CHEMICAL AND MORPHOLOGICAL STUDIES OF BACTERIAL SPORE FORMATION

IV. The Development of Spore Refractility

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ABSTRACT

From the stage of a completed membranous forespore to that of a fully ripened free spore, synchronously sporulating cells of a variant *Bacillus cereus* were studied by cytological and chemical methods. Particular attention was paid to the development of the three spore layers—cortex, coat, and exosporium—in relation to the forespore membrane. First, the cortex is laid down between the recently described (5) double layers of the forespore membrane. Then when the cortex is ⅓ fully formed, the spore coat and exosporium are laid down peripheral to the outer membrane layer covering the cortex. As these latter layers appear, the spores, previously dense by dark phase contrast, gradually "whiten" or show an increase in refractive index. With this whitening, calcium uptake commences, closely followed by the synthesis of dipicolinic acid and the process is terminated, an hour later, with the formation of a fully refractile spore. In calcium-deficient media, final refractility is lessened and dipicolinic acid is formed only in amounts proportional to the available calcium. If calcium is withheld during the period of uptake beyond a critical point, sporulating cells lose the ability to assimilate calcium and to form normal amounts of dipicolinic acid. The resulting deficient spores are liberated from the sporangia but are unstable in water suspensions. Unlike ripe spores, they do not react violently to acid hydrolysis and, in thin sections, their cytoplasmic granules continue to stain with lead solutions.

INTRODUCTION

The high refractility of the bacterial spore as seen in the light and dark phase contrast microscope is a characteristic which develops during the very late stages of spore formation. Preliminary evidence has associated the development of refractility in time with the laying down of those structures of the mature spore—the cortex, spore coat, and exosporium—which lie peripheral to the membrane-enclosed spore body (23).

Chemically, these advanced stages of spore development have also been associated with the synthesis of dipicolinic acid (8, 17), the uptake of calcium (17, 21) and a change in the distribution of hexosamine-containing peptides (17).

In an attempt to understand better the development of refractility we have correlated the structural with some chemical changes which occurred in populations of *Bacillus* cells during the late stages of reasonably synchronous sporulation. We have also extended these studies to cells sporulating in media varying in calcium concentration.

MATERIALS AND METHODS

Culturing Techniques

Two strains, A^+ (crystal-forming) and A^- (non-crystal-forming), of *Bacillus cereus* var. *alesti* were the organisms studied. Their origins and characteristics have been described previously (6).

Both organisms sporulated with the degree of synchrony reported in the earlier papers in this series (24, 25) when cultured in the medium and under the conditions already described (25).

Microscopy and Cytological Techniques

The procedures involved in the use of the light and phase contrast microscopes have been described (24).

Samples for electron microscopy of thin sections were fixed in osmium tetroxide buffer and soaked in uranyl acetate as described by Kellenberger, Ryter, and Séchaud (13). The fixed samples, in small blocks of agar, were dehydrated in acetone solutions and embedded in a polyester (Vestopal) (13). Sections were cut onto distilled water with a glass knife and picked up on grids filmed with carbon. In some preparations contrast was augmented by staining the cut sections with lead hydroxide by the method of Watson (22) or Dalton and Zeigal (4); in others partial staining of the spore cortex was achieved by floating sections on phosphotungstic acid solution (1 per cent) and rinsing with water (14). Further details of the methods used in electron microscopy have been described (6).

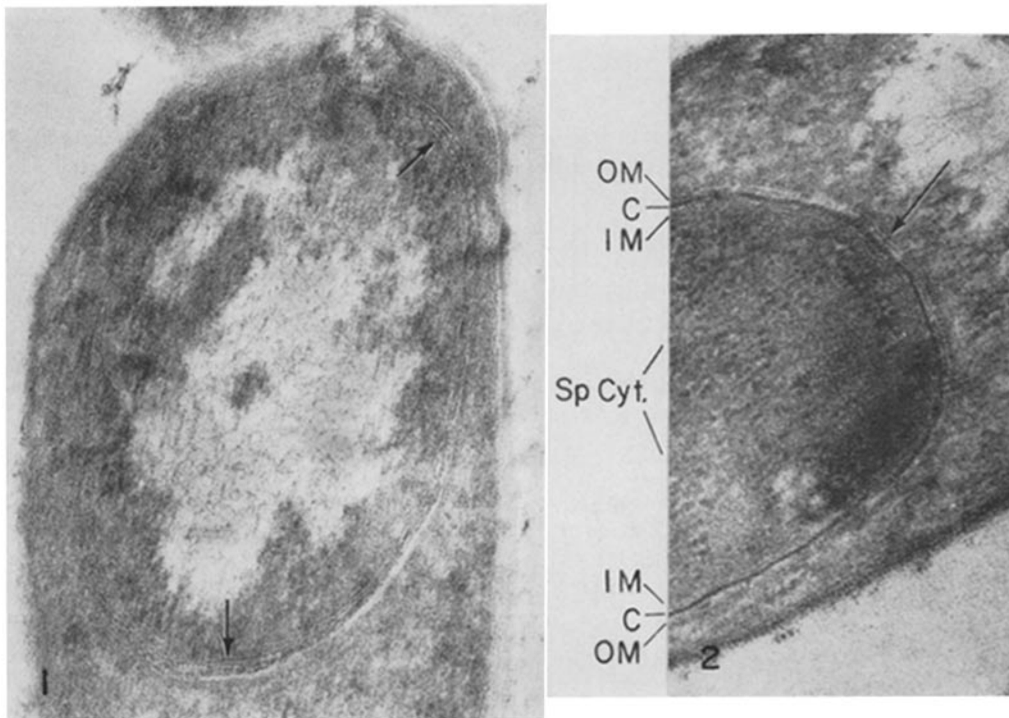


FIGURE 1

The completed double-membraned forespore (arrows) in *B. cereus* var. *alesti* A^+ strain after 12 hours' aeration at 30°C. × 88,000.

FIGURE 2

Early stage in the development of the cortex in *B. cereus* var. *alesti* A^- strain after 13½ hours' aeration, lead-stained section. The early cortex (*C*) is seen as a dense material lying between the inner (*IM*) and outer (*OM*) layers of the double membrane of the forespore. These three layers are clearly displayed at the arrow. The spore cytoplasm (*Sp Cyt*) stains well with lead. × 97,000.

Chemical Analyses

DIPICOLINIC ACID: At each time of analysis ten ml of culture were chilled and centrifuged in the cold. The pellet of cells was kept in the frozen state until analyzed for their content of dipicolinic acid (DPA) by the method of Janssen, Lund, and Anderson (12). Occasionally, the original supernatant, as well as the DPA-containing extract from the cells, was further analyzed with the Beckman Model DU spectro-

photometer in the wavelength region of 260 to 280 mμ.

CALCIUM: The uptake of calcium into the cells was detected by following the incorporation of radioactive calcium ($Ca^{45}CO_3$, Atomic Energy of Canada, Ltd.; converted to $Ca^{45}Cl$ by dissolving in dilute HCl) from the medium (approx. 0.08 microcuries per ml) into the cells. At the time of analyses, samples in duplicate of an appropriate volume of culture (25 to 100 μl) were rapidly filtered through Millipore filters (Millipore Corporation, Bedford, Massachusetts). The thin layer of cells, on the surface of the filter, was washed with four successive 5 to 10 ml volumes of the salts solution used in the culture fluid (7 and 25) which contains 10^{-3} M calcium chloride. The wet filters were pasted with a thin layer of rubber cement onto aluminum planchets and dried. A windowless gas flow counter designed to measure soft radiation (Nuclear Chicago Corp.) was used with a Berkeley decimal scaler unit (Berkeley Scientific, California) to measure the radioactivity present. This rapid and simple procedure, developed by others to study the incorporation of other radioactive compounds (18), gave excellent duplication and greatly increased the sensitivity of the determination of calcium as compared to the more conventional chemical assays. Within an experiment, the number of counts per cell was a direct measure of the calcium content of the cell. The cellular concentration could be calculated, where necessary, from the known specific activity of the calcium in the medium.

