

Chemical and Morphological Studies of Bacterial Spore Formation

I. The Formation of Spores in *Bacillus cereus*^{*}, ‡

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PLATES 234 TO 237

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ABSTRACT

Experimental conditions were developed whereby a culture of *Bacillus cereus* formed spores with reasonable synchrony following a growth cycle of some 8 hours. The cytology of this metamorphosis was studied by dark phase contrast, bright-field microscopy and electron microscopy of thin sections. Particular attention has been paid to the changes in chromatin patterns and these have been correlated with quantitative chemical estimations of the nucleic acids.

The cell commencing sporulation contains two compact chromatin bodies and twice the spore amount of deoxyribonucleic acid. Following fusion of the two chromatin bodies, one-half of this chromatin becomes located at a cell end. A transverse septum growing inwards from, and remaining attached to, the inner surface of the cell wall separates this end-piece of chromatin and some associated cytoplasm from the rest of the cell to form the primordial spore. Although the synthesis of deoxyribonucleic acid ceases during the segregation process, it recommences in this organism and continues at a linear rate as the spore develops. Tracer studies with radioactive phosphorus indicated that this further synthesis is confined to the non-spore portion of the sporangium. Although the net synthesis of ribonucleic acid ceased prior to the onset of sporogenesis, some evidence of a turnover of this fraction during the sporulation process was found.

INTRODUCTION

The increasing interest in the bacterial spore as a subject for biochemical and genetical studies has emphasized the need for an understanding of the process of spore formation. The numerous descriptions of the cytology of the process remain largely inconclusive or contradictory, and what is known

concerning the chemical changes has not been related to precise stages of spore development. It was the absence of such a correlation which prompted the investigation reported in this series of communications.

The literature concerning sporogenesis can be assigned to two periods, of which the first begins with Koch's report on *Bacillus anthracis* (19) and culminates with the review of Lewis in 1941 (20). The second begins following this time when it became almost universally accepted that bacterial cells possessed nuclear equivalents (29). Thus, subsequent to 1941, descriptions of the process have been primarily devoted to an understanding of the role of these nuclear bodies in spore formation.

From these studies it is known that the spore develops about a small, brightly staining granule of chromatin, and although most bacterial cytologists agree that sporogenesis has commenced when this primordium can be recognized within the cell

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(2, 6, 7, 12, 18, 28) some have suggested that the process may begin even earlier with fusion of the several vegetative chromatin bodies into an axial filament (2, 12, 18). It is apparent, however, that only a portion of the cell's chromatinic material, whether present as a filament or not, becomes located within the spore; the fraction constituting this specialized portion being variously estimated as one-half to one-eighth of the total (2, 6, 7, 12, 18, 38). Such a range might be expected, as the number of chromatin bodies within the vegetative bacilli of even the same organism appears to be inconstant (7) and as no quantitative estimation has been made during sporogenesis of the chromatin or deoxyribonucleic acid content of these organisms.

On the other hand, two lines of evidence suggest that a more definite and predictable amount of chromatin is confined within the spore. Firstly, the average content of deoxyribonucleic acid in spores of several species belonging to the genus *Bacillus* has been found to be a characteristic and constant value for each type of organism (9, 11). Secondly, the amount of deoxyribonucleic acid in the average cell of an exponentially growing culture is twice that in the corresponding spore, although, depending on the stage of division, this amount varies between 1.5 and 3 times the "spore" amount in a synchronously dividing culture (36).

Since, in the first instance, the problem was concerned with the origin of the nuclear body of the spore, we determined quantitatively the changes in the nucleic acid fractions during sporogenesis and correlated these findings with the attendant cytological appearance of the cells. An interpretation of the sporulation process in *Bacillus cereus* has been derived from these results and is presented in this paper. A companion publication presents similar studies of sporulation as it occurs in *Bacillus cereus* var. *alesti* (37) wherein the process is complicated by the simultaneous formation of a parasporal protein inclusion.

Materials and Methods

Culturing Techniques:

A laboratory strain of *Bacillus cereus* showing smooth colony growth on agar was maintained in the sporulated state on the agar medium (supplemented with 0.5 per cent casamino acids) of Howie and Cruickshank (16).

The liquid medium used for the sporulation studies was a modification of the CCY medium of Gladstone and Fildes (13) in which the nutrient stock (glutamine,

100 mg.; acid casein hydrolysate, 5 gm.; enzymatic casein hydrolysate, 5 gm.; enzymatic yeast hydrolysate, 2 gm.; sodium glycerophosphate, 10 gm.; distilled water, 100 ml.) was diluted 1/20 in the salts solution described by Grelet (14). Twenty ml. of this medium in a litre Erlenmeyer flask were inoculated with a loopful of the sporulated culture and aerated in a constant temperature room (29°-31°) on an oscillating platform shaker (90 cycles per minute). When this starter culture was visibly turbid (about 2 to 3 hours from the time of inoculation), it was thoroughly mixed with the total volume of medium required, distributed into litre flasks and returned to the shaker. Adequate aeration was obtained when each litre flask contained 80 ml. or less of culture. Volumes smaller than 80 ml. had no obvious advantage and although adequate growth was obtained using larger volumes, lysed cells were often seen and sporulation was always asynchronous and incomplete.

Measurement of Bacterial Growth:

The increase in the number of cells, or growth, in the culture was determined by direct cell counts on a sample suitably diluted in 1 per cent formaldehyde in Grelet salts. Counts were made using a Petroff-Hausser counting chamber and the $\times 40$ objective of a dark phase contrast microscope.

The optical density at 650 $m\mu$ of a 1/10 dilution of the culture in 1 per cent formaldehyde in Grelet salts was used as another measure of cell proliferation since the ratio of the number of cells per ml. to the optical density of the growing culture was a constant (number of cells per ml. $\times 10^{-9}$: optical density at 650 $m\mu$ of 1/10 dilution = 2.4).

The increase in the dry weight of cells per ml. of culture was also determined. Duplicate samples were removed from the culture, centrifuged in the cold, and twice washed with distilled water before being transferred quantitatively to tared weighing bottles. They were dried overnight at 70°-90°, then stored to a constant weight in a vacuum desiccator over phosphorus pentoxide.

Within each experiment the appearance and number of the cells and the optical density of the culture were identical in all flasks throughout growth and sporulation.

Microscopy and Cytological Techniques:

For the preparation of fixed impression smears a sample of the culture was spread on the surface of a small block of agar (1½ per cent in Grelet salts) and placed in the vapours of 2 per cent osmium tetroxide for 2½ minutes. Two or three impression smears were made on grease-free coverslips. These were rinsed for 5 minutes in 70 per cent ethanol, removed, air-dried, and stored until studied (no longer than 48 hours).

The fixed cells, mounted in distilled water, were

photographed (Royal Pan film, Eastman Kodak) using either a Bausch and Lomb or a Zeiss dark phase contrast microscope which was illuminated by a tungsten ribbon filament lamp. At least two fields of cells on each smear were marked and photographed, although many were observed to ensure that these fields were representative.

The coverslip bearing the cells which had been photographed was removed from the slide. The chromatin within the cells was then stained by the HCl-Azure A method described by Huebschman (17). Brightfield photomicrographs of the marked fields were taken with standard equipment already described (9).

The coverslip bearing the cells which had been photographed was removed from the slide. The chromatin within the cells was then stained by the HCl-Azure A method described by Huebschman (17). Brightfield photomicrographs of the marked fields were taken with standard equipment already described (9). Cells for thin-sectioning were separated from the medium by centrifugation at 10,000 g for 1 to 2 minutes. They were suspended in one ml. Grelet salts and then fixed by the addition of an equal volume of 2 per cent osmium tetroxide in Grelet salts. After 1 hour of fixation at 37° the cells were sedimented, washed twice with Grelet salts, and then dehydrated with ethanol (70, 90, and 95 per cent and absolute). Standard procedures for embedding the cells in methacrylate (90 per cent butyl methacrylate + 10 per cent methyl methacrylate) were employed using a prepolymerized mixture for the final suspension of the cells. Polymerization was completed by 6 to 8 hours' exposure to ultraviolet light (Hanovia, Slough, England). Thin sections were cut to a gray or silver colour with a glass knife in the Porter-Blum microtome onto either 10 per cent acetone or 0.5 per cent lanthanum nitrate in distilled water. The sections were picked up on carbon-filmed grids and washed with one drop of distilled water. They were observed in a Philips 100 A electron microscope which was fitted with a 50 μ objective aperture and used at an accelerating voltage of 60 kv.

Chemical Fractionation Procedures:

Nucleic Acids.—Both the Schneider (31) and the Schmidt and Thannhauser (30) methods as modified by Fitz-James (9) were used for the fractionation of the phosphorus-containing compounds.

Results for both types of nucleic acid were calculated in terms of nucleic acid phosphorus; *i.e.*, deoxyribonucleic acid phosphorus (DNA-P) and ribonucleic acid phosphorus (RNA-P).

