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Spore Refractility in Variants of *Bacillus cereus* Treated with Actinomycin D

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Refractility as indicated by light microscopy, electron microscopy of thin sections, and freeze fracture etching was increased and maintained in a cortexless mutant, A(−)1, of *Bacillus cereus* var. *alesti* by the addition during sporulation stage 4 of actinomycin D, which prevents the terminal lysis of spore core associated with sporulation in this organism. ^{45}Calcium uptake levels and dipicolinic acid (DPA) content were similarly maintained. The location of these components appears to be in the spore protoplast. In the parent A(−), treated with actinomycin D during stage 4, spore particles with similar morphology to the mutant, that is without a cortex and with the characteristics of refractility, were obtained. A major difference in sensitivity to actinomycin D between the processes of ^{45}Ca uptake and DPA synthesis was observed. Some heat resistance in A(−) made cortexless by actinomycin D could be observed. These studies indicate that the role of the cortex is not to produce the dehydrated refractile spore state but to maintain it.

The cortex of bacterial spores has been implicated in the mechanism of partial dehydration of the spore core and, consequently, in the acquisition of refractility and heat resistance (10, 12, 16). In the widely discussed hypothesis of the contractile cortex, calcium or calcium dipicolinate (Ca-DPA) is assumed to have a cortical location, thus causing the reduction of electrostatic repulsion between the carboxyl groups of the cortical mucopeptide (11, 19). Fitz-James (3) has briefly described the occurrence of refractility and heat resistance in cultures containing spores with much-reduced cortices produced by addition of antibiotics during the sporulation process. In this paper, we reinvestigated this problem using a cortexless mutant, A(−)1, of *Bacillus cereus* var. *alesti* and its parent, A(−), modified by treatment with actinomycin D.

MATERIALS AND METHODS

Organisms and cultivation. A non-crystal-forming strain of *Bacillus cereus* var. *alesti*, A(−), described previously (5), and a mutant, A(−)1, derived from this (15), were used. Growth and sporulation conditions were those detailed by Young and Fitz-James (21).

Microscopy. Phase-contrast microscopy was used to follow the stage of growth and sporulation. The per cent of phase-whitened spores was estimated by simple counting of those spore particles in any stage of phase whitening. Nigrosin smears (4) examined by bright-field microscopy were also used for the detection of refractility. Samples for electron microscopy were prepared essentially by the method of Kellenberger et al. (9) with minor modifications detailed elsewhere (15). Freeze fracture etching of samples quick frozen in Freon 22 was done in a Balzers unit (AG, Furstentum Lichtenstein). A Phillips 200 electron microscope was used for all electron micrographs.

Heat resistance. Diluted samples of culture were heated at 60 C for 30 min, and 1-ml samples were plated with nutrient agar by using the pour plate technique. Unheated samples were plated as a control. Since the cultures were often badly clumped, a 10-sec sonic oscillation period in a sonic oscillator (Measuring & Scientific Equipment, Ltd.) running at 110 v was employed. This procedure broke up the clumps into single cells, some doublets, and occasional chains of three or four cells. Thus, heat resistance values are approximate, since only colony-forming units were counted.

Chemical analysis. Dipicolinic acid (DPA) was determined by the method of Janssen et al. (8). ^{45}Ca (New England Nuclear Corp.) uptake was measured as described previously (22), with slight modifications. A final concentration of 0.2 μCi of ^{45}Ca/ml was used. The membrane filters (Millipore Corp.) were placed in vials with 10 ml of scintillant [4 g of 2,5-diphenyloxazole per liter and 0.1 g of 1,4-bis-(5-phenyloxazolyl)-benzene per liter in toluene]; radioactivity was counted in a Phillips scintillation analyzer.

Actinomycin D (Calbiochem) was dissolved in a small volume of 35% ethanol and kept in a warmed (30 C) flask covered with black paper. At the required

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stage of sporulation, sufficient volume of culture to give a final actinomycin D concentration of 10 μg/ml was added to the darkened flask, and shaking was continued.