RESULTS

Structural Changes During the Development of Refractility; The Formation of the Cortex

In a recent study, the forespore membrane has been shown to arise by an invagination of the cytoplasmic membrane of the sporulating cell to form first a double-layered membrane septum which subsequently proliferates within the cell into a double-layered forespore enclosure (5). Thus, in sections of osmiumtetroxide- or permanganate-fixed cells, the profile of the forespore membrane could be traced back to a doubling of the unit plasma membrane of the cell. The membrane surface facing out toward the cell wall corresponds to the inner surfaces opposing each other in the forespore double structure (5) (Figs. 1 and 8). Only after the complete enclosure of the forespore region by this double membrane, as indicated in Fig. 1, does cortex formation

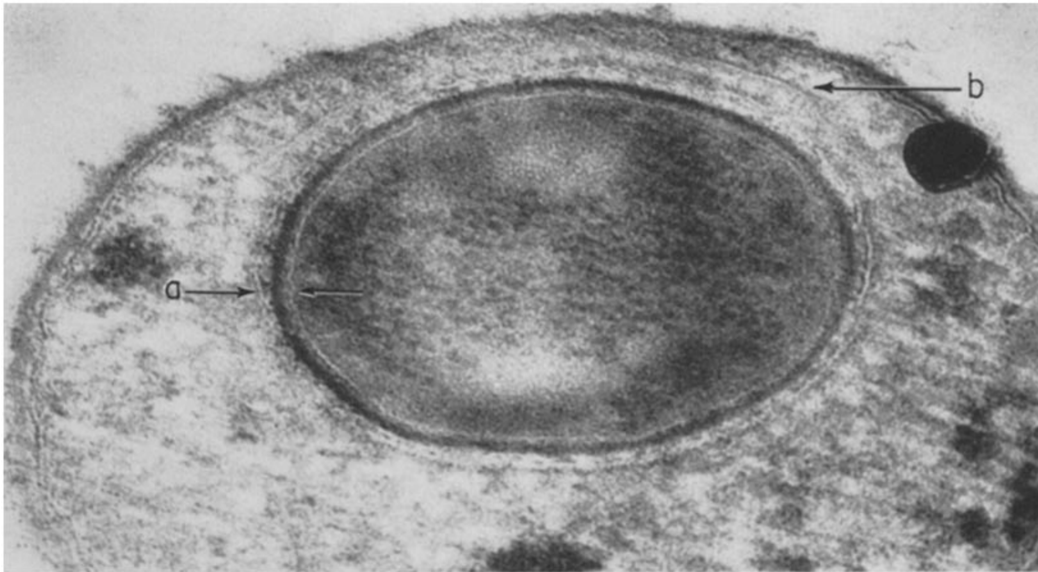


FIGURE 3

A lead-stained section of a cell from the A^- culture at 14 hours. The membrane covering both cortical surfaces is seen at *a*. Some early formation of the spore coat and exosporium can be made out at *b*. The cytoplasmic granules of the spore are stained with lead. × 97,000.

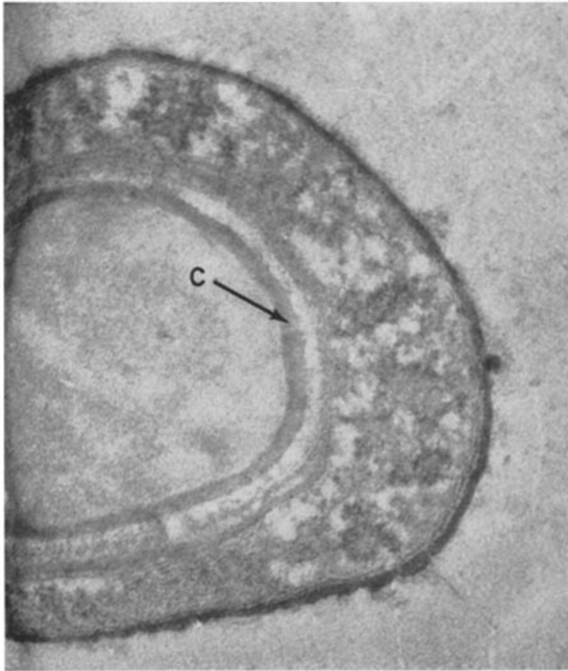


Figure 4

A section of a sporulating cell of the A^- strain at 14½ hours' aeration, stained with phosphotungstic acid. The cytoplasmic granules do not stain but the developing cortex (*c*) does. The developing coat and what may be exosporium can be seen in places. × 77,000.

begin. Then, at the site of these opposing membrane surfaces, cortex material is first detected as a fine, dense, and probably osmiophilic band (Figs. 2 and 8). Over the subsequent 2½ hours this zone, which has an affinity for lead and phosphotungstic acid, increases in width and so further separates the bordering membranes (Figs. 3 and 4) until it has the dimensions of the cortex of the mature spore (Figs. 5 and 6). Finally, as the spore becomes completely refractile, the cortex loses, along with the interior of the spore, much of its affinity for heavy metals (Fig. 7) and appears as a uniform band of low density.

The location of the cortex between two membranes is structurally similar to that of the developing transverse septum of a bacillus. In fact, in some unidentified bacteria, the developing transverse septum even appears to be preceded by the formation of a membrane septum (3).

Early stages of cortex formation are accompanied by a progressive increase in density of the spore region when viewed by dark phase contrast. When the cortex appeared, in sections, to be ⅓ to ½ its final dimension, the spore area, in dark

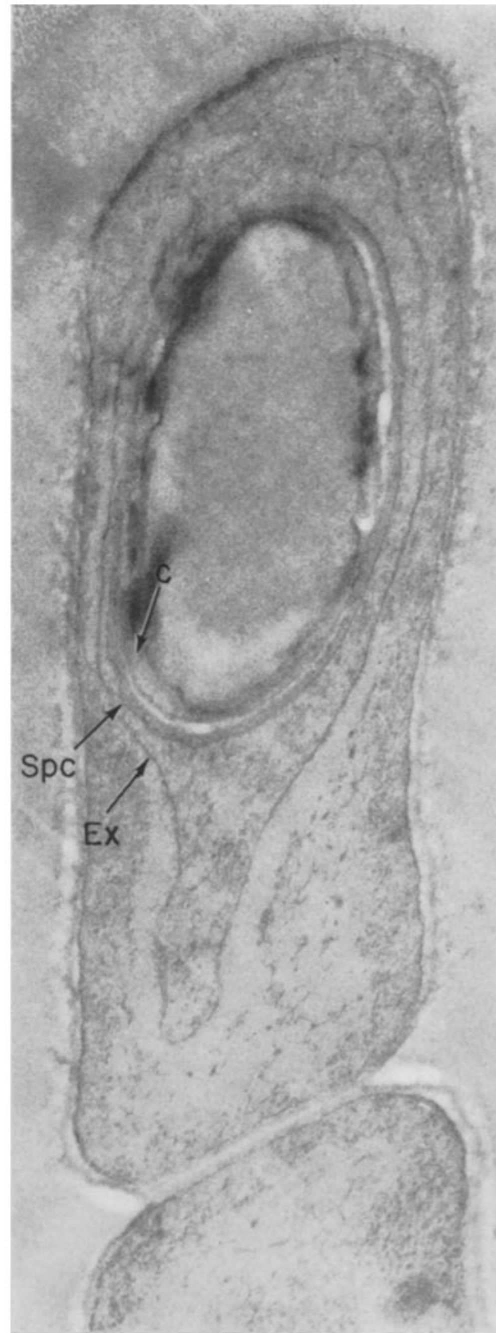


Figure 5

A phosphotungstic acid-stained section of a normally developing spore in the A^- culture at 16 hours of aeration. The late stage of developing refractility characterized by partial staining of the cortex (*c*) and the early signs of lysis; sparse cell cytoplasm and lessened contrast of the cell wall. The cytoplasmic membrane of the cell is still intact. Spore coat (*Spc*) and exosporium (*Ex*) are well developed. × 56,000.

phase contrast, began to turn "white," indicating an increase in refractive index. The whitening begins in the center or thickest part of the spore, and progresses peripherally to include the entire spore body except for a rather diffuse rim (see Fig. 11 *a*, reference 25).

The Formation of the Spore Coat and Exosporium

When the first whitening of the spores appears, that is, when the cortex has developed to approximately one-half its final width, the beginnings of the spore coat can be detected on the periphery of the forespore (Fig. 3). At this time the outer membranous layer of the cortex often appears poorly fixed and gaps containing remnants of membrane may appear between the forming coat and the cortex (Figs. 4 and 5).

The completed coat of spores of varieties of *B. cereus* appears by these techniques to be a single structure composed of two zones of differing density; an outer dense layer composed of two fine lines, some 70 A apart, covers a less dense matrix (Figs. 6 and 7). This inner zone is often difficult to distinguish from the cortex and may appear to merge with it without cleavage, or, to be separated from it by only a remnant of the outer forespore membrane (Fig. 7).

The exosporium, that thin, membranous-like sac which forms the outermost layer of spores of *B. cereus* varieties, develops at approximately the same time as does the spore coat. It can be readily seen in Figs. 6 and 7 and in Fig. 5 in which the cell is showing early lytic changes. Here it projects beyond the ends of the spore into the sparse sporangial cytoplasm displacing the remnant nuclear material.