For each time period of analysis, 20 ml. of culture in duplicate were required for each of the two fractionation procedures. The samples, rapidly cooled by swirling in an ice-brine bath were transferred to chilled cups and centrifuged in the cold (2°–4°) at 10,000 g for 2 minutes. One wash of the pellet of cells in ice cold bicarbonate buffer (0.1 M pH 7.0) was followed by further washes in ice cold 50 per cent ethanol in buffer and finally in cold absolute ethanol.

The cells, suspended in absolute ethanol or methanol, were disrupted by 10 to 15 minutes of vibration in the Mickle tissue disintegrator (24) with Ballotini glass

beads (No. 10 size for vegetative cells; No. 14 size for sporulated cultures or free spores).

Incorporation of Radioactive Phosphorus (³²P) into the Nucleic Acids.—In all studies of the incorporation of ³²P into the nucleic acids the phosphate level of the Grelet salts in the medium was reduced to 25 $\times 10^{-3}$ M. This reduction did not alter the pattern of growth or of sporulation.

1. *Addition.*—Radioactive phosphorus (as H₃³²PO₄) was added to the cultures (5 μ c. per ml.) at various times during growth and sporulation. Spore formation and lysis of the sporangia went to completion before the spores were cleaned of detectable vegetative debris by several washes in Grelet salts, saline, and water. The spores were washed into absolute ethanol or methanol, disrupted, and then fractionated by the Schmidt and Thannhauser procedure (30) as modified (9). A sample of the trichloroacetic acid extract containing the DNA was digested with one ml. of 60 per cent perchloric acid and the total phosphorus determined following extraction into isobutanol:benzene by the method described by Ernster, Zetterstrom, and Lindberg (8). After the colour had been read, the radioactivity of the phosphorus in this sample was determined in a liquid counter and the specific activity of the original extract calculated and expressed as counts per minute per μ g. DNA-P.

2. *Transfer.*—The nucleic acids of the vegetative cells may be labelled with ³²P by an addition of the active phosphorus to the medium at an early stage of growth. If the cells are removed from the active medium at subsequent times during sporulation and allowed to complete sporogenesis in an unlabelled medium, certain information can be drawn concerning nucleic acid syntheses from a comparison of the specific activities of the nucleic acids in the cells at the time of transfer with those in the spores formed following the transfer.

Effective removal of the cells from the labelled phosphate could not be achieved simply by the addition of an excess of unlabelled phosphate or unlabelled medium for such additions would alter the cultural conditions and could provoke further vegetative growth rather than sporogenesis. Therefore, cells from a labelled culture were transferred to media which had supported the same degree of growth and sporulation. That is, at various times during late growth or sporulation half the culture from each of two flasks, identical except that one contained labelled (5 μ c. per ml.) and the other non-labelled culture, was centrifuged (10,000 g for 2 minutes) at room temperature. The pellet of labelled cells was suspended in Grelet salts; the suspension was recentrifuged and the pellet then suspended in the supernatant of the non-labelled cells. This suspension was transferred to a sterile litre flask and returned to the shaker. Fifteen minutes were required to effect the transfer.

The other half of labelled culture had, in the meantime, been chilled and centrifuged (2°–4°). The cells

were washed in the cold and disrupted prior to fractionation. The remaining 40 ml. of unlabelled culture were discarded. For each time period of analysis the specific activity of the DNA-P of the labelled cells at the time of transfer was compared with the specific activity of the DNA-P of the spores resulting from the transfer. The spores were prepared and fractionated as described above.

In these experiments RNA was estimated in the supernatant remaining following precipitation of the DNA (30). This value for RNA-P (derived from the value for total phosphorus less that for inorganic phosphorus) was comparable to that derived from the orcinol reaction on the trichloroacetic acid extract of the Schneider procedure (31). The specific activity of the RNA-P of the labelled cells at the time of exchange was compared with the specific activity of the RNA-P of the spores resulting from the exchange.

Estimation of Synchrony:

Before the sequence of events attendant on spore formation in a single bacterium can be reliably inferred from observations on a whole culture two experimental requirements must be met. Firstly, there must be no further growth in the culture; *i.e.* all and only those cells beginning spore formation should be present at the completion of the process and should contain a spore. Secondly, there must be a reasonable degree of synchrony of the process; *i.e.*, essentially all cells should begin spore formation in phase, remain in phase, and finally contain a refractile spore at the same time. Furthermore, in achieving this ideal situation, the cells should not be subjected to any treatment which could unwittingly alter the pattern of events from the normal.

Whether the first requirement is met by an experimental procedure can be determined on objective grounds. The determination of the second, as in all determinations of synchrony, cannot be so precise. The criteria used here were the similarity of chromatin patterns from cell to cell, as well as the spread in time of the initial appearance of the fore-spores and of the fully refractile spores in all cells. Since the latter event was a distinctive end process requiring only a portion of the total time, it served as the best indicator of synchrony. It is thus estimated that all cells were within 15 to 30 minutes of each other in a process requiring some 5 hours. It was not necessary to induce sporulation by an artificial treatment; hence certain conclusions may be validly drawn about the normal sporulation process as it occurs in a single cell.

RESULTS

General Observations

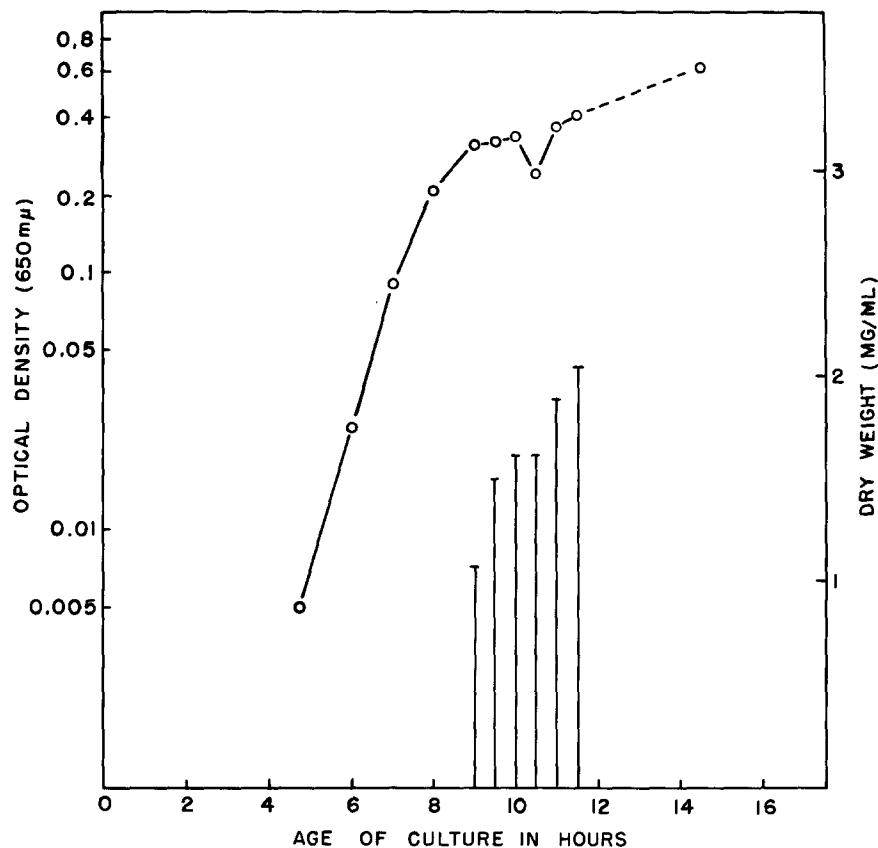
The rapid growth of *B. cereus* which followed germination of the spore inoculum produced a vis-

ible turbidity in the culture within 2 to 3 hours. During the exponential phase of growth which followed, the cells were generally longer and more slender than those seen during sporulation and occurred singly or in chains of two bacilli. During this period, the increase in optical density of the culture was directly related to the total cell count if each walled-off unit as seen in the phase contrast microscope was considered a single cell. Some 9 hours (this time varied slightly among experiments) from the time of inoculation of the culture, this relationship was invalidated by an aggregation of the cells into small clumps which altered the optical properties of the suspension and made total cell counts impossible. However, the number of spores eventually formed was always equal to the number of cells present just as clumping occurred. There was no evidence of cell lysis prior to the appearance of the refractile spores or of lysis or regrowth of the spores once formed. Thus, since each cell gives rise to only one spore, it was concluded that no further increase in cell numbers occurred following clumping. Thus, the exponential phase of growth ceased approximately 1 hour prior to the onset of sporulation although growth continued at a slower rate until sporulation commenced (at 9 hours in Text-fig. 1) With this complete cessation of growth distinctive cytological changes could be detected within each cell; these are here associated with the sporulation process (see below).

