacterial spore formation. II)

PLATE 241

FIG. 11. Cells of *B. cereus* var. *alesti* just prior to the development of complete refractility (15 hours of aeration). *a*, dark phase contrast of osmium-fixed smear. × 3880. *b*, the same cells after hydrolysis and staining with azure A showing the distribution of chromatin peculiar to this state of spore development. *c*, counterstaining with basic fuchsin stains the ripening crystal less deeply. There is some deposition of the cell chromatin on the periphery of the crystal. Figs. 11 *b* and *c*, × 3600.

FIGS. 12 to 17. Electron micrographs of thin sections of *B. cereus* var. *alesti* at different stages of sporulation. Sections cut onto 0.5 per cent lanthanum nitrate. × 30,000.

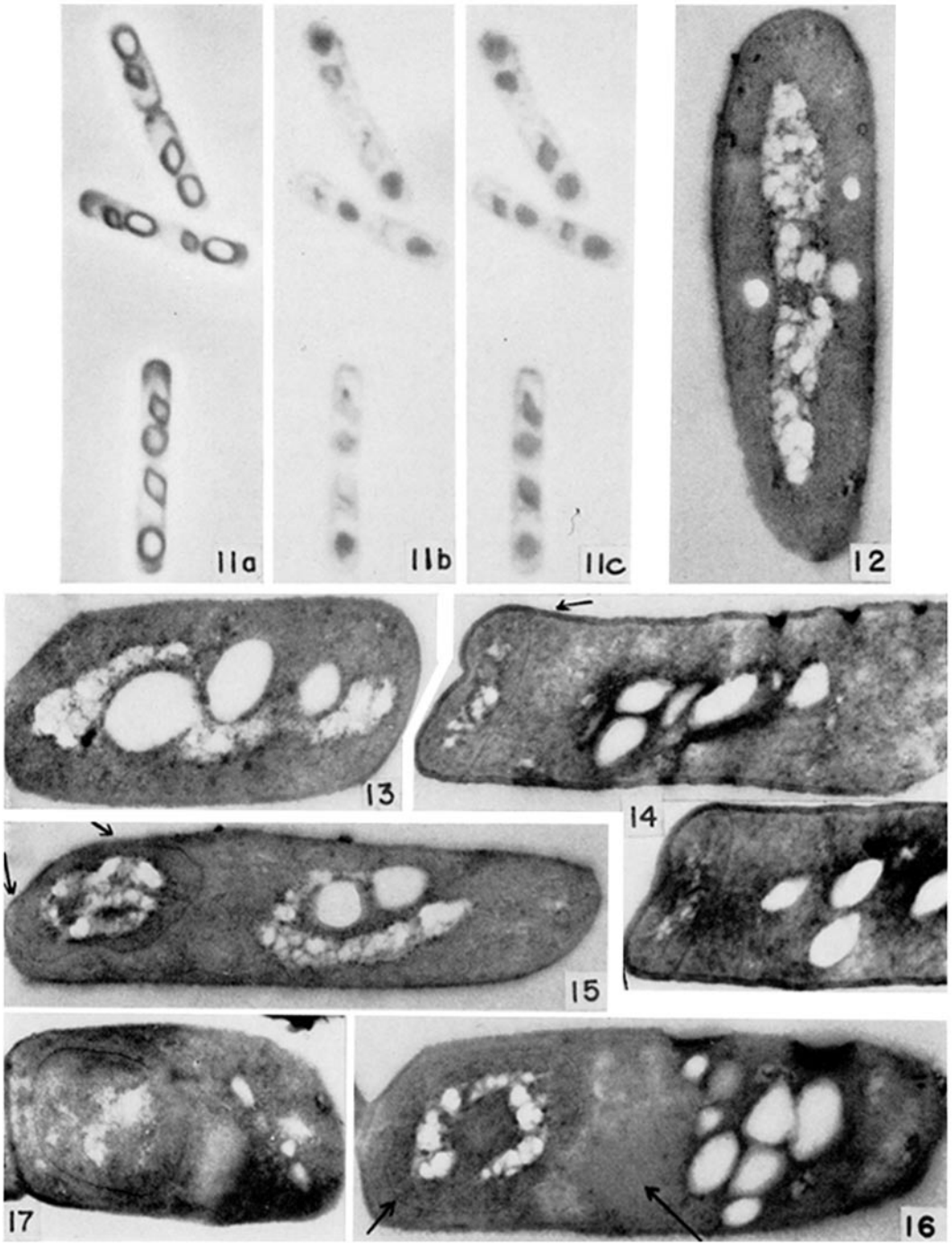
FIG. 12. The axial filament of chromatin appears as a relatively transparent gel which is interspersed with more electron-dense material some of which radiates from a central disc. Two of the lipid-containing bodies closely associated with the chromatin at early stages of sporulation are visible as empty holes in the section.

FIG. 13. Fifteen to 20 minutes later the chromatin is separating into two masses, one of which will become the nuclear body of the future spore.

FIG. 14. Two serial cuts of a cell showing the appearance of embeddings made 20 to 30 minutes after the stage shown in Fig. 13. Half of the chromatin has become walled off by a thin septum which is continuous with the cell wall (arrow).

FIG. 15. The development of the septum into the spore wall. The left one-third of the circumference is still continuous with the inside surface of the cell wall (arrows).

FIGS. 16 and 17. Two cells showing the further progress of sporulation found 1 hour after that represented in Fig. 15. The crystal appears as a low density area near the developing spore coats. In Fig. 16 the left arrow indicates the early spore wall on the outside of which other layers will or are forming, and the right arrow indicates the protein inclusion. Lipid-containing inclusions are crowding to the opposite pole. In Fig. 17 the outer spore envelopes are being laid down outside the well stained spore wall.



(Young and Fitz-James: Bacterial spore formation. II)