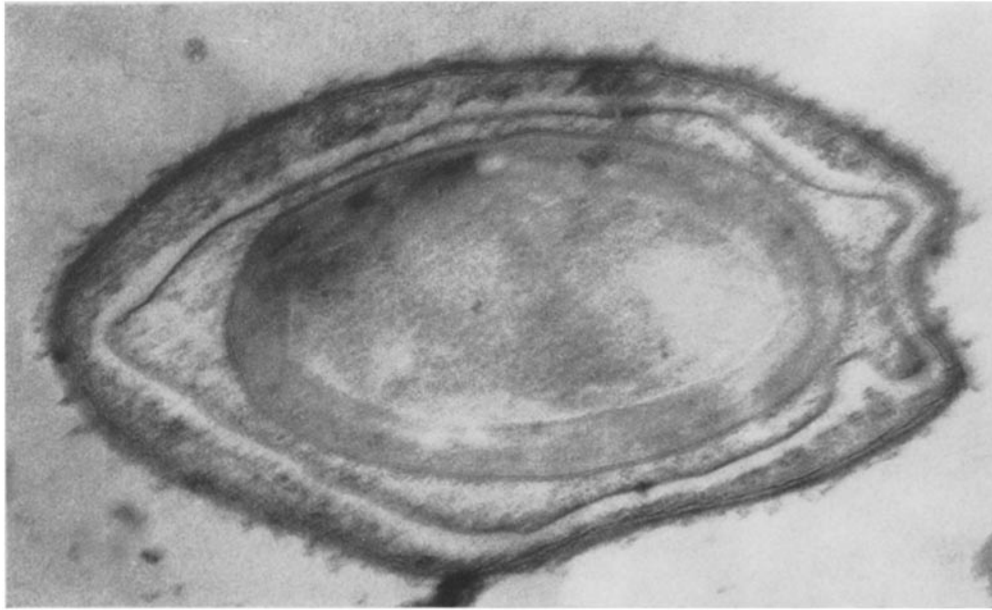


FIGURE 6

A lead-stained section from the same embedding as that shown in Fig. 5. The cortex is slightly stained while the spore cytoplasmic granules are only faintly stained. × 78,000.

During these late stages of cortex development and of spore coat and exosporium formation, the spores in dark phase contrast become progressively more white and lose the dark diffuse border. They now have a characteristic brilliance, and by such cytological standards as their appearance in nigrosin-smear preparations, their affinity for stains and their response to acid-hydrolysis, are fully refractile.

A diagrammatic summary showing the sequence of the formation of the various covers of the spores of *B. cereus* varieties is shown in Fig. 8.

Chemical Changes During Development of Refractility Synthesis of DPA and the Uptake of Calcium

The synthesis of DPA and the uptake of calcium into the spore have been shown to be terminal processes of sporulation (8, 17, 23). However, it has not been clearly shown how these two processes are related in time nor whether their until the whitening of the forespore area has begun. The synthesis of DPA, once begun, continues for a 2-hour period until a maximum level in the cell is reached (Fig. 9). As is true for calcium, all of the DPA synthesized can be found in the free, refractile spores.

The data in Fig. 9 imply that the uptake of calcium commences ahead of the synthesis of DPA. Although this apparent separation of these

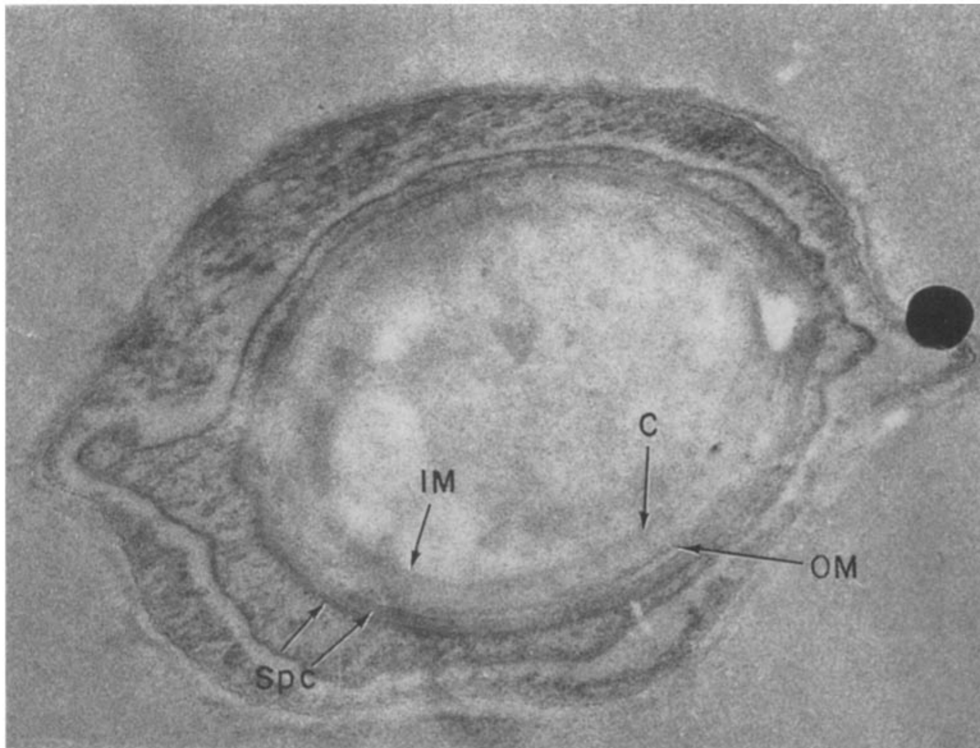


FIGURE 7

Full refractility after 17 hours' aeration. The spore cytoplasm has lost most of its affinity for lead. The density of the cortex (*C*) has greatly diminished. Spore coat (*Spc*), inner (*IM*) and outer (*OM*) layers of double membrane of forespore. × 97,000.

appearance in the spore can be related to the development or alteration of a specific structure. The data presented in Fig. 9 indicate that calcium is present in the sporulating cell in a low but constant amount until the forespore is dense blue in dark phase contrast. As the forespore whitens, calcium is taken into the cell at a linear rate over the subsequent 2 hours. Cessation of intake at this time is abrupt and all the calcium so accumulated is eventually found entirely in the freed spores.

There is no DPA present in the sporulating cell processes could be attributed to the relative sensitivities of the measurements used for the detection of the two compounds, evidence to the contrary was gained in other experiments. Thus, the data presented in Fig. 10 were derived from analyses of a culture of *B. cereus* var. *alesti* which was undergoing highly synchronous sporulation but at a slower than usual rate. In this situation it is readily apparent that the onset of calcium intake precedes the synthesis of DPA by 1 hour. Since this observation is incompatible with the

often expressed view that the calcium intake during sporulation is secondary to the synthesis of DPA, and is due to the strong chelating powers for calcium of the DPA, the experiments to be reported below were completed in an attempt to define their relationship.

ever, it is apparent that traces of the metal were carried into the medium in the organic components and as a contaminant in the other salts. The rate and degree of growth in this medium did not differ from the control, nor was the morphology of the cells altered. A form of sporulation occurred in all cells, but the "spores," when observed by phase contrast microscopy, did not achieve the bright refractility of normal spores (Fig. 11 *a*); rather, the majority of them, even when liberated from the cells, appeared less bright and possessed a heavier or more diffuse border (Figs. 11 *b* and *c*). The subtle difference in refrac-

