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Structure and Morphogenesis of the Bacterial Spore Coat

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INTRODUCTION

Various aspects of spore formation, structure, and germination have been the subjects of several recent reviews (31, 38, 70). There has been extensive work on asporogenous mutants, many of which are not well characterized phenotypically, numerous reports on spore-related events such as antibiotic and protease production (70), and detailed comparisons of various enzymes found in exponentially growing and sporulating cells (49). The latter studies have revealed few, if any, differences. Although further isolation and characterization of mutants altered in specific enzymes will be necessary, it appears that most enzymes found in sporulating cells are the same as those corresponding activities found in exponentially growing cells.

There has also been extensive work on the regulation of spore formation (38), including patterns of nucleic acid synthesis that are presumably essential for the process (20). Although there are some intriguing results, especially in regard to alterations of ribonucleic acid (RNA) polymerase possibly needed for the formation of spore-specific components (54), definitive studies require the understanding of the synthesis and assembly of spore-specific components.

Concerning this last problem, there have been a number of studies of spore components such as mucopeptide, phospholipids, dipicolinic acid (DPA), and exosporium as reviewed by Tipper and Gauthier (82). These have been primarily chemical characterizations and comparisons to analogous components in vegetative cells. There are very striking differences in the mucopeptide structure (89), but the enzymatic and control mechanisms have not yet been elucidated (53). The *Bacillus cereus* exosporium appears to be a unique spore structure, rather complex chemically (56), and an obvious challenge in terms of synthesis and assembly. The difficulty of readily obtaining sufficient material has precluded rapid progress. DPA, another spore-specific component, is synthesized via part of the lysine biosynthetic pathway (1) plus a unique spore enzyme, DPA synthetase (9, 14). The interrelationship of this enzyme with the lysine biosynthetic pathway, especially the differential regulation, is an intriguing problem actively being studied.

Clearly, a detailed knowledge of the synthesis of these spore-specific components is needed to understand the assembly of structures and the regulation of the overall process. As a part of this program, we have been studying the composition and mechanism of assembly of the spore coat layers. A combination of morphological, chemical, and biochemical techniques has resulted in considerable progress in unraveling this complex problem. We feel that progress is sufficient at this time to warrant compilation of

relevant data. Perhaps our understanding of the process of coat formation will serve as a model for the synthesis and assembly of other spore structures and permit us to define, in a limited way, possible spore regulatory mechanisms. Overall, our studies provide some insight into a morphogenetic process in a prokaryotic system.

MORPHOLOGY OF THE SPORE COAT LAYERS

The basic structural features of the spore coat have been well reviewed (43, 71, 88). Here, the emphasis will be on new features revealed by a study of chemically altered and germinating spores, coat mutants, a comparative study of freeze-etched and sectioned preparations, and a survey of some unique crystal or inclusion-forming species.

Freeze-Etch Preparations and Sections of Control, Sulfite-Treated, and Mutant Spores

As first revealed by Holt and Leadbetter (43), spores of several species contain a unique layer beneath the exosporium that has a species-specific appearance. In *B. cereus* spores, this layer is extensively cross patched (CP) (Fig. 1). Holt and Leadbetter found that the CP layer in *B. cereus* and in *B. cereus* var. *alesti* was located above a pitted (P) layer (Fig. 4). We did not find that the revelation of the P layer was a result of extensive etching (8 min). Preparations illustrated here (Fig. 4) were routinely etched for 1.5 min (at −100 C).

The CP layer is composed of stacked, parallel bars or rods that show a repeating dimension of 6.8 to 7.0 nm and form a layer some 10 nm deep. At the edges of a patch, the individual rod appears as a flattened structure 13 nm wide and some 3 nm thick (Fig. 2). These are stacked at a sufficient slope to form the patches as indicated above. With optimal shadowing, there is an indication of repeating units along the rods (Fig. 3).

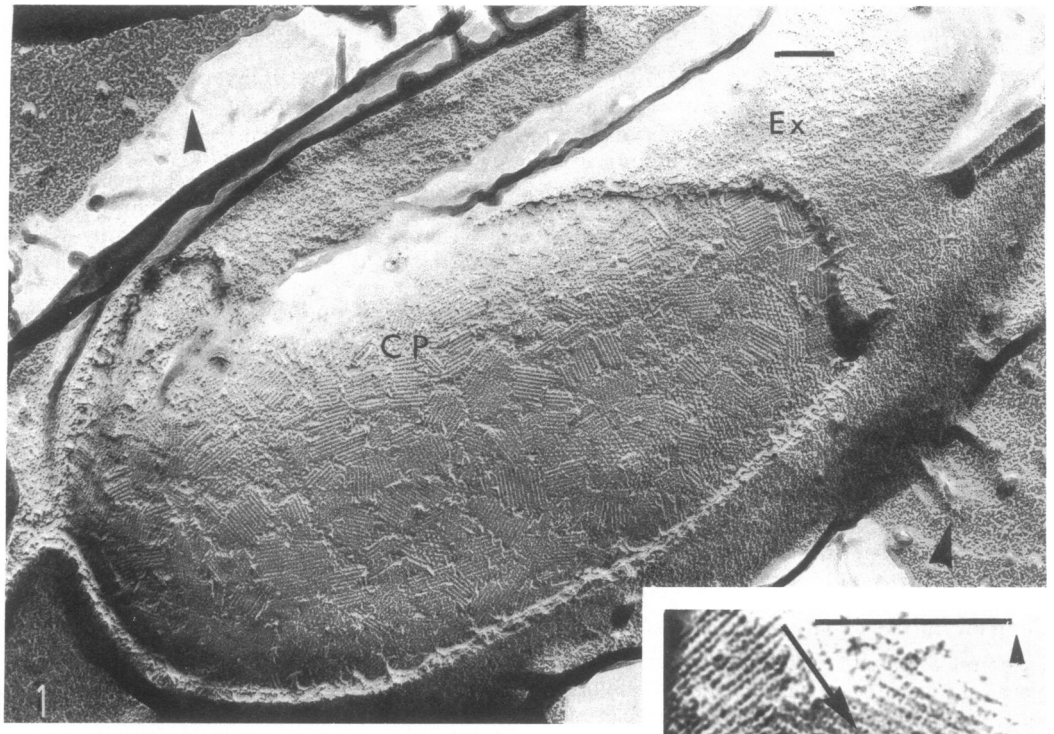
The addition during late coat formation of 0.05 M Na_2SO_3 inhibits the uptake of cystine (2) and formation of the CP layer, and also leads to the deposition of somewhat longer patch pieces in this layer (Fig. 4). In untreated spores of *B. cereus*, the patches are rather uniform in length, between 65 and 90 nm, whereas in sulfite-treated spores, the remaining or surviving patches vary in length from 70 to 140 nm. It should also be noted that spores formed on agar medium have more robust CP layers than those formed in the same medium as an aerated fluid (as in Fig. 4; compare with Fig. 1), even though the total extractable coat protein content is the same (Table 6). The CP layer seems to be most variable among species and conditions of spore formation (Table 1).

The P layer, and the underlying less dense undercoat, are the first coat layers to form during sporulation (Fig. 5). Cleave etchings of this basic coat reveal edges rich in this P layer and devoid of CP (Fig. 6). With the completion of coat formation in wild strains, the P layer is more rarely seen in freeze-cleave-etch preparations, being hidden by under coat on one side or the CP layer on top. In several coat mutants, synthesis of the CP layer is defective or deficient (8). One of these, *B. cereus* 10 LD, is shown in Fig. 7 at the time in stage VI when the CP layer should be forming. Further inhibition of CP has been achieved by the addition of sulfite. The P layer is clearly exposed, covered only by some poorly organized coat fibers.

The under coat surface is rarely exposed by freeze cleavage of whole spores of *B. cereus*. A visualization of the under surface of the coat of a resting spore is shown in Fig. 8. It has no distinctive architecture and, in some areas, it is sufficiently thin to reveal the under surface of the P layer and, where this was torn, the under surface of the CP layer (Fig. 8).

The thin-sectional array of coat layers corresponding to those elucidated by freeze etching are shown in Fig. 11 for an intact spore, and in Fig. 12 for an intact spore coat. The sulfite inhibition of late uptake of half-cystine into the coat, and particularly the CP layer, inhibits the formation of CP and alters the thin-sectional profile of the coats (Fig. 14). In sections near the plane of untreated spore coats, the pattern of CP rods can be seen (Fig. 13). In similar segments of sections of sulfite-treated wild or mutant spores, the P layer may be distinguished (Fig. 15).

The P layer may also be seen to advantage in some negative stain preparations of isolated coats (8) or in incompletely cleaned freeze etchings (Fig. 16). The pits or holes are in rows 6 to 8° off the short axis of the spore some 8.6 nm apart. The rows in another orientation show an 8.85-nm spacing that are some 35° off the long axis of the spore. The diagonal spacing of the holes is about 10.1 nm center to center. Each hole or pit is from 6 to 7 nm in diameter, which may mean that 35 to 40% of this layer is composed of holes or filled pits (on the basis of model construction). Despite the presence of cytoplasm between the outer forespore membrane and under coat during formation (Fig. 5), there is no evidence for a layer of trapped cytoplasm becoming the inner coat of mature spores as proposed by Ohye and Murrell (61). In addi-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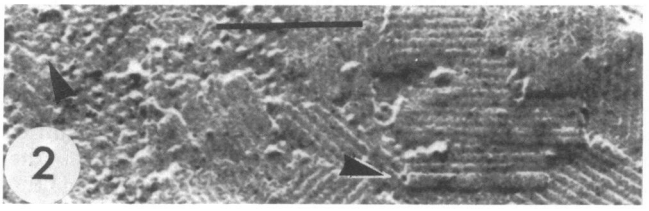
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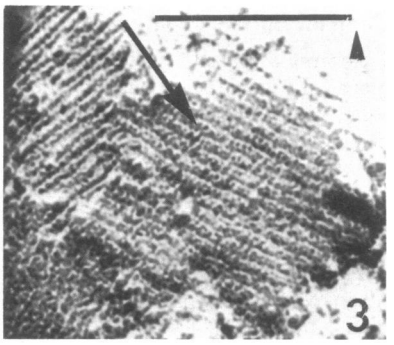
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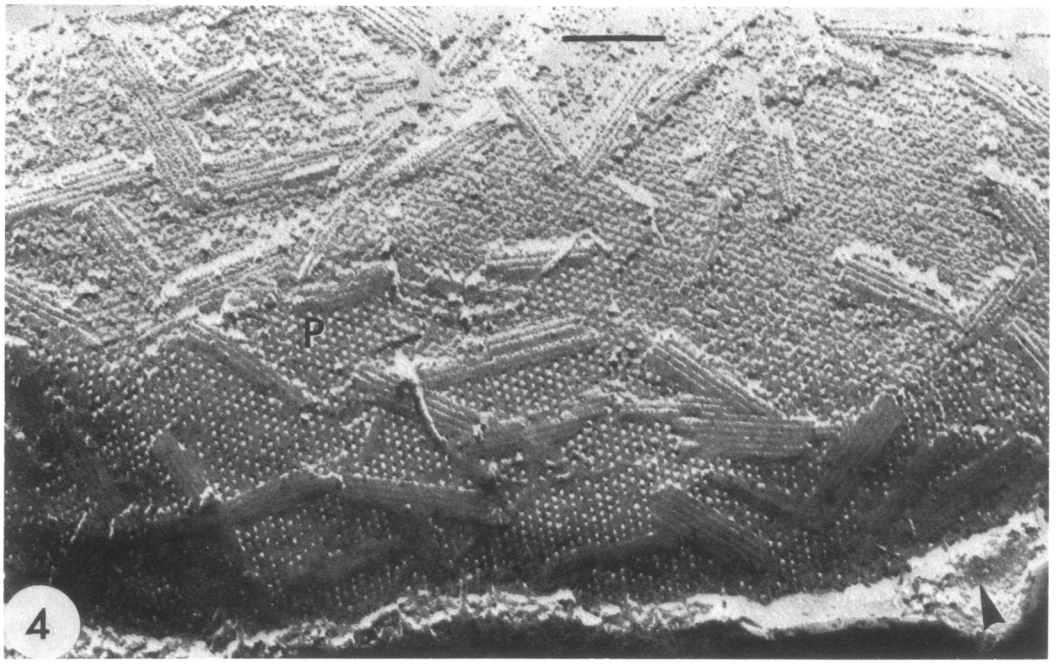
P
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TABLE 1. *Summary of the morphological features of various spores*

Strain	Presence of coat layers			Crystal or inclusion location	Source	Other features
	CP	P	Under coat			
Bacillus cereus T	+	+	+		Fig. 1-3, 11, 13	
B. cereus T, germinated	+	−	Depleted		Fig. 8-10, 20, 21	
B. cereus, SO_3 treated	Weak	+	+		Fig. 4, 14	
B. cereus, extracted with DTE	+	?	Depleted		Fig. 19, reference 5-7	
B. cereus, extracted with DTE-SDS	Modified?	−	−		Fig. 17a, b, 18, reference 5-7	
B. cereus 10^{LD} (lysozyme-dependent germination)	Weak	±	±		Fig. 7, reference 8	
B. cereus 13^{LS} (lysozyme sensitive)	Weak	±	±		Reference 8	
B. cereus 10^{ts} (temp-sensitive GSH reductase)[a]	Weak	+	+		Fig. 15, 21, reference 15	Altered coats and lysozyme sensitivity in spores produced at 38 C
B. thuringiensis var. *alesti*	Weak[b]	+	+	Outside exosporium	Fig. 32, 33	
B. cereus var. *fowler*	?[c]	+	+	Within exosporium	Fig. 34, 35, 37	
B. thuringiensis var. *finitimus*	?[c,d]	+	+	Within exosporium	Fig. 36	
B. popilliae	?	?	+	Within exosporium	Fig. 38-40	Very dense outer coat like *B. subtilis* and related species (43)
B. thuringiensis var. *schwetzova*	Sparse	+	+	Outside exosporium	Fig. 41-43	Accessory inclusion with coat-like covering
B. medusa	−	+	+	Outside exosporium	Fig. 44-48	Formation of the inclusion at late vegetative stages
B. cereus var. *lewin*	?[d]	+	+	Within exosporium	Fig. 49, 52	

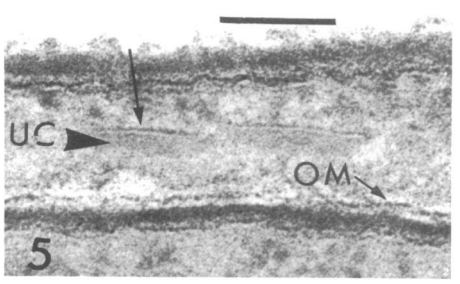
[a] GSH, Reduced form of glutathione.
[b] Less-defined rods (72); always weaker than in acrystalloferous varieties.
[c] Only a few fibers form under exosporium (72).
[d] Absent or not seen by freeze etching.

FIG. 1. *An intact, untreated spore of B. cereus harvested from an agar-grown culture showing the exosporium (Ex) cleaved off the underlying outer CP layer of the spore coat. This is interpreted as layer CP in the corresonding thin section (Fig. 11). In some regions (arrows), the underlying P layers show through the CP covering. ×78,500. Bar represents 100 nm in this and subsequent electron micrographs and line drawings.*

FIG. 2. *A portion of CP layer in B. cereus at high magnification showing the form of the flattened rods that stack to make this coat layer. The rod indicated by the arrow is 13 nm wide by 105 nm long. On the left portion of the figure the underlying P layer is exposed. ×191,000.*

FIG. 3. *Optimally shadowed part of a freeze-etch preparation of B. cereus spore coat showing repeating ringlike units (arrow) along the edge of the stacked CP rods some 4.6 to 5.0 nm apart. ×253,000.*

FIG. 4. *A spore of B. cereus treated with 10 mM Na_2SO_3 during coat formation showing the marked inhibition of the CP layer and revealing the underlying P layer of the spore coat. This corresponds to the double-track layer seen in thin sections. The long axis of the spore runs from left to right. ×136,000.*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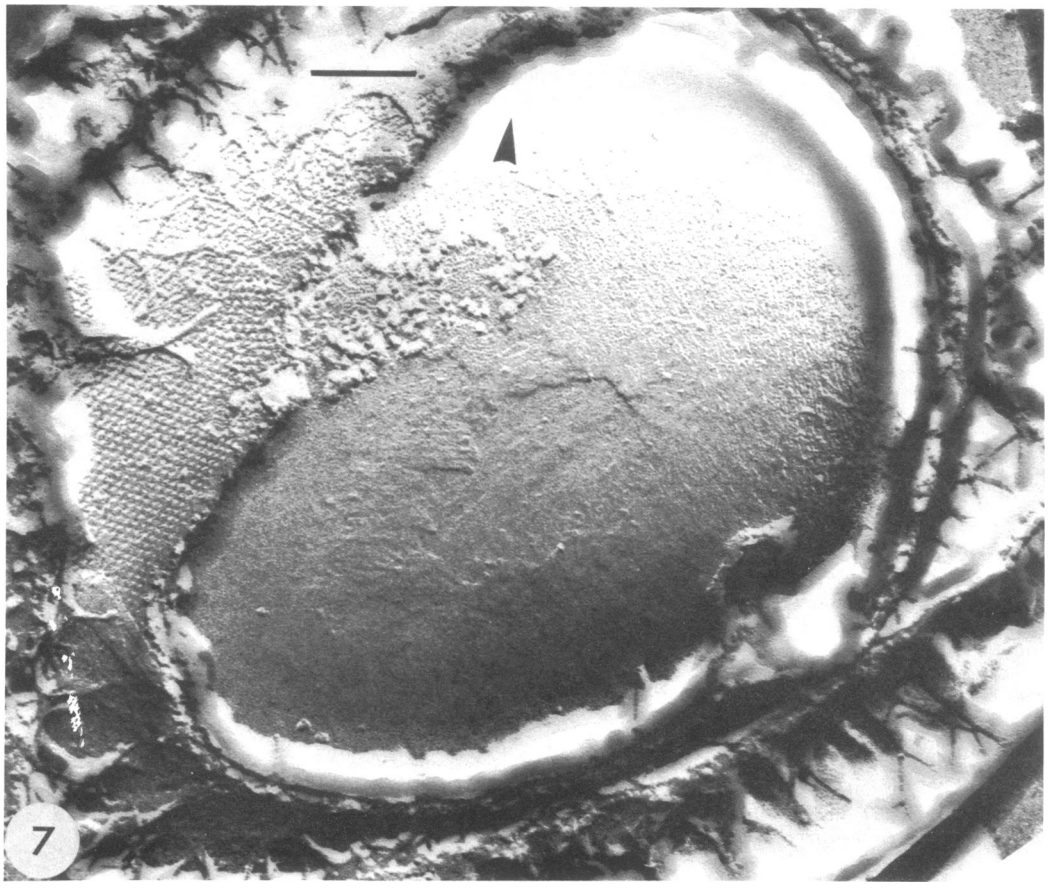
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tion, Walker et al. (88) found no labeling of *B. subtilis* spore coat layers with ferritin-labeled antivegetative protein antibody and only occasional labeling of *B. cereus* coat fractions (87). The latter may be due to adhering cortical material. A summary of the *B. cereus* morphological data is diagramed in Fig. 51 and 52.

Germinating Spores

Coat alterations on germination help to define the morphological layers, as well as the changes associated with germination. The most notable change, as observed in freeze etches (Fig. 9 and 10), is the early loss of the pitted pattern, thinning of the under coat (see references 40 and 69), and the persistance of the half-cystine-rich (sulfite-sensitive) CP layer. Careful comparison of thin sections of coats of germinated and resting spores support the freeze-etch observations and pinpoint the localization of the sectional coat profile. The coat profile of a germinated *B. cereus* spore is identical to that of the resting spore except for the loss of density of the inner half of the P layer (compare Fig. 20 with Fig. 11 and 12). Similarly, in a temperature-sensitive mutant of *B. cereus* formed at nonpermissive temperatures to produce coat-defective (marked absence of CP layer) and lysozyme-sensitive spores (mutant 10^{ts}, see Table 1), the same loss of the dense inner track of the P layer is seen (Fig. 21). Decrease in under coat density may also occur. From the freeze-etch studies alteration of the P layer appears to be a very early germination change.

This loss of pitted freeze-etch pattern and the inner dense profile of the germ cell wall (Fig. 21), raises the possibility that, as in phospholipid membrane cleavage (11, 59), the freeze-etch cleavage plane lies in the low-density zone (perhaps hydrophobic) between the two dense lines of the P layer and that the hexagonal-pitted sheet is the lower leaflet of this triplet. In the diagram presented (Fig. 52), the holes are shown traversing the entire layer.

Comparative Structural Features of Various Species

Structures of other spore coats indicate several similarities and differences to those of the *B. cereus* group (see reference 43). The spores of *B. megaterium* appear to exist in two-coat forms; those of *B. megaterium* KM and 899a have a single-multilayered covering in which under coat, P, and CP (outer) profiles can be seen by thin-section preparations (Fig. 22) and which in surface replica shows a very loose CP deposit of coat fibers on the curved surface (Fig. 23). On the other hand, most other species of *B. megaterium* form a dense outer coat or hull, as well as an inner coat basically similar to that on KM and 899a (Fig. 24).

The outer hull appears to be formed by the deposition of a dense matrix between two structured layers (Fig. 24 and 25). Solubilization of the spore coats of *B. megaterium* KM (see next section) offer a coatless spore useful in spore protoplast studies (24). However, the outer dense covering of the double-coated spores resists the action of coat-solubilizing agents. The inner coat is essentially dissolved by dithiothreitol-sodium dodecyl sulfate (DTE-SDS) at pH 10.0 (Fig. 25). The persistent outer coat interferes with spore protoplast fractionations in these types of spores.

Freeze cleavage of a double-coated *B. megaterium* spore displaying the outer coat, inner coat, and multilayered cortex is shown in Fig. 26. The repeating structure of the outer coat surface shown at the extreme left of Fig. 26 is more clearly seen in Fig. 27. The fine repeating pattern of the spore covering (arrows in Fig. 24) is seen to be fitted as a patch work array over the curved surface of the spore. Although the protein nature of the inner coat for this species has been demonstrated, the chemical nature of the dense outer hull has yet to be studied.