RESULTS

Experiments with the cortexless mutant A(−)1. The characteristics of sporulation in this mutant have been described previously (15). Briefly, sporulation as seen by phase microscopy appears to proceed normally to stage 5; however, full refractility is not observed, and a drastic lysis terminates the sporulation process, leaving lysed spore hulls as the final product. No cortical layer is seen in electron micrographs. In the present experiments, actinomycin D was added at various times through stages 4 and 5 to prevent synthesis of lytic enzyme, thus blocking the terminal lysis.

Morphology. Figure 1 at 21 hr of aeration shows the effect of addition of actinomycin D (10 μg/ml) at 30% phase whitening (11.5 hr of aeration). Many of the spore particles are held in a phase-bright stage. The control spores (Fig. 2) have undergone terminal lysis with lysed spore hulls as the final product of sporulation. In most cultures of A(−)1 treated with actinomycin D, at times varying from no phase whitening in the culture to 75% phase whitening, only 50 to 60% of the spore structures remained in a phase-bright state. This presumably is due to lack of complete synchrony in the culture. At any time of addition there would be only a proportion of cells sufficiently advanced to attain phase refractility yet not so far advanced that lysis would occur. Use of the nigrosin smear technique to test for refractility showed that those spores maintained in a whole state by actinomycin D appeared quite refractile (Fig. 3). They also appear smaller and rounder than the normal A(−)1 spore. If addition was delayed until most of the culture had phase whitened, an actual decrease in size of the spore forms resulted.

In electron micrographs of thin sections, refractility is characterized by a lack of detail within the spore core. Concurrently, the deoxyribonucleic acid (DNA) condenses into semi-crystalline masses at the periphery. In the mutant A(−)1, some refractility is seen at peak phase whitening (15). In this work, the degree of refractility was increased and maintained by the addition of actinomycin D, since synthesis of the lytic enzyme was prevented, thus halting terminal lysis. Figure 4 shows a dense, presumably dehydrated spore core within the sporangium which is seen in some areas to be lysing internally. The germ cell wall is seen as a narrow band between the forespore membranes. Freeze fracture etching also revealed the characteristic dense appearance of refractile spore core cytoplasm compared to the more granular "spongy" sporangial cytoplasm (Fig. 5). Figure 6 shows a cell quick frozen at a later time than that in Fig. 5. The sporangial cytoplasm has lysed, leaving the refractile spore core intact. A freeze etching of a fully coated refractile spore of the parent strain A(−) is shown for comparison of the degree of refractility obtained (Fig. 13).

Isotope and chemical analysis. DPA synthesis and ^{45}Ca uptake in A(−)1 approached that of the parent A(−), but these components were lost during the terminal lysis (15). In the present study, ^{45}Ca levels were maintained and sometimes increased by the addition of actinomycin D at appropriate times during the sporulation