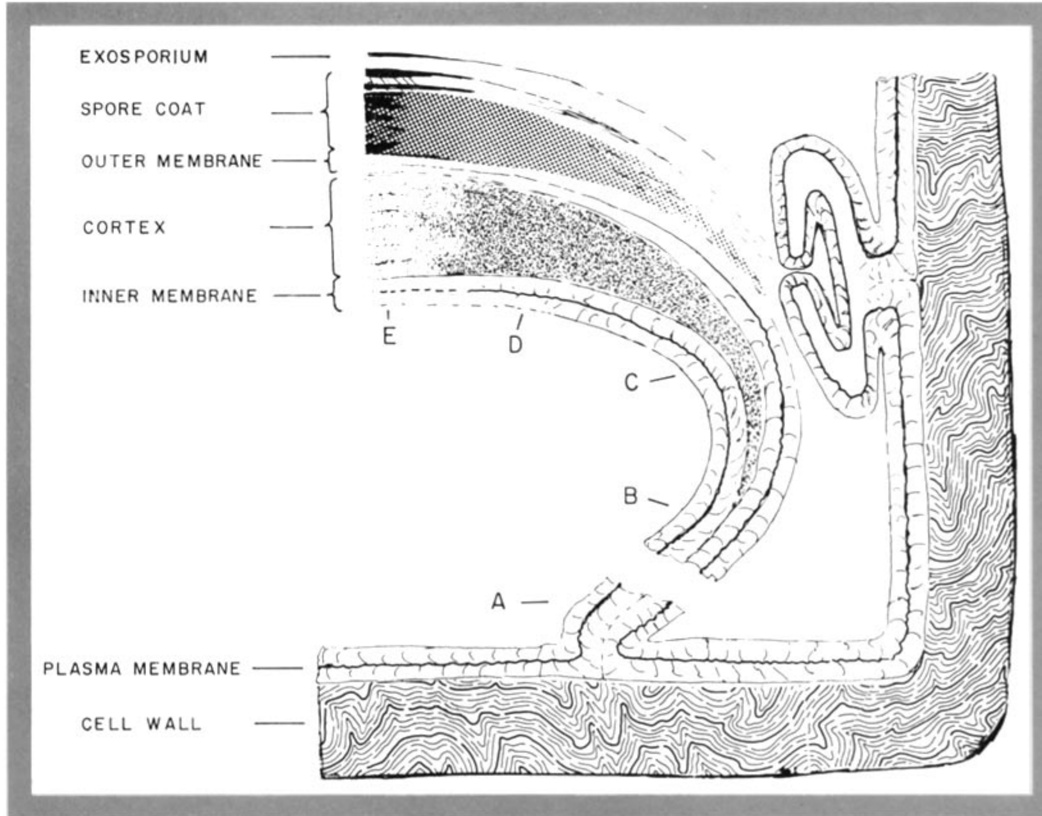


FIGURE 8

A diagrammatic summary showing the profiles found at different times (*A* to *E*) during the development of the layers on a spore of *Bacillus cereus*. The initial development of the spore septum (*A*, see reference 5), proceeds to the formation of an enclosed forespore membrane (*B*). Between the two layers of the forespore membrane the cortex is laid down (*B* to *C*). The spore coat and exosporium are seen when the cortex is ⅓ to ½ its full width (*C* to *D*). Now the spore appears to whiten in phase contrast optics. Full refractility and ripening of the spores (*D* to *E*) is accompanied by further differentiation of cortex, coat, and exosporium (as well as spore soma). A perisporal mesosome (5) is shown at the right.

Chemical Changes During Development of Refractility in Media Deficient in Calcium

The calcium-deficient medium employed by us differed from that used in the control experiments only in that the calcium chloride (10^{-3} M) normally included in the salts solution (7) was omitted. How-

tility between deficient and normal spores could also be detected in nigrosin-smears of water-washed spores (Fig. 12).

These deficient spores, when washed free of nutrients, were unstable to storage in water, that is, many rapidly lost the degree of refractility attained during sporulation (Fig. 11 *d*). As can be seen from the data in Fig. 13, the rate of loss of refractility by the deficient spores varied with the temperature of storage.

Deficiency of calcium in the medium does not, however, lead only to a deficiency of calcium in the resulting spores. From the data presented in Fig. 14, it is apparent that spores formed in the deficient medium have only one-fifth of the control amount of DPA. While synthesis in the deficient medium is linear in rate over a 2¾ hour period, that in the control is nearly exponential. It might be suggested that DPA was synthesized at a normal rate and to the full amount in the calcium-deficient system but that it was not bound in the cell fraction. However, all efforts to locate DPA in the medium during the sporulation process and following liberation of the spores from the sporangia yielded negative results.

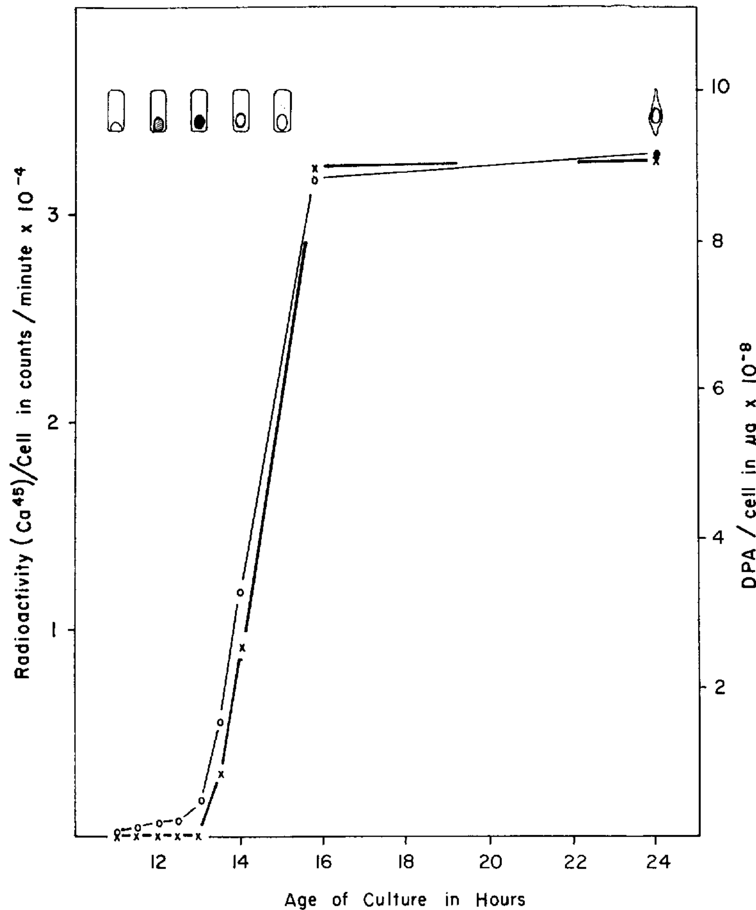


FIGURE 9

The amount of dipicolinic acid (x—x) and the relative amounts of calcium (o—o; measured by intake of Ca^{45} from the medium) in cells of *B. cereus* var. *alesti* A⁻ at various stages of sporulation. These stages are indicated by the line drawings.

In a subsequent experiment varying amounts of calcium in the form of $Ca^{45}Cl_2$ were added to lots of the deficient medium and the calcium and DPA contents of spores formed in these media were analyzed. In the medium with no added calcium, spores were again formed with one-fifth the full content of DPA. The maximum amount of DPA

was synthesized and the near-maximum content of calcium attained when 0.06 μg calcium per ml of culture was added. Below 0.05 μg added calcium per ml, the amount of DPA synthesized and the calcium taken into the spores were proportional to the added calcium (Fig. 15).

These data imply that complete elimination of calcium from the sporulation medium would result in "spores" which contained not only no calcium but also no DPA.[1] However, such a complete elimination, if technically possible, would conceivably affect the balance of enzyme systems not associated with this terminal process.

With the evidence that maximum DPA synthesis was dependent upon the presence of a specific amount of available calcium in the sporulation medium, we next determined whether there was a critical point in time when the presence of calcium would effect maximum DPA synthesis. A full complement of Ca^{45} was added to the deficient medium at different times during sporulation and calcium intake and DPA synthesis were followed as before. When calcium was added while the spores were phase-white with a diffuse rim (13½ hours, Fig. 16) there followed an

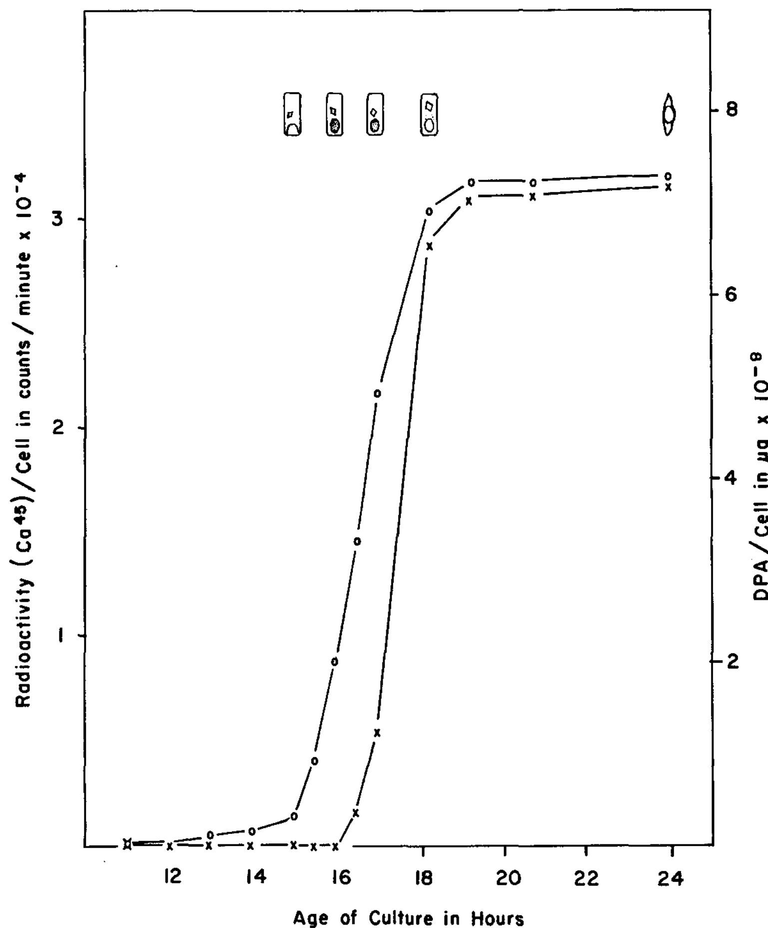
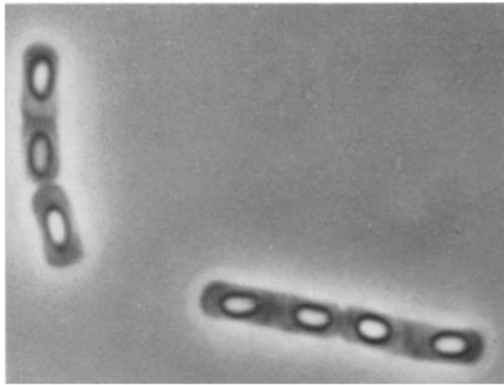