Although there was no increase in cell numbers following 9 hours of growth, an increase in the dry weight of cells per ml. of culture was observed (Text-fig. 1).

The living bacilli at the cessation of growth were short, plump, and quite homogeneously phase-dark. An hour and a half later, however, the cells became more phase-transparent; now, small, phase-dark granules and a distinct terminal fore-spore could be detected within the cytoplasm (Fig. 7). This "clearing" stage could also be detected as a drop in the optical density of the culture (Text-fig. 1, 10½ hours). This was a transitory stage, for the cells soon reverted to their phase-dark appearance and began to re-aggregate into clumps. Finally, the optical density rose with the increase in spore density and the development of refractility.

Since each cell contained a refractile, mature spore 14½ to 15 hours from the time of the original inoculation, some 5 to 6 hours elapsed be-



TEXT-FIG. 1. Changes in the optical density (plotted on a logarithmic scale) (○—○) and dry weight of a culture of *B. cereus* during growth and sporulation. The fall in optical density at 10½ hours indicates the clearing stage peculiar to this organism.

tween the cessation of vegetative division and the emergence of the mature spores. A period then followed during which lysis of the sporangia occurred. Only free refractile spores were seen in the culture 24 hours from the time of inoculation. A yield of approximately 0.7×10^9 spores per ml. was found for *B. cereus* under these growth conditions.

Cytology

Changes in Cytology Prior to the Appearance of the Fore-Spore:

Alteration of Phase Contrast Due to Cell Proximity.—With the cessation of growth, phase-dark granules could be detected within the living cells which on fixation could appear either phase-dark or phase-white,¹

¹ In dark phase contrast, objects of greater optical path appear darker than their surroundings whereas those of lesser optical path appear lighter. Objects

depending on the proximity of the cells. This effect is illustrated in Figs. 2, 3 a, and 4 a. The phase-light areas in the two cells crowded on the left (Fig. 3 a, arrows at x) were found to be the sites of chromatin following hydrolysis and staining (Fig. 3 b). This same phase appearance is more apparent in all of the more crowded cells from another field of the same preparation (Fig. 2) in which the phase-light chromatin is on either side of a more phase-dark object. Conversely, in the well isolated cell (Fig. 3 a, bottom arrow) it is the phase-dark material surrounding the phase-white body which stains for chromatin (Fig. 3 b). Similar relationships are apparent in the closely

can also appear phase-white. The physical basis for such an appearance is uncertain but may be due either to an increase in the refractive power of the object or due to interfering diffracted light. In the original preparations photographed above, objects which appeared phase-white could be readily distinguished from those which appeared phase-light. This difference, however, cannot be distinguished in many of the photomicrographs.

grouped cells of Figs. 4 *a* and 4 *b* and in the well isolated cells of the same figures (inserts).

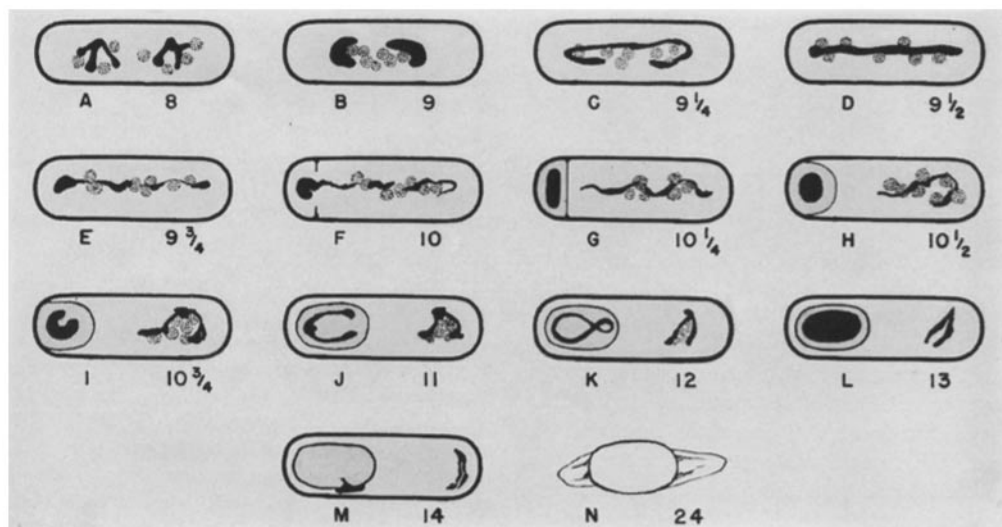
Initial Changes in the Chromatin Patterns.—During periods of cell multiplication bacterial nuclear bodies appear as clusters of interconnected granules when stained by Feulgen procedures (28, 29). In these cultures of *B. cereus* such configurations were observed throughout the exponential phase of growth and were still apparent during the final hour of less rapid growth (Fig. 1; Text-fig. 2, *A*). Although the phase contrast photomicrographs at these early stages were relatively featureless, slightly more phase-light areas could be detected in the original preparations. Similar areas have been shown by others to correspond to the chromatin sites (21, 29, 32).

When growth ceased, all of the cells began a sequence of cytological changes which terminated with the formation of spores. Thus, at 9 hours the

delicate, open, divisional figures of chromatin had become remarkably compact aggregates in the shape of bars or small c's (Fig. 3 *b*; Text-fig. 2, *B*). Each cell contained two of these condensed chromatin bodies in close association with, and yet separated by central inclusions. This stage of condensation was fleeting for within the next half-hour the nuclear material opened out (Figs. 4 *a* and *b*; Text-fig. 2, *C*), extended, and fused into a continuous axial filament (Figs. 5 *a* and *b*; Text-fig. 2, *D*).

Simultaneously, the central non-chromatinic inclusions fragmented into a network of smaller connected bodies, giving the impression that the chromatin was pushed into an axial filament by this shifting apparatus.

These non-chromatinic inclusions can be seen in the phase-photomicrographs as dark beads (Fig. 5 *a*) and in the corresponding brightfield photomicrographs as glistening areas in the cytoplasm



TEXT-FIG. 2. Diagrammatic representation of the sequence of morphological changes which occur when a cell of *B. cereus* forms a spore; summarized from dark 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 and the dotted bodies the lipid-containing inclusions. *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*, the completed spore septum encloses the spore chromatin in an end-pocket. *H*, growth of the septum around the cell end with cleavage from the cell wall (clearing stage). *I*, continued growth of the septum; the spore chromatin is opening into a ring while the non-spore chromatin is re-organizing about the lipid-containing inclusions. *J*, completion of the spore wall; spore chromatin as an open, beaded ring. *K*, spore chromatin as a figure 8. *L*, the body of the prerefractile spore (phase-white with a dark outer rim) stains for chromatin. At the stages *K* to *L* spore coats and a cortex are formed. *M*, refractile spore within the sporangium; the chromatin has been extruded to the periphery by acid hydrolysis. *N*, free refractile spore with surrounding exosporium.

Fig. 5 *b*; Text-fig. 2, *C*, *D*) When such preparations were mounted in Sudan black B (0.3 per cent in ethylene glycol-ethanol-water, 2:2:1, by volume), the inclusions showed an affinity for the dye indicating that their surface layers at least contained a lipid component. In living cells, observed in the phase contrast microscope, the 6 to 10 small globules appeared connected by fine strands (Fig. 7). This network could be seen slowly swaying and twisting within the cell but the rapid, independent jostling of the separate granules characteristic of Brownian movement was not seen to occur. In electron micrographs of thin sections of cells at this stage of development "vacuoles" of comparable size and distribution were observed within the cytoplasm (see below).

Twenty to 30 minutes after its formation the chromatin thread became more tortuous and resembled a broken strand of beads (Figs. 6 *a* and *b*; Text-fig. 2, *E*). Concomitantly, the intensity of the chromatin stain lessened; this was due either to a decrease in the number of dye-binding sites, or more probably, to the partial dispersion of the chromatin itself (Fig. 6 *b*). Then, the portion of the chromatin destined to become the nuclear body of the future spore became apparent as an intensely staining dot at one end of the cell (Fig. 8 *b*). Immediately prior to this, all that could be distinguished was a somewhat hyperchromatinic swelling at one end of the tortuous axial thread (Fig. 6 *b*; Text-fig. 2, *E*).

The Formation of the Fore-Spore.—Electron microscopy of thin sections of cells removed from the sporulating culture at 10 to 15 minute intervals was extremely useful in clarifying the sequence of events which occurred at this decisive phase in the life cycle. The thin-section images revealed the same arrangements of chromatin as had been seen in the light microscope, but further revealed that the segregation of the future spore chromatin to one end of the cell was timed to the rapid growth of a thin wall or septum. This septum grew centripetally from the inner surfaces of the cell wall some 0.2 to 0.3 μ from the cell end (arrow, Fig. 12; Text-fig. 2, *F*). The appearance of some cuts suggested that its formation was not always symmetrical, being further advanced on one side than the other (Fig. 13). The pocket formed between the new septum and the cell end enveloped that portion of the chromatin destined for the spore. In the sections, this chromatin can be recognized as foamy or feathery material of relatively low

electron density. The non-spore chromatin has a similar appearance and can be seen coursing throughout the cytoplasm in close association with apparent "vacuoles" corresponding in size and distribution to the beaded bodies described above (Figs. 13 and 14).