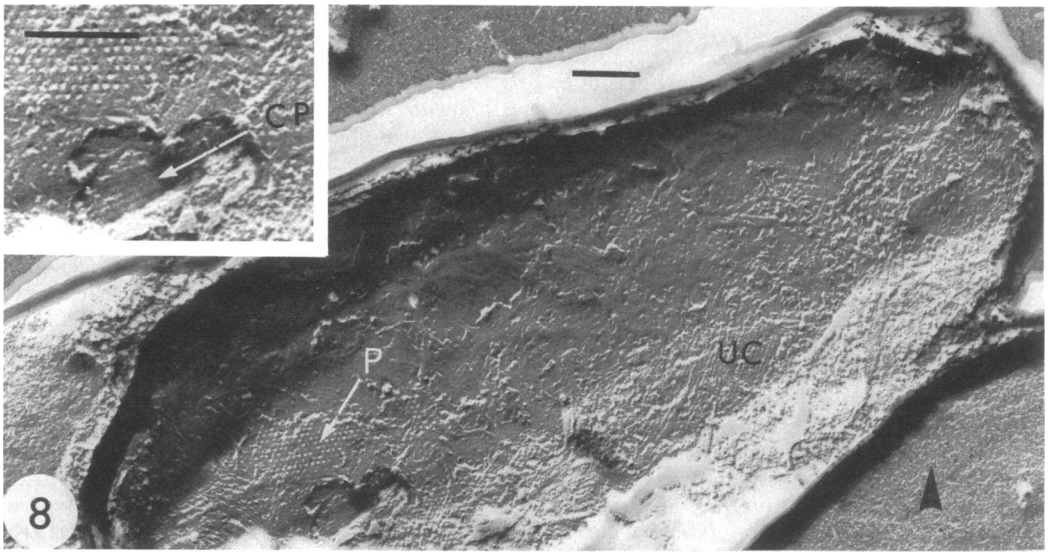
The spore coat of *B. subtilis* is also composed of two components of different density, a dense outer coat and a lamellar inner coat closely applied to the cortex (Fig. 28 and references 43, 71).

The late addition of sodium sulfite to sporulating cells of *B. subtilis* reduces the density and, in some spores, the thickness of the outer coat revealing the loose lamellar array within (Fig. 29) and the closely aligned surface lamellae (Fig. 30). It would appear that cleavage just through or into this surface layer (exosporium

FIG. 5. *Coat formation in B. cereus. Early stage IV in a B. cereus showing that the appearance of the spore coat in the sporangial cytoplasm consists of a double dense line (unlabeled arrow) and associated under coat (UC) with no close association with the outer forespore membranes (OM). ×145,000.*

FIG. 6. *Coat formation in B. cereus. Freeze etching of later stage IV showing the cortex (Cx), exosporium (Ex), and the extensive P layer of developing spore coat devoid of CP material. ×134,000.*

FIG. 7. *Coat formation in mutant B. cereus 10LD (see Tables 1, 8) that forms lysozyme-sensitive spores and shows a deficiency in the CP layer (8). Further inhibition of the CP layer, but not the P layer, has been accomplished here by coat formation in the presence of 0.01 M Na_2SO_3 (2). The cleavage has also revealed the outer surface of mucopeptide cortex. ×136,000.*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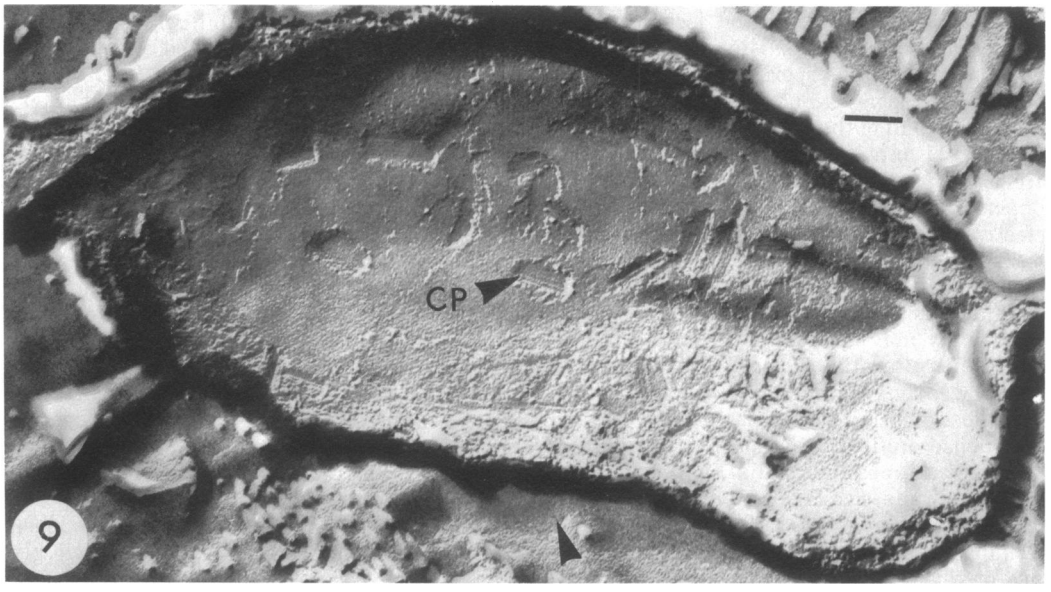
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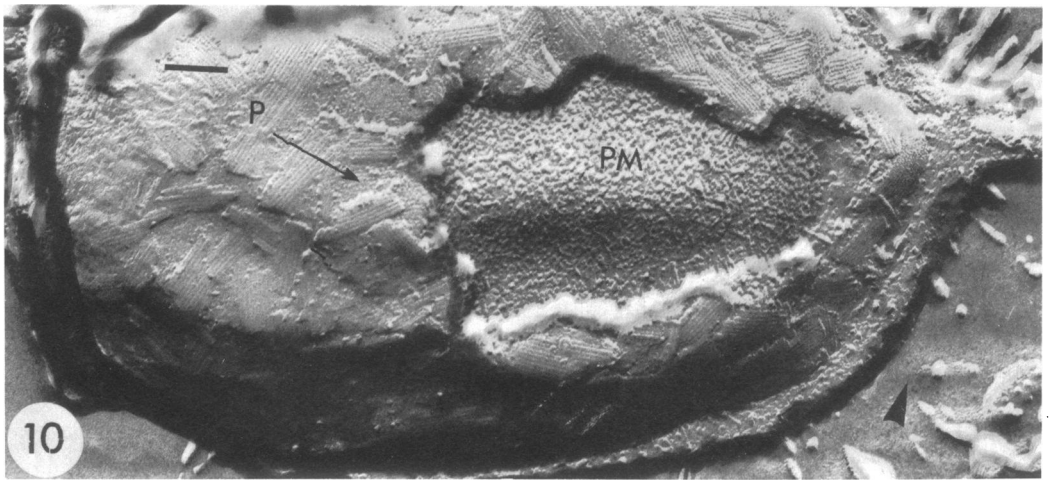
P
PM
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in reference 43) reveals the very open CP array of the surface molecules on the outer coat (Fig. 31). Inner coat lamellae are not revealed by freeze etchings of whole spores of this species. To further elucidate the fine structure of the interesting coat layers of this and other spore formers, the limitation imposed by electron densities and by the planes of cleavage occurring in ripe frozen spores will have to be overcome by treatments before negative staining, fixation, embedding, and freeze etching. Treatments that have already proved useful are perturbations of coat formation (sulfite addition), germination, partial coat extraction, and spore disruption.

Parasporal Inclusions in Relation to Spore Coats

Parasporal inclusions are a secondary major structural product of many sporulating bacillus species (36). Most of those associated with *B. cereus* type spores (or *B. thuringiensis* and its variants) carry a toxin that breaks down the midgut barrier of lepidoptera larvae and ultimately causes paralysis. Besides the interesting area of insect toxicology and mode of action of the toxin (16), microbiologists have become concerned with the significance and origin of these inclusions within the sporulating cell. A brief discussion of the relation of these proteins to spore coat is included in the section Spore Coat-Related Proteins.

The structural studies of early inclusion formation in a *B. thuringiensis* and *B. medusa* suggest the parasporal inclusion is a bulk-phase deposition of some membrane protein that has escaped control during closure of the forespore or septal membrane (22). In 1970, Sommerville and James (75) suggested from the proximity of crystals at stage III with what appeared to be developing exosporium, that parasporal protein may be related to spore coat protein. Sommerville and his colleagues have since accumulated immunological and structural evidence (73, 75) to support this contention.

Because "terminal" synthesis of coat and parasporal proteins plus lysis within the confines of the sporulating cell could well lead to cross-mixing or cross-deposition of antigens (see Fig. 41-43), care should be used in basing an origin concept on immunological evidence alone. Most structural conclusions of parasporal inclusion origin seem to be based on studies of either rather late stages of formation or on micrographs of sections of indifferent quality. We therefore present here some stages of inclusion formation of several parasporal formers all of which indicate that regardless of the ultimate coat relationship, the commencement of inclusion formation is always membrane associated.

To start, we should point out that based on location, parasporal inclusions are of two types; those that end up outside the exosporium and those that end up associated with the exosporium, but outside the spore coats proper (Table 1). To date, in all except one, the inclusion is first deposited on the sporangial surface of the forespore membrane at or near its closure. In *B. medusa*, however, the inclusion first appears as a deposit against the membrane of a transverse septum following the final cell division, preceding spore formation by several hours (Fig. 44-46).

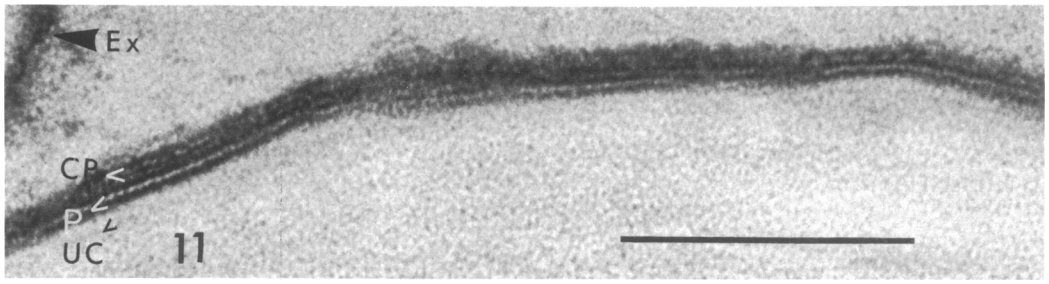
Those *Bacillus* species with parasporal inclusions exterior to the exosporium are most of the typical *B. thuringiensis* and *B. medusa*. The group with enclosed inclusions are "*cereus*-type" spore formers: *B. thuringiensis finitimus*, *B. cereus* var. *fowler* or Fowler's bacillus (37), and Lewin's bacillus (*B. cereus*/var. *lewin*). Likewise, *B. popilliae*, which causes "milky disease" of potato beetle and June bug larvae, produces a parasporal inclusion enclosed by a surface spore layer.

The formation of parasporal inclusions in a stage II sporulating *B. thuringiensis* is shown in Fig. 32a and b. The crystal is already well formed. Although the exosporium is not normally seen at stage II, an additional layer that could be the beginning of exosporium is seen in one section, but not the other, interposed between the forespore membrane and the formed crystal (Fig. 32 a and b). Subsequent stages of sporulation do indeed show exosporium sepa-

FIG. 8. *A freeze etching of the concave undersurface of a B. cereus spore coat showing under coat (UC) layer, the undersurface of the P layers, and through a hole in this, the undersurface of the CP layer. ×78,000. The rods of the CP layer are faintly visible in an insert at higher magnification. ×145,000.*

FIG. 9. *A similar cleavage showing the under surface of a coat from a B. cereus spore that was germinated for 2.5 min. The P layer is largely free of under coat and its pits have all but disappeared (compare with Fig. 4). Through breaks or holes in this layer, the outer CP material is still intact. ×78,000.*

FIG. 10. *A freeze etch of the outer surface of a spore from the same preparation as shown in Fig. 9. The loss of structure in the P layer and the persistence of the CP are characteristic of early germination. The usual cleavage to the surface of the cortex (Fig. 7) is now gone and replaced by what appears to be a cleavage through a highly granular plasma membrane (PM). ×78,000.*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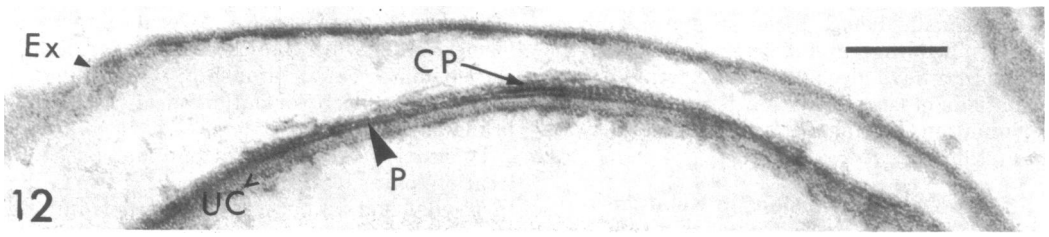
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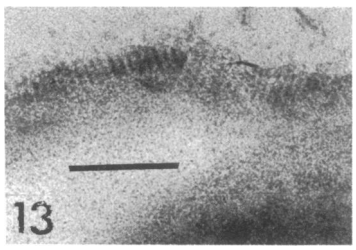
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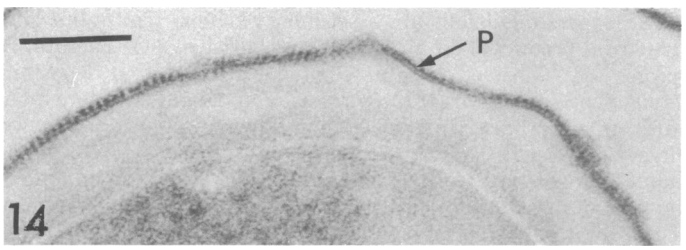
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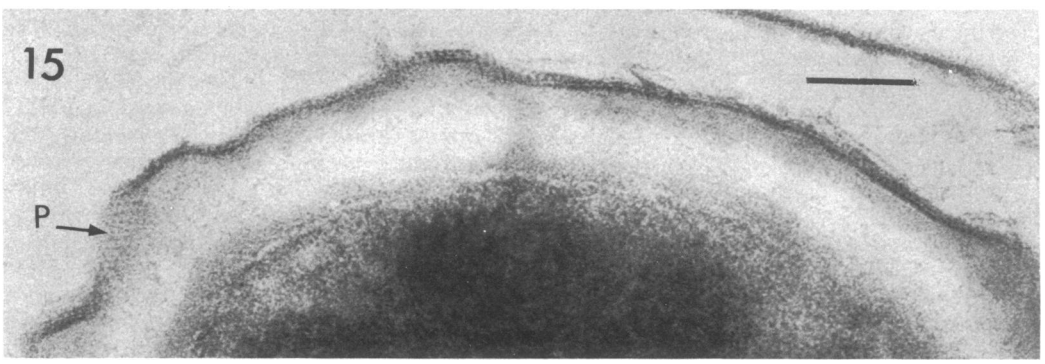
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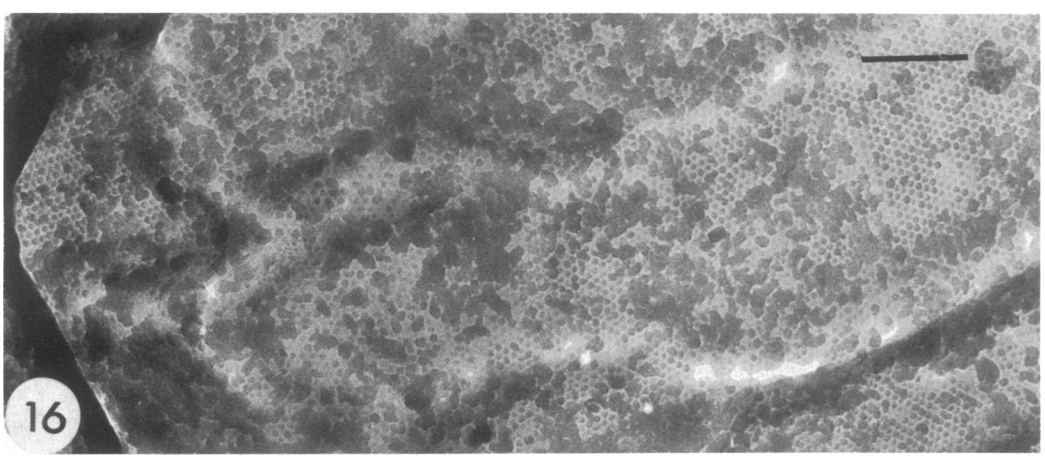
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rating the crystal from the developing coat (Fig. 33). In some sections, the relation of exosporium to crystal seems sufficiently intimate to suggest that the crystal is synthesized and assembled on the exosporium. In well-preserved cytoplasm, however, a narrow space is usually seen between the exosporium and the crystal.

Early deposition of parasporal protein on the developing forespore membrane of Fowler's bacillus (*B. cereus* var. *fowler*) is also evident well before the appearance of the exosporium (Fig. 34). When the exosporium does appear, the deposition of the spore coat (P layer and under coat) is well established. The exosporium when first deposited shows no intimate contact with the growing inclusion (Fig. 35). The inclusion ultimately matches the spore in size and becomes multicrystalline. A similar sequence of deposition occurs in *B. thuringiensis* var. *finitimus* leading to enclosure of the inclusion and spore within the exosporium (Fig. 36). Later in sporulation some adhesion of the inclusion to the coat develops. In freeze etchings, this adhesion between coat and inclusion is well displayed in both *B. finitimus* (43) and Fowler's bacillus (Fig. 37).

B. popilliae, the insect pathogen, sporulates well in and causes milky disease of Japanese potato beetles and June bug larvae. Thirty-six days after injection of *B. popilliae* spores into the latter, various stages of sporulation are encountered (Fig. 38–40). The inclusion's first appearance is again membrane associated (Fig. 38) and ultimately it comes to lie in a pocket between the outer spore coat and an exosporium-like outer covering (Fig. 39). The spore coat architecture of this highly motile bacillus has many characteristics common to the coats of *B. subtilis* (Fig. 28–30) and *B. sphaericus* (44), such as the lamellar inner and dense outer coats (Fig. 40). Freeze etchings reveal the hexagonal packing of the persistent cell wall enclosing the entire spore and inclusion, the highly crystalline structure of the inclusion, and very thick and almost featureless fractures of the spore coat down to the inner lamellae and cortex.

B. thuringiensis var. *schwetzova* is of particular interest because, besides a spore and typical crystalline inclusion, it also forms an ovoid inclusion usually covered by, and associated with, what appears to be in thin section whorls of redundant coats (Fig. 41). Many of the beads become free of the crystals on shaking and sedimentation. Freeze etchings reveal CP coat material deposited on many of the crystalline inclusions, presumably at the former sites of the bead structure (Fig. 42). In spite of the deposition of CP coat on the crystals, the spores were deficient in this layer and, like the sulfite-treated spores, showed considerable areas of P layer under the exosporium (Fig. 43).

The large filamentous *B. medusa* begins the formation of its parasporal inclusion at the end of vegetative growth. Against the base of the transverse septal membrane of each cell, a crystalline deposit of parasporal protein appears about 1.5 h before the decline of optical density increase characteristic of t_0 (Fig. 44). For the following 2.5 h, the nondividing filaments synthesize parasporal protein. When the inclusions are about half their ultimate volume, forespore is formed (Fig. 45). Sporulation continues along with the continued growth of the inclusion until, by stage VII (t_9), the filaments appear as strings of spores and inclusions (Fig. 46). The lytic stage of sporulation is very prolonged in *B. medusa*: over 30 h is often needed before any spores or inclusions are released from the cell casings.

In spite of their proximity during formation, the surfaces of the inclusion are strikingly different from that of the spore. The crystalline inclusions of *B. medusa* are covered with a skin composed of a condensed fibrous protein. On

FIG. 11. *Spore coat on intact B. cereus (agar grown) showing coat layers. Under coat (UC) in most well-fixed ripe spores is poorly defined from the cortex but occupies the region indicated. The profiles of the CP and P layers are indicated by arrows. ×390,000.*

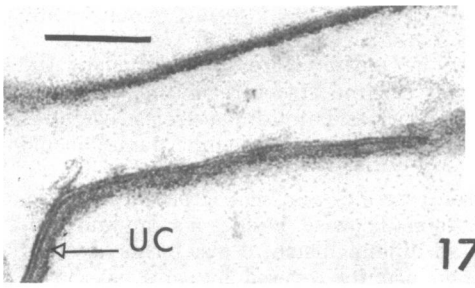
FIG. 12. *A separated coat of a B. cereus spore showing the same layers of coat as in Fig. 11 plus the undercoat (UC). ×136,000.*

FIG. 13. *An area of thin section near to the plane of the spore coat of an untreated B. cereus spore showing the arrays of CP rods. ×146,000.*

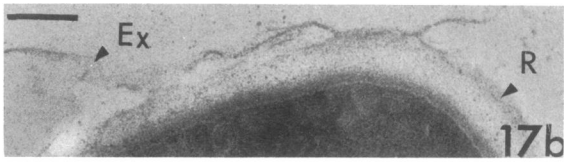
FIG. 14. *Sections of sulfite-treated spores (spore coats where maturation has been altered by the late addition of Na_2SO_3) are usually low in, or devoid of, the outer covering of CP material. Slightly obliquely cut regions reveal more clearly the fine structure of what appears to be the P layer. ×146,000.*

FIG. 15. *A section of a mutant B. cereus 10^{ts} spore (Table 1) formed at the nonpermissive temperature (38 C) in 0.05 M Na_2SO_3. The lysozyme-sensitive spore coats are generally free of CP layer and only the P coat is seen in an area cut parallel to the coat (compare with Fig. 13). ×146,000.*

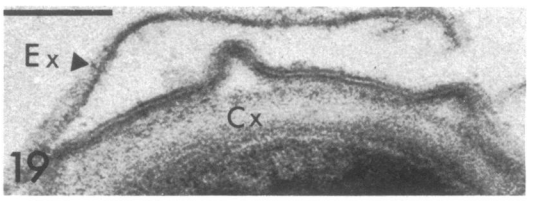
FIG. 16. *A piece of B. cereus-liberated spore coat encountered in an incompletely cleaned freeze etching and showing the hexagonal net of the P layer. ×136,000.*



17a



17b



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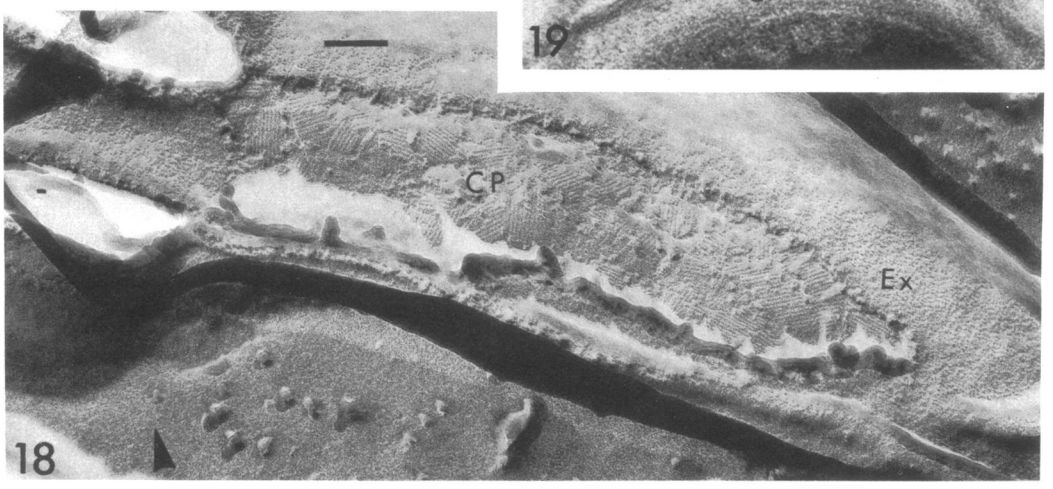