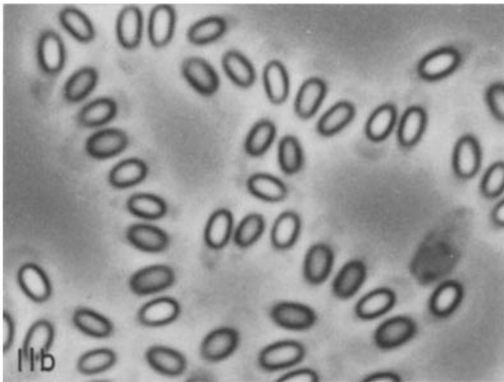
FIGURE 10

The amount of dipicolinic acid (x—x) and the relative amounts of calcium (o—o; measured by intake of Ca^{45} from the medium) in cells of *B. cereus* var. *alesti* A^+ at various stages of sporulation. These stages are indicated by the line drawings.

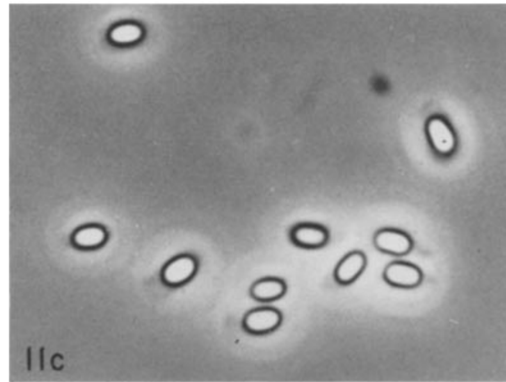
[1] If this be so, then from the data in Fig. 15, it can be calculated that the DPA synthesized in the deficient medium corresponds to a basic calcium content of 0.008 μg/ml.



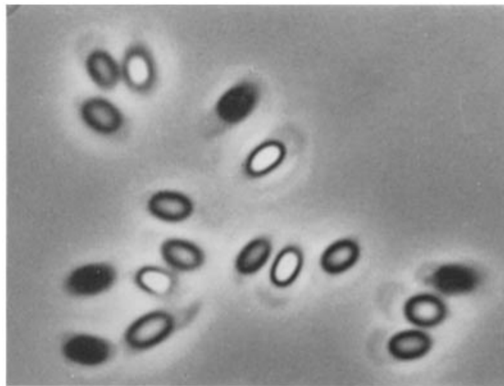
11 *a*. Developing calcium-deficient cells with spores near the maximum state of refractility before cell lysis and spore liberation.



11 *b*. Calcium-deficient spores 2 hours after becoming freed from the cells.



11 *c*. Normal spores—this refractility persists.



11 *d*. Spores from the same sample shown in Fig. 11 *b*, after 24 hours' storage at room temperature.

FIGURE 11

Phase contrast micrographs of calcium-deficient and normal spores *B. cereus* var. *alesti* A^-. $\times$ 3,880.

immediate and normal uptake of calcium and synthesis of DPA. However, if the addition of the calcium were delayed until the spores had reached the maximum degree of refractility possible in the deficient system (16 hours, Fig. 16), then no uptake of calcium and no further synthesis of DPA occurred. The precise, time when this system fails to respond to calcium has not been determined.

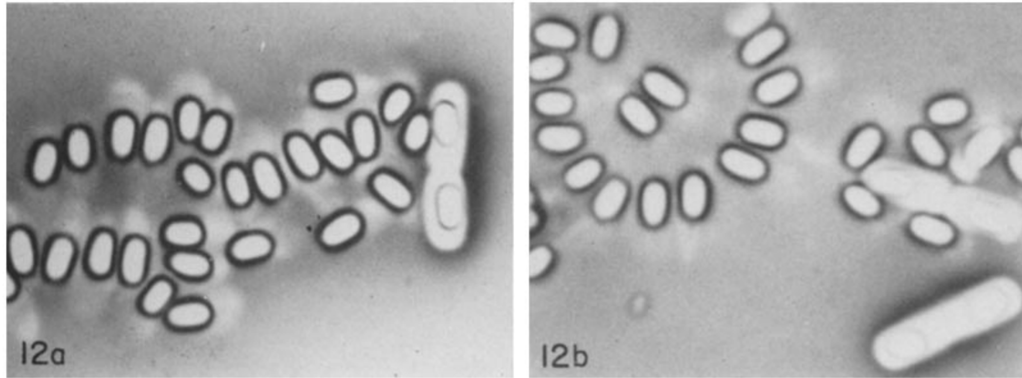


FIGURE 12

A comparison of the refractility of normal (*a*) and calcium-deficient (*b*) spores as seen by air-mounted nigrosin smears. Conditions of smearing, photography, and printing were identical in both. The lessened refractility of the deficient spores is also evident in those still in the sporangia. × 3,600.

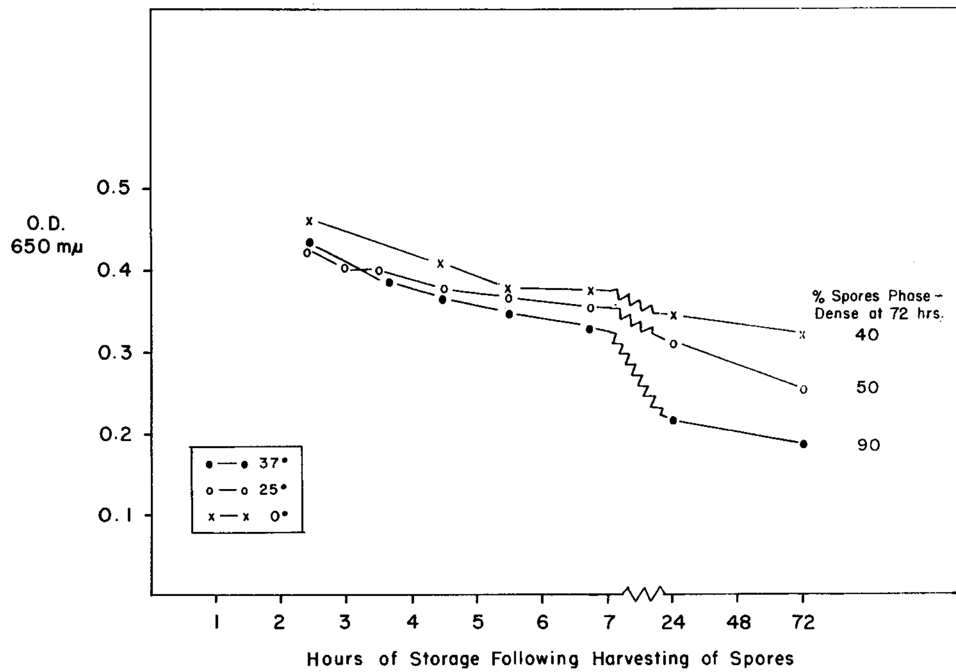


FIGURE 13

The effect of the temperature (●—●, 37°C; ○—○, 25°C; ×—×, 0°C) and length of storage on the refractility of "spores" of *B. cereus* var. *alesti* A⁻ formed in calcium-deficient medium. The change in degree of refractility was measured as a change in the optical density of the suspension at 650 mμ.

Structure of Spores Grown in Calcium-Deficient Media

The studies of normal spore formation reported above lead one to speculate that since calcium uptake and DPA synthesis coincide in time with the formation of the cortex these compounds might form an integral part of this structure (23). Such a speculation is supported by both chemical (16) and morphological (14) studies of spore germination and by earlier electron microscopic studies of methacrylate-embedded spores formed in media deficient in calcium or containing versene or high levels of cysteine (23). There is now, however, some evidence which tends to refute such a speculation (10). Thus, these deficient spores were re-observed with the present improved methods of electron microscopy in an attempt to determine any alteration in structure associated with a deficiency in these compounds.