This early formation of a spore wall, not previously recognized, has also been observed in *B. cereus* var. *mycoides*, *B. cereus* var. *alesti*, and *B. cereus* var. *thuringiensis*. In a thin section of *B. cereus* var. *mycoides* (Fig. 14) the forming spore wall can be seen sequestering the spore chromatin in the end-pocket. Cells from the same sample are shown in Fig. 15 stained for chromatin.

Vegetative cross-wall formation differs considerably from the formation of the spore septum; the cross-wall is normally formed centrally in the cell, and, like the cell wall, is approximately three times the thickness of the septum although all are lanthanophilic (*cf.* Figs. 13 and 14 with 16). Furthermore, the break in the outer surface of the cell which accompanies the completion of the vegetative cross-wall (5) does not occur during the formation of the spore septum. During its initial stage of formation the septum appears to be continuous with the substance of the cell wall by a slightly thickened base. When completed, the transverse septum rapidly develops into a spore wall. This appeared to be accomplished by the proliferation of the septum from its attached edges around the cell end, as is shown in Text-fig. 2, *G* to *I*. The spore wall was not free from the cell wall until the entire spore spheroid had been enclosed (Text-fig. 2, *J*). During the enclosure, the full and characteristic outline of the spore protoplast is achieved. A more detailed series of electron micrographs showing the chromatin changes and the mode of formation of the spore septum was obtained with *B. cereus* var. *alesti* and is presented in a companion publication (37).

It was during the final stages of completion of the spore wall that "clearing" occurred in *B. cereus*. The round to oval fore-spore, which could be recognized as a phase-light zone at the end of the cell (Figs. 7 and 8 *a*; Text-fig. 2, *H* and *I*), contained the densely staining dot of confined spore chromatin (Fig. 8 *b*). On the other hand, the more lightly stained chromatin in the vegetative portion of the sporangium was still wound about the phase-dark (Fig. 7) or phase-white bodies (Figs. 8 *a* and *b*, arrows; Text-fig. 2, *G*, *H*). It is interesting to note that these bodies did not ap-

pear to be enclosed in the spore pocket with the chromatin (*cf.* 3).

Changes in Cytology During Maturation of the Spore.—With the development of the elliptical fore-spore the enclosed chromatin moved back slightly from the cell-end but remained temporarily as a condensed bar. Then around the 11th hour it began to expand to form an open, beaded ring or triangle (Fig. 9 *b*; Text-fig. 2, *I, J*). As maturation progressed, this ring widened, assumed the form of a figure 8, and lost its beaded appearance (Fig. 10 *b*; Text-fig. 2, *K*). In the corresponding phase contrast photomicrographs (Fig. 10 *a*) it can be seen that at this time the spores were phase-dark (*i.e.* deep and dense blue) although a few spores were showing the earliest signs of increasing refractility and were thus becoming phase-white (Fig. 10 *a*).

In the vegetative portion of the sporangium the non-chromatinic, lipid-containing beads so apparent at "clearing," reaggregated to form an apparently single inclusion (Fig. 9 *a*); however, electron micrographs of thin-sections indicated that this inclusion was still composed of several small closely aggregated "vacuoles." At this same time, while remaining in close association with the lipid-containing inclusions, the non-spore chromatin reorganized to assume configurations typical of nuclear bodies in dividing cells (Figs. 9 *a* and *b*). Eventually, this chromatin appeared to be pushed against the opposite cell end by the developing spore. By this time the lipid-containing inclusions had vanished (Figs. 10 *a* and *b*; Text-fig. 2, *K, L*).

The development of refractility was rapid. Fore-spores at the stage in which their chromatin was shaped as an open figure 8 were either phase-dark or just becoming phase-white (Figs. 10 *a* and *b*). When the main body of the spore was entirely phase-white but outlined with a dark rim (Fig. 11 *a*, upper arrow) the central portion appeared to stain for chromatin (Fig. 11 *b*, upper arrow; Text-fig. 2, *L*). Presumably this diffuse staining is an artefact of hydrolysis. Such an arrangement of chromatin is also encountered when spores in the initial moments of germination are hydrolysed and stained (27). However, with the development of the full refractility characteristic of the mature spore (Fig. 11 *a*, lower arrow), the dark diffuse outline was no longer apparent in phase contrast. Now the spore chromatin was extruded during acid hydrolysis (Fig. 11 *b*, lower arrow; Text-fig. 2, *M*) (26, 27) and the spores appeared fully re-

fractile in air-mounted nigrosin smears and possessed the heat resistance characteristic of the mature form (39). Only such mature spores still confined by the sporangia were present in the sample removed $\frac{1}{2}$ hour later. Eight to 9 hours later the spores had been completely liberated (Text-fig. 2, *N*).

More complete chemical and morphological studies of the development of spore refractility and heat resistance will be published separately (39).

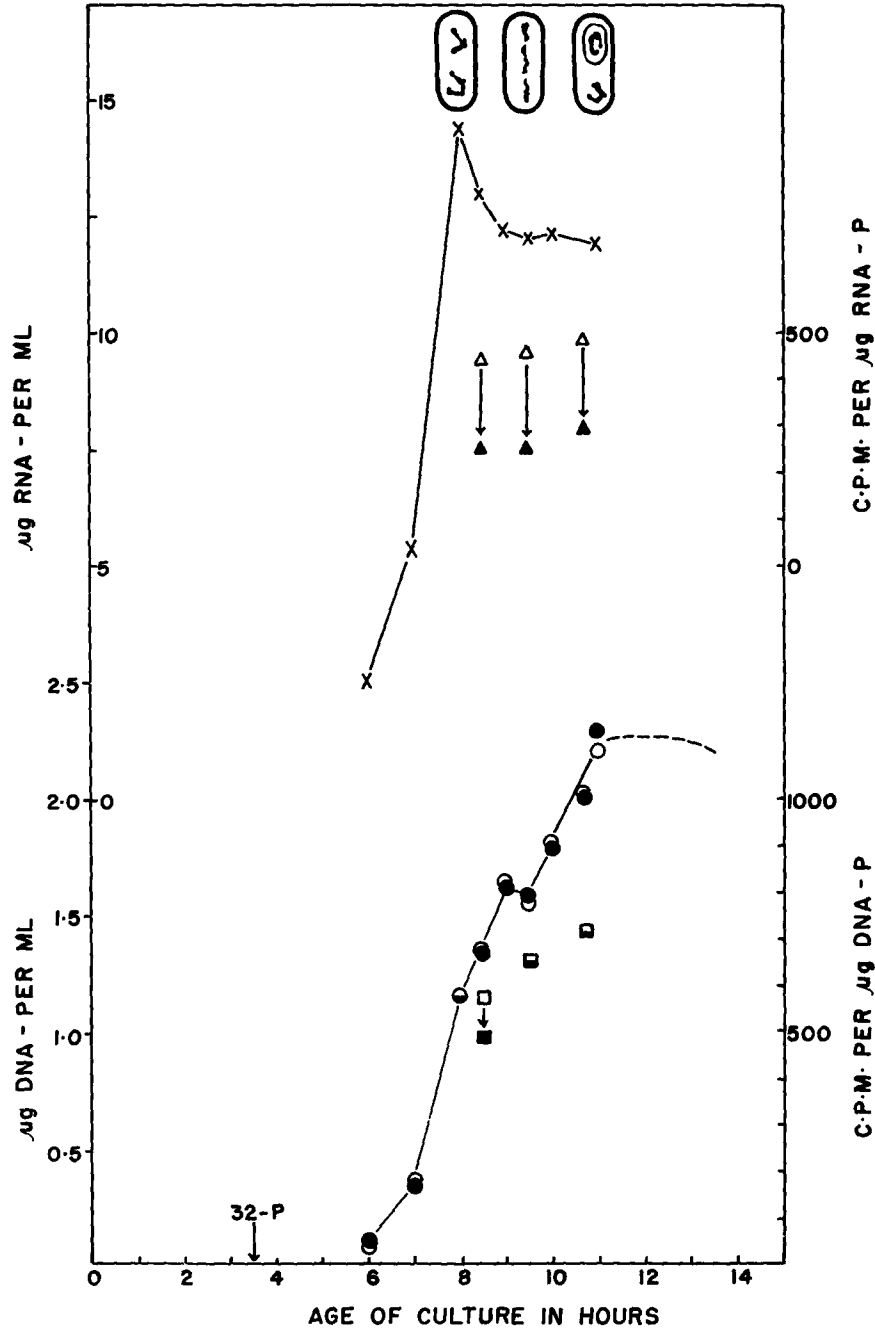
Chemistry

The DNA Content of Cells Commencing Sporulation:

Direct analyses of the DNA content of the cells of *B. cereus* harvested during asynchronous, exponential growth and of the spores resulting from such growth showed that each cell contained an average of 2.30×10^{-9} $\mu\text{g.}$ and the spore 1.05×10^{-9} $\mu\text{g.}$ DNA-P. This is in agreement with the earlier comparisons of spores and cells grown in other media (9, 11) and with the changes in DNA-P associated with germination (10) and synchronously dividing cells (36).