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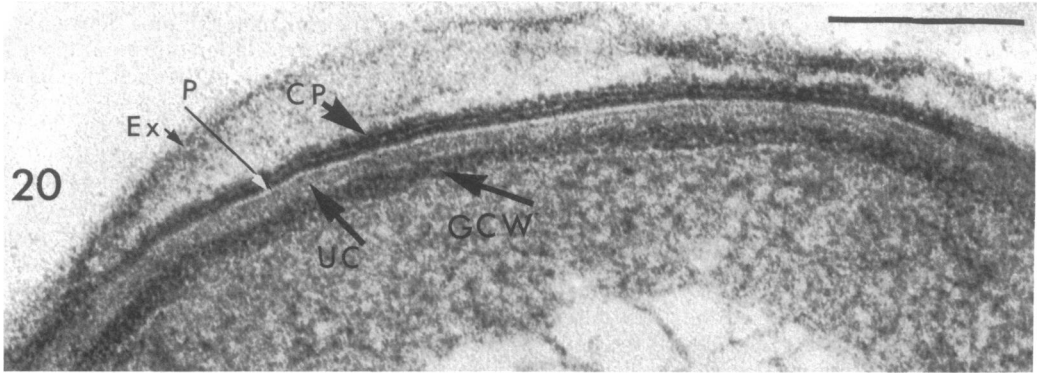

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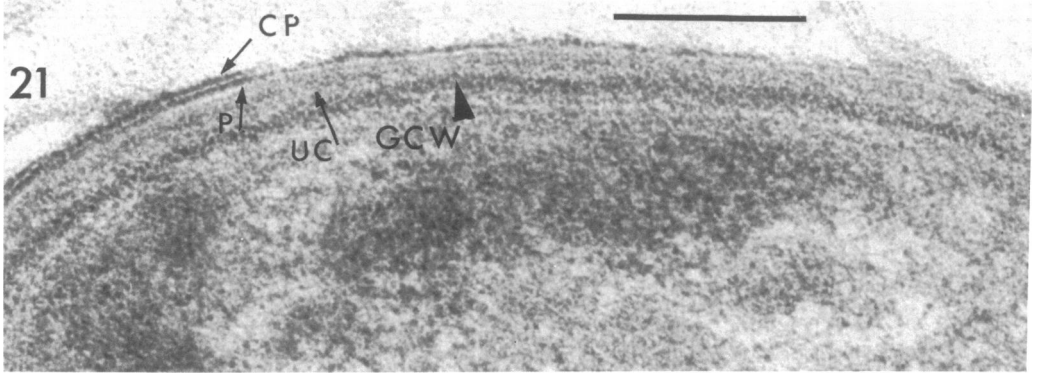

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the surface of the skin, the long fibrils of protein show a helical array (Fig. 47). In contrast to the crystalline core, these skin fibers are trypsin and keratinase resistant and their presence appear related to the occurrence of a small RNA-containing phagelike particle. Mutants devoid of the particle produce skinless inclusions (G. S. Hendry, J. B. Gillespie, and P. C. Fitz-James, J. Virol., in press). The inclusion skin fibers are rarely seen on the spore surface. Moreover, the coat of these *B. cereus*-type spores appears devoid of the CP layer. Replicas show only the basic P coat layer where the exosporium is cleaved (Fig. 48).

In 1961, a parasporal inclusion-forming bacillus was isolated from hot-spring water in Iceland by Ralph A. Lewin (Scripps Institute of Oceanography). This *B. cereus*-type sporeformer also develops a crystalline ovoid parasporal inclusion that is enclosed within the exosporium but covered by a lamellar skin (Fig. 49). The spores have a thin coat composed of a P layer and are apparently devoid of the CP surface layer. The lamellar inclusion surface has a distinctive fine structure, unlike that found in the coats of the spore (Fig. 50).

This structural analysis (summarized in Table 1) indicates the difference between parasporal inclusion protein, which first deposits or crystallizes on the forespore or transverse septal membrane, and coat protein that deposits into sheetlike layers on the forespore some hours after the beginning of the crystal. In the case of *B. medusa*, the parasporal deposition begins some 3 h before the beginning of the forespore in cells which are not yet committed to sporulation.

The time of formation and the solubility properties of these inclusion proteins overlap with those of spore coat (see the section Spore Coat-Related Proteins). In addition, there is immunological evidence, at least for *B. thuringiensis*, for cross-reactivity (51, 73), although considerable differences between crystal and spore coat structure are evident from the morphological studies. As noted for *B. thuringiensis* var. *schwetzova* (Fig. 41–43), there may be coatlike deposits on the crystal surface. Such possibilities must be considered when concluding cross-reactivity of crystal and spore coat proteins by immunodiffusion or ferritin staining of sections (51, 72).

EXTRACTION AND CHARACTERIZATION OF COAT POLYPEPTIDES

Coat removal initially involved rather drastic procedures for breaking spores (2, 58, 80) and a somewhat arbitrary washing protocol. The latter is primarily for removing mucopeptide cortical material, although readily soluble coat polypeptides would also be lost. On the basis of reconstruction experiments and electron micrographs, there appears to be little soluble contamination, although remnants of exosporium are usually present (2). The loss of minor structural proteins that do not noticeably alter the coat morphology would not have been detected. More recently, protein has been extracted directly from spores that should obviate many of these preparative problems and does provide preparations with properties very similar to those from isolated coats. The conditions required to solubilize the protein from either preparation are summarized in Table 2. Contamination or artifacts may result from: (i) solubilization of exosporium or outer membrane proteins. These are the most likely contaminants from extraction of purified spores, but there may be other sources in isolated coat preparations. Neither structure should be a major contaminant. The exosporium is still in-

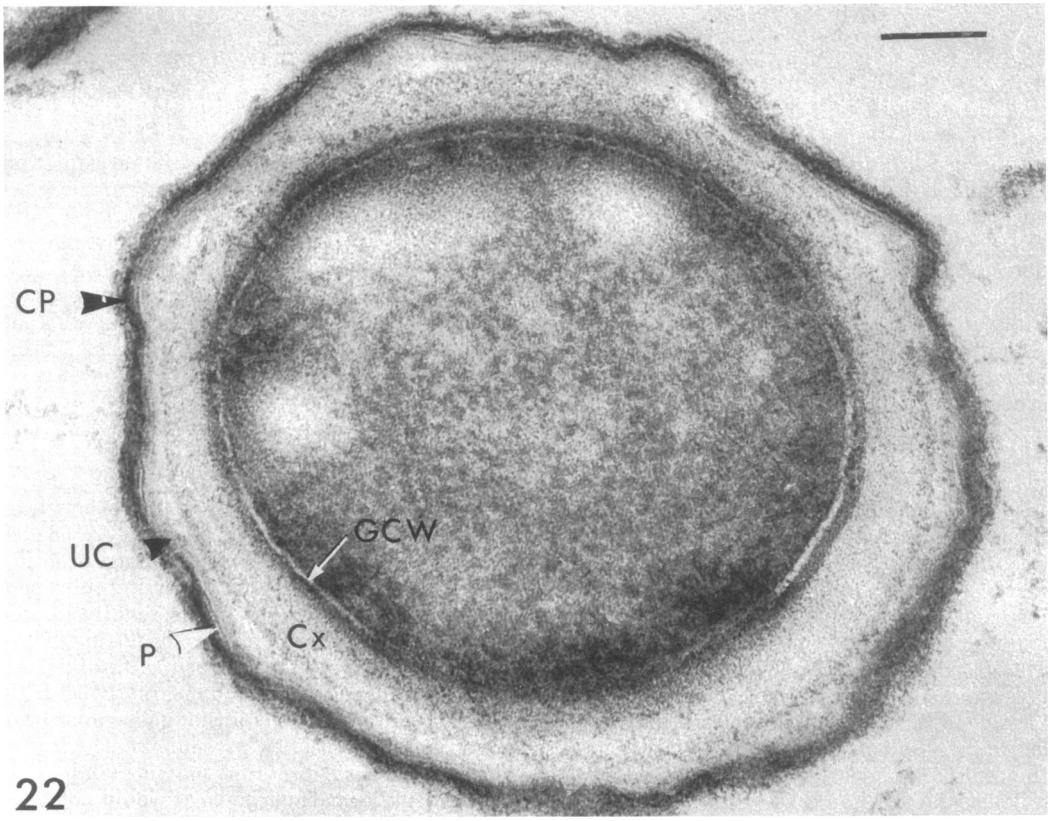
FIG. 17. *(a) A section of an isolated coat of an agar-grown B. cereus spore after 1.5 h of extraction with DTE-SDS at pH 10 (see Table 2). Some overall thinning of the coat has occurred. The under coat (UC) is partly dissolved (compare with Fig. 12). ×136,000. (b) A section of a spore of B. cereus produced in liquid medium extracted for 12 h with DTE-SDS at pH 10 to 10.5. Only the exosporium (Ex) and a remanant of coat protein (R) on the cortex have survived extraction. ×78,000.*

FIG. 18. *A freeze-etching preparation of the same material shown in Fig. 17a (compare with Fig. 1). Under the persistent exosporium, the CP layer, in spite of its apparent dissolution in the sections, is still evident after cleavage. The pits of the P layer are completely absent after treatment with DTE-SDS. ×78,000.*

FIG. 19. *A section of a whole spore of B. cereus after extraction of coat protein with DTE (0.05 M) in 4.0 M urea for 3 h (twice). Under coat appears to be the layer most readily attacked by this treatment. ×146,000.*

FIG. 20. *A thin section of a spore of B. cereus germinating for 30 min in inosine (0.001 M) and L-alanine (0.01 M). The cortex is gone and the germ cell wall (GCW) now lies against the under coat (compare with Fig. 11 and 12). The CP layer persists (see also Fig. 10). The inner dense profile of the double-track P layer is gone. ×252,000.*

FIG. 21. *A spore of B. cereus 10^{ts} formed in fluid medium at 40 C in the presence of 0.05 M Na_2SO_3 and germinated for 8.5 min in inosine (0.001 M) and L-alanine (0.01 M) (for control see Fig. 14). Only small segments of CP layer are present. The changes shown in Fig. 20 also occur here, particularly the loss of the dense profile at the junction of undercoat and pitted layer. ×252,000.*



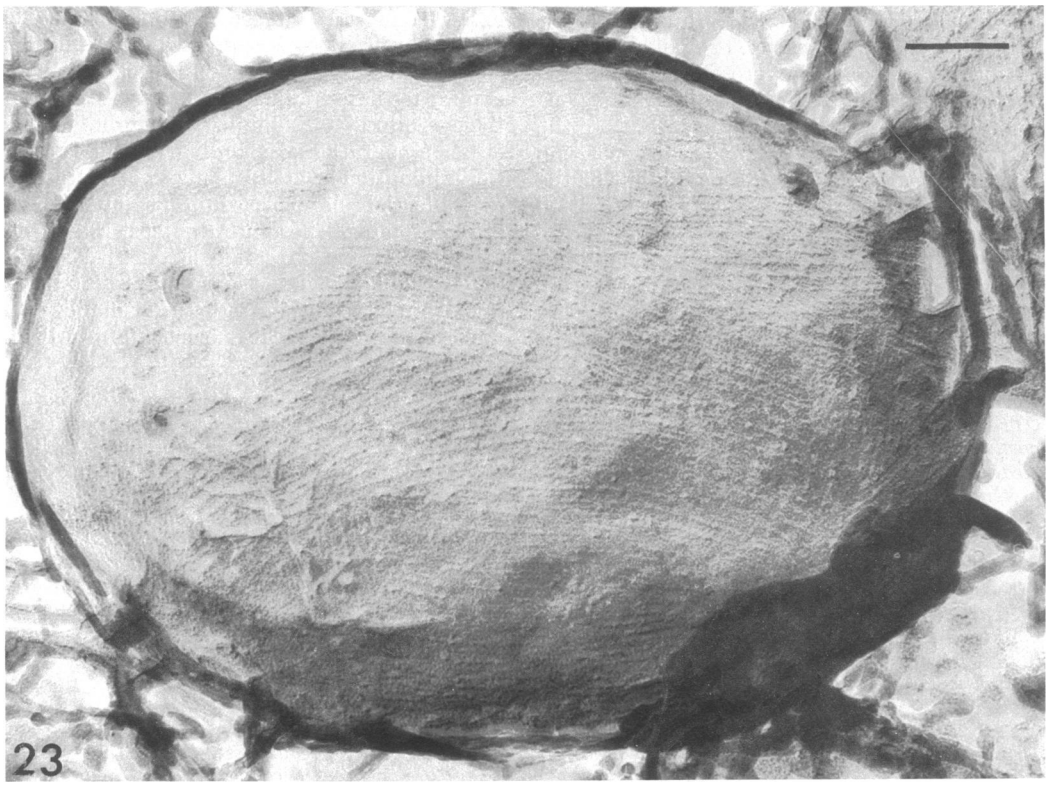
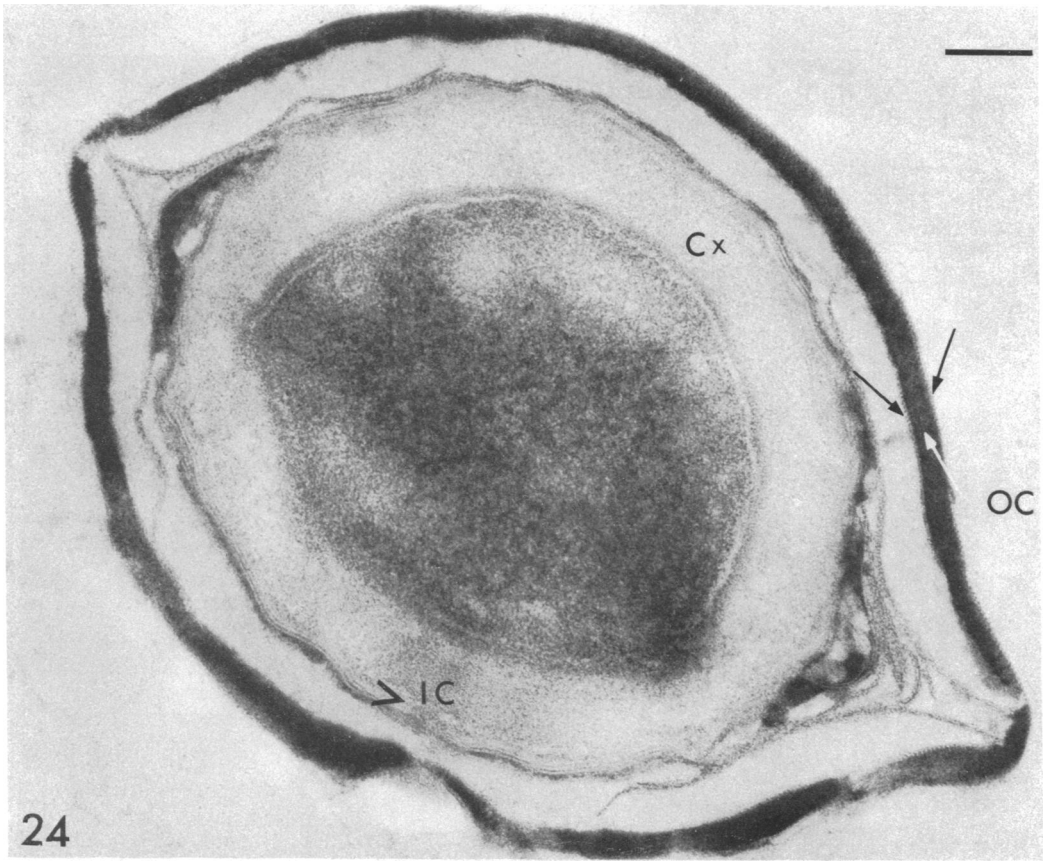


FIG. 22. *A single type of spore coat found on B. megaterium 899a and KM. A thin section of B. megaterium KM showing the arrangement of coat protein somewhat like that of B. cereus in under coat (UC), double-track (P), and a dense over layer (CP). ×136,000.*

FIG. 23. *A single type of spore coat found on B. megaterium 899a and KM. A freeze etching of the surface of a spore of B. megaterium KM. The CP packing of protein is less well defined than in B. cereus. ×136,000.*



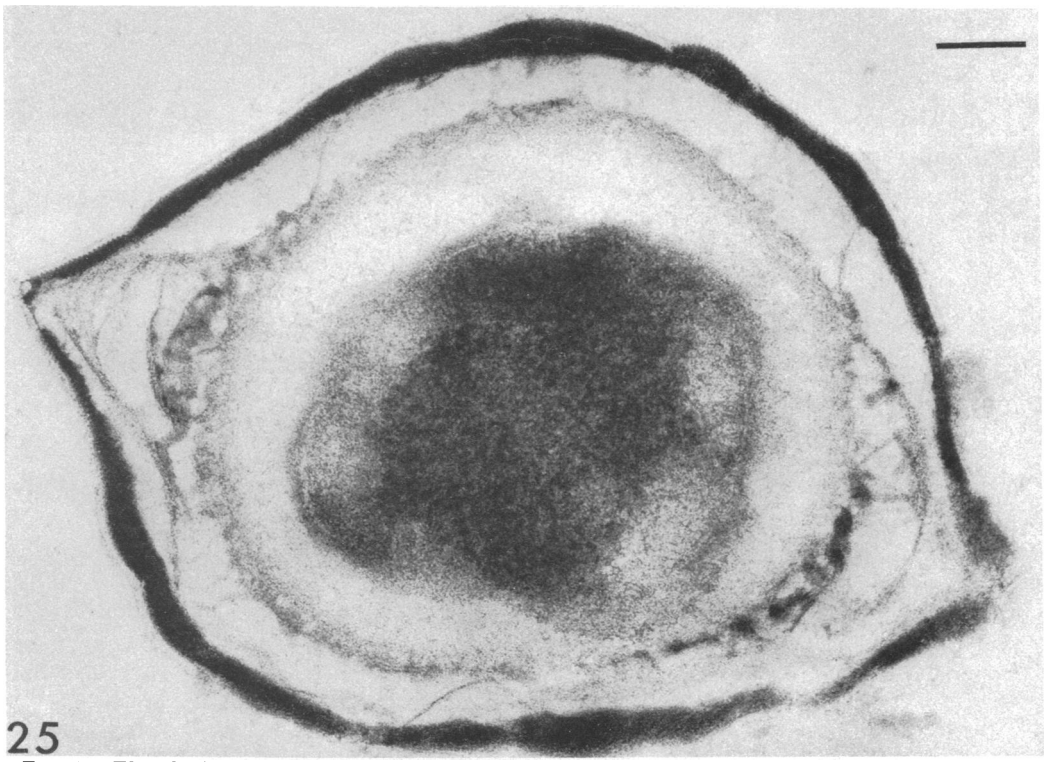
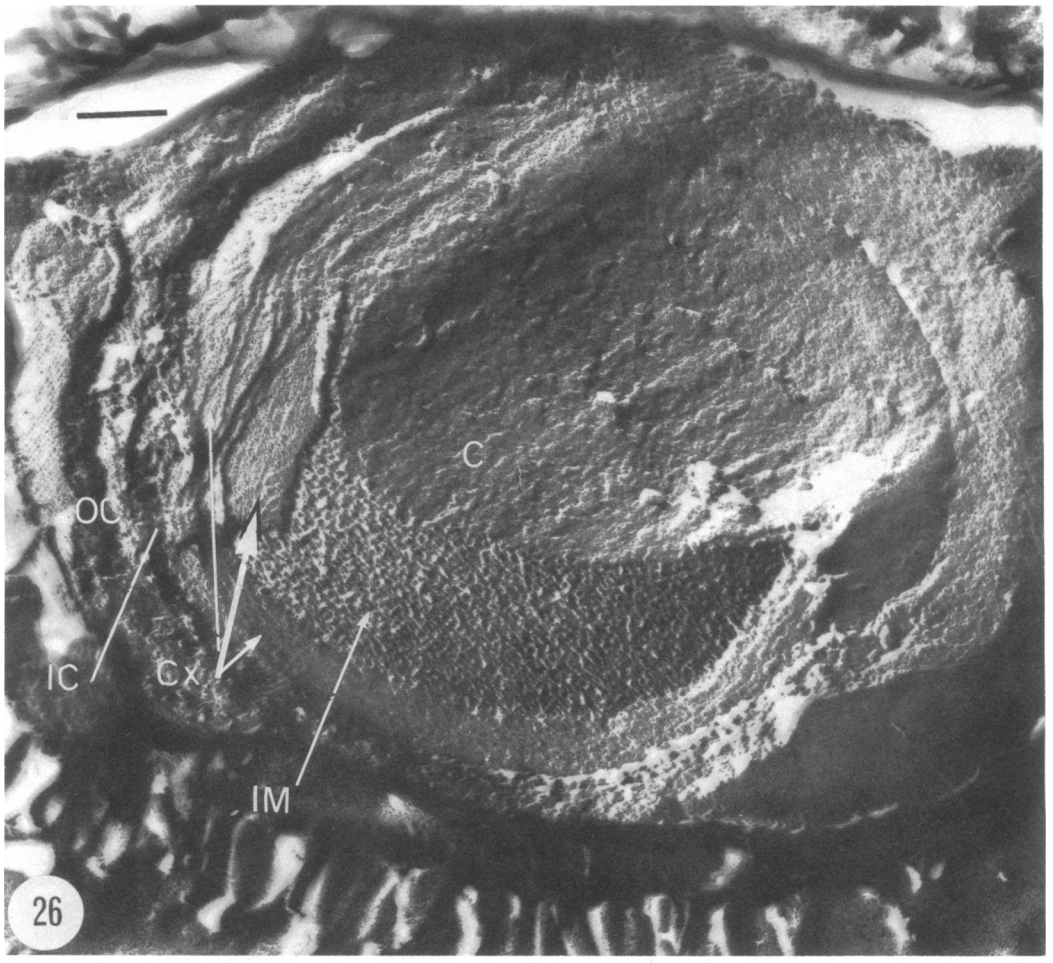
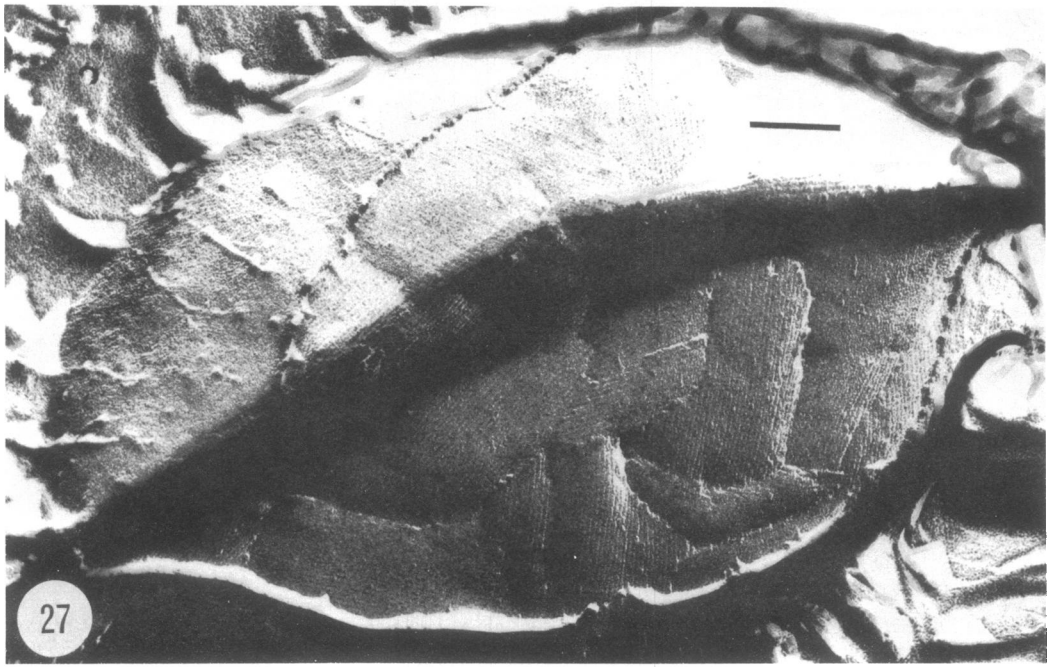


FIG. 24. *The "double" type of spore coat found on "typical" B. megaterium spores. The inner coat (IC) lying on the cortex (Cx) is essentially similar to that found on B. megaterium KM and 899a (Fig. 22). The dense and thick outer coat (OC) appears as a deposition between two highly structured layers (arrows). ×112,000.*

FIG. 25. *A spore similar to that shown in Fig. 24 after extraction with DTE in 1.0% SDS at pH 10.0. The inner coat has nearly all dissolved, the outer one persists. ×106,000.*


C
OC
IC
Cx
IM
26


27

tact albeit modified after coat extraction. On the basis of the data of Matz et al. (56), the exosporial proteins are only about 3% of the total coat protein (70% of total spore protein, reference 58), which would be the maximum contamination possible.