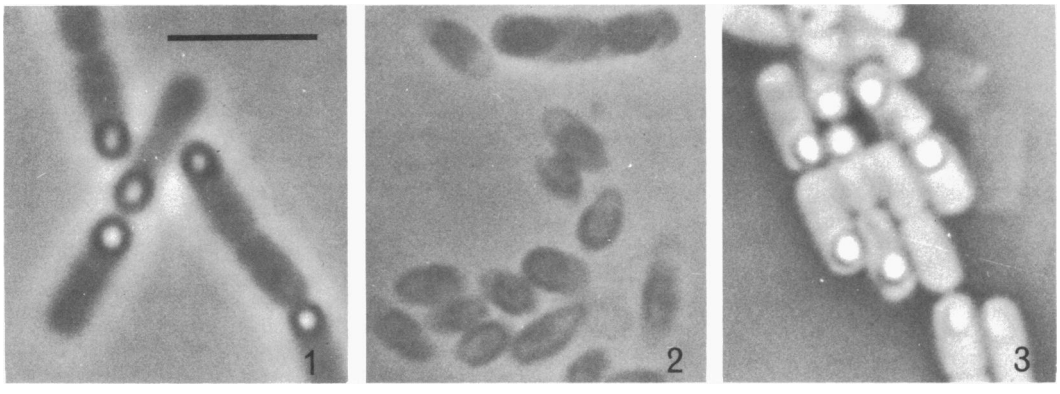

FIG. 1-3. *Magnification as shown by the 5-μm marker in Fig. 1.* Fig. 1. *Phase-contrast photograph of B. cereus var. alesti strain A(−) 1 10 hr after treatment with actinomycin D at 30% phase whitening of the culture. The forespores have remained phase bright.* Fig. 2. *Phase-contrast photograph of untreated A(−)1, taken at the same time as Fig. 1. Mainly lysed spore hulls are seen.* Fig. 3. *Nigrosin smear of A(−)1 photographed 8 hr after addition of actinomycin D at 1% phase whitening in the culture, showing bright refractile forespores in many cells.*

process (Fig. 7). However, even at the third addition, some lysis does occur, causing a slight drop in the ^{45}Ca level. This effect becomes more marked if actinomycin addition is further delayed. The effect of actinomycin D on DPA synthesis was much more marked in that synthesis was inhibited within 90 min or less after addition (Fig. 8), whereas Ca uptake continued for many hours (Fig. 7 and 8). However, the levels of DPA attained were held for many hours. Levels of DPA higher than those shown in Fig. 8 could not be obtained by delaying actinomycin D addition, since later additions allowed some synthesis of lytic enzyme, causing a drop in DPA and ^{45}Ca content and a decline in numbers of phase-bright spore particles.

Heat resistance of these spore forms could not be tested, since germination of A(−)1 or of A(−)1 treated with actinomycin D could not be accomplished.

Studies on the parent strain A(−). Since A(−) does germinate, we reinvestigated the effect of actinomycin on the parent (3) to further study the relationships between heat resistance, refractility, and lack of cortex.

Morphology. The addition of actinomycin D at various times during stage 4 produced spores similar in phase microscopy to those previously described (3). Again, in thin section electron micrographs, the typical refractile cores were seen without a surrounding cortical layer if actinomycin D was added before or during early phase whitening in approximately 20% or less of the culture (Fig. 9). The germ cell wall was formed but coat deposition was prevented. A(−), thus treated, looks very similar to the cortexless mutant at this stage of formation (*see* Fig. 4). If actinomycin D is added after further whitening has occurred, those spores most advanced proceeded to form some cortex and coats (Fig. 10). In a limited number of forespores, an unusual and uneven thickening of what was apparently germ cell wall was seen (Fig. 11). The significance of this is not known. A similar appearance was also seen occasionally in actinomycin D-treated cells of A(−)1. Freeze fracture etching revealed the expected smooth textured cytoplasm of the spore core (Fig. 12), as in the untreated mature spore (Fig. 13).

Isotope and chemical analysis. ^{45}Ca uptake and DPA synthesis were studied in A(−) under conditions in which little or no cortex was formed, due to actinomycin D addition. Figure 14 shows the effect of addition at approximately 8% phase whitening on these processes. ^{45}Ca uptake reached over 80% of the control level, whereas DPA levels remained very low. Other experiments showed that addition of actinomycin D just before phase whitening still allowed ^{45}Ca uptake, whereas DPA content was negligible. To obtain higher levels of DPA, addition had to be considerably delayed, and thus some cortical synthesis occurred.

Heat resistance studies showed that, if actinomycin D was added before whitening in 20% of the cells, heat resistance ranged up to 30% of the control. The number of cells that germinated before heating after actinomycin D treatment was much lower than control levels. Hence, of those cells capable of germinating, usually 50% (at least) were heat resistant. The higher values previously reported (3) could not be obtained if actinomycin D addition was kept to early phase whitening to ensure little cortex formation.