In the representative experiment of growth and sporulation of *B. cereus* shown in Text-fig. 3, the amount of DNA-P measured in the cell fraction up to 9 hours is also a direct measure of the number of cells present. When clumping of the cells was just beginning at 9 hours there were 0.7×10^9 cells per ml., (direct cell count) a number remarkably close to the number of spores eventually produced (0.69×10^9 per ml.). Each of these cells contained two distinct, but somewhat condensed chromatin bodies. One hour later (at 10 hours) the nascent spore chromatin could be detected in all cells at one end of the axial thread. Thus, growth ceased and sporulation began at 9 hours from the time of inoculation at a time when each cell contained two chromatin bodies and a DNA-P content (2.33×10^{-9} $\mu\text{g.}$) which was twice that of the spore.

Analyses of a sample removed from the culture at $9\frac{1}{2}$ hours revealed that upon cessation of growth there was also a cessation of DNA synthesis; this period of no net synthesis occurred as the condensed bars of chromatin extended into an axial filament (Text-fig. 2, *B* to *E*). It is apparent, however, that synthesis of DNA recommenced and continued at a linear rate during the development of the spore (Text-fig. 3). In six experiments in



TEXT-FIG. 3. Changes in the DNA-P (●—●, by Dische reaction; ○—○, by total-P) and in the RNA-P (X—X) content of a culture of *B. cereus* during growth and sporulation. The line drawings derived from photomicrographs of the cells indicate that sporulation began at 9 hours. Radioactive phosphorus was added (↓) to lots of this same culture and the cells were transferred to non-labelled medium at 8½, 9½, and 10¾ hours. The specific activities of the DNA-P (□) and RNA-P (△) of the cells at the time of transfer are compared with the specific activities of the DNA-P (■) and RNA-P (▲) of the spores resulting from the transfer.

which the same degree of synchrony was achieved, a net increase of DNA occurred during sporogenesis which was equivalent in amount to that eventually found in the spores. In those experiments in which samples were removed at later stages of spore development it was found that this synthesis ceased when each cell contained three times the basic or "spore" amount of DNA. The DNA content of the cells plateaued at this level and then fell as lysis of the sporangia began (dotted line, Text-fig. 3).

Such results suggested that the spore DNA was synthesized *de novo* and did not arise by partitioning of vegetative cell chromatin to the fore-spore area as had been surmised from the cytological studies. However, the information gained from labelling of the DNA-P with ^{32}P clarified this apparent discrepancy.

The Origin of the Spore Chromatin:

Transfer of Cells Labelled with ^{32}P to a Non-Labelled Medium.—Tracer amounts of radioactive phosphorus had been added to three flasks of this same culture at $3\frac{1}{2}$ hours of growth (Text-fig. 3). A transfer of the labelled cells to non-labelled medium was then effected at $8\frac{1}{2}$, $9\frac{1}{2}$, and $10\frac{3}{4}$ hours (see Methods). The specific activities of the DNA-P in the cells at the time of transfer and of the spores resulting from the transfer are shown in Text-fig. 3. In spite of the further synthesis of DNA-P between $9\frac{1}{2}$ and $10\frac{3}{4}$ hours there is no difference between the specific activity of the DNA-P in the cells and in the spores formed following either transfer. On the other hand, the DNA-P of spores derived from labelled cells transferred to non-labelled medium before the end of vegetative growth ($8\frac{1}{2}$ hours) had a somewhat lower specific activity than that from the progenitor cells (Text-fig. 3). Such decreased activity can be accounted for by the further synthesis of DNA occurring between $8\frac{1}{2}$ and 9 hours and associated with the growth in the non-labelled medium.

Therefore, in agreement with the cytological appearances, these results suggest that it is chromatin within the cells at the end of vegetative growth which is incorporated into the spore. Hence, it is inferred that the further synthesis of DNA which occurred during sporulation must be confined to the vegetative or non-spore portion of the sporangium.

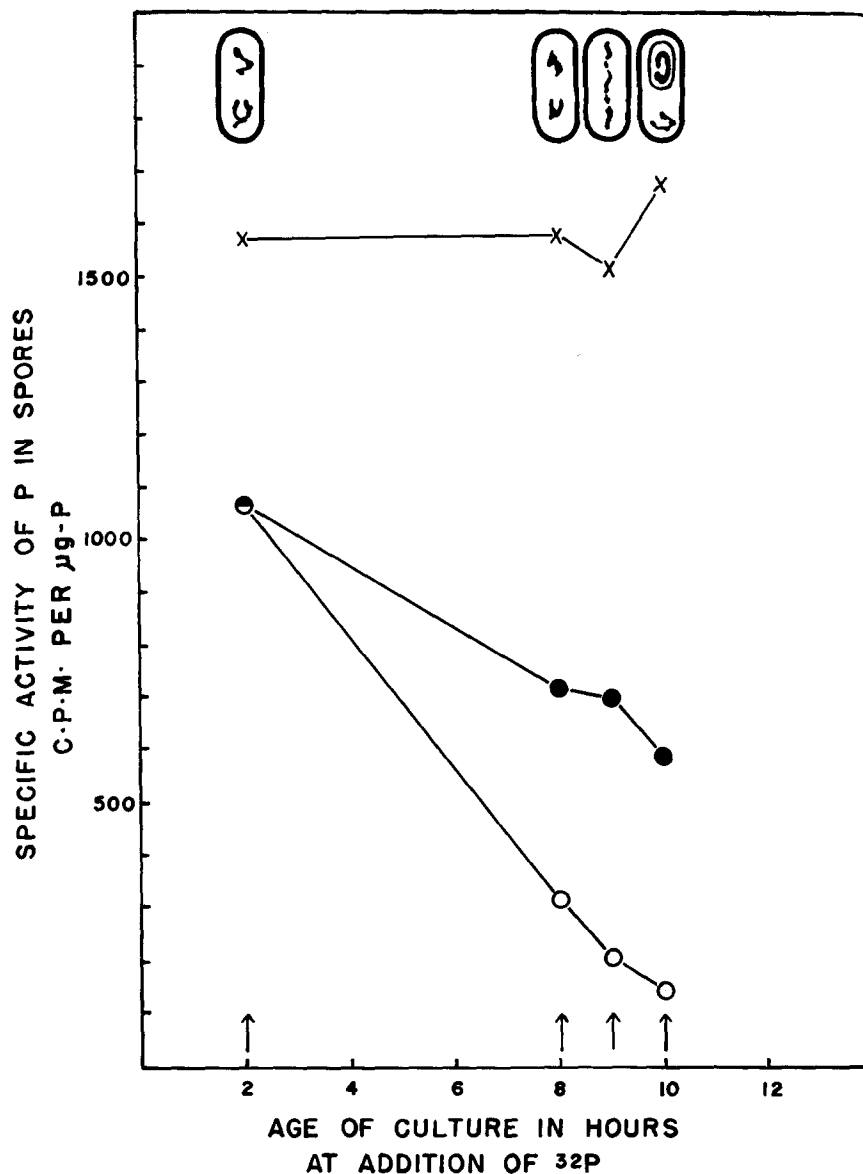
Addition of ^{32}P to Cells at Various Times During

Growth and Sporulation.—In the above experiments the possibility remained that despite transfer of the cells to a non-labelled medium the specific activity of the phosphorus in the precursor pool for DNA would not be altered. If this were so, one need not expect a decrease in the specific activity of the spore DNA-P upon a further synthesis of this compound. To investigate this, ^{32}P was added to the unlabelled medium at various times during growth and sporulation. The specific activities of the DNA-P of the spores formed following the additions were measured as before.

Addition of ^{32}P to the culture at 2 hours of growth resulted in a spore DNA-P which was highly labelled (Text-fig. 4). Addition of the same amount to another flask of the same culture at 8 hours when the chromatin in the cells was just becoming condensed, resulted in an incorporation into the spore DNA-P of 189 counts per minute per μg . At 9 hours of growth the spore chromatin was either condensed or opening into a beaded ring. Additions of ^{32}P at these times did result in an apparent incorporation into the spore DNA-P (121 and 99 counts per minute per μg ., respectively). However, during the period of sporulation, when the major synthesis of DNA occurred, there was no significant change in the already low rate of incorporation of ^{32}P into the spore DNA-P. Since no further synthesis of DNA would be expected once the spore chromatin had expanded to an open, beaded ring (following 10 hours), it is probable that the apparent incorporations following additions at the 9th and 10th hours are due to contamination of the DNA extract by the highly active residue or spore-coat phosphorus which remains unextracted following the Schmidt and Thannhauser fractionation (9) (Text-fig. 4). If 2 per cent of the total phosphorus in the DNA extract were this residue phosphorus, it would account for the total activity measured. The uniformly high activity of the residue fraction even following late additions of ^{32}P (Text-fig. 4) attests to the late formation of the spore coats which make up this residue fraction. Indeed, electron microscopy had indicated that spore coats were formed in this experiment between the 12th and 13th hour.