(ii) Although extraction from intact spores may be more desirable, the possibility of contamination from lysing or germinating spores must be considered. We have counted spores before and after extraction, but there is sufficient error so that a 10% loss would go undetected. Many of the steps that are useful for removing cells and debris may lead to activation and subsequent germination. These include a two-phase polymer system (66) or pelleting spores through a dense (1.25 to 1.30 g/cc) solution of sucrose or Renografin. We found that the latter material was quite satisfactory for removing cells and debris, but it often resulted in activation and subsequent phase darkening of 10 to 20% of the spores. This activation may be due to pressure, a topic discussed by Gould and Sale (32).

(iii) The requirement for a high pH to solubilize coat protein (Table 2) can lead to formation of lanthionine and/or lysinoalanine (10). The latter compound results from the formation of dehydroalanine (usually from cysteine) and the subsequent reaction with the ϵ-amino group of lysine. Formation of either amino acid may result in inter- or intramolecular crosslinking and the creation of new species of polypeptides with altered solubility, etc.

Because lysinoalanine formation generally occurs under milder conditions than those required for lanthionine formation, we have monitored only for the former amino acid (Table 2). If we keep the pH of extraction below 10, no lysinoalanine is detected and, therefore, we assume that by extracting at pH 9.5 to 9.8 at 37 C for 90 min or at 27 C for 3 h, no secondary modifications are occurring.

(iv) Modification of the coat proteins by contaminating proteases does not seem very likely. Although most sporeforming organisms excrete at least one extracellular protease and contain an intracellular species (usually a serine protease), neither of these enzymes digests isolated spore coat protein (as judged by release of material soluble in 12% trichloroacetic acid). In addition, coat protein prepared in the presence of 2 mm of ethylenediaminotetraacetic acid and phenylmethylsulfonyl fluoride to inhibit metal and serine proteases, respectively, had identical electrophretic mobilities.

The extraction conditions as listed in line ($\bar{e}$) of Table 2 result in solubilization of at least 95% of the total protein present in isolated *B. cereus* T. coats. There is always a residue (2; Fig. 7a, b) that may consist of altered coat polypeptides or proteins from other sources such as membranes, exosporium, or lysed spores. There is also a small amount of carbohydrate and lipid present in the residual fraction (6).

Treatment of intact *B. cereus* (or *B. megaterium* KM) spores with these reagents appears to remove virtually all of the undercoat and P layers (Fig. 17-19; reference 5, 24). The remnants of the CP layer (Fig. 18) have the double-track pattern of the P layer in thin sections (Fig. 17, 19), suggesting an alternation of conformation during extraction. As discussed below (Functions of the Spore Coat), the properties of these extracted spores are certainly altered, especially in regard to sensitivity to lysozyme.

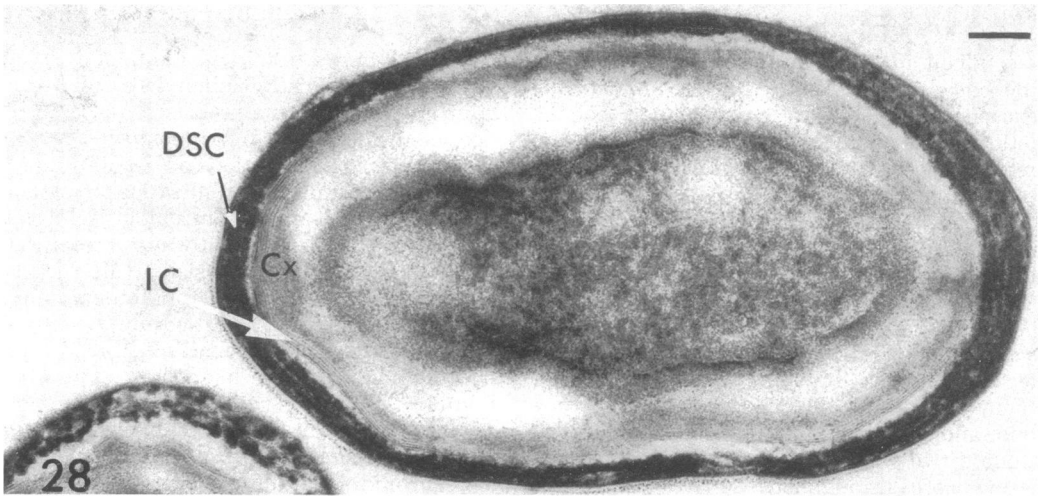
A comparable amount of protein was solubilized by Sommerville et al. (74) employing similar treatments. Interestingly, attempts to solubilize the coat from *B. subtilis* spores with these reagents were not successful, although 70 to 80% of the protein in isolated coats could be solubilized (A. Uchida and A. I. Aronson, unpublished data). We suspect that *B. subtilis* Marburg spores are surrounded by a very impenetrable exosporium or outer layer around the coat (Fig. 29, 30; reference 43), although about 50% of the protein was solubilized from intact spores of *B. subtilis* SB 153 by Spudich and Kornberg (79).

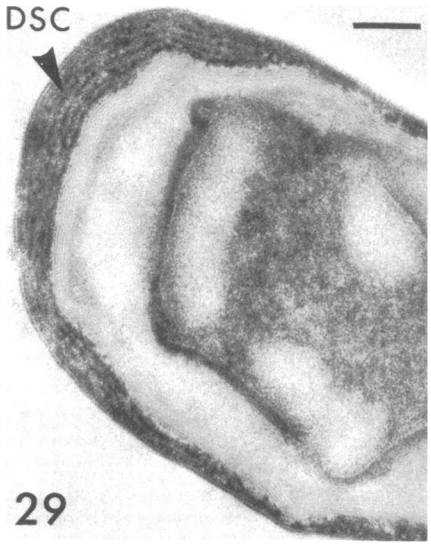
To attempt to further purify the coat protein of *B. cereus*, we have precipitated with 25% saturation ammonium sulfate (0 C) and passed this preparation over *O*-triethylaminoethyl)-cellulose (6) or a Bio-Rad P-10 column (8). With these procedures, we have obtained a major species of polypeptide but may have lost minor coat components as well as contaminants. We have, therefore, compared the polypeptides in the crude extract with those in purified preparations.

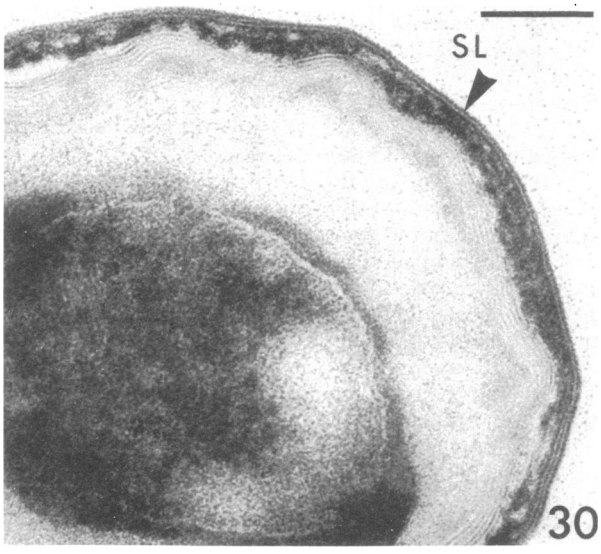
The chemical data we have obtained on the

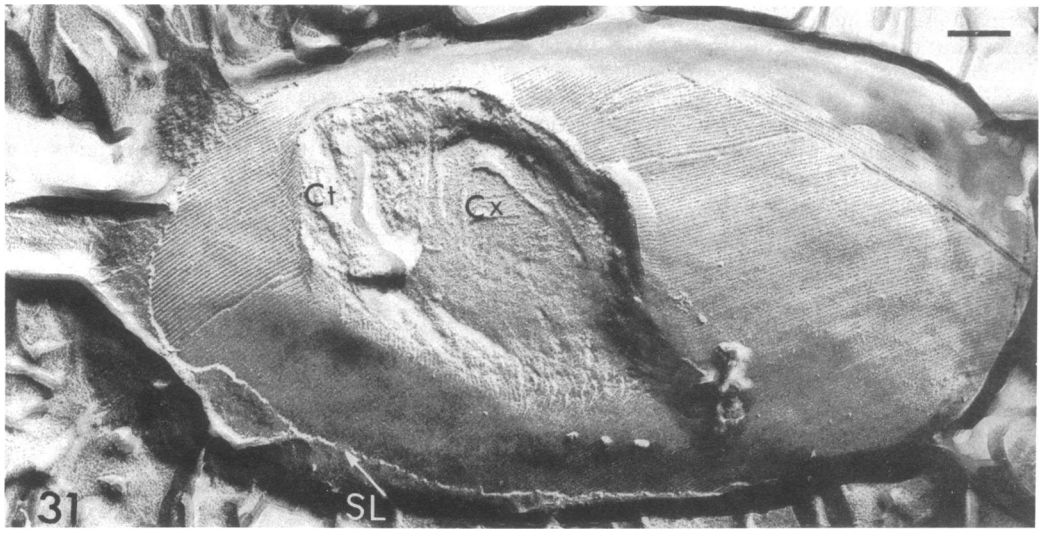
FIG. 26. *Freeze-cleave-etch preparations of B. megaterium L. The surface of the outer heavy coat (OC) shows a linear-repeating array. The inner coat (IC) is not advantageously exposed; the thick cortex (Cx) has been torn to show six or seven layers on the cleaved plasma membrane (IM) covering the spore core (C).* ×*114,000*.

FIG. 27. *A good view of the surface of the outer coat of B. megaterium L showing the more open CP array of rods accomodating to the spore surface.* ×*114,000*.


DSC
IC
Cx
28


DSC
29


SL
30


Ct
Cx
SL
31

coat fraction is summarized in Table 3. We find one major component in gel electrophoresis that accounts for 80 to 90% of the radioactivity in a labeled preparation if we electrophorese immediately after preparation (Fig. 53). As shown in Fig. 53, there is one major band of 12,000 to 13,000 daltons when protein is extracted with DTE-SDS, but there are two bands in this molecular weight region when urea-DTE-SDS is used for extraction. The reason for the difference is not known, but it may be a reflection of the poor SDS binding at alkaline pH with a resulting separation by charge difference (and possibly size) in the urea extracts. The other major component most noticeable in the urea extracts of Fig. 53 is a 28,000-dalton species. We suspect this may be a coat precursor found in variable amounts associated with mature spores, but definitive kinetic experiments have not yet been done. Several other minor bands are also present, but rough calculations of the molar amounts indicate less than 100 molecules per spore. Some of these bands are aggregates since extensive heating of the extracts results in increased amounts. Others may be due to protein contaminating the spores or spore lysis during extraction. Storage of the extracts at any temperature inevitably results in some aggregation and a less well-defined profile. While some investigators have published pictures purporting to show one major protein species (51, 74), others have reported several bands to be present (27, 83). Artifacts occurring during extraction, contaminants, spore germination, and aggregation of the extracted protein (especially during dialysis) may all contribute to the variation in results. The fact that primarily a single band may be obtained with good recovery of input protein does argue, however, for restricted heterogeneity among the major coat protein species.

As previously mentioned (6), both serine and glycine were found as amino termini in protein solubilized from isolated coats. Because the fraction of the two varied (qualitative analysis of both dinitrophenyl and dansyl derivatives), we suspected secondary alterations, possibly due to exopeptidases, during coat preparation or extraction. More recently, we have extracted the protein directly from spores, carried out an analysis of amino termini, and have sequenced manually the first four residues employing the procedure of Weiner et al. (91) (Table 4).

Primarily, amino terminal glycine, with considerably smaller amounts of serine is found. The same results are obtained with total coat preparations enriched for inner or outer coat (see section on solubilization of spore coat layers), or coat protein purified by fractionation on *O*-(triethylaminoethyl)-cellulose or P-10 columns. Because of the large quantitative differences, sequencing was continued on the mixture of at least two polypeptides. Unfortunately, the results from the second round of hydrolysis were equivocal, presumably because of the incomplete hydrolysis of valine dipeptide(s) from the amino termini (34). If this assumption is correct, two sequences may be derived that differ only in the absence of amino terminal glycine in a minor fraction, i.e., NH_2 Gly-Ser-Val-Ala(ala) and NH_2-Ser-Val-Ala-Ala. Although these are not unique solutions to the data, they are compatible with a minimal amount of polypeptide heterogeneity and, in fact, are consistent with a single species with loss of amino terminal glycine from a small fraction.

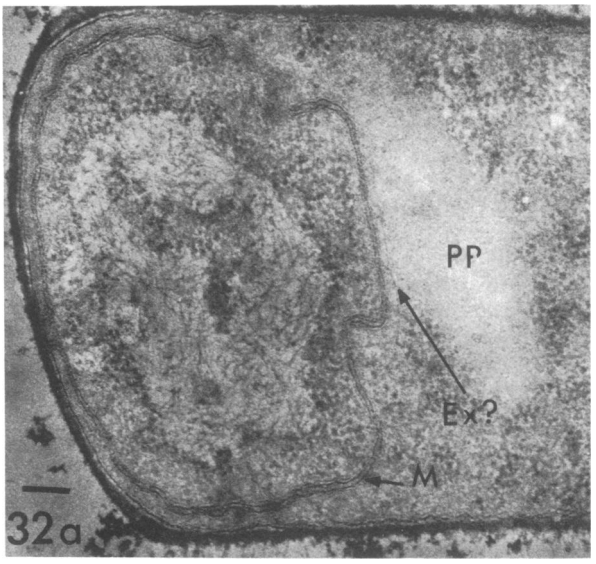
The early amino terminal analyses of Salton and Marshall (67) are consistent with these results in that primarily dinitrophenol (DNP)-glycine was found. Lecadet et al. (51) found phenylalanine as the principle amino terminal amino acid in solubilized coat preparations (about 40 to 45% of the total coat) isolated from *B. thuringiensis* spores, whereas the insoluble fraction contained a more heterogeneous mixture. Hiragi (41) studied the coat fractions of *B. subtilis* (ATCC 6051) prepared according to Kondo and Foster (48), i.e., an alkali-soluble fraction, a fraction solubilized by sonic treatment, and the residue. He found very similar physical and chemical properties among the three fractions, including the spectrum of amino terminal residues. The latter included lysine (which may be ϵ-DNP lysine) and a large amount of serine with lesser amounts of several other amino acids.

FIG. 28. *A thin section of a B. subtilis 168 spore showing the wide, multilayered, and dense outer coat (DSC) and the more tightly layered inner coat (IC) closely applied to the outer surface of the cortex (Cx). ×78,500.*

FIG. 29. *Sections of B. subtilis spores found in fluid-shake cultures after the late addition of 0.05 M Na_2SO_3. The sulfite treatment has decreased the electron capacity of the outer dense spore coat (DSC) improving the visualization of the layers, including the outer most closely organized layer. ×78,000.*

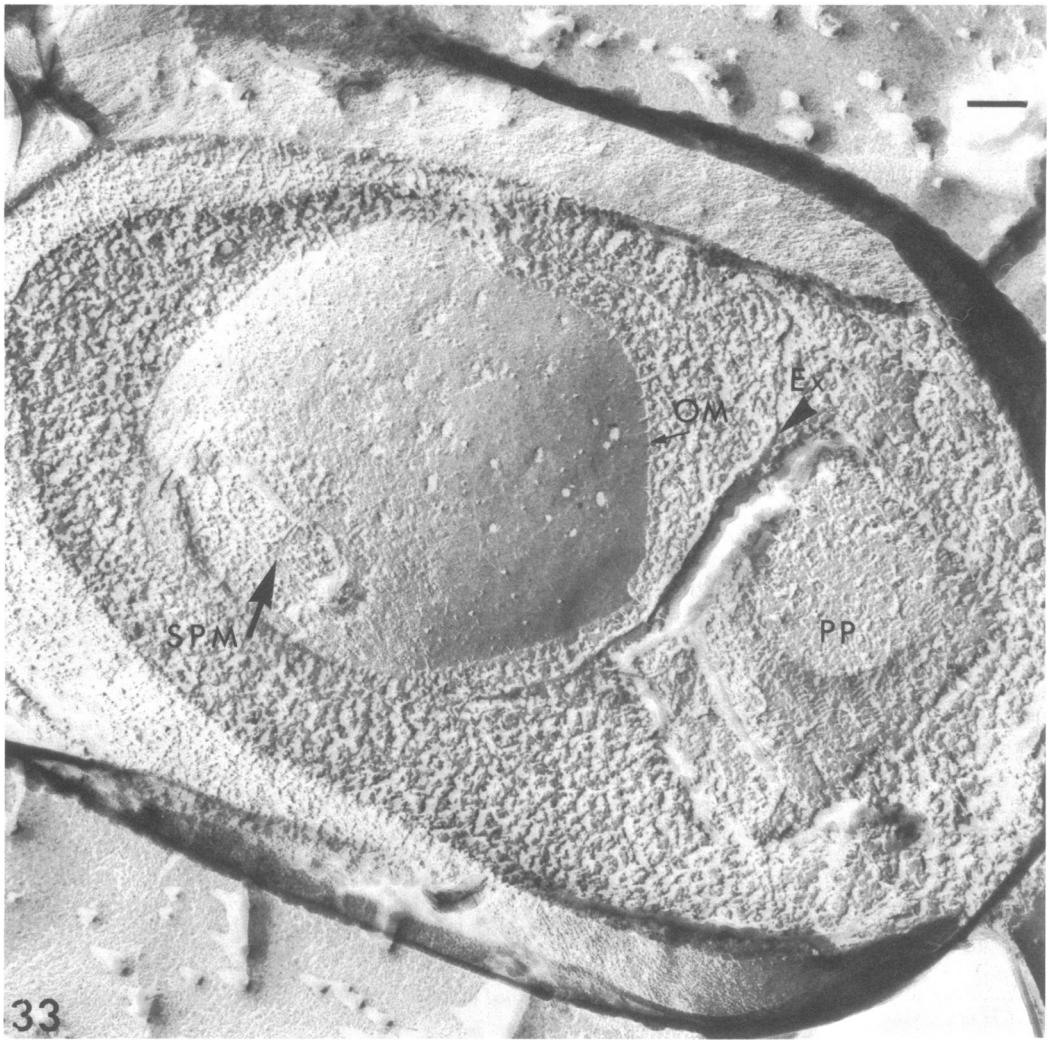
FIG. 30. *Same as Fig. 29, only it reveals more clearly the surface layer (SL). ×144,000.*

FIG. 31. *A freeze-cleave-etched replica of an untreated B. subtilis spore. The rigid, loosely CP surface lies just under the surface layer (SL). Where the spore is cleaved out down to the cortex (Cx), the thickness of the coat (Ct) is revealed. ×78,000.*



32a

32b



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Other data consistent with one or a few species of coat polypeptides include the molecular weight analyses of Aronson and Horn (6) and Fig. 53. Here, again, some heterogeneity was found that was partially attributable to aggregation (protein in the void volume of the agarose column, peak A) and partly attributable to formation of lysinoalanine during extraction. The aggregation was alleviated by reducing and carboxymethylating the protein before chromatography. Much of the protein used in these experiments was extracted at pH 10 to 10.5 and heated, conditions favoring lysinoalanine formation (Table 2). In fact, amino acid analyses of peak B and C in Fig. 54 showed enrichment in the former peak for lysinoalanine. We assume that the altered elution is due to the formation of this amino acid and intramolecular cross-linking of polypeptides. As a result, these altered polypeptides may not be totally denatured even under the conditions employed (legend to Fig. 54), and thus an altered elution profile is seen. Because both the coat protein in the void volume and peak B can accounted for, we assume that there is one major molecular weight species of about 12,000.

Results of immunological studies are also consistent with very few species of polypeptides. Antibody prepared against isolated spore coats of *B. cereus* showed two precipitin bands on an immunoelectrophoretic analysis of spore coat solubilized with alkaline thioglycollate (Fitz-James, unpublished data). As mentioned previously, aggregation of the spore coat may lead to spurious electrophoretic patterns and certainly render quantitative analysis very difficult. If we assume that a large fraction of the coat protein migrated in the agar, then these results argue for a minimal complexity of at least the highly antigenic species of coat proteins. In fact, one of the two bands may be due to aggregation or noncoat material.

The protein in coat preparations is resistant to hydrolysis by proteolytic enzymes (Pronase treatment results in 30% becoming soluble in 12% trichloroacetic acid), except for a keratinase prepared from enrichment cultures of *Streptomyces fradiae* (60). The peptides produced by treatment with this enzyme may be resolved into 12 to 13 spots in a two-dimensional system leaving a 30 to 40% core (8). The purity of only four of these peptides has been determined. When the digests of coat protein labeled either with radioactive arginine or [^{35}S]cysteine (i.e., sporulating cells incubated with $^{35}SO_4$ plus methionine) were analyzed, only a few radioactive peptides were found (Table 5). On the basis of the amino acid composition of *B. cereus* T coat protein (2), a maximum of four peptides containing arginine and three with half-cystine residues would be expected if a single protein species were present. As a control, the spore-soluble proteins (see footnote to Table 5) were treated in an identical fashion and at least four peptides with half-cystine and nine with arginine were found (some of these spots may contain two or more peptides). In the latter case, only peptides yielding detectable color with ninhydrin were counted. Because recovery was poor (25%), there were probably many other labeled peptides either poorly resolved or present in minor amounts.