Spore formation in the deficient system progressed normally through to the formation of the initial spore membranes, the cortex, spore coats, and exosporium. However, many of these spores, even when freed from the vegetative remnants, structurally resembled immature normal spores and indeed were liberated from the lysed sporangia some 2 to 3 hours before the control. Hence the deficient spores often possessed cleavages between the cortex and spore coat, both of which occasionally appeared partially incomplete (Fig. 17). The cortices, like those of immature spores, still stained with phosphotungstic acid (Fig. 19).

The most notable difference, however, was associated with the spore cytoplasm. In spores developing in a complete medium the cytoplasm appears to contain organizations of granules (presumably ribosomes) which are characterized by their marked affinity for lead but not for phosphotungstic acid (Figs. 2 to 4). As the spore becomes mature these structures are less readily stained with lead (Fig. 6) and, with full refractility, completely lose this affinity (Fig. 7). Thus the interior of the ripe spore is of a low uniform density; the contained nuclear material is often barely visible as an even less dense area. On the other hand, the cytoplasm of every spore formed in the calcium-deficient medium retains its affinity for lead, and the granular structures remain prominent (Fig. 18) even in those spores which survive a period of storage (Fig. 20).

This differential staining by lead was most

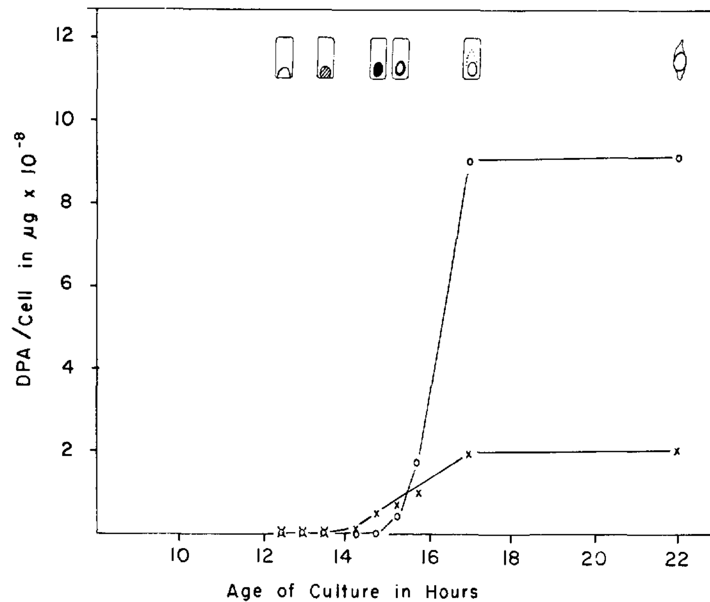


FIGURE 14

The amount of dipicolinic acid synthesized in cells of *B. cereus* var. *alesti* A⁻ during sporulation in normal medium (O—O) and in a medium deficient in calcium (X—X).

striking after 3 to 5 minutes' exposure to lead solutions. Afer a longer period of staining (20 to 30 minutes) the ribosomes of resting spores showed some faint staining.

EFFECT OF ACID-HYDROLYSIS: In an earlier study it was observed that the majority of spores formed in a calcium-deficient medium did not respond in the usual way to acid hydrolysis minutes) were used and gave similar results. However, fixation with osmium tetroxide prior to hydrolysis preserved the protoplast structures of both types of spores. As can be seen in Fig. 21 *a*, there is little alteration in structural arrangements from the unhydrolyzed control (Fig. 20). There is, however, some lead staining at the sites of the chromatin strands.

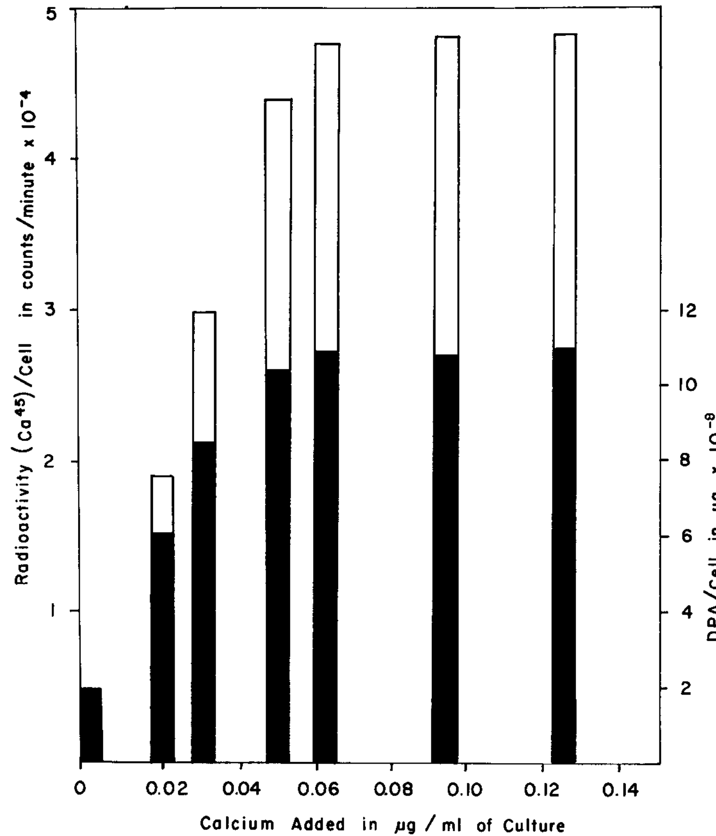


FIGURE 15

The amount of dipicolinic acid (▮) and the relative amount of calcium (▯), measured by intake of Ca^{45} from the medium) in "spores" of *B. cereus* var. *alesti* A^{-} formed in media differing only in content of calcium.

(23). This treatment of normal spores results in an extrusion of the protoplasm to the periphery, which ruptures the cortex (14) and usually leaves much of the chromatin as a displaced mass (19). Such a rearrangement of chromatin or breaking of the cortex does not occur on acid-hydrolysis of calcium-deficient spores. This striking difference is shown in Fig. 21. Both HCl (1 N at 60°C for 10 minutes) and HNO_3 (1 N at 25°C for 30

DISCUSSION

It is now evident from this and other work that the development of refractility in the bacterial spore coincides in time with an intake of calcium, synthesis of dipicolinic acid, and with the appearance of the peripheral envelopes: the cortex, spore coat(s), and exosporium. However, prior to any of these changes, the protoplasm of the future

spore is covered by two layers of membrane, which apparently proliferate from, and are continuous with, the cell cytoplasmic membrane. If one assumes that each of the layers in this double forespore membrane possesses the functional characteristics of a plasma membrane—selective permeability and high enzyme activity (15)—then the subsequent events in the development of refractility become more meaningful. Perhaps the failure of other workers (9, 11, 2, 20) to detect the initial forespore membranes has led to interpretations of sequences of the development of spore layers different from that presented here.

If it can be assumed that each half of the double forespore membrane, besides being functional, has the polarity suggested by its mode of formation, then the laying down of the cortex between the opposed surfaces can be viewed as the formation of a specialized layer in an extra-cytoplasmic zone completely separated from both spore and cell cytoplasm by barriers which are osmotically and presumably enzymatically active. Thus, it becomes possible to understand how the large amounts of calcium and dipicolinic acid which accumulate in the spore at this time could be distributed in either, or both, the spore body and cortex.