Synthesis of RNA During Growth and Sporulation:

Although there was a marked increase in the amount of RNA in the cell fraction during growth (Text-fig. 3) the synthesis of this nucleic acid



TEXT-FIG. 4. The specific activities of the DNA-P (O—O), RNA-P (●—●), and residue-P (X—X) of spores formed following additions (↑) of radioactive phosphorus to lots of a culture of *B. cereus* at 2, 8, 9, and 10 hours of aeration. The line drawings derived from photomicrographs of the cells indicate sporulation began here between 8 and 8½ hours.

did not keep pace with that of DNA. Thus, the decreasing ratio of RNA-P/DNA-P indicates a decreasing amount of RNA per cell during later growth. The sharp cessation of net RNA synthesis which occurred between ½ and 1 hour prior to the onset of spore formation (Text-fig. 3, between 8 and 8½ hours) was followed by an absolute de-

crease in the amount of this compound per cell. Throughout sporulation the RNA per cell continued to fall in spite of the recommencement of synthesis of DNA.

The specific activity of the RNA-P of the vegetative cells at the time of transfer to non-labelled medium was compared with that of the spores re-

sulting from the transfer (Text-fig. 3). Although there was no net synthesis of RNA-P during sporulation, the 50 per cent lower specific activity of the RNA-P of the spores found following each of the last three times of transfer suggests that some turnover of this fraction occurred. Similarly, the considerable incorporation of ^{32}P into the spore RNA following additions of ^{32}P during sporulation supports this suggestion (Text-fig. 4).

DISCUSSION

The Origin of the Bacterial Spore Chromatin.—The investigations reported above have sought to elucidate the origin of the bacterial spore chromatin and the cytological sequence associated with its appearance within the cell. In all *Bacillus* species so far investigated the spore arises in a cell containing two chromatin bodies, twice the spore amount of DNA and at a time when there is no net synthesis of this nucleic acid. Thus, the fusion of these two chromatin bodies into a single axial filament and its subsequent apparent fragmentation are interpreted as successive events leading to the segregation of one-half of the cell's chromatin to the developing spore site.

It is necessary now to reconcile these results with the somewhat contradictory observations of earlier investigators.

In these previous descriptions of the sporulation process, each discrete Feulgen-positive mass was referred to as a "chromosome," a "nuclear granule," or a "chromatin or nuclear body," of which each cell commencing sporulation could contain two to eight or more (2, 7, 12, 18, 28). This range in numbers of chromatin masses per cell could occur within the same population and so was independent of species variation, cultural conditions, or the objectivity of the individual investigator. Although Delaporte (7) cautioned that "inability to discern a morphological heterogeneity does not necessarily imply a similarity of function of all nuclear granules," the impression is gained from other reports that each of the chromatin granules was equivalent quantitatively and perhaps genetically. In this connexion, Flewett (12) considered that the bacterial individual contained only one nuclear body and proposed that the presence of several nuclear bodies in a cell should be interpreted as "several individuals still sharing a common cytoplasm and cell membrane". Accordingly, he reasoned that the fusion cell of *B. anthracis* (*i.e.*, one containing an axial filament of chromatin) "represents the fusion of four individual organisms" (12).

In spite of this apparent range in numbers of chromatin granules within the cell there was agree-

ment that the spore developed about only one of them. Consequently, the spore received only a portion of the cell's chromatin depending on the number of chromatinic bodies originally present. The problem was thus two-fold: Is there a predictable number of chromatinic granules within the cell about to commence sporogenesis and does the spore receive a precise amount of this chromatin?

It now appears that growth ceases and sporulation commences when each cell possesses two fully separated chromatin bodies and twice the spore amount of DNA. In the period between this cessation of cell division and the appearance of the fore-spore the chromatinic material exhibits such a degree of plasticity that at one time it may appear as several discrete granules and at another as a continuum within the cell (28, 29). During this interval there is no alteration in cellular content of DNA. Contrary then to chromosome-nuclei (1) the *number* of chromatinic masses need not correspond to the *amount* of chromatinic material actually within the cell.

If there is a common mechanism of sporulation, at least within the same species, then one could expect to observe the same cytological sequence occurring within all cells. How, then, can the variability in the number of chromatin bodies previously observed in cells prior to the appearance of the fore-spore be explained? Failure to recognize individual cell limits leads to an erroneous estimate of the number of chromatin bodies per cell. In cells which do not form chains or in those already possessing spores, demarcation of individual cell limits is not difficult. But, in organisms such as *B. anthracis* (see Flewett, 12) which remain attached following division and so form chains containing many cells, it is impossible, or at best difficult, to determine the outline of each individual (*i.e.*, each walled-off unit) especially in nuclear-stained preparations. That this is so becomes evident by a comparison of "cells" in Fig. 4 *b* with the same ones in Fig. 4 *a*. Moreover, the study of non-synchronous cultures may contribute to confusion since in such preparations, cells representative of the various stages in the life cycle (containing from one to several chromatin granules) may be present at any one time. Since in stained preparations no single cell can be observed at the various stages in the process, the determination of the proper sequence of events becomes a somewhat arbitrary procedure.

The Axial Filament of Chromatin and Commitment to Sporulation.—In all cells of *Bacillus* species so far investigated (*B. cereus*, *B. cereus* var. *mycoides*, *B. cereus* var. *thuringiensis*, *B. cereus* var. *alesti*, *B. anthracis*) the chromatin has formed a continuous axial filament prior to the appearance of the fore-spore chromatin. Strains of *B. anthracis* have been reported, however, in which this does not apparently occur (12). As yet, no significance can be attached to this formation, although similar arrangements of chromatin are of common occurrence in bacteria undergoing periods of stress (28, 34).

Nevertheless, it is while the chromatin is in this arrangement that the cells become committed to the sporulation process, although they are possibly not irrevocably committed to sporogenesis until the future spore chromatin is about to be enclosed by the fore-spore membrane. During this interval there is no net synthesis of DNA, the level of RNA per cell falls, and the oxygen consumption drops markedly (unreported results). Moreover, additions of purine analogues such as 8-azaguanine, at or following this time, fail to inhibit sporogenesis, although prior additions completely prevent the initiation and thus the completion of sporulation (38).

The Formation of the Initial Spore Septum.—The newly formed spore septum not only encloses the hereditary material for the next generation but also eventually occupies the site of the primordial cell wall of the future bacillus. Although the presence of such a limiting wall about the developing fore-spore has long been suspected it has required electron microscopy of thin sections to demonstrate its formation. Both Bayne-Jones and Petrilli (3) and Flewett (12) could not detect a limiting membrane about the developing spore area. However, the former workers described the failure of rapidly moving granules to penetrate this zone. On the other hand, Lewis (20) in agreement with Peters (25) and Meyer (23) described the spore as being set off from the rest of the cell by a membrane. Similarly, Klieneberger-Nobel (18) and DeLamater and Hunter (6) described the formation of a septum or wall which separated the spore part from the remainder of the cell. Since, in these instances, the spores were already in an advanced stage of development it is probable that the formation of spore coats was observed rather than that of the initial septum. A recent study employing electron microscopy of thin sections failed to demonstrate the presence of a wall about the developing spore body in *B. cereus* and *B. megaterium* (4). That such might exist was suggested from observations by Mayall and Robinow (22) on thin

sections of acid-treated and germinating spores of *B. megaterium*. In pictures of acid-treated spores they frequently detected a narrow band of relatively high affinity for lanthanum forming the inner table of the cortex and suggested that it corresponded to the "cell wall of the dormant bacillus" (22). In another study in which similar techniques were employed the appearance of a very thin membrane layer was described as the first indication of spore formation in *Clostridium sporogenes* (15). This was not detected, however, until the enclosed fore-spore was already ovoid to spherical in outline and lying free within the cytoplasm. Similarly, Tokuyasu and Yamada (33) described the formation of a membrane about the fore-spore of *B. subtilis*. In permanganate-fixed material they observed two membranes developing in a clear interspace between the already oval fore-spore and the sporangial cytoplasm. One of these was tightly attached to the pre-spore and was named the "spore cytoplasmic membrane"; the other layer, called "spore core wall," although forming on the primordium, showed occasional attachment to the cell wall.

