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Paracrystalline Sheets Reaggregated from Solubilized Exosporium of *Bacillus cereus*

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Fragments of exosporium, isolated from dormant spores of *Bacillus cereus*, were disintegrated by treatment with sodium dodecyl sulfate (SDS) or with phenol and acetic acid. After centrifugation of each preparation, proteins in the supernatant fractions were resolved by disc gel electrophoresis into either two or eight bands, respectively. The SDS-solubilized fraction contained spheroidal particles 11 to 44 nm in diameter. When centrifuged until clear, this fraction after dialysis still gave rise to crystal-like sheets which had the same lattice symmetry and major chemical components (protein, lipid, and carbohydrate) as fragments of the native exosporium.

The formation of exosporium in the sporangium of *Bacillus cereus* progresses from a localized point concurrently with, but external to, morphogenesis of the spore proper (18, 19). Since the basal layer of exosporium is structured as a close-packed hexagonal lattice (8), its mechanism of assembly could be analogous to crystallization. The in vitro formation of sheetlike aggregates from solubilized integument is described below. The reaggregated pieces were found to resemble isolated fragments of native exosporium physically and chemically.

MATERIALS AND METHODS

Spores of *B. cereus* T were cultured, harvested, and cleaned, and fragments of exosporium were isolated and cleaned, by methods previously described (16). The preparation (1 mg of exosporium per ml of distilled water) was stored at −20 C and thawed just before use.

Disaggregation of the exosporium was accomplished by incubating the suspension for 1 hr at 37 C with 1% (w/v) final concentration of sodium dodecyl sulfate (SDS). At first, 1.0% (w/v) β-mercaptoethanol also was added but subsequently was omitted without affecting the disaggregation. Alternatively, phenol-acetic acid-water (2:1:0.5; w/v/v) was used instead of SDS.

Disc gel electrophoresis of the SDS-treated preparations was carried out by the procedure of Weber and Osborn (25), with the exosporium solubilized in 0.01 M sodium phosphate buffer (*p*H 7). After centrifugation of the preparation at 10,000 × *g* for 15 min, the supernatant fluid was mixed with 0.05% (w/v) bromphenol blue and a drop of glycerol. The mixture was layered on top of a 7.5% (w/v) acrylamide gel in a glass tube (6 by 75 mm). The sample was covered with 0.1 M sodium phosphate buffer (*p*H 7.2) containing 0.1% (w/v) SDS. Both the upper and lower compartments of the electrophoresis unit were filled with the 0.1 M phosphate buffer. Electrophoresis was maintained at about 22 C for 2 hr, with the anode in the lower reservoir and a constant current of 8 ma per tube. Afterwards, the gels were stained with 0.125% (w/v) Coomassie brilliant blue R250 (Colab Laboratories, Inc.) for 2 hr. Excess stain was removed electrophoretically with a solution containing 75 ml of acetic acid, 50 ml of methanol, and 875 ml of water. The molecular sizes of the protein bands in solubilized exosporium were extrapolated from a plot of the relative mobilities of the following reference proteins (25): bovine serum albumin, molecular weight 68,000 (Sigma Chemical Co. St. Louis, Mo.); ovalbumin, 43,000 (Nutritional Biochemicals Corp., Cleveland, Ohio); pepsin, 35,000 (Worthington Biochemical Corp., Freehold, N.J.); trypsin, 23,000 (Worthington Biochemical Corp.); and lysozyme, 14,300 (Sigma Chemical Co.).

Electrophoresis of the preparations treated with phenol plus acetic acid was carried out by the procedures of Rodwell et al. (22). After centrifugation of the preparation at 10,000 × *g* for 15 min, one volume of the supernatant fluid was mixed with one volume of 40% (w/v) sucrose in 35% acetic acid. The mixture was layered on top of a gel, plus an overlay of 75% (v/v) acetic acid. The gel contained 7.5% (w/v) acrylamide, 35% (v/v) acetic acid, and 5 M urea. Both the upper and lower reservoirs of the electrophoresis unit were filled with 10% (v/v) acetic acid. Electrophoresis was maintained at about 22 C for 60 to 90 min, with the cathode in the lower reservoir and a constant current of 5 ma per tube. Rhodamine 6-G was used as the front marker. Afterwards, the gels were stained for 60 min with 1% (w/v) Amido black 10B (Merck & Co., Inc., Rahway, N.J.) in 7% (v/v) acetic acid. Excess stain was removed electrophoretically with 7% acetic acid.

