ULTRASTRUCTURE OF THE EXOSPORIUM ENVELOPING SPORES OF *BACILLUS CEREUS*

PHILIPP GERHARDT AND EDGAR RIBI

Department of Microbiology, The University of Michigan, Ann Arbor, Michigan, and Rocky Mountain Laboratory, National Institute of Allergy and Infectious Diseases, U.S. Public Health Service, Hamilton, Montana

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ABSTRACT

Gerhardt, Philipp (The University of Michigan, Ann Arbor), and Edgar Ribi. Ultrastructure of the exosporium enveloping spores of *Bacillus cereus*. J. Bacteriol. 88:1774-1789. 1964.—Structural details in the outer envelope of spores, such as those of *Bacillus cereus* and *B. anthracis*, were studied by electron microscopy and by X-ray diffraction analysis. Procedures were developed for isolating homogeneous fragments of the membrane with minimal damage to or germination of the spore proper. Exosporium of *B. cereus* appeared to embody two main layers. An outer layer was made up of a nap of hairlike projections, irregularly distributed and about 250 A deep; these arose from an intermediate covering, about 60 A in depth and similarly lead-stainable. An inner basal layer had a hexagonally perforate surface pattern of holes, averaging 76 A from center to center, and was made up of four lamellae, which could fragment into crystal-like elements. The intact basal membrane was about 190 A thick and the thinnest elements, 45 A. Microscopic observations of a crystal-like nature of the exosporium basal membrane were confirmed by X-ray diffraction analysis; the pattern of reflection lines in powder diagrams of exosporium fragments or paracrystals, or intact spores, corresponded to a hexagonal, close-packed crystal structure. The unit cell was calculated to have dimensions of 7.6 A along the a axis and 11.9 A along the c axis of the space lattice.

A filmy membrane loosely envelopes certain bacterial spores. In a classic paper on the etiology of anthrax, Koch (1877) sketched the basic structure of the spore and described the outer zone and membrane as a "round transparent mass which appeared like a small, light ring surrounding the spore." This identification preceded the discovery usually attributed to de Bary (1885) and the name "exosporium" given by Flügge (1886). The membrane is now recognized as the primary physiological barrier between spore and environment, and as a specific spore component rather than a sporangial remnant. "Exosporium" also has been used to designate different structures on the spore surface, e.g., a capsule-like zone on spores of *Bacillus megaterium* (Tomesik and Baumann-Grace, 1959). Reviews of pertinent literature are available (Knaysi, 1948; Robinow, 1960; Tomesik, 1962).

As a first step in the selective fractionation of spore components of *B. cereus* strain terminalis, we sought to slip off the exosporium with minimal damage to the spore proper, and without causing germination. Among the isolated fragments we discovered delicate crystal-like elements, and so were prompted to scrutinize this remarkable membrane by various optical means. Chemical analysis was also undertaken (Matz and Gerhardt, 1964).

**Materials and Methods**

* Cultures. Spores of a phage-resistant mutant of *B. cereus* strain terminalis were cultivated and prepared as described previously (Black and Gerhardt, 1961). A similar procedure was used to obtain spores of the Sterne strain of *B. anthracis*.

* Isolation of exosporium. Spores were ruptured by extruding a suspension (50 mg or less of wet spores per ml) through a refrigerated pressure unit similar to that described by Ribi et al. (1959). Although maximal disruption occurred with repeated passage at 50,000 psi, single treatment at 30,000 psi removed practicable amounts of exosporium and left the spore proper reasonably intact and ungerminated, as judged by sectioned appearance in the electron microscope and by refractivity. However, about half of the dipicolinic acid originally present in the spore
appeared in the suspending liquid. Sonic treatment with or without glass beads, blending with alumina, or shaking with glass beads removed exosporium, but caused progressive diminution of the fragments and abraded the coat of the spores (cf. Berger and Marr, 1960).

Fractals of exosporium were separated from spores and debris by centrifugation (30 min at about 400 × g) through a linear gradient of glycerol (Ribi and Hoyer, 1960). This concentrated the desired material in a trailing but well-separated zone, which was removed and centrifuged for greater purity. Differential centrifugation in water at similarly low force was useful for isolating larger amounts of exosporium.