In this present study the spore septum projected across the cell from the inner surfaces of the vegetative cell wall. When the septum was complete, it had separated the spore chromatin and associated cytoplasm from the remainder of the cell. Subsequent development of the septum led to the formation of the spheroidal fore-spore which lay free in the cytoplasm. A cortex, spore coats, and an exosporium were then laid down peripheral to the spore body and at a later stage in spore development (39).

Synthesis of DNA During Spore Development.—The further synthesis of DNA associated with the development of the spore in *B. cereus* was initially interpreted as a requirement of the vegetative portion of the cell to maintain its normal quantity of DNA (35). More recent studies indicate that this phenomenon is not universal among *Bacillus* species. Although both a rough and smooth strain of our *B. cereus*, exhibiting synchronous sporulation, did synthesize this further amount of DNA, other variants (*B. cereus* var. *mycoides*, *B. cereus* var. *alesti*, and *B. cereus* var. *thuringiensis*) did not (37). This chemical evidence suggests that although similar in appearance the spore-forming process may not be identical in all organisms.

We wish to thank Mrs. Sheila Newton for her most careful assistance during these investigations. We are also grateful to Professor C. F. Robinow for his criticism of the manuscripts.

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EXPLANATION OF PLATES

PLATE 234

All photographs are of cells of *Bacillus cereus* removed from the same culture at various times during growth and sporulation. The dark phase contrast photographs (Figs. 2, 3 *a*, 4 *a*, 5 *a*, 6 *a*) are of the cells fixed in osmium tetroxide vapours and mounted in water ($\times 3880$). The brightfield photographs are of the same cells hydrolysed in N HCl and stained for chromatin with SO_2 -azure A. $\times 3600$.

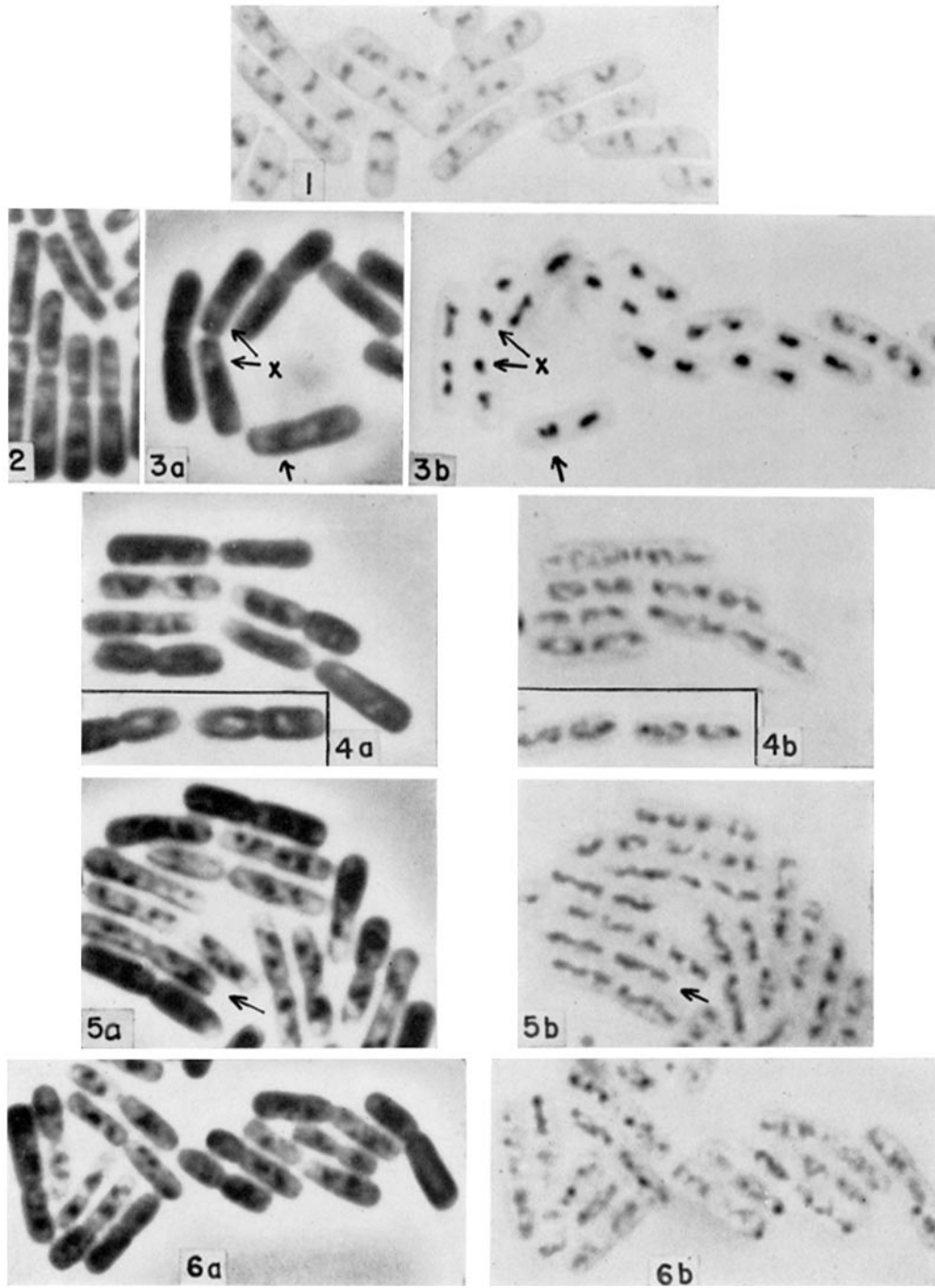
FIG. 1. Cells at a late stage of vegetative growth (1 hour prior to the cessation of growth). Note the open, divisional-type of chromatin bodies.

FIGS. 2 to 3 *b*. Cells at the end of vegetative growth ($8\frac{1}{2}$ hours of aeration). In the crowded cells in Fig. 2, and 3 *a* (arrows at *x*) the chromatin appears phase-light and is found on either side of a phase-dark body. Photographed from the same impression smear as Fig. 2, the lower, uncrowded cell in Fig. 3 *a* (arrow) contains phase-dark material which stains for chromatin in Fig. 3 *b*. It is found on either side of a phase-white body. Note that each cell contains two markedly compact chromatin bodies.

FIGS. 4 *a* and *b*. Cells at 9 hours of aeration. The altered phase contrast dependent upon the proximity of the cells is again evident. In the uncrowded cells (insert, Fig. 4 *a*) the phase-dark material stains for chromatin (insert, Fig. 4 *b*). The chromatin in all cells is now beginning to extend into an axial filament.

FIGS. 5 *a* and *b*. Cells at $9\frac{1}{2}$ hours of aeration. The chromatin in most cells in Fig. 5 *b* now appears as an axial filament. In the same cells in Fig. 5 *a*, prior to hydrolysis and staining, the chromatin can be recognized as phase-light material. Note also the several phase-dark bodies in each cell and the interwound chromatin (arrows).

FIGS. 6 *a* and *b*. Cells at 10 hours of aeration. The chromatin stain is now less intense and the strand appears broken into fragments by the phase-dark bodies.



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

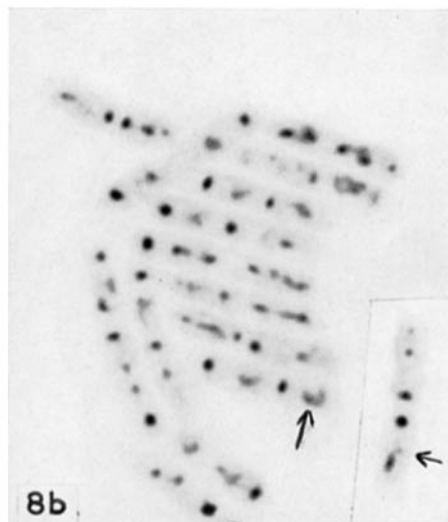
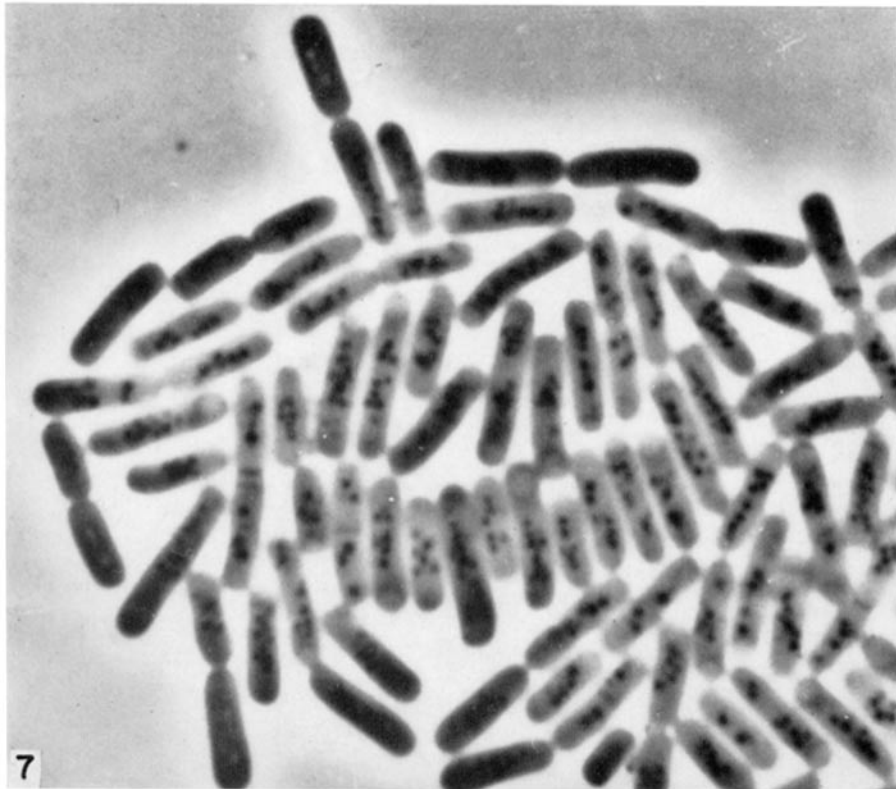
PLATE 235