DISCUSSION

There is no doubt that the cortex is an essential spore layer for the maintenance of the resting spore state. The instability of the cortexless mutant (15) and of spores treated with penicillin (3) and cycloserine (12), which hinder cross-linking of mucopeptide, clearly demonstrate this. However, the precise roles of the cortex, calcium, and DPA in the formation of refractile heat-resistant spores are not clearly understood. The "contractile cortex" hypothesis (11) proposes that pressure is exerted on the spore core by a contraction of the cortical mucopeptide, caused by neutralization of the electrostatic repulsions of the carboxyl groups by calcium or calcium dipicolinate. This contraction would dehydrate the spore protoplast. The hypothesis has received support from morphological and chemical studies on the cortex (7, 14, 19) and from the reversion of germination by basic peptides (17). Our results on the cortexless mutant A(−)1, treated with actinomycin D to prevent the terminal lysis, show that refractility with its associated changes in the spore cytoplasm, presumably due to dehydration, can occur in spite of the lack of a cortex. These spores are typically bright, when viewed by phase-contrast microscopy, and typically refractile, when examined by bright-field microscopy, in air-dried and air-mounted smears negatively stained with nigrosin. Moreover, the lack of detail in the spore core and the semicrystalline form of the spore DNA seen in electron micrographs of thin sections, and the "hard" appearance of the fractured spore cytoplasm in the freeze-etch studies bear this out.

A similar development is seen with the parent A(−) treated with actinomycin D during early stage 4 to prevent normal cortical synthesis. Phase-contrast studies (3) and our present freeze-etch and thin-section electron micrographs again