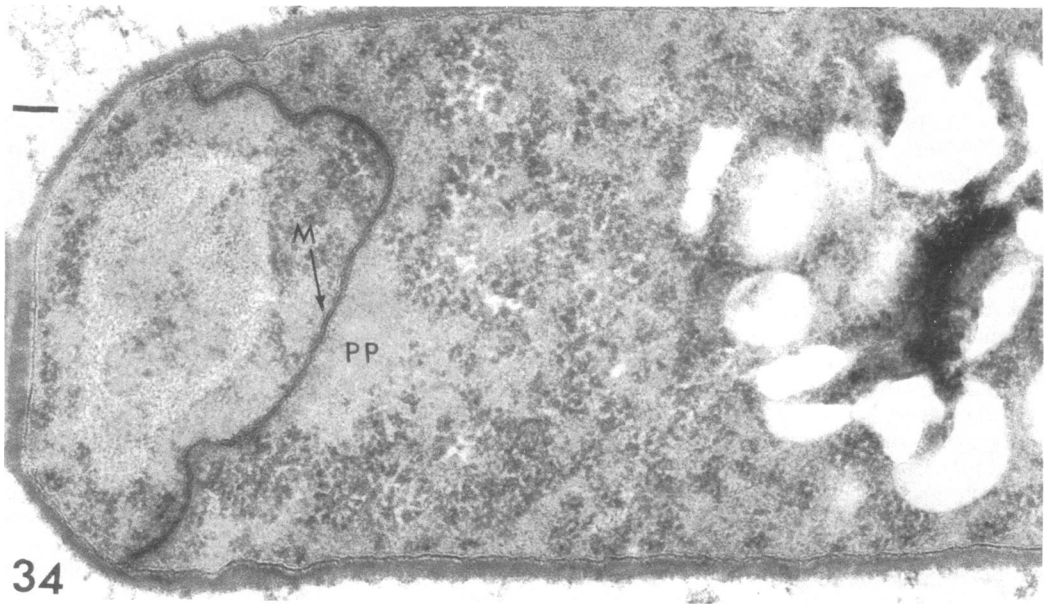
The purity of the peptides from coat digests containing half-cystine residues has been confirmed by chromatography in two other solvents. Although similar analyses must be done with the arginine peptides, the data at present are consistent with a single major species of coat protein.

Despite the arguments for minimal heterogeneity in coat polypeptide species as summarized above, there are claims for more extensive heterogeneity based on a number of criteria, primarily differential solubility. For example, Kondo and Foster (48) and Kawasaki et al. (47) have used sequential extraction with NaOH and sonic treatment to define three coat fractions in six species. Extreme alkalinity should certainly be avoided to prevent alterations leading to changed solubility and amino acid content. In addition, contamination of coats was not monitored. Surprisingly, Hiragi (41) employed the same procedures on *B. subtilis* spores and found the three coat fractions to be very similar on the basis of several physical and chemical criteria. As implied for *B. cereus* T, solubility differences may not be adequate for concluding differences among constituent polypeptides (see section on solubilization of spore coat layers).

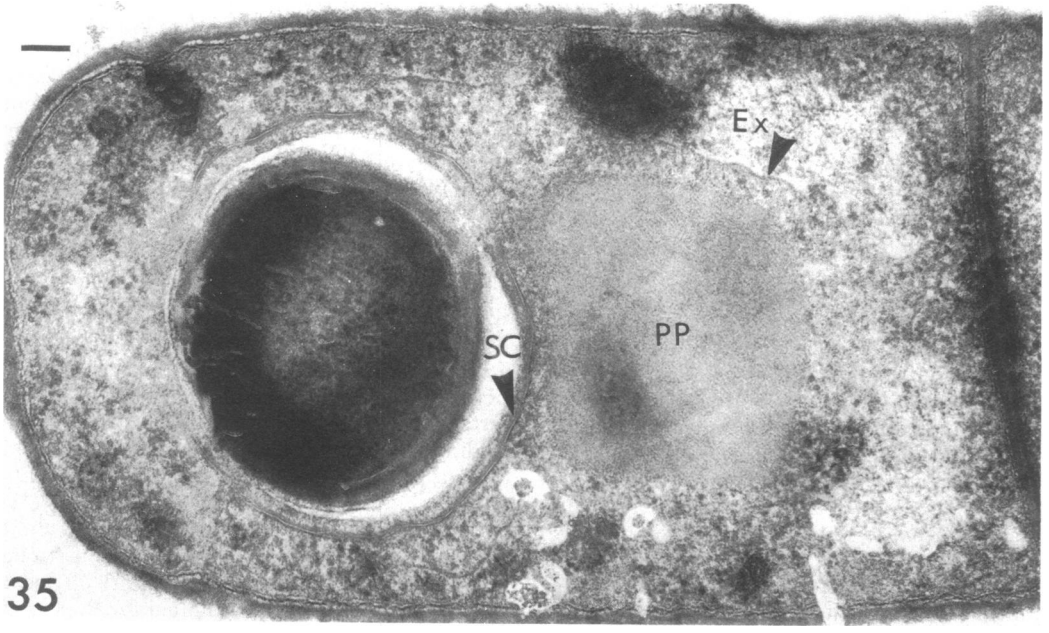
Spudich and Kohnberg (79) also defined two coat fractions in *B. subtilis* on the basis of

FIG. 32. *Sections (serials 1 and 3) showing the early deposition of parasporal crystal protein (PP) closely applied to the late stage III forespore membrane (M) in B. thuringienses var. alesti. The exosporium is not seen; a faint profile (arrow) in (a) may be the beginning of this layer. (a) ×60,000; (b) ×60,000.*

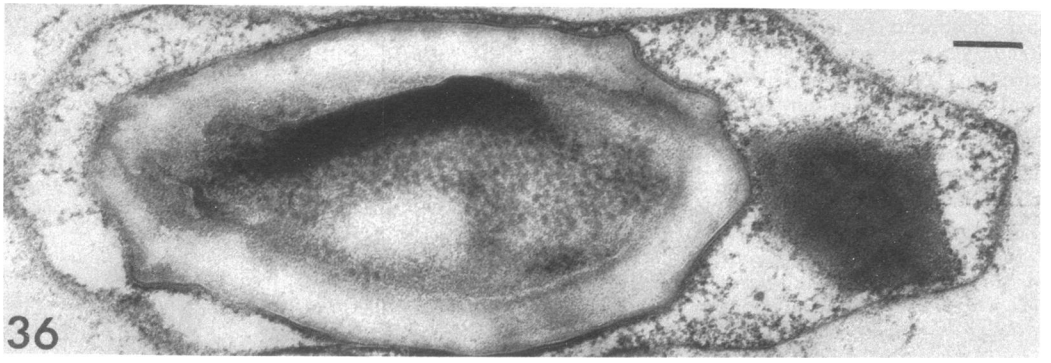
FIG. 33. *A freeze-etch preparation of a late stage III sporulating B. thuringienses var. alesti showing the early development of the exosporium (Ex) separating the parasporal crystal (PP) from the forespore membrane (M). The inner half of the outer membrane (OM) has been cleaved in one area down to the convex inner leaflet of the granular spore plasma membrane (SPM). ×78,000.*



34



35



36

differential solubility and amino acid composition. *B. subtilis* spores do appear to have a thick coat (43; Fig. 28–31), including a fraction with a relatively high tyrosine content and may indeed be more heterogeneous than *B. cereus* and related species. Caution should be exercised, however, in drawing conclusions from fractions obtained from isolated coats, especially insoluble material. Rather drastic procedures are required to break spores and may result in denaturation and aggregation of protein. Such proteins are likely to end up in the insoluble fraction and may bias chemical analyses. In our experiments, the insoluble fractions of isolated *B. cereus* T coat fractions had a very varied amino acid composition (2), possibly reflecting preparative artifacts. As mentioned previously, however, there is even an insoluble coat residue after direct extraction of B. subtilis spores (24, 79). The amino acid composition of the insoluble residue seems to differ significantly from the soluble coat protein, indicating very different protein species in the two fractions. In addition, electrophoresis of *B. subtilis* coat protein on 15% acrylamide-SDS-urea gels shows at least four bands, a 40,000-dalton species, and three components of 9,000 to 12,000 daltons (N. K. Pandey and A. I. Aronson, unpublished data). Munoz and Doi have also found extensive heterogeneity in their *B. subtilis* coat preparations (personal communication) and, in both cases, the gel patterns differ from those obtained with *B. cereus* coats (Fig. 53).

Heterogeneity in amino termini (41, 51) also implies an extensive number of polypeptide species. As previously discussed, however, there may be problems in preparing pure coat preparations, especially if some spores germinate or lyse during extraction. The presence of trace amounts of several amino termini may mean contaminated coat preparations due to either inadequate washing procedures or spore lysis (or germination).

In summary, while the evidence for only a single species of coat polypeptide is not complete, there is no compelling reason to assume more than one major species at least in *B. cereus* and *B. megaterium*. There is suggestive evidence for at least two species in *B. subtilis*. The difficulty in solubilizing and handling the coat protein has made quantitative chemical and physical analyses rather difficult. Perhaps the best hope for determining the number of polypeptide species required for coat assembly is to isolate and analyze mutants altered in coat formation and/or structure (see section Analysis of Presumptive Spore Coat Mutants).

ATTEMPTS TO DIFFERENTIALLY SOLUBILIZE SPORE COAT STRUCTURAL COMPONENTS

The morphological distinction between inner and outer (both CP and P layers) spore coat layers provided impetus for attempting to differentially solubilize the constituent polypeptides (2, 6, 7). We now know the outer coat layers are morphologically heterogeneous (Fig. 1–4), whereas the under or inner coat coat has not been well resolved in *B. cereus* in either thin sections or by freeze etching. However, on the basis of appearance of isolated coat preparations in thin section, extraction with reducing reagents (DTE, thioglycollate, or mercaptoethanol) at alkaline pH results in solubilization of about 80% of the protein present in washed isolated coats without marked disruption of the characteristic morphology as seen in sections (2). There was some indication that a soft under coat had been removed, as well as distortion or disruption of some of the fine features of the outer coat (Fig. 17a). A similar result was obtained when intact spores were treated, i.e., about 30 to 35% of the total spore protein was solubilized (Table 2), with no major alteration in outer coat morphology as seen in thin sections or by freeze etching (5, 6).

The major structural features of the coat were disrupted, and an additional 8 to 12% of the spore protein was solubilized by subsequent treatment with a reducing reagent at alkaline pH plus either 1% SDS or 4 to 8 M urea (6 M guanidine hydrochloride was less efficient; reference 5, 6). Initial extraction with this latter combination of reagents results in solubilization of virtually the total coat as judged by appearance of the extracted spores (5, 24; Fig. 17–19, 25).

The sequential extraction procedure thus provided a basis for comparing fractions rich in inner and outer coat polypeptides (6). On the basis of gel electrophoretic mobilities, elution profile from agarose columns (as in Fig. 53),

FIG. 34. *Stage III (engulfment) in sporulating Fowler's bacillus showing the deposition of parasporal protein (PP) on the sporangial surface of the forespore membrane (M). Exosporium is not evident.* ×60,000.

FIG. 35. *A section of an early stage V sporulating Fowler's bacillus showing the deposition of the exosporium in the sporangial cytoplasm enclosing both spore and parasporal inclusion. The spore coat (SC) is now forming between spore and inclusion.* ×60,000.

FIG. 36. *A thin section of B. thuringiensis var. finitimus during stage VII sporulation showing the location of the crystal inclusion within the exosporium.* ×78,500.

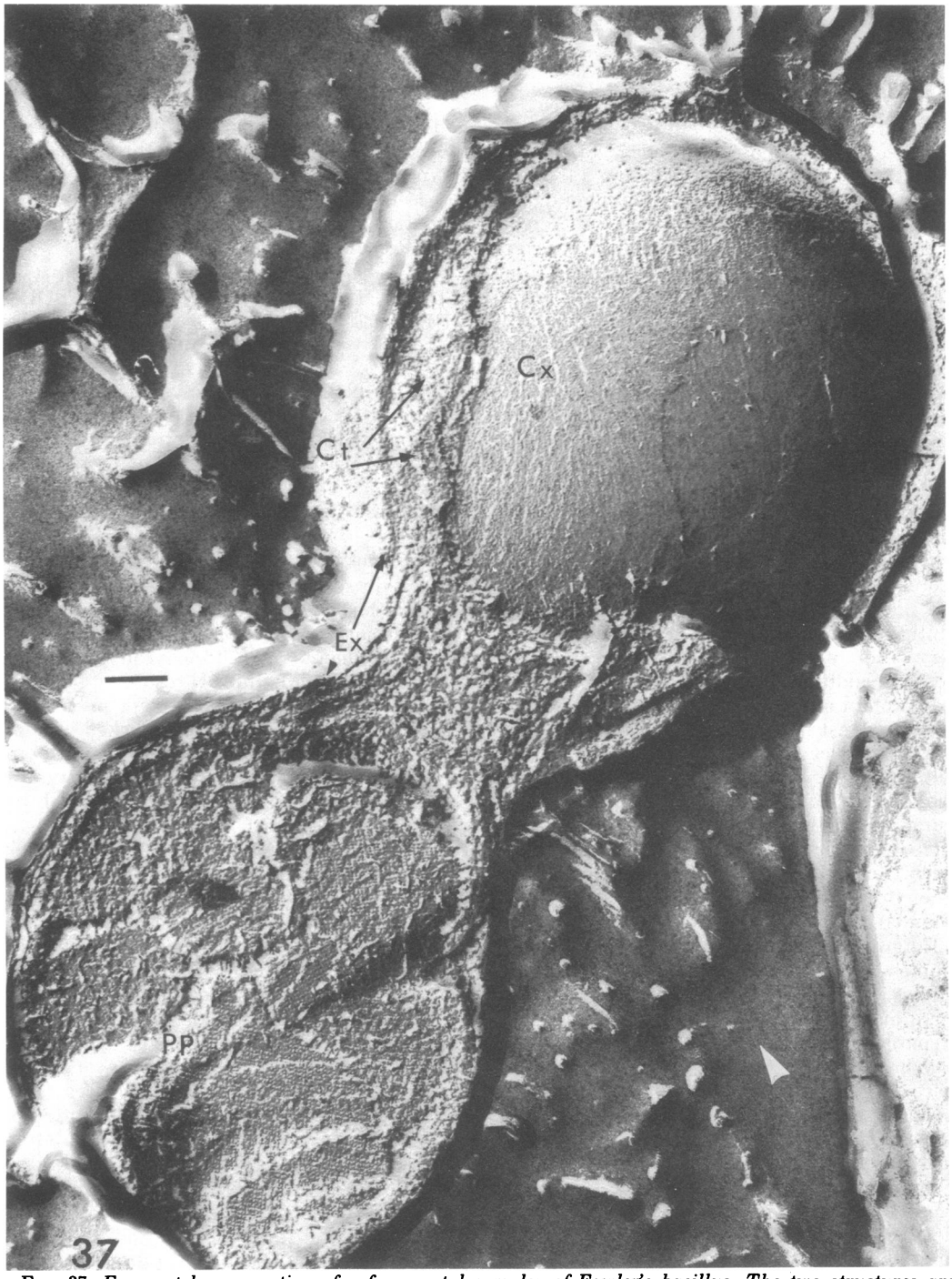
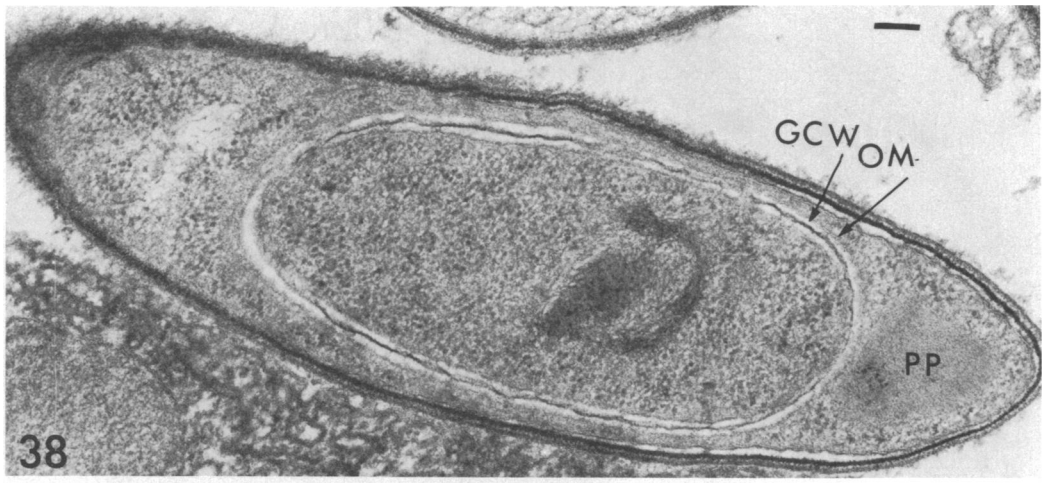


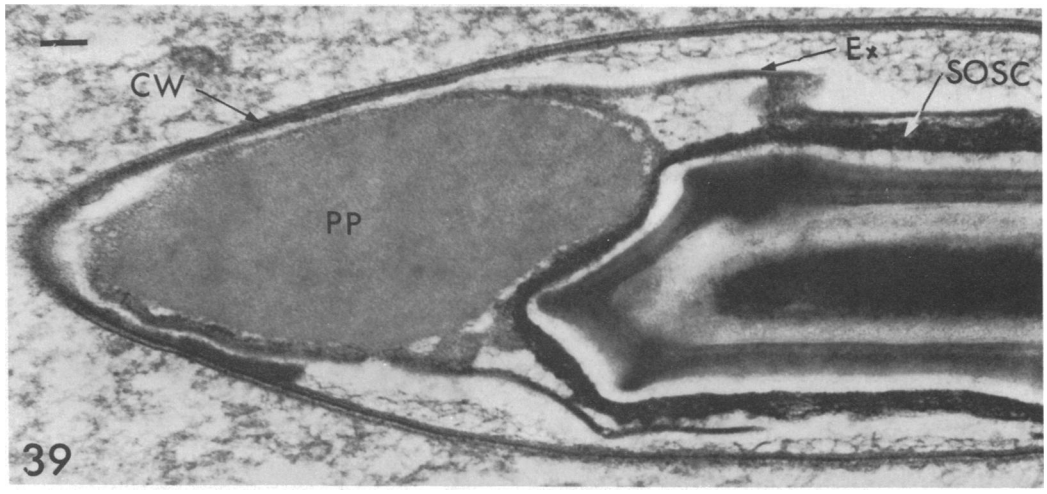
FIG. 37. *Freeze-etch preparation of a free-crystal complex of Fowler's bacillus. The two structures are contained within the exosporium (Ex) and are bridged by material continuous with spore coat (Ct). The parasporal body (PP) is composed of multiple crystalline aggregates that present different crystalline faces in this preparation (lower left). The cleavage has exposed much of the multilayered cortex (Cx). ×78,000.*

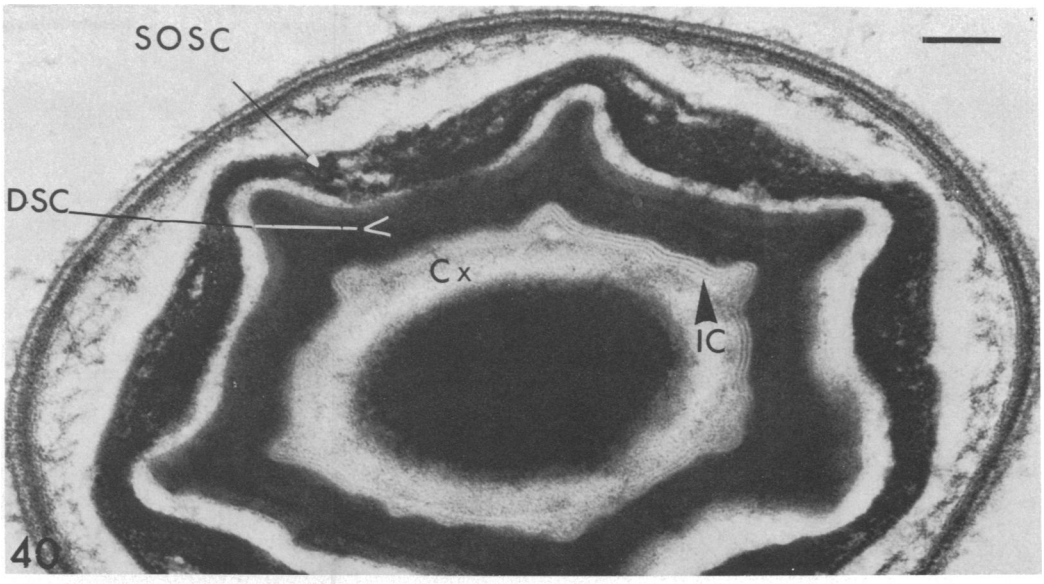
FIG. 38. *B. popilliae in various stages of sporulation in the tissues of a June bug larvae 36 days after injection. The germ cell wall (GCW) is forming (stage IV), no definite coat layers are seen, and the parasporal protein (PP) is developing adjacent to the outer forespore membrane (OM). ×59,500.*

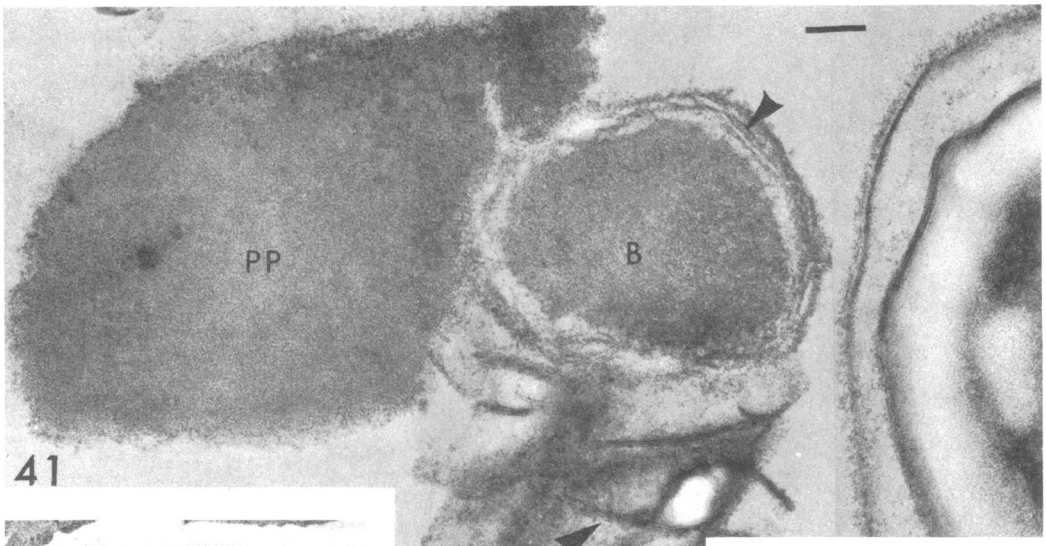
FIG. 39. *The same preparation as Fig. 38, showing the relation of the parasporal inclusion (PP) to the spore coats at stage VII. The persistent cell wall (CW) covers the two products of sporulation. The parasporal inclusion lies outside the spongy outer spore coat (SOSC) and is enclosed by the thinner outer exosporium (Ex). ×59,500.*