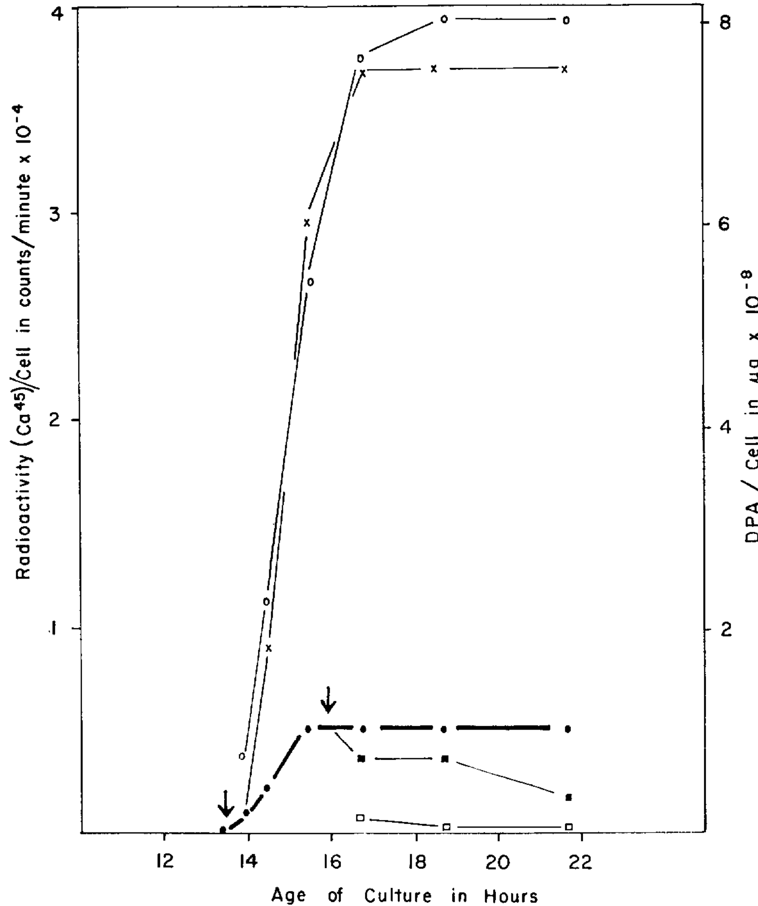


FIGURE 16

The effect of the time of addition of calcium on the subsequent intake of calcium and synthesis of dipicolinic acid in cultures of *B. cereus* var. *alesti* A^- sporulating in a calcium-deficient medium. Synthesis of dipicolinic acid without addition of calcium (control, ●—●); following addition of calcium (to 10^{-3} M $CaCl_2$) at 13½ hr. (×—×); following addition of calcium (to 10^{-3} M $CaCl_2$) at 16 hrs (■—■). Relative intake of calcium following addition at 13½ hr. (○—○) and 16 hr. (□—□).

In earlier studies with methacrylate embeddings,

the cortex in deficient spores appeared incompletely developed (23). These results lent some support to the suggestion that calcium and dipicolinic acid are specifically located in the cortex. However, the present reinvestigation with the improved techniques does not support such a conclusion. Although accurate localization of these calcium in the medium determines the degree and rate of synthesis of dipicolinic acid. That spores formed in media deficient in available calcium were also deficient in dipicolinic acid was suggested by earlier results in spores formed in versene- and cyst(e)ine-containing media and more recently has been demonstrated in studies of "endotrophic"

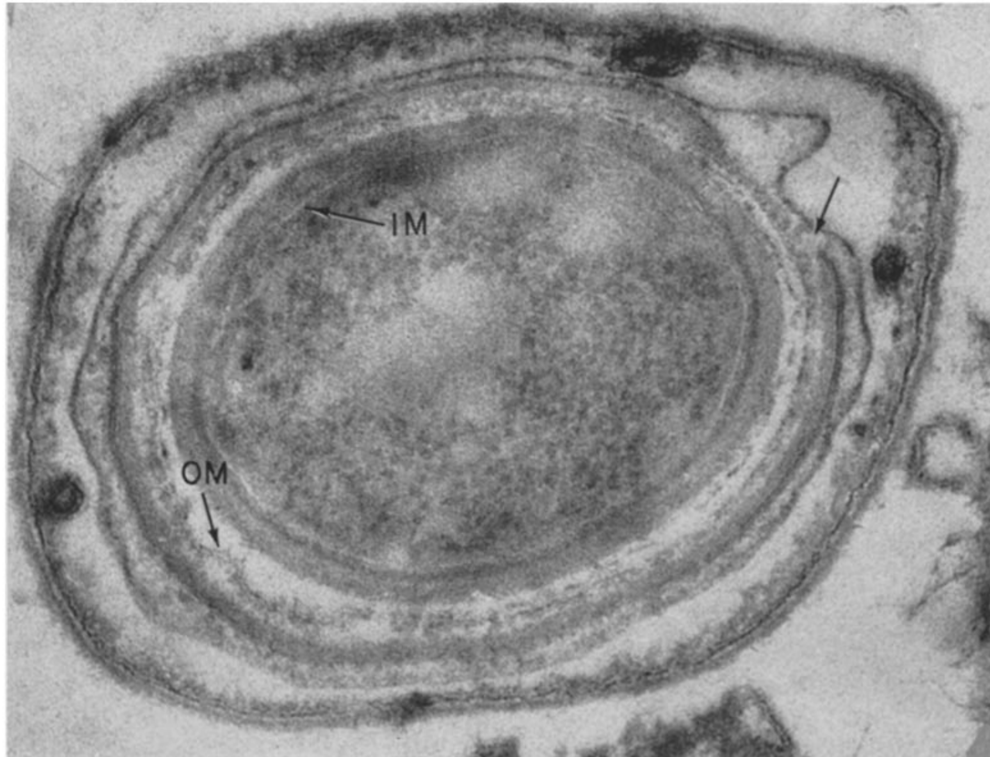


Figure 17

A calcium-deficient spore of *B. cereus* var. *alesti* A^- from a sample where 50 per cent of the spores were free. Sporangial lysis is underway but, as is often the case with calcium-deficient systems, the characteristic structures seen are still those of "unripe" spores: the lead staining of the spore cytoplasm, the instability towards embedding shown in the outer zone of the cortex, and the incompleteness of the spore coat (unlabeled arrow). In places the inner (*IM*) and outer (*OM*) layers of the forespore membrane can be seen on either side of the cortex. This section should be compared to that shown in Fig. 7. × 120,000.

chemical components is not possible from the present results, the observation that the intake of calcium precedes the synthesis of dipicolinic acid is further evidence that this metal does not appear in the spore solely as a consequence of the chelating powers of the acid; thus, it is possible that these components may occupy unrelated sites in the resting spore. However, it must be emphasized that the presence, as well as the quantity, of sporulation (1). In this latter condition, however, it is apparently necessary to have an adequate amount of calcium available in the medium prior to the appearance of the forespore (*i.e.*, "granular cells" of reference 1) if the metal is to direct the maximum synthesis of dipicolinic acid. This is not so for cells sporulating normally in a complex medium. The foregoing results have shown that the low level of calcium in the vegetative and

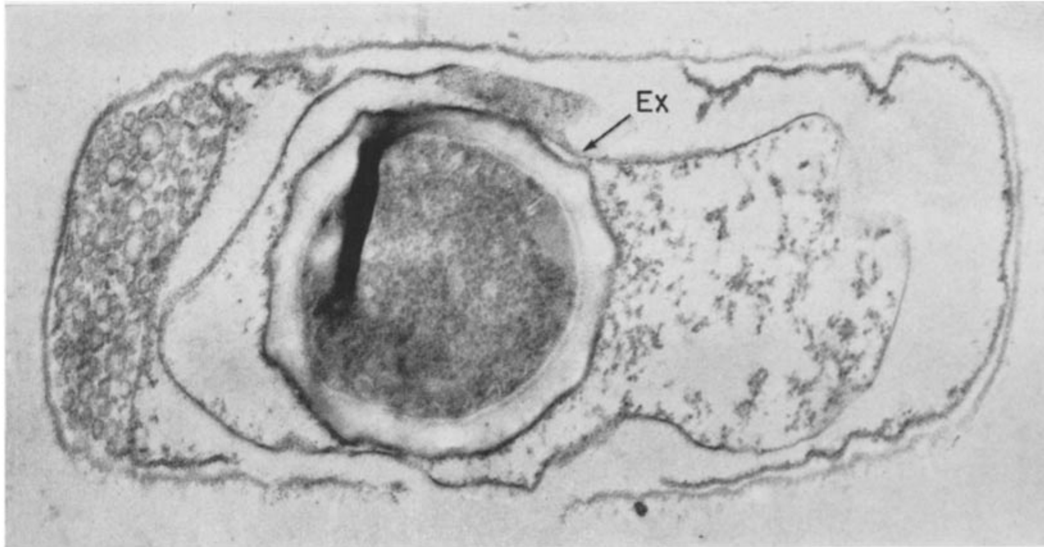


FIGURE 18

Showing the persistence of lead staining even at the time of release in a spore formed in calcium-deficient medium. Internal lytic changes have now left the cell wall and cytoplasmic membrane as sole major cell constituents. The perisporal mesosomes (5) are often, as here, found converted to a mass of circular profiles trapped between the cell membrane and the cell wall of the disintegrating sporangium. In several areas the section plane is parallel to that of the exosporium (*Ex*). × 50,000.