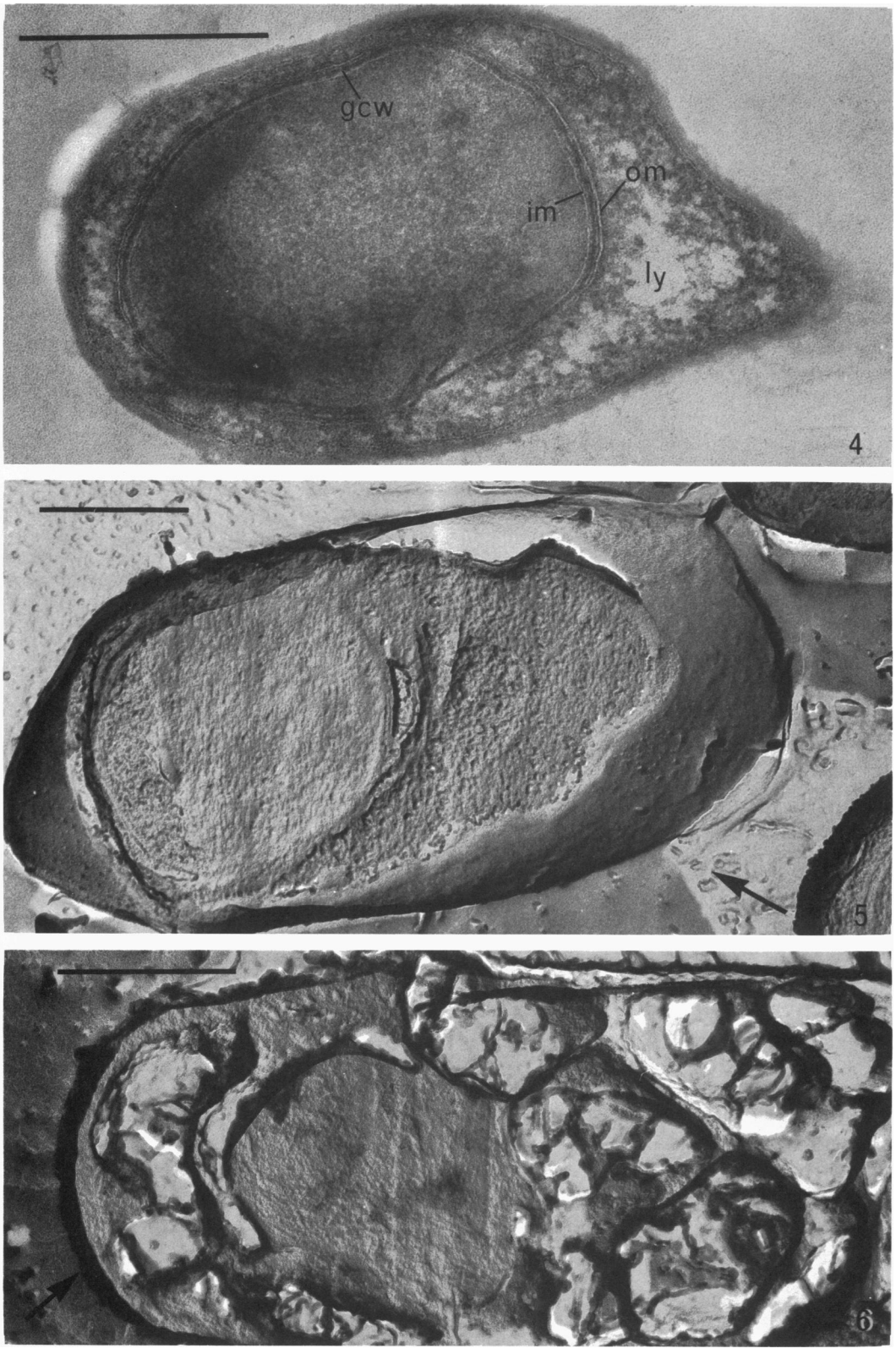

FIG. 4–6.

show characteristics associated with refractility, although we know from the corresponding thin-section micrographs that the cortex is absent in all specimens. Thus, the mechanism for refractility is not dependent on the presence of a cortex and is set up early in the sporulation process, since it is resistant to additions of actinomycin D during sporulation stage 4. Walker (18) has demonstrated that, at the same time, changes occur in the character of the spore ribosomes, making them resistant to digestion from ultrathin sections by ribonuclease. These changes are suggested to be due to core dehydration, again indicative of initiation of the dehydration mechanism early in cortex development.

The studies of ^{45}Ca uptake show that, even with addition of actinomycin D to cultures of both A(−) and A(−)1 before phase whitening begins, uptake of ^{45}Ca proceeds. Since only 50% of A(−)1 spores treated with actinomycin D usually become phase bright, these spores would have a greater content of calcium than that normally accumulated. The process of Ca uptake can be separated from that of DPA synthesis by using actinomycin D addition (Fig. 14). The Ca transporting system must be organized early in stage 4 and be no longer dependent on continued ribonucleic acid (RNA) formation, whereas DPA synthesis appears to be inhibited comparatively soon after actinomycin D addition, showing a dependence on continued messenger RNA production. A similar inhibition of DPA synthesis by actinomycin D has been described by Chasin and Szulmajster (1). It should be noted that, in these cultures with low DPA levels, refractility is still seen (Fig. 9). Earlier work with chloramphenicol (3) led to similar conclusions as to the time of initiation of the refractility mechanism. These studies are in accord with the recent hypothesis of Fitz-James (4) that some of the spore Ca^{2+} is required to bridge and stabilize ionic phosphate groups of the spore protoplast membrane when dehydration occurs, in that Ca uptake would be necessary for refractility to be maintained, whereas DPA would not be required.

Heat resistance cannot be studied in the mutant (A−)1, since it is incapable of germinating. Studies of the parent strain show that some heat resistance (up to 30% of the control) can be obtained in cultures treated with actinomycin D at early phase whitening. However, it is difficult from these results to distinguish the possible need for some cortical development from the definite