Electron microscopy. Specimens intended for surface examination were deposited as droplets on beryllium- or carbon-filmed grids, dried, and shadowed in a vacuum with platinum-palladium alloy or osmium tetroxide (Murphy and Goodman, 1960). Specimens for negative staining were suspended in 2% (final concentration) neutralized phosphotungstic acid containing 0.004% sucrose (Murray, 1963a), deposited as droplets on carbon films, and dried. Specimens for sectioning were fixed and stained in buffered (pH 6) 2% osmic acid with 1% potassium permanganate, stained after fixation in saturated uranyl acetate, embedded in Vestopal W (Martin Jaeger, Geneva, Switzerland), sectioned with a glass knife in an LKB ultramicrotome, deposited on an unfilmed grid, post-stained with saturated lead hydroxide for 15 to 30 min, washed in distilled water, and dried. Three different electron microscopes were employed.

X-ray diffraction analysis. Debye-Scherrer powder diagrams were made by using copper-Kα rays filtered through nickel foil, with a North American Philips (New York, N.Y.) camera, type no. 52057-B. The specimens were rotated and consisted of powdered lyophilized material which had been pressed into sticks 0.5 mm in diameter and about 8 mm in length. The angle of reflection of each line was determined from the known diameter of the film (57.3 mm) and the distance of the symmetrical lines (2θ).

Results

Exosporium usually is only faintly discernible in a light microscope, even with phase-contrast optics, but was accentuated by using a cell-wall stain (Webb, 1954) and contrast coloration of the background (Fig. 1).

Electron micrographs revealed the structure clearly, especially after fixation with formaldehyde for direct examination, or after shadowing with a metal (Fig. 2). The surface texture seemed smooth, although a narrow fringe appeared beyond the main boundary of the folded exosporium. Shadowing with osmium oxide instead of the usual metals has been recommended for high resolution of surface topography (Murphy and Goodman, 1960); applied here, the method did not show any features of the inner and outer surfaces, but did reveal scalloped edges where the exosporium had flattened. Negative staining with phosphotungstic acid showed the protrusions more clearly (Fig. 3). Carbon replicas of spores showed main wrinkles where the exosporium had flattened, but provided no further clues.

Sectioning at first disclosed little about the fine structure of exosporium, but did reveal new details among recognized components of the intact spore (Fig. 4). In the space between exosporium and spore proper, one or more discontinuous lines (Fig. 4 and 5), which occasionally were laminated (Fig. 6), were frequently observed. Apparently they were flakelike parasporal inclusion bodies, analogous to the granular inclusions observed by Warth, Ohye, and Murrell (1963) in the same strain, and to the globular body observed by Hannay (1961) within the
FIG. 2. (top) Metal-shadowed spores, possibly germinated. In addition to the prominent exosporium, a coat and a core of the spore proper can be discerned. The fibrillar material seemingly emanating from the spore may represent polymeric material known to be released upon germination. Especially at "illuminated" portions, a narrow fringe appears to extend beyond the folded exosporium boundary (arrow). × 29,600. Micrograph by W. R. Brown.

FIG. 3. (bottom) Portion of exosporium negatively stained with phosphotungstic acid: the protrusions beyond the main boundary are more apparent. × 92,600. Micrograph by Betty J. Moberly.

FIG. 4 to 6 on following page.

FIG. 4. (top) Entire spore, medially sectioned and stained with permanganate, uranyl, and lead ions. The main structural components are successively identifiable: exosporium, coat, cortex, and core. The coat consists of a lamellar outer layer, the dark bands of which measured about 115 Å from center to center, and a dense unresolved inner layer. Discernible in the core are: a core wall and perhaps an underlying membrane, light zones believed to represent nuclear material, and granular cytoplasm with particles corresponding in size (about 100 Å) to ribosomes. Note the discontinuous parasporal inclusion bodies (arrows) that frequently were found in the space between exosporium and spore proper. × 96,200.
FIG. 5. (middle) Portion of a sectioned and triply stained spore, showing unusual comblike structure associated with the lamellar outer coat. The dark bands measured about 80 A from center to center. Another inclusion body also occurs. X 137,800.

FIG. 6. (bottom) Portion of another spore, prepared as above, showing unusually laminated parasporal inclusion body. The dark bands measured about 120 A from center to center, although not uniformly. Such bodies with two or three layers also have been observed. X 129,800. Micrographs by Betty J. Moberly.
exosporium of the Fowler variant of *B. cereus*. The parasporal flakes could represent the same material as exosporium. Another new feature, found rarely, was a comblike extension from the typically layered outer coat (Fig. 5); its nature is unknown.