Densitometric tracings of the stained gels from both systems were made in a Beckman DU model 2400 spectrophotometer with a Gilford model 220 scanner. The bands were also traced visually.

The determinations of apparent buoyant density were made by centrifugations on preformed linear gradients of sucrose, 20 to 65% (w/w), in 0.05 M tris-(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.5). Samples were layered on top of the gradients, which

were centrifuged for 2 hr at 25,000 rev/min in an SB-110 rotor with a model B60 ultracentrifuge (International Equipment Co., Needham Heights, Mass.). The fractions were analyzed by the method of Brakke (2).

Protein was determined by the method of Lowry et al. (15). Total carbohydrate was measured by the anthrone procedure (24), after hydrolysis of the sample in 2 N H_2SO_4 at 100 C for 120 min. Lipid was extracted in chloroform-methanol (2:1), washed successively with 1 M KCl and distilled water, dried, and weighed (6).

Shadowed specimens for electron microscopy were mounted on Formvar or Formvar carbon-filmed grids and coated with platinum-palladium (80:20) alloy in a vacuum evaporator at an angle of approximately 30°. Negatively stained specimens were mounted on collodion carbon-covered grids and stained by flooding with 2% (w/v) phosphotungstic acid (*p*H 7.2), with bovine serum albumin as a spreading agent. Excess stain was drawn off with filter paper. The specimens were examined with a Hitachi Hu-11 or a Philips 300 electron microscope.

RESULTS

Intact exosporium is readily fractured by physical forces such as sonic treatment, friction, or abrasion. The structure is relatively resistant, however, to enzymes such as Pronase, trypsin, pepsin, lipase, or lysozyme and to reagents such as acid, β-mercaptoethanol, or deoxycholate (L. L. Matz, Ph.D. Thesis, Univ. of Michigan, 1965). But partial susceptibility, as indicated by 20 to 30% reduction in the turbidity of suspensions of isolated exosporium, was observed after exposure to alkali, ethylenediaminetetraacetic acid, SDS, and phenol-acetic acid. The supernatant fraction from the SDS-treated exosporium, for example, contained about 15% of the original protein content and had a peak at 280 nm (with a shoulder at 260 nm) in the absorption spectrum. The disintegration products resulting from action of SDS and phenol-acetic acid were characterized further.

Disc gel electrophoresis was employed to determine whether exosporium contained more than one protein species. Eight bands (three major and five minor ones) were distinguished in the fraction prepared with phenol (Fig. 1A), and two bands were resolved in the fraction prepared with SDS (Fig. 1B). Compared with reference proteins in relative migration, the two subunits released by SDS treatment corresponded to 77,000 and 125,000 in molecular weight. When solubilized protoplast (cytoplasmic) membrane was isolated from vegetative cells of the same organism, five and eight bands were obtained with SDS and phenol preparations, respectively (Beaman and Gerhardt, Bacteriol. Proc., p. 51, 1970). Mirsky (17) has reported that a protein fraction from SDS-solubilized protoplast membranes of *B. megaterium* strain KM, after elec-