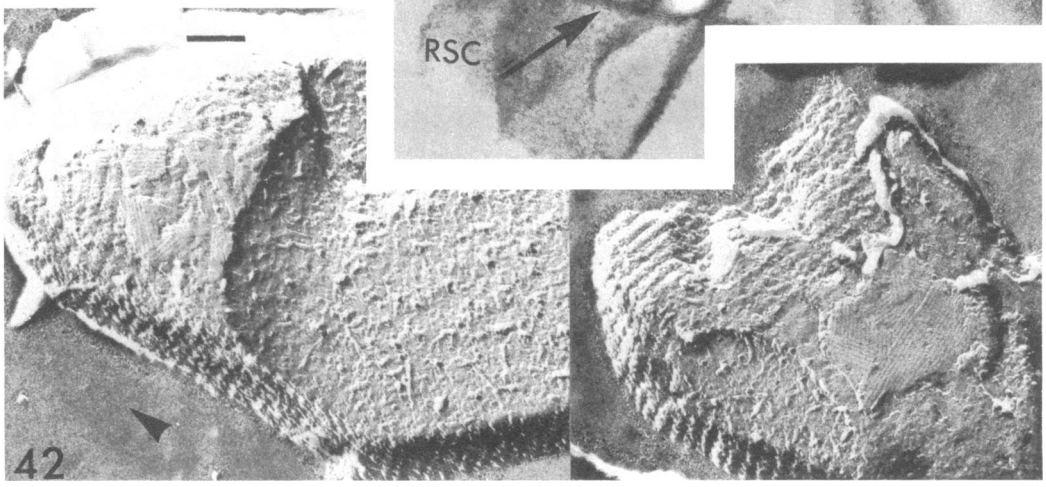
FIG. 40. *The same preparation as in Fig. 38; a cross section of a ripe spore showing the coat layers. The spongy outer spore coat (SOSC) lies on highly rigid and multilayered dense spore coat (DSC). Between it and the cortex (Cx) is a more delicate multilayered inner coat (IC). ×102,000.*

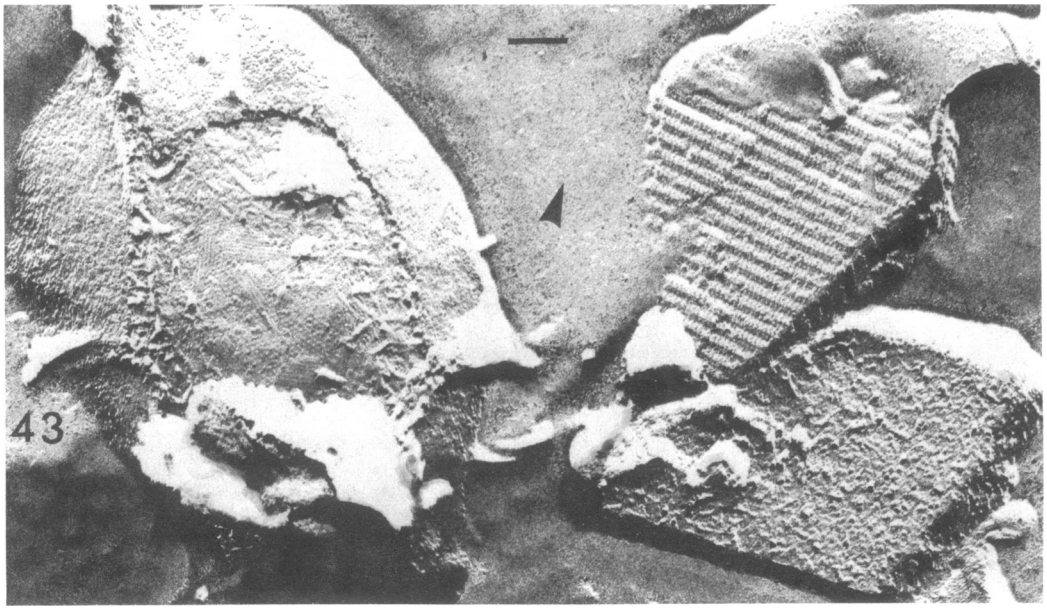

GCW
OM
PP
38


Ex
SOSC
CW
PP
39


SOSC
DSC
Cx
IC
40


PP
B
41


RSC
42


43

and sequencing of four residues from the amino termini, the two fractions contained very similar, if not identical, species of polypeptides. The keratinase peptide maps have also been compared by cross-mixing ^{3}H- and ^{14}C-labeled polypeptides extracted with either DTE or DTE plus SDS (after DTE) before denaturation, performic acid oxidation, and digestion. Within the limits of the resolution of this technique, no differences were detected supporting other evidence for the identity of the polypeptides in the two fractions.

Comparable studies have not yet been done with spores from other species, although as discussed in the section Morphology of the Spore Coat Layers, there are differences in solubility of coat fractions (48, 50, 79). The great similarity between polypeptides in the two-coat fractions prepared from *B. cereus* clearly implies, however, that differences in solubility are not adequate criteria for concluding differences in the constituent polypeptides. In the case of *B. subtilis* SB133, Spudich and Kornberg (79) did show that the amino acid composition of the soluble and insoluble coat fractions differed significantly. Aside from possible contamination of coat fractions (especially the insoluble material), and secondary reactions that may cross-link polypeptides, such as ϵ-(γ glutamyl) lysine linkages as in hair (39), the differential solubility may indeed be due in this case to different classes of polypeptides. Further studies will be necessary to resolve this problem.

In the case of *B. cereus*, however, the identity of the polypeptides in the two-coat fractions is consistent with the minimal heterogeneity found in the analysis of total spore coat protein (Extraction and Characterization of Coat Polypeptides).

KINETICS OF SYNTHESIS OF SPORE COAT PROTEIN

The appearance of spore coat in electron micrograph sections of sporulating bacilli is a fairly late event (stage IV). It is also known from the early work of Vinter (84, 85) that there is an increase in cystine uptake from the medium and its appearance in a crude spore coat fraction. We have confirmed these observations and, indeed, found that the coat fraction most resistant to solubilization was enriched for half-cystine. This fraction looks very similar in electron micrographs of thin sections to the outer spore coat (2). In addition, a quantitative immunological assay for coat protein confirmed the appearance of outer coat at phase whitening, i.e., stage V (45). Antibody prepared against solubilized total coat was enriched for outer coat antibody by adsorption to, and elution from, intact spores. This purified antibody was iodinated with ^{125}I to permit quantitation of outer coat formation in sporulating *B. cereus* T. A marked increase in outer coat antigen was found at the time of phase whitening of the forespore, a morphological change correlated with outer coat formation (i.e., stage V).

The deposition of the basic coat, i.e., under coat and P layer (80% of the total coat protein in *B. cereus* T) has been detected early in stage IV in *B. cereus* (Fig. 5). Formation of this layer is also noted earlier than the outer coat in other species (62, 65). Coat synthesis usually ascribed to stage V reflects, therefore, the final stages of coat assembly, i.e., the maturation of the outer coat.

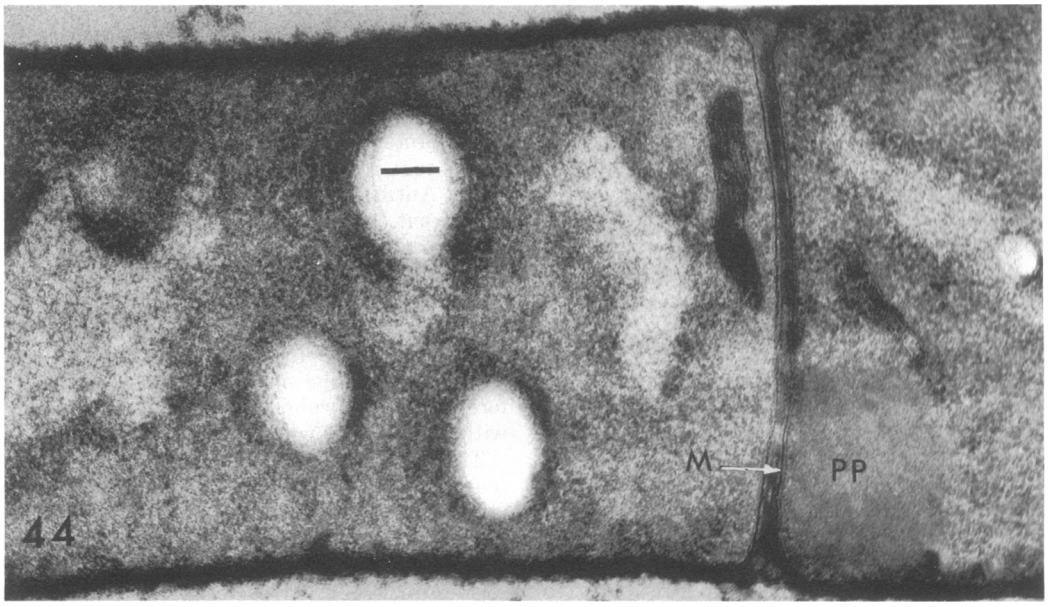
Because there was evidence for one major species of coat polypeptide in *B. cereus* T spores, a kinetic analysis of coat protein synthesis was undertaken (2). A pulse-chase protocol, i.e., incubating sporulating cells with labeled amino acids for short intervals and then "chasing" them in unlabeled "spent" medium (the supernatant from a culture incubated in parallel) supplemented with excess unlabeled amino acid, was used. The coat fractions were then solubilized from purified coats and specific activities were determined. We found that coat synthesis starts fairly early in sporulation (about the time of forespore formation) and continues at essentially a constant rate until completion. The incorporation of several amino acids into total coat or fractions enriched for inner or outer coat fitted this pattern, except for cystine which showed a marked increase in incorporation at the time of appearance of outer coat (stage V). The implications of this late cystine uptake will be discussed in detail later.

The interpretation of these experiments is dependent on: (i) good synchrony of sporula-

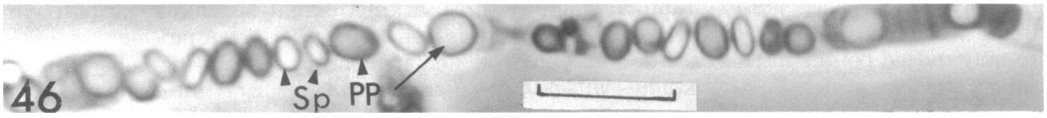
FIG. 41. *A composite micrograph showing sections of the products of sporulation in B. thuringiensis var. schwetzova (Gallaeria). The parasporal crystal (PP) usually carries with it a smaller ovoid inclusion (B) enclosed with what appears to be coat like layers (arrow). A whorl or fold of redundant spore coat (RSC) is also produced.* ×78,500.

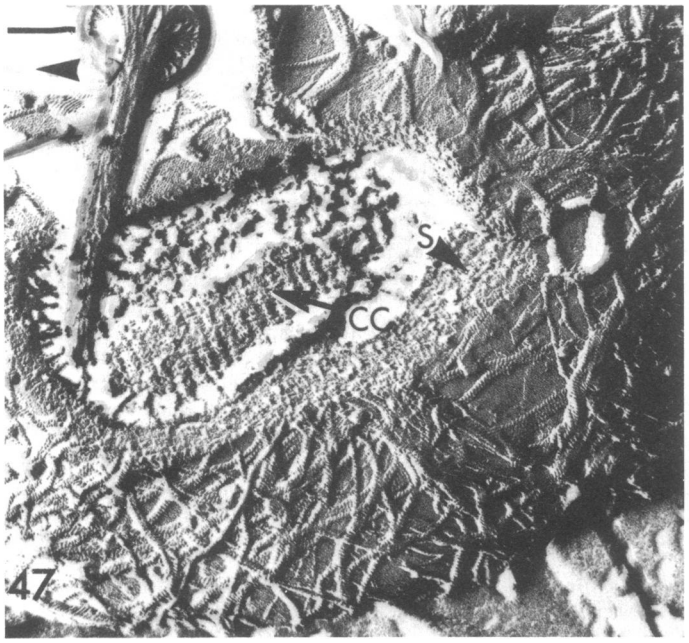
FIG. 42. *Freeze-etched replicas appear to confirm the spore coat nature of the crystal-associated layers. CP material is found plastered on many of the crystal surfaces.*

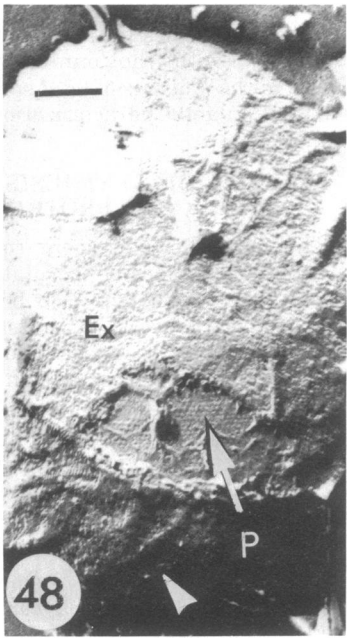
FIG. 43. *In spite of the spore coat deposition on the crystals, freeze-etch replicas of the spores themselves usually show a deficiency of the CP layer.*


M
PP
44


45
PP
TS


46
Sp
PP


S
CC
47


Ex
P
48

tion. We found it necessary to use small volumes relative to the flask volume (less than 10%) and vigorous aeration. The frequency of appearance of forespore septa and "phase-white" endospores was monitored, and only cultures with at least 80% of the cells at the same stage at a given time were used.

(ii) It was assumed that the rates of protein synthesis during the various pulse periods were the same. We did monitor initial rates of amino acid incorporation into trichloroacetic acid-insoluble material and these did not change by more than ± 10% over the period studied. Subsequently, we checked the specific activity of the labeled amino acid in the pool (cold 5% trichloroacetic acid-soluble fraction) and found very little fluctuation (within experimental error) for those employed (primarily leucine). If amino acids are withdrawn randomly from the pool, then the rates of protein synthesis are the same and thus the amount of protein synthesized during each pulse period is identical.

(iii) We have also confirmed that the resuspension of sporulating cells in spent medium with an excess (at least 100-fold) of unlabeled amino acid is a satisfactory "chase" by (a) measuring the decrease in specific activity of the amino acid pool, and (b) following the change in incorporation into trichloroacetic acid-insoluble material after resuspension. While there is protein turnover in sporulating cells (6 to 7% per hour), the rate does not change after the end of exponential growth, so loss of pulse-labeled protein should be essentially equal at all times. In addition, we have found the coat protein to be most resistant to proteolysis in vitro and would suspect that presumptive precursors are very stable.

(iv) After the end of growth (to) for at least several hours (6 to 7 h through stage V), there appears to be little change in total protein. Unfortunately, these measurements are not highly accurate (±20%) due to cell clumping and resulting sampling problems, as well as to insolubility in 0.2 N NaOH of some cellular material. If there were little or no change in total protein, then measurements of specific activities of spore coat fractions do indicate the relative amounts of coat protein synthesized at different stages. It should be noted, however, that Mandelstam et al. (55) did find continued increase in total protein after resuspension of *B. subtilis* in a sporulating medium.

Kinetic experiments similar to these were performed by Lecadet and Dedonder employing *B. thuringiensis* (50). If the conditions discussed above were satisfied, then the results were qualitatively similar, i.e., in the noncrystal-forming mutant, coat synthesis starts early and increases two to threefold overall to stage V. There is also an enhanced incorporation of cystine late in sporulation. The quantitative discrepancies between the two species may be due to lack of fulfilling the four criteria discussed above, especially synchrony. In addition, the conditions used for solubilizing coat proteins differ significantly from those used for the studies with *B. cereus*. A pH of at least 9.5 is essential for maximum coat protein solubilization (Table 2) and a more meaningful separation of coat fractions in terns of morphological studies is achieved by extracting in the absence and then presence of a reagent that weakens hydrophobic interactions (SDS, urea, or guanidine hydrochloride) (see discussion on solubilization of spore coat layers).

Comparable studies were carried out by Spudich and Kornberg (79) to measure rates of synthesis of spore coat (soluble and insoluble after treatment with alkaline DTE plus SDS) and "core" fractions of *B. subtilis* SB133 (a leaky phenylalanine auxotroph). Many of the potential problems of measuring rates, as discussed above, were bypassed by using labeled phenylalanine and measuring the specific activity of this amino acid isolated from protein hydrolysates. No special precautions were taken to obtain synchrony, although nutrient exhaustion ensures some degree of synchrony so their conclusions would be affected only in a quantitative way. One inherent problem was that the phenyalanine auxotroph used was "leaky" with endogenous synthesis of phenylalanine, resulting in a continual decrease of

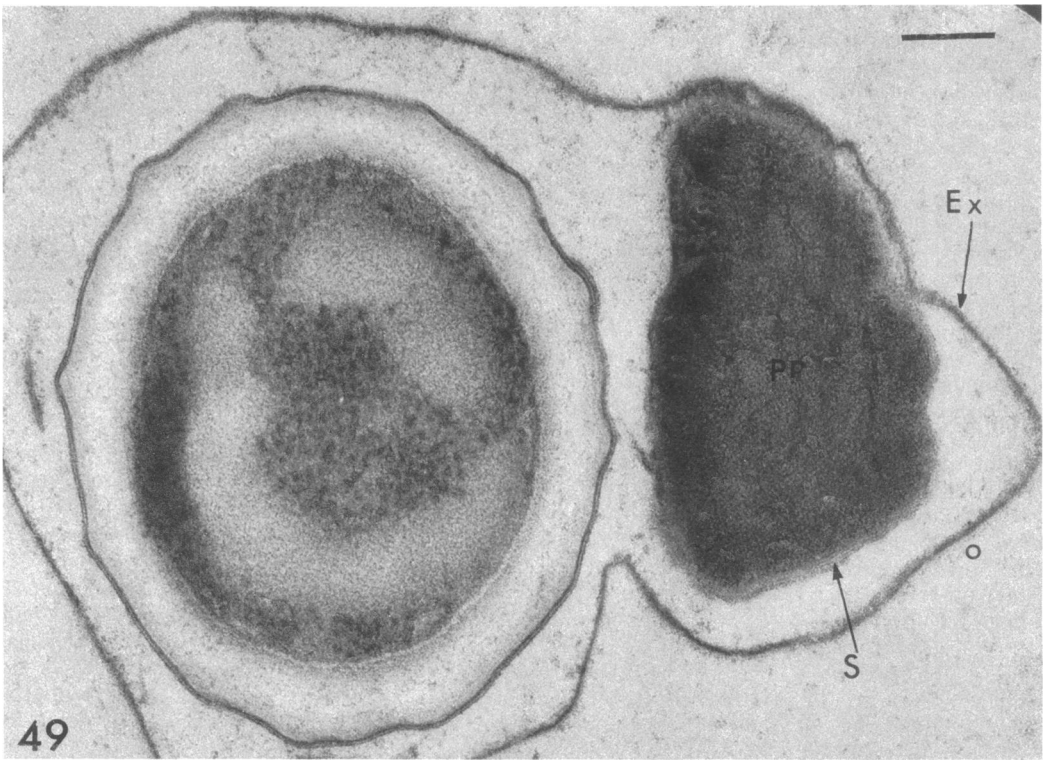
FIG. 44. *A thin section of B. medusa at t_1 showing the early deposition of parasporal protein (PP) against the base of the transverse septal membrane (M). ×70,000.*

FIG. 45. *Phase-contrast micrograph of a filament of B. medusa. (Magnificiation as indicated by 5-μm marker). At stage III of sporulation, the parasporal inclusions (PP) are about three-quarters their final volume. The developing forespore membranes (arrows) and transverse septa (TS) are more faintly visible.*

FIG. 46. *The same preparation as in Fig. 45; at stage VII the refractile spores (Sp) and ovoid inclusions (PP) fill the remnants of the filaments.*

FIG. 47. *A freeze-cleave replica of the surface of a B. medusa inclusion showing the heavy skin (S) covering the crystalline core (CC). Helically arranged fibers cover the surface of the inclusion. ×78,000.*

FIG. 48. *A spore replica from the same preparation shown in Fig. 47. Fibers are not deposited on the spore surface. A cleavage through the exosporium (Ex) reveals P layer. ×78,500.*



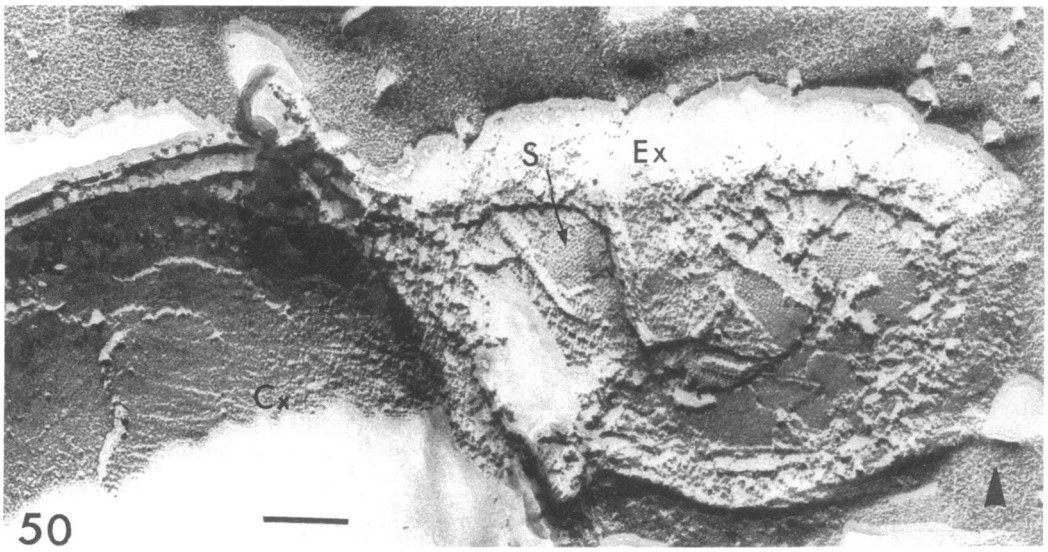


FIG. 49. *A thin section of the products of sporulation of Lewin's bacillus showing the crystalline parasporal inclusion (PP) covered by a multilayered skin (S) and enclosed within the exosporium (Ex). ×112,000.*

FIG. 50. *A freeze-cleaved replica of a spore and parasporal inclusion (PP) of Lewin's bacillus. Under the exosporium (Ex), the structure of the multilayered inclusion skin (S) is seen. The spore part of the replica shows the concave under surface of cortical layers (Cx). ×112,000.*

the precursor-specific activity.