Structural details of the sectioned exosporium became apparent, by accident, when the period of staining with lead hydroxide was prolonged to 30 min. A hairlike or spinelike nap was revealed on the outer surface of the structure (Fig. 7), especially on minimally washed spores. In selected fields (Fig. 8), the exosporium was seen to consist of a basal membrane measuring 180 to 200 A (average 190 A) thick, an indistinct intermediate covering of 45 to 70 A (average 60 A), and, projecting from the latter, a fairly even nap of 230 to 270 A (average 250 A). The total

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**FIG. 7.** (top) Portion of a spore, sectioned and triply stained but with extended exposure in lead hydroxide staining solution. As a result, a nap of hairlike projections was disclosed on the outer surface of the exosporium. X 105,000.

**FIG. 8.** (middle) Greater enlargement of another segment of sectioned and heavily lead-stained exosporium. In places (arrow), an underlying basal membrane was distinguished from the hirsute layer, which in turn seemed to be made up of an intermediate covering and hairlike projections. X 155,000.

**FIG. 9.** (bottom) Still greater enlargement of an exosporium segment, prepared as above. Lamination within the basal membrane was faintly observable (arrow), but such instances were rare. The lamination consisted of three dark bands, separated by two lighter bands; the dark bands measured about 25 A in thickness and 50 A from center to center. X 245,000. Micrographs by Betty J. Moberly.
FIG. 10. (top) Portion of a spore of *Bacillus anthracis*, in which a longer and more apparent nap occurs on the exosporium. X 167,700.

FIG. 11. (bottom) Section through exosporium nap of *Bacillus anthracis* spore. In the center, the nap appeared essentially cross-sectioned; around the periphery, the projections were cut more longitudinally. X 188,700. Micrographs by Betty J. Moberly.
thickness of exosporium was about 500 A. Faint laminations of the basal membrane were seen infrequently in heavily lead-stained sections (Fig. 9).

The nap of hairlike projections on exosporium was more apparent on spores of B. anthracis (Shafa, Moberly, and Gerhardt, unpublished). A portion of anthrax spore, excessively lead-stained, is shown in Fig. 10. Here the exosporium basal membrane measured 90 to 110 A (average 100 A) thick, and the nap, 560 to 690 A (average 620 A); the intermediate covering was not resolved. A section through the nap (Fig. 11) showed the hairlike projections both transversely and longitudinally. They appeared irregular in shape but of fairly even length.

Fractionation of terminalis spores provided homogeneous exosporium fragments. High-pressure extrusion of a spore suspension removed the exosporium and left the spore proper reasonably intact and ungerminated; exosporium fragments were then isolated by centrifugation through a linear gradient of glycerol. In the dense center of the gradient zone were found large characteristic fragments of exosporium (Fig. 12), slightly contaminated with bits of granular membrane believed to be coat material. Also among these characteristic fragments were some remarkable thin smooth sheets that occasionally had hexagonal outlines. A search for their identity ensued.

The trailing edge of the gradient zone, at first discarded, proved to be rich in hexagonal crystal-like elements (Fig. 13). Their relative concentration was increased by centrifugation, but they were still associated with characteristic exosporium fragments. Fractions mostly containing characteristic exosporium fragments became progressively richer in crystal-like elements with

FIG. 12. Isolated fragments of exosporium from dense part of glycerol-gradient zone, dispersed on beryllium-coated film and shadowed with platinum-palladium. A few bits of more granular material were apparent, probably representing contaminating fragments of spore coat. Also apparent were some thin sheets (arrow), which came to be recognized as structural elements of exosporium. X 33,000. Micrograph by W. R. Brown.
repeated passage through the pressure cell. Thus the elements seemed to be structural components of exosporium.

Selected fields of the paracrystals, viewed at higher magnification (Fig. 14), contained some almost perfect hexagonal pieces, with angles of 120°. Others had highly irregular edges. The thinnest pieces were only faintly distinguishable from the background. The single element shown in Fig. 15 was folded like a membrane; there were small tears; the edges were not all perfectly straight; and the surface seemed unpatterned even at high magnification. Often, there were vertical stacks of four or five pieces in integral arrangement with parallel or common edges. When elements were stacked together, the electron density increased but remained less than that of the characteristic exosporium fragments; the surface appearances also differed.

The ratio of shadow-length cast by characteristic exosporium fragments to that by the thinnest elements was about 3.6:1 instead of 4:1.
or perhaps more; where two elements were stacked with a common edge, the measured shadow ratio was about 1.7:1 instead of 2:1. This lack of unit proportionality could be due to a nestled or closed type of stacking, with elevations in the lattice of one surface fitting into corresponding depressions of another. Such an arrangement also would explain the rare appearance of lamination in sections of exosporium (Fig. 9).