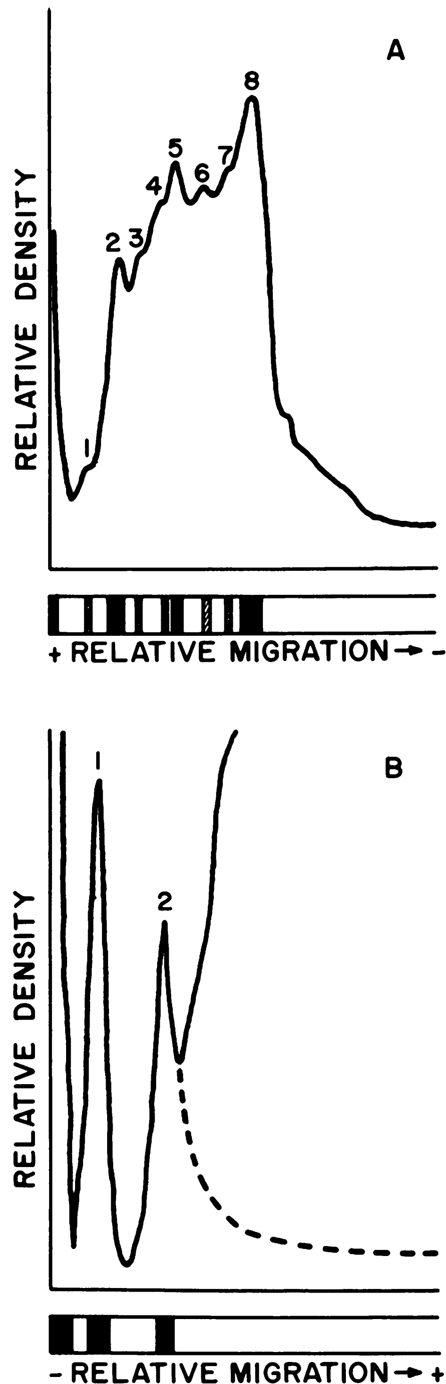

FIG. 1. *Densitometric (top) and visual (bottom) tracings of polyacrylamide gels after disc electrophoresis of the supernatant fraction from phenol-solubilized (A) and SDS-solubilized (B) exosporium. The broken line in (B) indicates the projected extension of the second band of protein, which was masked by the diffusion of dye.*

trophoresis in polyacrylamide gel, resolves into one main band, a second minor band, and a diffuse-staining band. When solubilized with phenol-acetic acid and urea, however, the membrane protein fraction resolves into at least six discrete bands. Thus, exosporium and cytomembrane appear to be similar in the multiplicity of protein species that comprise their structure.

The supernatant fraction from the SDS-solubilized integument was examined by electron microscopy and found to contain spheroidal particles which varied from about 11 to 44 nm in diameter (Fig. 2). Evidence of substructure was not detected either in the shadowed or in negatively stained preparations.

When a preparation of these particles was dialyzed against water for several days, electron microscopy revealed the formation of a few sheetlike aggregates. The SDS-solubilized exosporium then was centrifuged at 27,000 × *g* for 30 min to obtain a clear supernatant solution (A_{660} < 0.01). When this supernatant fraction was dialyzed for several days against repeated changes of Tris-hydrochloride buffer (*p*H 7.2) containing 0.01 M $MgCl_2$, profuse numbers of the sheetlike aggregates were formed. These reaggregated pieces (Fig. 3) appeared similar to the isolated fragments of exosporium basal layer, as previously depicted by Gerhardt and Ribi (8). Just as in fragments of native exosporium, the edges of reaggregated sheets occasionally were hexagonal (Fig. 4). Furthermore, with negative staining at high magnification, a lattice ultrastructure with hexagonal periodicity was discerned (Fig. 5). The average distance between the dark points in either the reaggregated or native exosporium approximated the range of 6.8 to 8.6 nm, previously reported for native exosporium by Gerhardt and Ribi (8).

The chemical composition of the sheets reag-

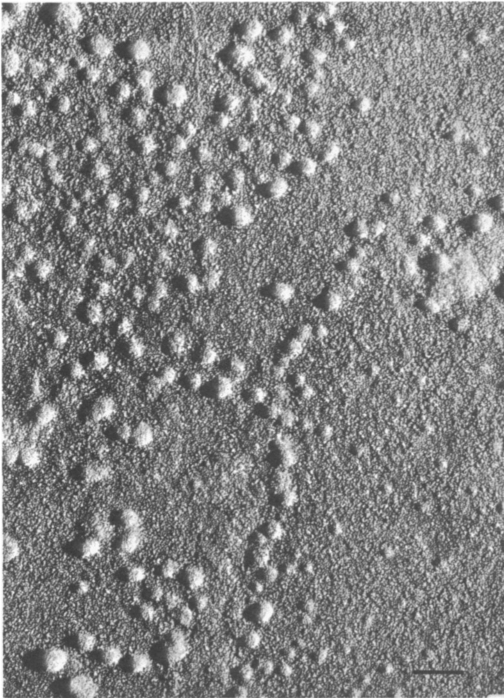

FIG. 2. *Electron micrograph of (metal-shadowed) particles in the supernatant fraction of SDS-solubilized exosporium. Bar = 100 nm.*