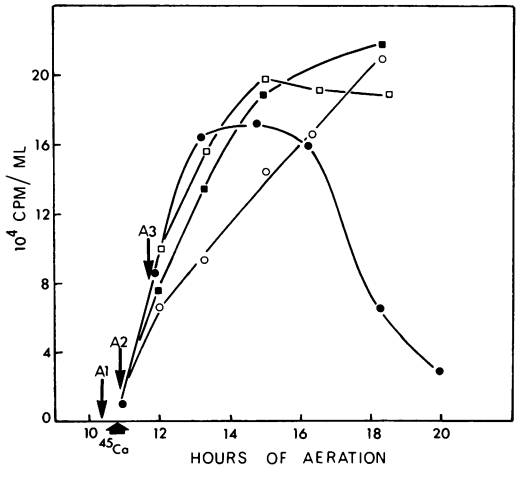

FIG. 7. *Uptake of ^{45}Ca by A(−)1 in control (●) and in portions of the stock culture treated with actinomycin D. Symbols: ○, represents addition at time indicated by arrow A1 (30 min before phase whitening); ■, arrow A2 (5% whitened); □, arrow A3 (35% whitened).*

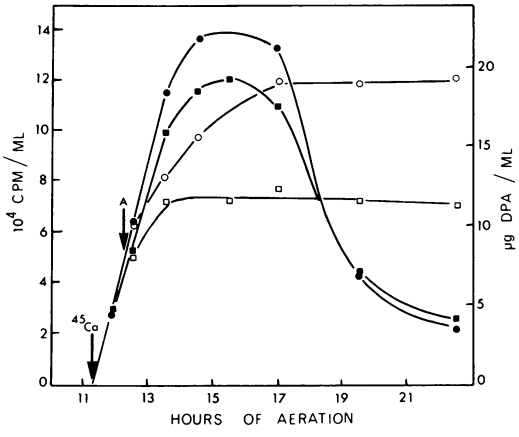

FIG. 8. *Uptake of ^{45}Ca by A(−)1 in control culture (●) and in portion treated at point A with actinomycin D (○). DPA content is shown for control (■) and actinomycin D-treated culture (□).*

FIG. 4-6. *Magnification as shown by the 0.5-μm markers.* Fig. 4. *Thin section electron micrograph of A(−)1 9 hr after addition of actinomycin D at 5% phase whitening. The spore core has become refractile. The germ cell wall (gcw) is seen lying between the inner (im) and outer (om) forespore membranes. The sporangial cytoplasm has partly lysed (ly).* Fig. 5. *Freeze etching of A(−)1 7 hr after actinomycin D addition at mid-phase whitening. The spore core appears more dense in texture than the sporangial cytoplasm. The direction of shadowing is shown by the arrow.* Fig. 6. *Freeze-etching of A(−)1 12 hr after actinomycin D addition just before phase whitening. The sporangial cytoplasm has lysed, whereas the spore core remains intact. The direction of shadowing is shown by the arrow.*