They found that the rate of synthesis of the spore coat fraction (relative to the core) increased markedly very late in sporulation such that 70 to 80% of the total coat was synthesized after "refractile forms" were present. Because these were identified by using phase-contrast rather than bright-field optics, they were, in all

TABLE 2. *Solubilization of spore coat protein*

Method[a]	% of total spore protein solubilized	Mol% lysino-alanine[b]
(a) 0.05 M DTE, pH 10.0	34	
(b) 0.05 M DTE-0.5% SDS, pH 10.0	42	
(c) 0.05 M DTE-0.5% SDS, pH 8.5	30	<1
(d) 0.05 M DTE-0.5% SDS, pH 9.0	34	<1
(e) 0.05 M DTE-0.5% SDS, pH 9.5	39	<1
(f) 0.05 M DTE-0.5% SDS, pH 10.0	40	1.0
(g) 0.05 M DTE-0.5% SDS, pH 10.5	45	2.3

[a] All extractions of purified *B. cereus* spores was done at 27 C for 3 h. Second extraction results in additional solubilization of less than 10% of initial amount. Similar results are obtained by extracting at 37 C for 90 min or by using 8 M urea in place of SDS. A 6 M amount of guanidine HCl is somewhat less satisfactory (6).

[b] Performic acid-oxidized protein was hydrolyzed in 5.7 N HCl at 108 C for 22 h. Amino acids were separated on a Technicon analyzer. Position of elution of lysino-alanine according to Bohak (10).

probability, phase-white (late stage IV) and not refractile spores in the true sense (stage VI). Hence, their observations with *B. subtilis* may not be greatly different from ours with *B. cereus*. Moreover, 80% of the total spore proteins are in the coat of *B. subtilis* (versus about 40% in *B. cereus* T spores), which is consistent with the very thick coat seen in thin sections (43; Fig. 28-31).

Although these studies were done most carefully, the rate measurements were dependent upon differential corrections of the precursor phenylalanine-specific activity due to the endogenous synthesis in this leaky auxotroph. In particular, the values for spore coat fractions isolated from cultures incubated with radioactive phenylalanine for 11 to 12 h before chasing were corrected by about 50% and the values from the unchased culture were corrected by 100%. These latter points are most critical in the studies supporting a marked increase in the rate of coat protein synthesis late in sporulation. Without these extensive corrections, the specific activity of the coat would increase linearly with time, a result consistent with a constant rate of coat synthesis (i.e., in continuous labeling experiments).

In fact, Wood (92) did find that an alkali-extractable protein, presumably from the spore coat of *B. subtilis* 168, was synthesized at a constant rate throughout sporulation. The experimental procedure was formally similar to that employed by Spudich and Kornberg (79), i.e., continuous labeling with an excess of radioactive amino acid, addition of an excess of unlabeled amino acid at various times, and extraction of proteins from the purified spores. Unfortunately, this alkali-soluble fraction represents only a small fraction (less than 10%) of the total coat protein.

In summary, it appears that at least for *B. cereus* and related species, the synthesis of coat protein commences early (about stage II) at essentially a constant rate. In *B. subtilis*, there may be a marked increase in the rate of coat synthesis later in sporulation, perhaps to accomodate the relatively thick coat in this species. In no case is there substantial evidence for different times of synthesis of coat polypeptides present in inner or outer coat fractions, probably reflecting the inadequate procedures for fractionating coat. An increase of cystine incorporation into the insoluble coat fraction does occur late but the bulk of the evidence, at least in *B. cereus*, suggests that this incorporation is primarily due to disulfide interchange rather than synthesis of cystine-rich proteins (see next section).

One other calculation worth commenting on is the amount of protein present in a spore coat of *B. cereus* and *B. subtilis* (Table 6). Interestingly, the amount of coat protein per spore (1.6×10^{-13} g/spore) is the same with the thicker appearance of the *B. subtilis* coat, probably reflecting the smaller spore size. If we assume that the major spore coat monomer has a molecular weight of 12,000 (6), there would be 5×10^6 to 10×10^6 molecules per spore. With a rate of protein synthesis as in the exponentially growing culture, it would take about 1 h to synthesize the required number of monomers if this were the only protein being made. Even if these species represented 30% of the protein being synthesized in sporulating cells, at least 3 h would be required. In addition, rates of protein synthesis probably decrease in postexponential cells and may be as low as 30% of the exponential rate (21, 25). If these assumptions are valid, a constant rate of synthesis throughout sporulation may be necessary to form the required number of monomers. As previously mentioned, the visualization of coat formation at stage IV is thus likely to be the culmination of synthetic events begun some hours previously.

MORPHOGENESIS OF THE OUTER COAT: INCORPORATION OF HALF-CYSTINE RESIDUES

If the same species of polypeptide is the principle component of all the coat layers in *B. cereus* spores, then the presence of at least three morphologically distinct layers must be accounted for by either secondary modification of the protein or the presence of minor structural proteins unique to each layer. There is evidence in support of the former hypothesis based primarily on the unique incorporation

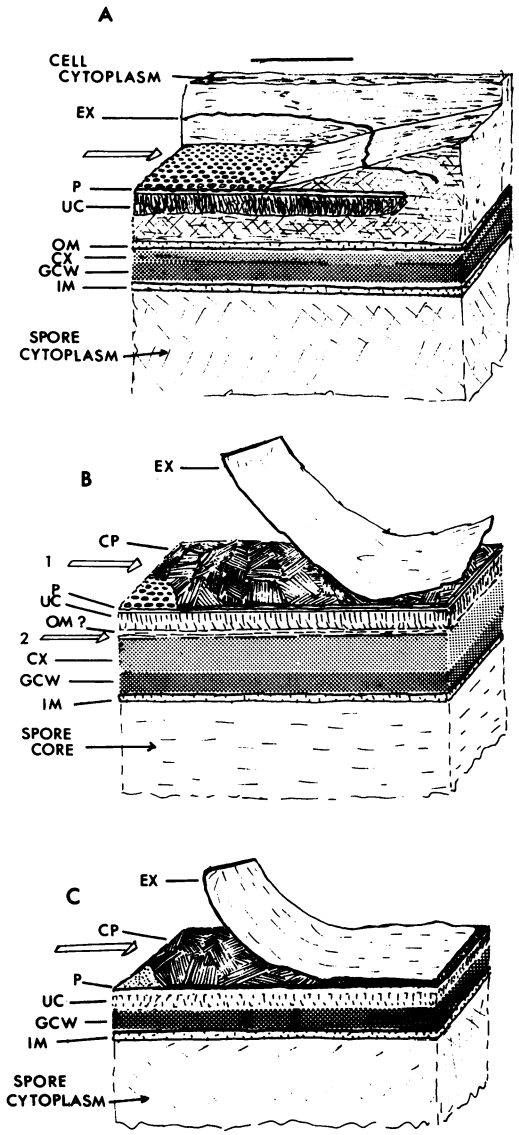
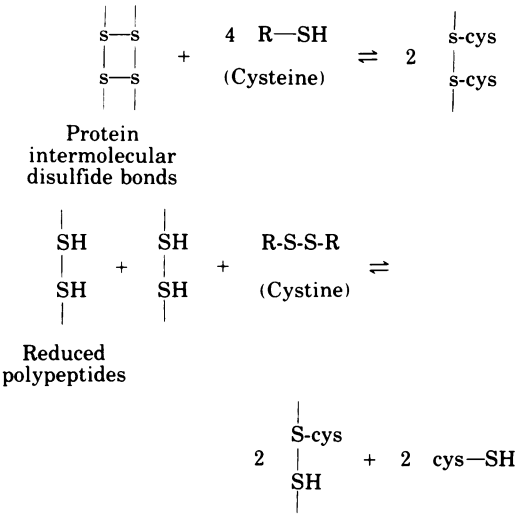


FIG. 51. *Diagramatic representation of the spore coat of B. cereus in: (A) developing spores; (B) ripe spores; and (C) spores germinated for 2 to 6 min. In (A), the developing spore coat showing P layer (P) and under coat (UC) is suspended under the forming exosporium (Ex) in the cell cytoplasm. Between the forespore membranes (OM-IM), germ cell wall (GCW) is fully formed and the cortex (Cx) is forming. A frequent plane of cleavage in freeze-etch preparations (flat arrow) reveals the pitted surface of the coat. In the ripe spore (B), the plane of cleavage illustrated (flat arrow) reveals the CP layer under the exosporium (Ex) and on the P layer. Outer membrane (OM) separating the under coat and cortex is probably not an intact layer at this stage. Another frequent plane of cleavage reveals the surface of the cortex (arrow 2). In the germinated spore (C), the cleavage plane indicated (flat arrow) reveals an unaltered CP layer. The P layer looses its pits, the inner line of that layer is lost, and the under coat disintegrates. A second frequent cleavage plane splits the inner membrane (see (Fig. 10).*

pattern of half-cystine residues. The enrichment for this amino acid in the outer coat relative to the inner coat fraction, and the correlation in time of selective incorporation with the appearance of the outer coat layers (2, 3, 45), clearly implies a special role for this amino acid in coat morphogenesis. There is considerable evidence that the late incorporation of much if not all the half-cystine occurs via disulfide interchange:



(i) Sulfite addition during stage V selectively inhibited incorporation of cystine into sporulating cells (2). The sulfite was incorporated and enriched in the outer spore coat. These spores were morphologically similar to the control, but differed in some intriguing fine points of structure and solubility of the the CP or outer coat layer (Fig. 4, 7, 14, 15).

(ii) Very late in sporulation (late stage V to early stage VI), the rate of incorporation of cystine increased whereas the rate of incorporation of other amino acids was less than 10% of that in early stage V (2).

(iii) A double mutant unable to synthesize either cystine or generate half-cystine residues from glutathione was specifically altered in outer coat assembly (15). This interesting mutant will be discussed further in the section Analysis of Presumptive Spore Coat Mutants.

(iv) Some coat deposition (with the appearance of outer coat in electron micrograph sec-

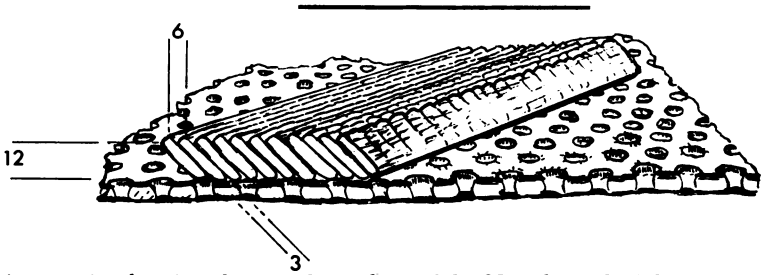


FIG. 52. *An interpretive drawing showing the packing of the CP rods on the P layer in a ripe spore of B. cereus. Approximate dimensions of the rod units are shown in nanometers. Under coat, an integral part of the P layer, is not shown.*

TABLE 3. *Summary of data regarding the heterogeneity of spore coat protein*

Method used	Proteins observed
Gel electrophoresis, pH 8.7	One major band (80–90% recovery)
Gel electrophoresis in SDS, pH 8.7[a]	One major band (80–90% recovery)
Isoelectric focusing in acrylamide (pH 3–9, ampholyte)	One band (30% recovery)
Agarose column[b]	One peak about 12,000 daltons
Immunoelectrophoresis[c]	Two bands
NH_2 termini[d]	Major Gly, minor Ser
Peptide maps[e]	Data consistent with one major species

[a] See Fig. 53.
[b] See Fig. 54.
[c] Fitz-James, unpublished data.
[d] See Table 4.
[e] See Table 5.

tions) was achieved by incubating stripped spores with soluble coat and dialyzing against a buffer containing cystine. Dialysis against buffer with no cystine resulted in an amorphous deposition of coat protein on the exosporium and cortex (5).

(v) Addition of $^{35}SO_4$ to sporulating cultures at stage V (phase whitening) results in synthesis of coat polypeptides that are identical on the basis of gel electrophoretic and keratinase peptide patterns to coat protein formed earlier. No new sulfur-rich coat polypeptides were detected in these experiments.

(vi) An antibody preparation, highly reactive to outer coat, provided a very sensitive monitor for the presence of these polypeptides (Table 7). The antigenic reactivity of outer coat was markedly reduced in vitro by denaturation in SDS plus a sulfhydryl reagent. Conversely, the reactivity of inner coat was increased somewhat by dialysis in the presence of cystine (7, 45), but significantly more by dialysis of untreated coat against cysteine.

Model for Coat Assembly

These factors, plus the great similarity if not identity of the polypeptides in the inner and outer coat fractions, have led to a model of coat formation (Fig. 55). Because sulfhydryl reagents preferentially solubilize inner coat, it is assumed that this layer forms by intermolecular disulfide bond formation. At the time of appearance of outer coat (stage V), there is a disulfide interchange, probably between cysteine and protein disulfide, so that one or two half-cystine residues in each monomer are converted to cystine (7). The resulting disruption of the intermolecular disulfide bonds may permit at least portions of the polypeptide chains to assume a new tertiary configuration where hydrophobic interactions are relatively more important. These altered polypeptides could then form the outer coat and would account for the need for both a sulfhydryl reagent and a reagent that disrupts hydrophobic interactions for solubilization.

The role of glutathione was defined by analysis of a mutant with a temperature-sensitive glutathione reductase (15). It was first established that a functional cysteine biosynthetic pathway was not essential by growing an auxotroph (blocked in a late step in the biosynthetic pathway) on limiting cysteine and finding that the spores produced contained intact coats (3). Because neither the function nor regulation of this pathway was essential, an alternate sulfur source was sought. It has been known for some time that sulfur-starved *Escherichia coli* will degrade glutathione to provide the needed amino acids (63) and we found that the steady-state level of glutathione decreased markedly at the commencement of sporulation. To further confirm a role for glutathione, we attempted to isolate a temperature-sensitive mutant unable to hydrolyze cysteinyl-glycine. Because the dipeptide will serve as a sulfur source for the cysteine auxotroph, we selected from among the auxotrophs those unable to grow at

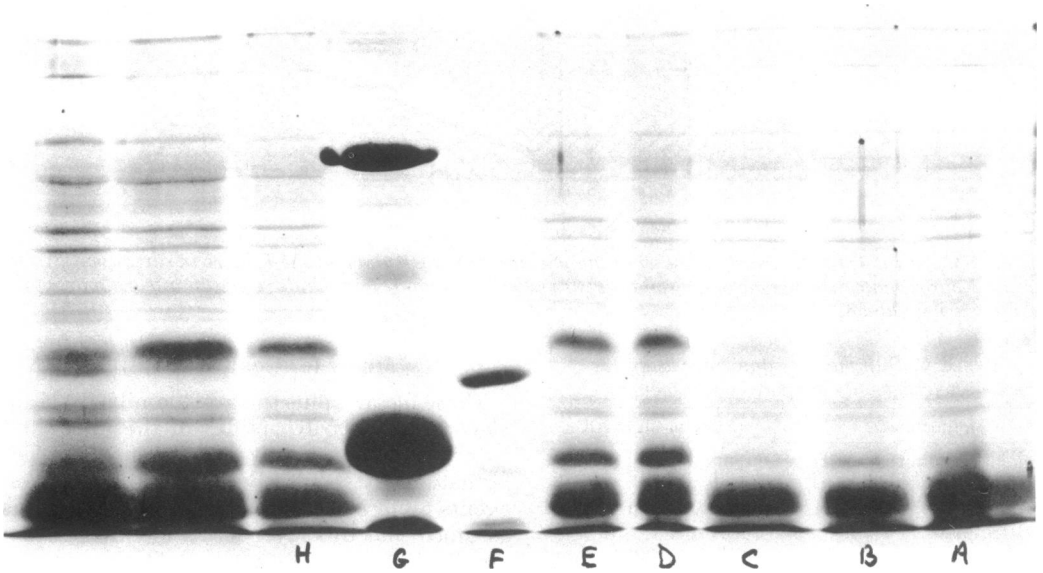


FIG. 53. *Gel electrophoresis profiles of B. cereus spore extracts. Electrophoresis in a slab apparatus at 25 mV for 3 h, 12.5% acrylamide-6 M urea-0.1% SDS gels employing 0.025 M tris(hydroxymethyl)aminomethane-0.19 M glycine-0.1% SDS-0.009 M mercaptoethanol, pH 8.5 buffer. (A) Spores extracted with 0.05 M DTE 1% SDS, pH 9.8; (B) as (A), but extract is adjusted to 3% SDS before electrophoresis, (C) as (B), with 5% SDS; (D) spores extracted with 7 M urea-0.05 M DTE-1% SDS, pH 9.8; (E) as (D), but extract adjusted to 3% SDS before electrophoresis; (F) trypsin; (G) mixture of bovine serum albumin, ribonuclease (overloaded), and cytochrome C; (H) as (E), but adjust to 5% SDS. Last two slots contained an excess of extracts as in (A) and (D).*

TABLE 4. *Amino terminal sequencing of spore coat polypeptides*[a]

Round of sequencing[b]	Dansyl derivatives		Sequences
	Major	Minor	
1	Gly[c]	Ser[c]	NH_2-Gly
			NH_2-Ser
2	Ser[c]	Val	NH_2-Gly-Ser[c](Val)
		Val-X[d]	NH_2-Ser-Val
3	Val-X[d]	Ala	NH_2-Gly-Ser-Val
			NH_2-Ser-Val-Ala
4	Ala		NH_2-Gly-Ser-Val-Ala
			NH_2-Ser-Val-Ala-Ala

[a] Same results were obtained with DTE extracts or DTE-SDS extracts of purified spores (see Table 2) and DTE extract of *B. megaterium* KM spores. Total coat was purified on *O*-(triethylaminoethyl)-cellulose (6).

[b] Manual procedure of Weiner et al. (91).

[c] Only found if hydrolysis in 5.7 N HCl at 108 C is for less than 6 h.

[d] Val-X is believed to be dansyl valine dipeptides that are not readily hydrolyzed to free amino acids (34).

38 C. Among such mutants were some that grew, as did the wild type, in an enriched medium, but produced spores with altered outer coat layers at 38 C. Although it was suspected that the mutation would be in a peptidase, the defect was found to be in glutathione reductase (15). The mutant grows well at 38 C (if cysteine is supplied) and contains primarily reduced glutathione in vegetative cells, implying alternate mechanisms for sustaining the reduced form of the tripeptide. During sporulation, however, there is a marked increase in the relative amount of oxidized glutathione (15). The oxidized form is a very poor substrate for the degradative enzymes, thus accounting for a starvation for cysteine (in conjunction with the block in the biosynthetic pathway) and the altered coat layers. Parenthetically, oxidized cystinylglycine is also a poor substrate for peptidases. When added to the medium, the dipeptide is rapidly oxidized (catalyzed by ferric ion and oxygen among others), and the inability of the altered glutathione reductase to reduce the dipeptide at 38 C accounts for the success of the selective procedure.

Temperature-shift experiments, immunological analyses, lysozyme sensitivity of spores, and electron micrographs (Fig. 15, 21) confirmed that a major phenotypic effect of this conditional mutation was on outer coat deposition. Reversion to either cysteine prototrophy or nontemperature-sensitive glutathione reductase resulted in normal spore coat formation. In the absence of glutathione degradation, there

TABLE 5. *Analysis of keratinase peptides of spore coat and core fractions*

Radioactive precursor	No. of radioactive peptides		
	Coat		
	Found	Maximum predicted[b]	Core[a]
^{3}H- or ^{14}C-labeled arginine	2(3)[c]	3-4	>9
$^{35}SO_4$ (+ unlabeled methionine)	2[d]	2-3	>4

[a] Digest of spore protein after coat removal. Only ninhydrin-reacting spots were counted. The purity of these spots has not been determined.

[b] Calculated on the basis of the amino acid analysis of *B. cereus* coat protein (2) and a molecular weight of 12,000.

[c] > 90% of input counts/min; ratio of counts/min in two peptides is 2:1.

[d] Core (origin) with 15 to 40% of input counts/min; most of this material is excluded from a Bio-Gel P10 column and is assumed to be undigested protein.

TABLE 6. *Coat protein content of spores*[a]

Species	Growth conditions	Grams of coat protein × 10^{-13}/spore
Bacillus cereus T	G-Tris[b]	1.60 ± 0.16
B. cereus T	CDGS[c] 30 C	2.15 ± 0.15
	37 C	1.60 ± 0.15
B. cereus T 9H[d]	G-Tris[b]	2.50 ± 0.25
B. subtilis	Reference 49	1.6

[a] For *B. cereus*, coat protein content of spores was determined by direct counts of the washed spores in a Petroff Hauser chamber and extraction of coat as in Table 2.

[b] A yeast extract-glucose medium (4). The same values exist as for spores produced in liquid or solid medium at 30 C or 37 C. The rate of aeration of the liquid cultures did not matter. Tris, Tris(hydroxymethyl)aminomethane.

[c] A glucose-salts-amino acid medium (4). Spores produced at 30 C (solid or liquid medium) are larger than those produced at 37 C or in G-Tris.

[d] A mutant producing large amounts of extracellular protease and larger spores than the parental strain (4).

may be compensation by the biosynthetic pathway to provide adequate cysteine. It appears that coordination of glutathione degradation and cysteine biosynthesis is normally required for this morphogenetic reaction, but either alone may suffice.

Although the scheme in Fig. 55 provides the outlines for coat formation, there are many possible steps not yet resolved, such as processing of coat monomers, an enzyme to catalyze disulfide-interchange reactions, etc. In addition, there is no mechanism for the regulation of outer spore coat formation implicit in the model. The time of appearance of outer coat is fairly precise as judged by (i) electron microscope studies (23); (ii) temperature-shift experiments of conditional mutants, such as the one with an altered glutathione reductase (15) and others producing lysozyme-sensitive spores only at 38 C (G. Stelma, personal communication); (iii) the rather abrupt increase in cystine incorporation in sporulating cultures (2); and (iv) the marked increase in outer coat antigen at the time of phase whitening (45). It is likely, therefore, that in addition to an adequate sup-

TABLE 7. *Antigenic reactivity of various forms of coat protein*

Source of coat (antigen)	% of ^{125}I antibody[a] bound per 0.2 μg of coat protein[b]
Inner (DTE extract)	13
Inner treated with 6 M urea and dialyzed[c] versus 3 μg of cystine/ml	48-55
Inner dialyzed[c] versus 3 μg of cysteine/ml	95-105
Outer (DTE-urea extract after DTE treatment)	140-160
Outer treated with 0.4% SDS-0.05 M DTE, pH 9.8, and dialyzed[c]	40-80

[a] Antibody was prepared and assayed as described by Horn et al. (45).

[b] Percentage of ^{125}I counts/min in pellet/(pellet + supernatant) (45). A control value (no coat preparation) of 9% was subtracted.

[c] All dialysis was against at least 200 volumes of 0.05 M sodium phosphate, pH 7.0, at 28 C and then versus 200 volumes of phosphate at 4 C.