All photographs are of cells of *Bacillus cereus* at the time of the formation of the fore-spore (10½ hours of aeration, "clearing" stage).

FIG. 7. Living cells photographed in dark-phase contrast while mounted in medium. The 6 to 10 phase-dark bodies in the cytoplasm and the clear zone at the tip of each cell (the fore-spore) are readily seen in the crowded cells. × 3880.

FIG. 8 *a*. Cells fixed in osmium vapours, mounted in water, and photographed in dark phase contrast. The phase-light fore-spore is more apparent following fixation, but the phase-dark bodies seen in the living cells can now appear either phase-dark or phase-white (arrows). × 3880.

FIG. 8 *b*. The same cells as photographed in Fig. 8 *a* following hydrolysis in N HCl and staining for chromatin with SO₂-azure A. Note the hyperchromatinic dot of chromatin situated in the fore-spore area. The non-spore chromatin can be seen closely associated with the phase-dark or -white bodies (arrows). × 3600.



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

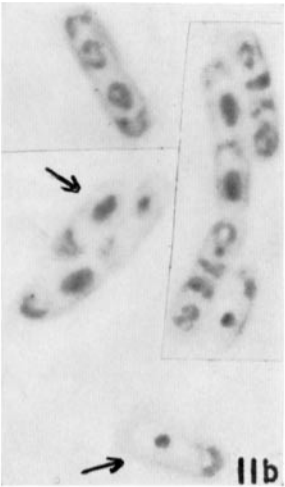
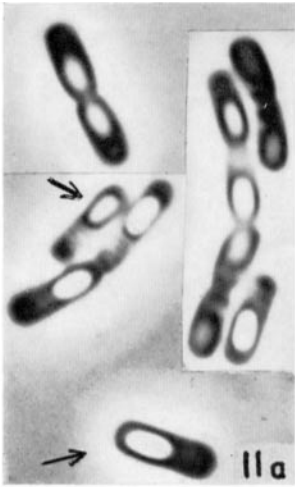
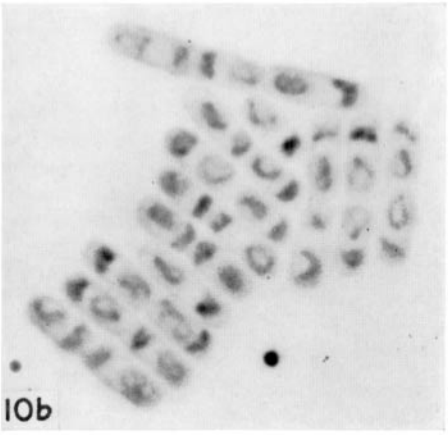
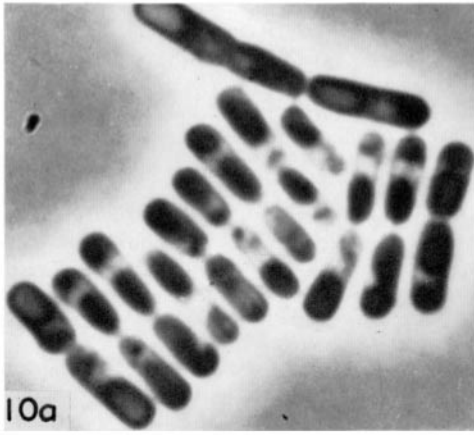
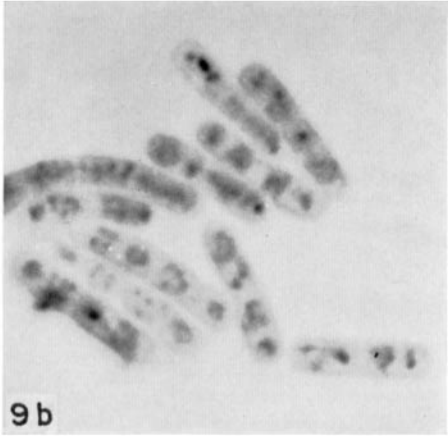
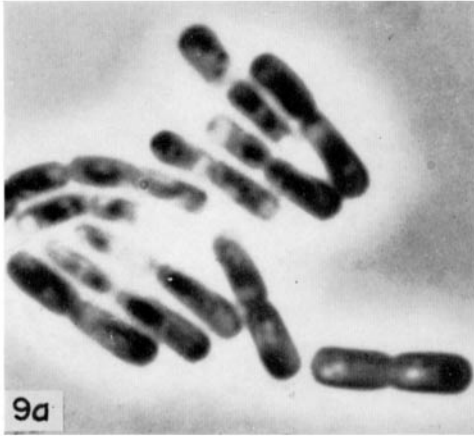
PLATE 236

All photographs are of cells of *Bacillus cereus* during the development of the spore. Figs. 9 *a*, 10 *a*, 11 *a* are of cells fixed in osmium vapours, mounted in water, and photographed in dark phase contrast. $\times 3880$. Figs. 9 *b*, 10 *b*, 11 *b* are correspondingly the same cells following hydrolysis and staining for chromatin with SO_2 -azure A. $\times 3600$.

FIGS. 9 *a* and *b*. Cells after $11\frac{1}{2}$ hours of aeration. The spore chromatin can be seen expanding into an open, beaded-ring while the vegetative chromatin shows arrangements reminiscent of replicating chromatin bodies and is closely associated with the phase-white beads.

FIGS. 10 *a* and *b*. Cells after $12\frac{1}{2}$ hours of aeration. Note that the developing spores are now phase-dark and contain chromatin shaped like a figure 8. The non-spore chromatin is now at the opposite pole of the cell to the spore.

FIGS. 11 *a* and *b*. Cells after $13\frac{1}{2}$ hours of aeration. All spores are in a stage of becoming refractile and appear phase-white. In those which are phase-white with a dark outline, the whole spore body appears to stain for chromatin (upper arrows). A spore which is completely refractile appears entirely phase-white and no longer has a darker, diffuse outline (lower arrow) and the spore chromatin is now extruded to the periphery during the hydrolysis procedure (lower arrow, Fig. 11 *b*).



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

PLATE 237

FIG. 12. An electron micrograph of a thin section cut obliquely through the end of a cell of *Bacillus cereus* showing the early stages of development of the spore wall (arrow). The chromatin of the future spore, appearing as areas of low electron density, has partly migrated into the pocket formed by the growing wall. Section cut onto lanthanum nitrate (0.5 per cent). $\times 30000$.

FIG. 13. An electron micrograph of a more centrally cut section of *B. cereus* at the same stage of development as that shown in Fig. 12. The development of the spore wall appears here to be asymmetrical. The lipid-containing bodies in the cytoplasm of the cell appear as "vacuoles." In close association with these are small flocs of low density chromatin. Section cut onto 0.5 per cent lanthanum nitrate. $\times 30000$.

FIG. 14. An electron micrograph of a thin section of *Bacillus cereus* var. *mycoides* following 11 hours of growth from spores. The forming spore wall can be seen "capturing" the low density spore chromatin (*scb*) in the end-pocket. Section cut onto 0.5 per cent lanthanum nitrate. $\times 35000$.

FIG. 15. Cells of *B. cereus* var. *mycoides* at the same stage of development as those shown in Fig. 14. The cells are fixed in the vapours of osmium, hydrolysed in N HCl, and the chromatin is stained with SO_2 -azure A. $\times 3600$.

FIG. 16. Electron micrograph of a thin section through a cell of *B. cereus* showing cross-wall formation during vegetative division. Note the relatively greater thickness and the position of this cross-wall as compared with that associated with spore formation. Section cut onto 0.5 per cent lanthanum nitrate. $\times 29000$.