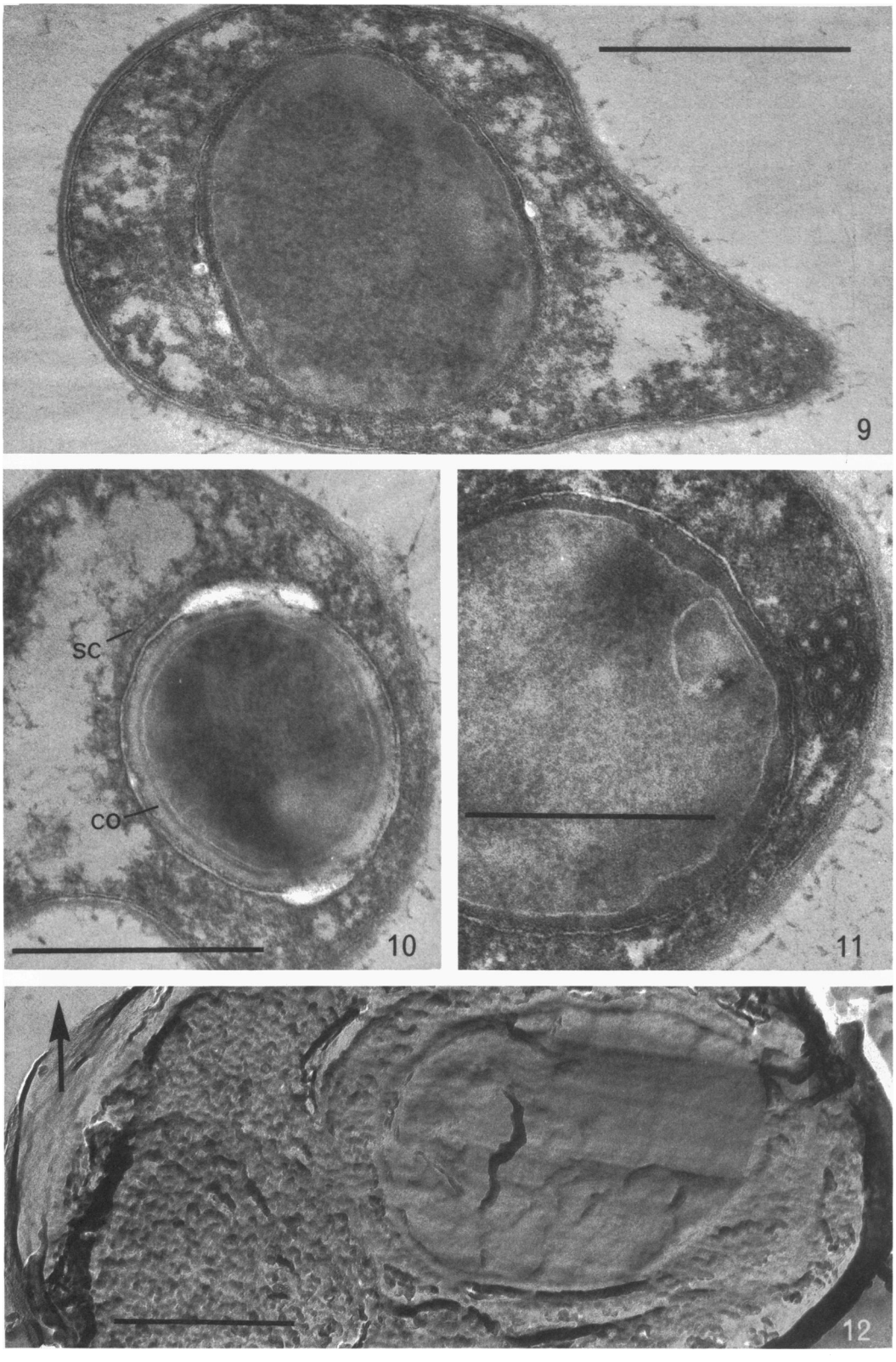

FIG. 9–12.