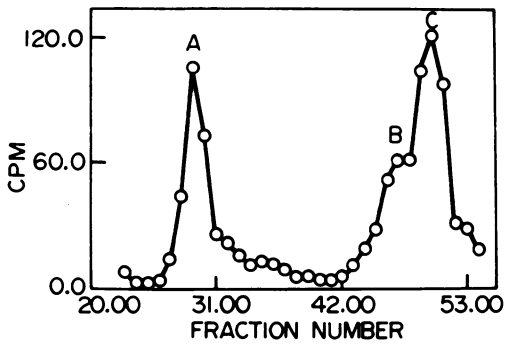


FIG. 54. *Elution of B. cereus spore coat (DTE-soluble fraction; [^{14}C]valine preparation) from an agarose column (for details of elution conditions and molecular weight calibrations see reference 6). Peak A elutes with the void volume. Peaks B and C are discussed in the text.*

ply of half-cystine residues (or cysteine) and coat monomers (both of which begin to accumulate several hours before outer coat completion), more precisely timed morphogenetic components are involved. As previously discussed, we have not been able to detect new sulfur-rich coat structural proteins at this time, which may reflect inadequate resolution in our techniques. A study of conditional mutants producing lysozyme-sensitive spores at 38 C may provide evidence for the role of other morphogenetic factors contributing to the rather precise timing of outer spore coat formation.

Analysis of Presumptive Spore Coat Mutants

There are a number of so-called stage V mutants that, on the basis of electron micrographs, appear to be altered in coat synthesis or assembly. Included in the extensive genetic studies of *B. subtilis* mutants by Schaeffer et al. (see reference 70 for a review) there are a few stage V mutants. Most of the mutants were detected by lack of pigment production and, coincidentally, were primarily blocked at early stages of spore formation. By selecting for chloroform sensitivity, Millet and Ryter (57) enriched for mutants blocked in late stages, some of which appeared to be altered in coat assembly. Sousa et al. (77) have also found two stage V mutants in *B. subtilis* that may be altered in the rate of coat production since they produce thicker coat plus cytoplasmic inclusions suggestive of coat deposits. Both mutants overproduce extracellular protease, a property exploited for the detection of a *B. cereus* mutant enriched for coat (4). The latter mutant, however, did not produce a thicker coat, but rather a larger spore.

Waites et al. (90) and Coote (17) have also found apparent coat mutants among mutagenized *B. subtilis* colonies initially screened for lack of pigment production. The isolation of a mutant of *Clostridium perfringens* with an apparent altered coat (12, 13) resulted from examination of one found to be dependent upon lysozyme for germination, a procedure exploited for the isolation of *B. cereus* coat mutants as described below. In all of the cases mentioned above, the only evidence for a coat mutation is appearance in electron micrographs. These mutants must be further studied once we understand the mechanism of coat formation more completely.

It has been difficult, however, to devise a variety of selective techniques suitable for isolating many different kinds of coat mutations. As will be discussed in more detail in the section Functions of the Spore Coat, extracted spores, i.e., those with little or no coat, retain many of the properties of intact spores, including DPA and heat resistance (2, 73, 83). They are unstable, however, and tend to turn phase dark upon storage (24 h, at 4 C). They are altered in density and are extremely sensitive to lysozyme (5). Lysozyme sensitivity of treated spores was first noted by Gould and Hitchens (30), although their treatments more likely altered coat structure rather than removed the coat since the sensitivity could be reversed by incubating the treated spores in aerated water.

We have employed lysozyme sensitivity as a means of enriching for mutants with altered coat structure (8). Lysozyme-treated spores turn phase dark and become much less dense than resistant or untreated spores. Sensitive spores may then be isolated from Renografin gradients (81). We have collected a large number of such mutants and our detailed studies of two of them indicate that there has been a mutation in the major coat structural protein. As shown in Fig. 7, the outer coat layers are fragmented or incomplete, a defect most easily seen in thin sections (8). If the model proposed in Fig. 55 were correct, then alterations in both inner and outer coat layers would be anticipated if the same species of polypeptide in both layers were mutated. Unfortunately, under or inner coat is not easily resolved. In addition, it is conceivable that a mutation affecting the structure of one coat layer could indirectly alter the packing in an adjoining layer, so micrographs per se cannot provide proof for the model.

The coat proteins extracted from the mutant shown in Fig. 7 differ from the wild type in gel mobility at pH 8.7 and in the distribution of sulfur-containing peptides in a keratinase digest (8). The same result was obtained whether total coat (DTE-SDS extract), enriched inner coat (DTE extract), or outer coat (SDS-DTE-soluble fraction after exhaustive extraction with DTE) were studied, implying that a major polypeptide present in both coat layers was altered. This result is consistent with the model proposed in Fig. 55. Because the reversion frequency to lysozyme resistance (and wild-type coat protein properties in two cases) is consistent with an original point mutation (Table 8), we assume that this is the only alteration in the mutant. As mentioned above and as summarized in Table 8, many other properties of these spores are unchanged but there is an alteration in germination response and sensitivity to octanol.

A variation of this screening technique involved selection for mutants unable to respond to normal germinants (L-alanine plus adeno-

TABLE 8. *Properties of lysozyme-sensitive mutants and chemically stripped spores of B. cereus*

Property	Wild type	Lysozyme-sensitive mutant	Chemically stripped spores	
			DTE only	DTE + SDS
DPA (grams × 10^{-15}/spore)	102–118	98–115	98–110	95–105
Resistance to 80 C/20 min	95–100	85–100	88–100	85–95
Lysozyme sensitivity[a]	–	+	–	+
Percentage of survival after treatment with octanol[b]	60–80	5–8	40–80	15–20
Storage in distilled water at 4 C	100% phase bright after 2 weeks	<50% phase bright in 48 h	100% phase bright in 48 h	<20% phase bright in 48 h
Germination rate (ΔOD_{650nm}/15 min)[c]:				
L-Alanine plus adenosine	0.48–0.55	<0.1	0.45	0.15–0.20
0.04 M calcium-DPA	0.35	<0.1	NT[d]	NT

[a] At least 90% phase dark after 30 min at 27 C with 10 μg/ml..
[b] A 1:20 dilution of octanol-mix on Vortex for 45 s at 27 C before dilution and plating.
[c] ΔOD_{650nm}, Optical density at 650 nm.
[d] NT, Not tested.

sine after a heat shock), but capable of germinating if lysozyme were present. Such mutants were first detected in *C. perfringens* by Cassier and Sebald (13) and appear to have an altered coat on the basis of electron micrographs (12). We have isolated such lysozyme-dependent mutants and the one studied in detail to date has properties very similar to the lysozyme-sensitive mutant. Here again, a major coat polypeptide extractable by either procedure is altered in a sulfur-containing peptide. Both the reversion frequency and spore properties are similar to those summarized in Table 8.

The limitation of the screening techniques has probably restricted the kinds of mutations selected. If there were other factors or proteins involved in coat formation, then clearly different selective procedures must be devised. We know, for example, that hydrolysis of glutathione is essential (at least under certain conditions) for providing half-cystine residues and a mutation affecting the hydrolysis of this tripeptide does indeed result in altered coat structure (Fig. 19, 21; also see section on the morphogenesis of the outer coat). In this respect, glutathione reductase would be an enzyme involved in coat formation. In the hope of obtaining alterations in a number of enzymes that may be involved in the morphogenetic process, we have selected mutants that produce lysozyme-sensitive spores at 38 C, but not at 30 C. Temperature-shift experiments already imply that the altered protein(s) functions during stages V and thus is probably directly involved in coat formation (G. N. Stelma and A. I. Aronson, unpublished data). We suspect that these conditional mutants will be altered in enzymes rather than in the major coat polypeptide since the latter protein is very resistant to denaturing solvents and proteolytic enzymes and probably not susceptible to the subtle changes involved in a temperature-sensitive phenotype. Hopefully, a detailed analysis of such mutants will unearth other morphogenetic factors and enzymes needed for coat formation.

FUNCTIONS OF THE SPORE COAT

Studies of chemically stripped spores, as well as some of the mutants described in the previous section, permit some insights into spore coat function. As found by others (74, 79, 83), and as summarized in Table 8, extracted spores retain their DPA, heat resistance, and refractility. They are generally very sensitive to lysozyme and solvents such as octanol, and do not keep well on storage. According to Vary (83), the germination response to L-alanine of *B. megaterium*-stripped spores is unaltered, although we have found a somewhat slower rate of germination for *B. cereus* spores (Table 8).

The behavior of the presumptive coat mutants is similar except that four of six lysozyme-dependent mutants respond neither to L-alanine plus adenosine nor to calcium-DPA (8). The altered germination pattern implies that some coat-related function is needed to respond to these germinants, although the results of chemically removing the coat are not entirely consistent with this conclusion. The availability of conditional lysozyme-sensitive mutants should permit a better definition of the relationship of germination response to coat assembly. It is possible that altered coat assembly has far-reaching implications for the assembly and arrangement of many spore components. If so,

the altered germination pattern may be a secondary consequence of this mutation.

It was noted that in the case of the mutants examined by freeze etching, a small fraction contained no coat. These "bald" spores occurred with a low frequency (less than 10%). When isolated on Renografin gradients (higher density than the mutants with coat), and subcultured, the bald spores produced a mixed population, i.e., they did not breed true. It is assumed that in some cases, the imperfect packing of the outer coat results in the loss of the inner coat. Aside from these general protective properties and a possible role in germination, no other functions for the coat have been found.

SPORE COAT-RELATED PROTEINS

Perhaps a brief comment about crystal proteins produced by *B. thuringiensis* and related species, and the toxin produced by clostridia, is appropriate since the production of both is correlated with spore formation and the proteins share some properties with coat polypeptides, especially conditions for solubilization. Although there is extensive literature on the properties of the crystal protein(s), it is beyond the scope of this treatise to review the work. The time of crystal formation is stage II or III of sporulation and the synthesis of this structure may even delay spore formation (2, 22, 50). The amino acid content is fairly similar to that of coat and there is some immunological cross-reactivity (50, 74; and Fitz-James, unpublished data). However, there are differences in peptide maps (Gillespie and Fitz-James, unpublished data), which argue for at least some of the polypeptides in a crystal being different from coat proteins, although some homology is not ruled out. A further characterization of the number of species of polypeptides in these crystals and their fractionation will be necessary before further comparisons can be made.

In addition, there are mutants of *B. thuringiensis* that are acrystalloferous, as well as those producing only crystals (50). Both classes argue for separate regulatory mechanisms of coat and crystal formation. Perhaps coat mutations, as described in the previous section, would help to resolve the relationship between the two structures. It should also be noted that crystal proteins have been reported at low frequency in a *B. subtilis* RNA polymerase mutant (68), some stage O mutants (78), and *C. perfringens* type A mutants (18). These crystals appear to form primarily in mutants altered in spore formation and may reflect crystallization of coat or other spore polypeptides under abnormal conditions. The chemical properties of these crystals have not been determined, so further speculation is premature.

There is evidence that a spore coat fraction is the enterotoxin produced by *C. perfringens* (27), since there is a correlation between the ability to sporulate and toxin production (19), and an antigenic cross-reactivity between coat protein extacted from purified spores employing procedures as in Table 2, and purified enterotoxin (27). Surprisingly, strains with no enterotoxin in cell extracts (*ent*$^-$ mutants) still contain toxin in spore coat fractions, as do spores of *B. thuringiensis* and *B. cereus* (76). Specific-activity values (toxin units per milligram of protein) for the coat fractions of clostridia are less than 10% of the specific activity of purified enterotoxin, indicating that only a small fraction of the extracted protein is toxin. The toxic activity is readily extractable from bacilli spores suggesting a very superficial association, perhaps bound to the exosporium (76). The gel electrophoretic patterns of the extracted *Clostridium* coat proteins are very complex, which may be due in part to aggregation of the proteins during dialysis before electrophoresis and/or may reflect the presence of a minor toxin species. In either case, a unique protein species comprising 10% or less of the total extracted coat could easily be present and represent a minor structural component of the coat. Alternatively, it could be a tightly bound contaminant. Only further biochemical and genetic studies can help decide.

A mutation in the enterotoxin structural gene that affects both coat formation and toxin activity would be most helpful. There are apparently mutations altering cost assembly (lysozyme dependent for germination) that do not affect enterotoxin production (19, 27). If this mutant is analogous to those discussed in our analysis of presumptive spore coat mutants, then it is unlikely that the major coat structural protein is the toxin, a result already implied by the relatively low specific activity of total coat extracts.

To further understand the relationship of the production of these toxic proteins to coat formation, a more complete analysis of coat synthesis is essential. Selection of mutants altered in toxin production and activity may provide a useful means for isolating new types of coat mutants.

CRITICAL REVIEW AND SUMMARY

To date, the working model in Fig. 55 is adequate to describe coat formation, at least in *B. cereus*, and we suspect in most species. Variations in the extent of the disulfide-interchange

reaction can provide altered protein interactions and different morphological components. Indeed, the outer coat of *B. cereus* may be viewed as containing at least two morphological components, the P layer formed originally with the under coat and the CP layer associated with the late uptake of cystine (see the last section and Fig. 52). They have not yet been separated but both layers can be at least partially solubilized by treatment with SDS plus DTE (after extensive extraction with DTE). This fraction seems to contain only one major polypeptide species identical to that found in the total coat extracts. We assume for the present, therefore, that secondary modifications, most likely disulfide interchanges, account for the two layers. The CP layer seems to be somewhat dispensable since it is deficient in sulfite-containing spores (Fig. 4), in spores produced in liquid versus agar medium, and in some inclusion-forming species (Table 1). In all cases, there are no obvious alterations of spore properties, although a more complete study is required.

Minor structural proteins have certainly not been ruled out but our chemical efforts to date to isolate such proteins have not been successful. A most likely possibility is a half-cystine-rich polypeptide synthesized late and presumably necessary for outer coat formation. As discussed previously, we have attempted to find such a protein by adding [^{14}C]cysteine or $^{35}SO_4$ to sporulating cells in late stage V (after DPA synthesis had commenced). The properties of the labeled coat protein extracted from such spores were identical to that of the major species in terms of electrophoretic mobility in gels and keratinase peptide profile. Because only 80 to 90% of the labeled coat protein was solubilized, however, a minor species could have been missed.

We feel there is considerable evidence in support of half-cysteine incorporation via disulfide-interchange reactions. Results of in vitro studies (Table 7) imply that a cystine exchange with protein disulfide is the most likely mechanism. If so, outer coat formation may result from an alteration in the structure of the outermost polypeptides present in the inner coat (i.e., cross-linked by disulfide bonds) and could account for the apparent late synthesis of outer coat protein (2, 79).

The catalyst for such an exchange is not known. We have looked for an enzyme that catalyzes disulfide interchanges as described by Fuchs et al. (28), but have not found any activity. Because the reaction occurs at a rather specific time in sporulation, and cannot be driven at an earlier time by adding sulfite, we suspect specific factors are involved. These may simply be the quantity of coat made (and thus the packing of subunits) and /or the internal cysteine concentration as dictated by biosynthesis, protein turnover, and glutathione degradation. The latter reaction appears to be essential and could augment the very low cysteine pool to a critical level. Hopefully, some of the conditional mutants that are altered at least in outer coat formation will help elucidate this mechanism.

There may indeed be a unique mechanism for regulating the synthesis of coat protein. As previously discussed in the section Kinetics of Synthesis of Spore Coat Protein, there may be a late burst of coat synthesis in *B. subtilis*, although an essentially constant rate throughout sporulation was found for *B. cereus*. Surprisingly, the total amount of coat protein in *B.*

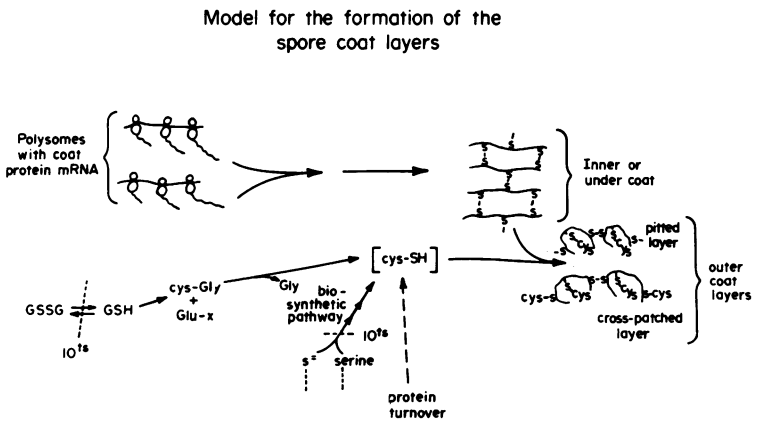


FIG. 55. *A model for the formation of the spore coat layers in B. cereus. The properties of mutant 10^{ts} are disccussed in the text and in Table 1.*

cereus and *B. subtilis* is the same despite a considerable difference in spore volume (Table 6).

This similarity is consistent with a constant and essentially identical rate of synthesis throughout sporulation (about the same total time period). Precision in coat deposition is also indicated by the fairly constant amount of extractable coat protein per spore for a given species grown under variable conditions (Table 6). Variation in the coat content per spore may result from production of larger spores as for a *B. cereus* mutant and spores produced in a synthetic medium at 30 C (Table 6). The size of *B. megaterium* spores may be varied by altering the carbon source in a replacement medium, i.e., those produced in acetate are considerably larger than spores formed in citrate and have an altered content of coat material (42).

There are also variations in the appearance of the coat layers, i.e., thickness, nature of the CP layers (43, 71), and in some cases, absence of a distinct CP layer (Table 1 and Fig. 48). These variations may in part be due to differences among the major coat structural proteins, presence of other structural proteins such as a tyrosine-rich component in *B. subtilis*, and differences in morphogenetic factors. All coats examined to date are relatively sulfur rich so disulfide interchange is probably an essential feature of the morphogenetic mechanism. Other morphogenetic reactions, such as the formation of γ aspartyl-lysine in *B. sphaericus* (82), may vary among species.

Variations in thickness may in part be attributable to spore size (Table 6), but may also reflect the time of culmination of the morphogenetic events involved in outer coat formation. There may be variation in the timing by changing the growth conditions of a given species (42), thus altering the time of function (or appearance) of one or more critical morphogenetic factors. Obviously, extensive studies of species other than *B. cereus* are necessary to determine the extent and significance of the variations involved in patterns of coat deposition.

The scheme proposed in Fig. 55 is only a first approximation to the mechanism of formation of coat layers. One may consider, for example, the maturation of coat precursor proteins possibly by proteolysis, such as that which occurs for the bacteriophage head polypeptides (50, 93). Another potential mechanism involved in coat formation is the covalent cross-linking of polypeptides. The formation of (γ-glutamyl) lysine cross-links has been shown in fibrin and hair proteins and is catalyzed by a transamidase (39). Because there are several properties of coat protein suggestive of a keratin-like structure, including low-angle X-ray patterns (46) and resistance to most proteolytic enzymes except a keratinase (8, 60), such a cross-linking mechanism may be important. In fact γ(aspartyl)-lysine has been isolated from spore coats of *B. sphaericus*, but not other species (82). We have looked for a similar dipeptide in acid hydrolysates of *B. cereus* T coats and have not found any. This possibility should be further explored, however, especially in species such as *B. subtilis* and some *B. megaterium* (Fig. 24–27) that contain a large insoluble residue of coat material.

Obviously, an extensive list of potential morphogenetic factors could be made. At present, however, a more detailed study employing a combination of morphological, biochemical, and genetic techniques, much as have been used so successfully for the elucidation of bacteriophage T4 assembly (93), are essential.

SOME CONCLUSIONS REGARDING SPORE FORMATION AND SPECULATIONS ON FUTURE STUDIES

Despite our incomplete knowledge of spore coat formation, we feel enough is known to speculate and generalize. The major outline of coat formation as summarized in Fig. 55 implies a rather simple mechanism with relatively few proteins and, thus, genes involved. All of the genetic information is assumed to be necessary for the synthesis and modification of the protein monomers. Formation of larger aggregates and structures is believed to proceed by self-assembly, although morphogenetic factors acting at higher orders of assembly have not been excluded. On the basis of this scheme, coat-related genes would include the enzymes required for glutathione synthesis (probably two), glutathione reductase, and probably two enzymes for degradation (a transpeptidase and a dipeptidase) and/or the enzymes in the cysteine biosynthetic pathway. Only one, the gene coding for the major spore coat polypeptide, is absolutely spore specific.

This interrelationship of cell and spore functions is not unique to coat formation. The biosynthesis of dipicolinic acid proceeds via the lysine biosynthetic pathway so that the first three enzymes in this pathway are essential for DPA formation (1, 29). There is apparently only one spore-specific enzyme, DPA synthetase (9, 14), needed for the synthesis of this unique spore compound, although derepression of one or more of the biosynthetic enzymes may be essential (14, 26). It is also likely that cortex formation depends on vegetative enzymes plus

some sporulating functions to account for the unique properties of the mucopeptide in the cortex (53, 82). Unfortunately, not enough is known about the biosynthesis of other spore structural components such as exosporium and membranes, nor of unique spore molecules, such as sulfolactic acid, to permit extension of this generality.

It is clear, however, that the close interplay between spore-specific and cell functions is essential for the formation of some spore components. Any resolution of the regulation of spore formation must consider mechanisms for the simultaneous "activation" of spore-specific operons and cell-related functions. Perhaps the operons of those cell-related functions that are either essential or dispensable (i.e., required only under certain conditions) for spore formation have two or more control switches (promoters) capable of responding to different signals, such as that implied from studies of regulatory mutants for alkaline phosphatase (33). RNA polymerase with higher affinity for these promoters relative to the polymerase from vegetative cells could then be an essential feature of spore regulation. Obviously, much of the research on new RNA polymerase factors is directed toward testing such a possibility (35).

The spore coat proteins may provide a means of directly testing the hypothesis of control at the transcriptional level. The major coat protein is the best characterized of all the spore-specific proteins and is produced in substantial quantities (Table 6). The rather small size of this polypeptide and the large amounts synthesized may be exploited for in vitro tests of unique sporulation transcriptional and translational signals.

The major coat protein should also be useful for testing hypotheses regarding catabolic control of sporulation. All studies to date have depended on the formation of a complete spore as a measure of catabolite control. Obviously, the formation of a spore is the culmination of many events only some of which may be under "sporulation catabolite control." Because the spore coat protein may be quantitated by immunological procedures (45), it should be feasible to examine regulatory mechanisms involved in the synthesis of a specific spore component. It is, of course, conceivable that the regulation of synthesis of this particular structural protein differs from other spore components, but that in itself is worth knowing.

We feel the start we have made in elucidating the mechanism of formation of a fairly complex structure is most promising. The intriguing interplay between vegetative cell and spore-specific components provides a unique system for studying the regulation of synthesis of a macrocomplex. In addition, the groundwork is provided for examining the basis for the extensive structural differences found in the spore coat layers of different species. We hope that some of the fundamental problems of spore formation will be solved by exploiting this system.

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