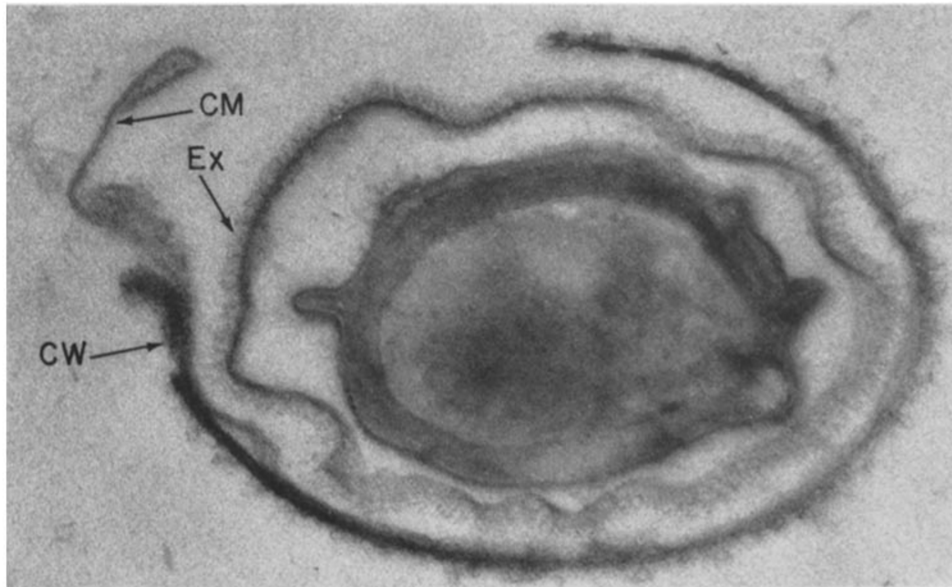


FIGURE 19

An escaping calcium-deficient spore stained with phosphotungstic acid. The cortex appears more heavily stained than that structure in the normal spore, but is well developed. The disintegrating cell wall (*CW*) and cell membrane (*CM*) are clearly distinguishable from the exosporium (*Ex*). × 76,000.

early sporulating cells is constant until refractility begins to develop, after which there is a rapid intake of the metal which is followed by the synthesis of dipicolinic acid. Furthermore, it has been possible to show that addition of calcium to a deficient medium, when the forespores are just beginning to whiten, results in an immediate intake of calcium and a maximum synthesis of dipicolinic acid. However, prolonged delay in the time of addition of the calcium can render the cell incapable of both activities.

calcium-deficient media are unstable in storage and, as in other cases (9), are relatively heat-sensitive. The failure of calcium-deficient spores to burst on acid hydrolysis suggests that, in this state, the products of hydrolysis can more readily escape from the spore and so prevent the rapid rise of internal pressure which in the normal spore leads to the explosive disruption of the cortex. Further comparisons of the fine structure and composition of normal and deficient spores, particularly of their cytoplasmic particles and cortical layers, may help to explain these differences in response to fixative stains and acid treatment.

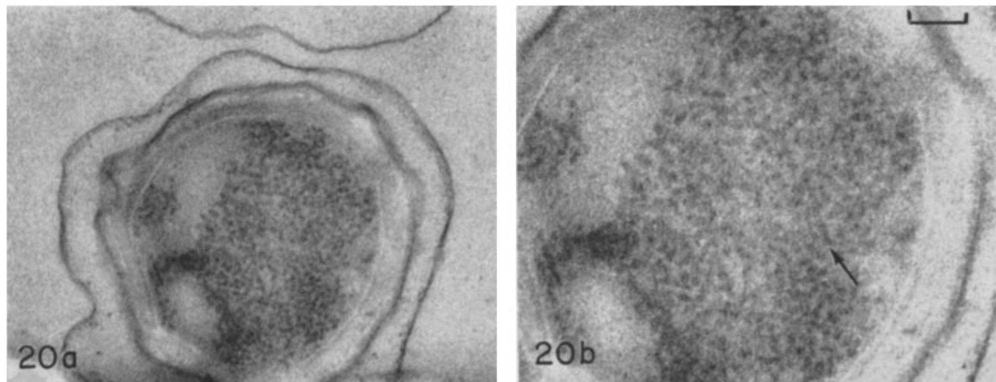


FIGURE 20

A lead-stained section of a calcium-deficient spore still semi-refractile after 72 hours of storage (50 per cent of the spores had autogerminated). The cytoplasmic granules stand out clearly with lead staining. Both the cortex and nuclear regions are unstained. (*a*) × 50,000; (*b*) part of the same micrograph enlarged (× 86,000, marker = 0.1 μ) to show the coil-like array of the stained structures (arrow) some 160 A wide.

The striking persistence of lead staining in the cytoplasm of the calcium-deficient spores might suggest a localization of either of the deficient components in this region. However, the content of these components in fixed material is yet to be investigated. Hence, although it is tempting to speculate that calcium dipicolinate is binding spore ribosomes or the protein-synthesizing mechanism so that lead cannot stain, this result could also be some secondary effect of ripening in the presence of sufficient calcium.

Physiologically, we have found that spores in

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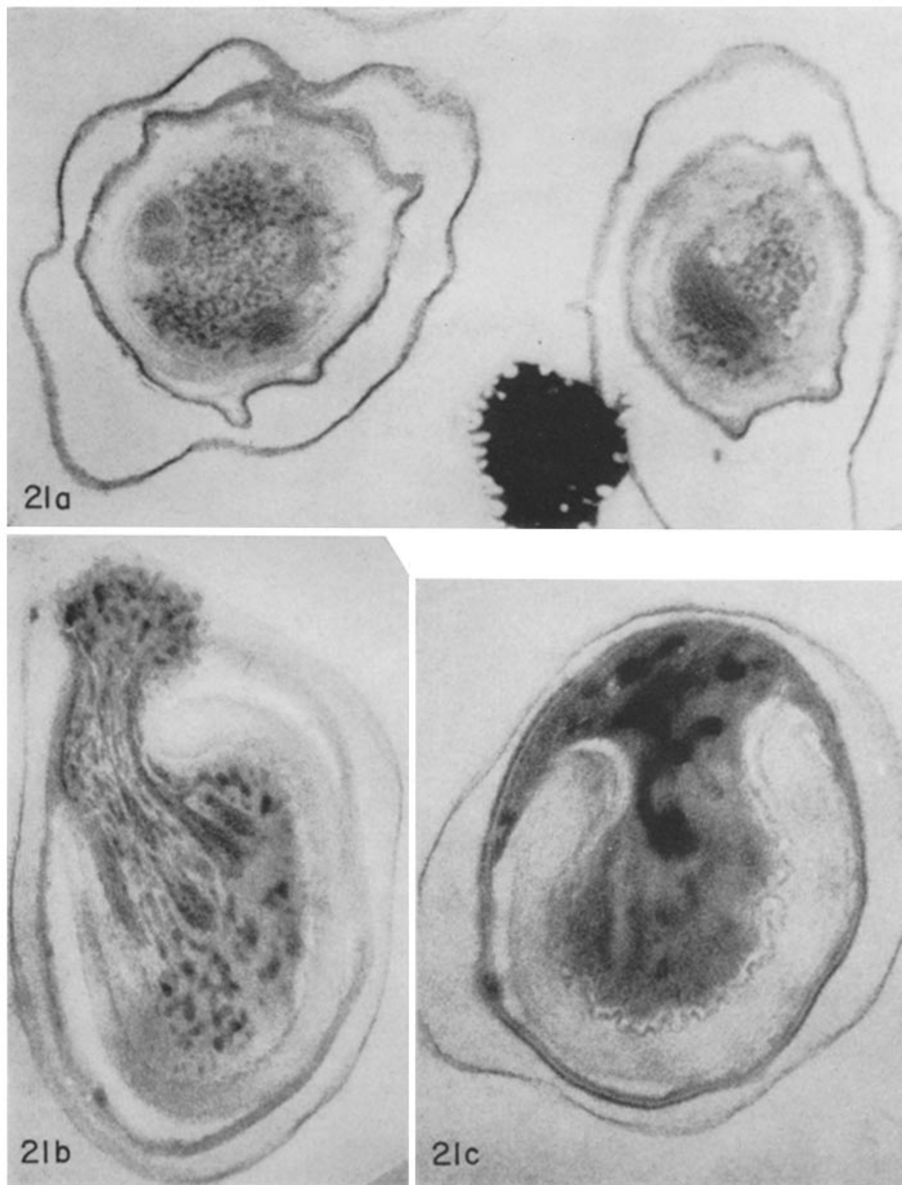

FIGURE 21

Comparison of the effect of acid hydrolysis (1 N nitric acid at 25°C for 30 minutes) on (*a*) calcium-deficient and (*b* and *c*) normal spores. The usual extrusion of the spore soma through the fibrous cortex (14) has not occurred in the calcium-deficient spores (compare with Fig. 20). Spores in *a* and *b* were fixed in buffered osmium tetroxide for 1 hour before hydrolysis and for 3 hours after hydrolysis. Those in *c* were fixed after hydrolysis only. All were washed with uranyl acetate before embedding. Contrast was accentuated by lead staining in *a* and *b*. × 68,000.

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