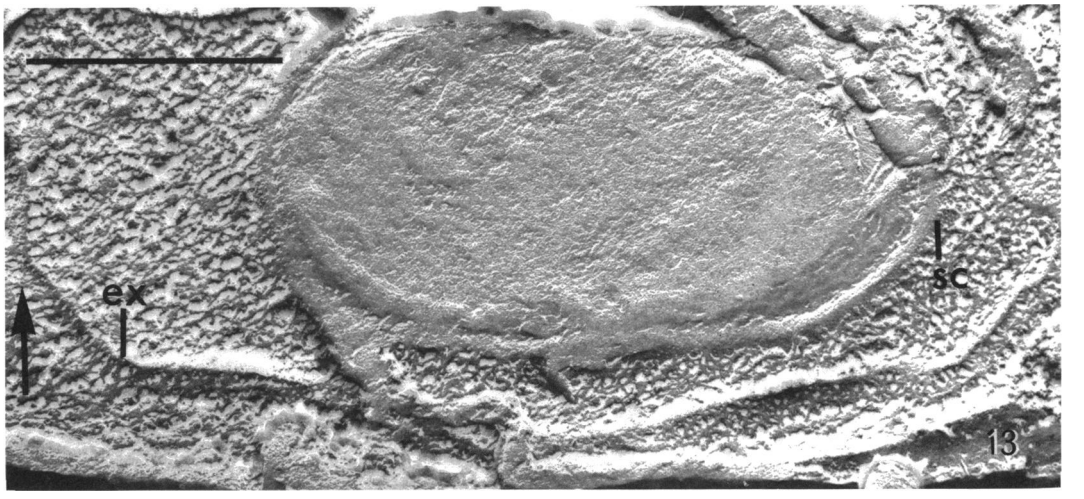

FIG. 13. *Freeze-etching of a mature spore of A(−) showing the typical dense appearance of refractile spore cytoplasm. The exosporium (ex) and some detail of spore coat (sc) are seen. Magnification is shown by the 0.5-μm marker. The direction of shadowing is shown by the arrow.*

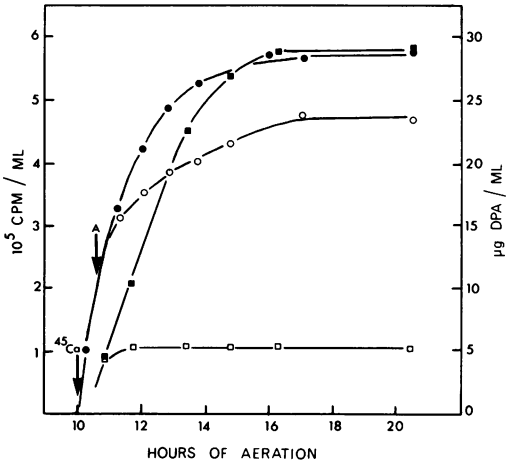

FIG. 14. *Uptake of ^{45}Ca and synthesis of DPA by untreated A(−), and A(−) treated with actinomycin D at time indicated by arrow A. ^{45}Ca uptake in control (●), in treated cells (○). DPA synthesis in control (■), in treated cells (□).*

need of DPA for normal heat resistance (6), since these early additions severely depress the synthesis of DPA (Fig. 14). To obtain higher levels of DPA and consequently higher levels of heat resistance, addition of actinomycin D has to be delayed. This also allows the synthesis of more cortical material. It would appear that heat resistance can be associated with spores possessing limited cortex provided they have sufficient DPA.

The location of DPA in the spore is indefinite although it is generally thought to be associated with calcium (12). Proponents of the contractile cortex hypothesis tend to place it with calcium as the Ca-DPA chelate in the cortex (13, 16, 19) although other work suggests a core location (2). Our results with the cortexless mutant indicate a core location, since the DPA levels attained in the presence of actinomycin D are maintained for many hours, even at a time when we know from freeze-etching and thin-section electron micrographs that the sporangial cytoplasm has lysed (Fig. 4 and 6). The DPA and calcium are presumably held within the spore particle. The actual state of DPA in the core is not known, but it may be in association with amino acids as

FIG. 9-12. *Magnification as indicated by the 0.5-μm markers.* Fig. 9. *Electron micrograph of the parent strain A(−) embedded 9 hr after actinomycin D addition at 1% phase whitening. The germ cell wall has formed but the cortex has not been synthesized. Note the similarity to the cortexless mutant A(−)1 in Fig. 4.* Fig. 10. *Electron micrograph of A(−) treated with actinomycin D when 25% of forespores had become phase white. Cortex (co) and some spore coat (sc) have developed.* Fig. 11. *Electron micrograph of A(−) treated with actinomycin D showing an unusual thickening of what appears to be germ cell wall material.* Fig. 12. *Freeze-etching of A(−) 9 hr after actinomycin D addition at 15% phase whitening. The spore core is dense and fine compared to the sporangial cytoplasm. The direction of shadowing is shown by the arrow.*

suggested by Young (20).

The precise mechanism for dehydration of the spore core remains unknown. From our results, we feel that the cortex is not essential for producing the initial dehydration and consequent refractility and heat resistance, and that it is not the major location of DPA and Ca^{2+}. Rather, its role appears to be that of maintaining and protecting the dehydrated stable state of the resting spore protoplast.

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