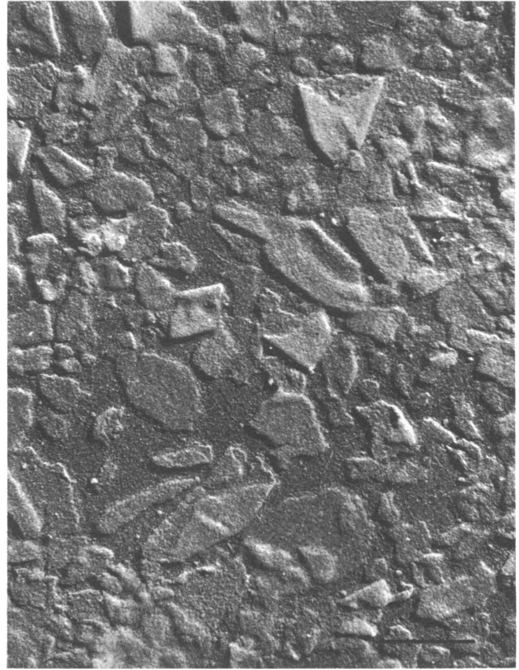

FIG. 3. *Electron micrograph of (metal-shadowed) sheets reaggregated from the supernatant fraction of SDS-solubilized exosporium. Bar = 500 nm.*

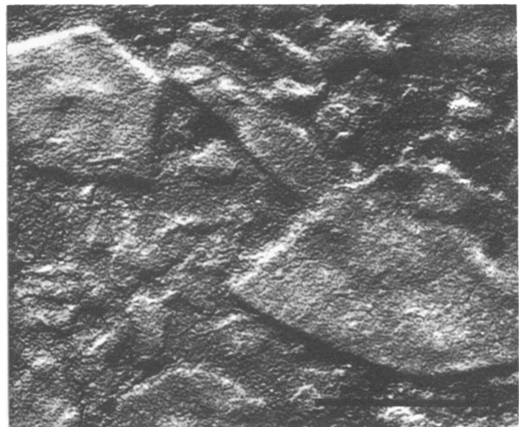

FIG. 4. *Selected field of reaggregated sheets (metal-shadowed), one (upper left) with a 120°-angled outline. Bar = 500 nm.*

gregated from SDS-solubilized exosporium, compared with that of the isolated native integument, is presented in Table 1. Protein, lipid, and carbohydrate were in both, but with a higher proportion of lipid in the reaggregated form. A similar change has been observed in reconstituted membranes from *Mycoplasma*, in which the ratio of lipid to protein varies in proportion to the concentration of Mg^{2+} (23). The higher proportion of lipid in the reaggregated exosporium sheets was reflected in a lower apparent buoyant density (1.21 g/cm^3) than that of the native integument (1.24 g/cm^3), as determined by sucrose gradient centrifugation.

DISCUSSION

Symmetrical lattice-structured layers occur on the outside of the cell walls of both gram-positive and gram-negative bacteria (20), as well as in the exosporium of spores (8, 1). The lattice in the exosporium is structured differently from that in the vegetative cell wall, at least in *B. anthracis* (7). The spore coat also has been reported to contain a latticed layer (11). However, when it fits closely around the coat (9, 14; Beaman, Pankratz, and Gerhardt, *unpublished results*), the exosporium often goes unrecognized. The coat itself appears to be comprised of a patchwork of fibrillar micelles (12).

In *B. cereus* and related species, the exosporium apparently is formed from a vesicle on the outer forespore membrane, away from the cytoplasmic membrane and mesosomes of the sporangium (19). From the evidence described above, it is supposed that subunit macromolecules are synthesized within or on the forespore membrane and spontaneously assemble from a seed point into a close-packed hexagonal sheet, which grows until it completely envelops the spore. The smallest (11 nm) spheroidal particles obtained from SDS-solubilized exosporium presumably represent the specific lipoprotein subunits of exosporium basal layer. Their size is consistent with the interspace periodicity of 6.8 to 8.6 nm observed in negatively stained exospo-

TABLE 1. *Comparative chemical composition of isolated exosporium in native and reaggregated[a] forms*

Component	Dry wt (%)	
	Native fragments	Reaggregated pieces
Protein	43.3	38.9
Lipid	15.0	33.0
Carbohydrate	22.7	12.0

[a] From the supernatant fraction of sodium dodecyl sulfate-solubilized exosporium.

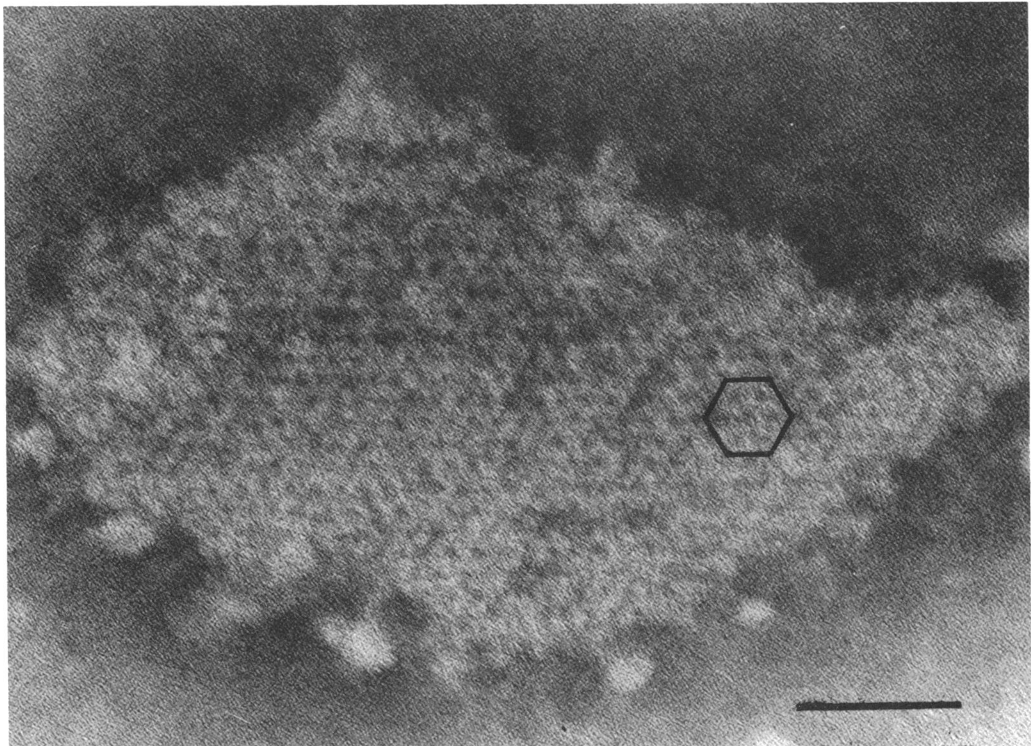

FIG. 5. *Piece of (negatively-stained) reaggregated exosporium in which the hexagonal lattice substructure is evident. Bar = 50 nm.*

rium, if allowance is made for differences in measurement with the two methods of preparation. The larger (22 to 44 nm) particles may represent the result of aggregation during the process of preparation for electron microscopy.

An analogous process of self-assembly has been proposed to account for the formation of diverse ordered biological structures, notably certain viruses but also bacterial ribosomes, flagella, pili, cell wall layers, and membranes (13). Self-assembly is envisioned as a process in which the macromolecules forming the structure fall spontaneously into a predetermined pattern by virtue of their stereochemical, configurational fit.

The studies on self-assembly of cell wall layers and cytoplasmic membranes have been the most directly analogous to the study on exosporium. Solubilized lipoprotein from the outer layer of *Spirillum serpens* cell walls can be redeposited as a hexagonal array on the surface of wall fragments if Ca^{2+} is made available (3). Similarly, the quadrangularly ordered protein subunits from *B. brevis* cell walls can be reconstituted in vitro as cylindrical tubes (C. C. Brinton, J. C. McNary, and J. Carnahan, Bacteriol. Proc., p. 48, 1969).

Considerable information is available about the solubilization and reaggregation of cytoplasmic membrane, from *Mycoplasma* (5, 21, 23) and bacterial protoplasts (4, 17), and these studies provided directly useful precedents for studying the molecular organization of exosporium. Among the several proposed models of membrane structure (13), the subunit model of Green et al. (10) postulates that membranes typically may be comprised of cuboidal repeating units formed in vivo through self-assembly into a two-dimensional sheet. If so, then the paracrystalline features of the glyco-phospho-lipoprotein exosporium "membrane" (16) may provide a reciprocally useful precedent for studying the ultrastructural organization of cytoplasmic membranes.

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