The crystal-like nature of the elements was examined by X-ray diffraction analysis. Debye-Scherrer powder patterns (Fig. 16) showed distinct reflection lines. A similar pattern was seen with three different preparations, the lines appearing sharper with a preparation rich in crystal-like elements. When a powder of intact spores was examined, moreover, several reflection lines were apparent, despite an intensive background scattering due to noncrystalline constituents of the spore. All of the lines corresponded to ones in the pattern from isolated exosporium, indicating that the pattern was not an artifact resulting from the isolation procedure. Furthermore, it seems as if exosporium is the prevailing, and perhaps the only, crystalline structure in the spores.

Ratios of the interplanar spacings \(d_{hkI}/d_{000}\) were calculated for each reflection in the powder pattern and were found to correspond to the theoretical ratios (Klug and Alexander, 1954) for a hexagonal, close-packed crystal structure (Table 1).

Dimensions of the smallest unit cell that would satisfy all of the observed reflections were determined by use of the quadratic form of the Bragg equation (Table 1). On the basis of the 100 and 110 prismatic reflections, the lattice...
constant $a_0$ (Fig. 17) was calculated to be 7.45 and 7.82 Å, respectively. Similarly, the lattice constant $c_0$ was found to be 11.95 and 11.78 Å on the basis of the 002 basal reflection and its second-order 004 reflection, respectively. With the averages of these lattice values, interplanar spacings then were calculated for all reflections, including the pyramidal ones; these calculated spacings matched with the observed values (Table 1). Thus, the indexing for a hexagonal, close-packed crystal structure was verified, with the smallest unit cell calculated to have dimensions of 7.7 Å along the $a$ axis and 12.1 Å along the $c$ axis of the crystal lattice.

Figure 17 is a diagram which corresponds to the doublet crystal-like element outlined in Fig. 15, and shows the lattice axes and the prismatic and basal planes. The $hko$ reflections in the exosporium diffraction patterns were relatively strong and narrow, independent of the proportion of elements in the preparation. But the 001 reflections were weak and broad, particularly in patterns from preparations rich in crystal-like elements. This is typical of laminar-dispersed, or plate-like, crystals having a limited number of 001 reflecting planes that diminish as the crystals become thinner.

The X-ray diffraction patterns and the often regular edges and angles of fragmented exosporium suggested the presence of a regular hexagonal pattern of subunits in the basal membrane, perhaps below the limit of resolution afforded by shadowing, replicating, or routine phosphotungstate staining. The manner of employing phosphotungstate was varied, and the electron microscope was adjusted for high resolution and contrast, with inorganic test material resolving at about 10 Å.

The predicted pattern was eventually found in exosporium fragments negatively stained with phosphotungstate (Fig. 18), especially in portions where the superficial convolutions were indistinct. Great magnification (Fig. 19) revealed a hexagonally perforate pattern of darkly stained centers, possibly representing holes or pits in a fabric. The shape of the holes could not be distinguished, nor could the form and arrangement of the strands comprising the fabric. The distance between the centers was 68 to 86 Å.
TABLE 1. Reflection lines and calculations* from X-ray diffraction powder pattern of isolated exosporium

<table>
<thead>
<tr>
<th>No.</th>
<th>Intensity</th>
<th>Diam</th>
<th>Reflection angle, sin θ</th>
<th>Interplanar spacing</th>
<th>Miller index, kkl</th>
<th>Lattice constant</th>
<th>Calculated interplanar spacing, d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>mm</td>
<td>A</td>
<td>a, b, c</td>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>1</td>
<td>Medium</td>
<td>13.7</td>
<td>0.1193</td>
<td>6.45</td>
<td>1.00</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Weak</td>
<td>14.8</td>
<td>0.1288</td>
<td>5.97</td>
<td>0.93</td>
<td>0.94</td>
<td>002</td>
</tr>
<tr>
<td>3</td>
<td>Strong</td>
<td>16.5</td>
<td>0.1435</td>
<td>5.36</td>
<td>0.83</td>
<td>0.88</td>
<td>101</td>
</tr>
<tr>
<td>4</td>
<td>Medium</td>
<td>20.2</td>
<td>0.1754</td>
<td>4.39</td>
<td>0.68</td>
<td>0.69</td>
<td>102</td>
</tr>
<tr>
<td>5</td>
<td>Strong</td>
<td>22.7</td>
<td>0.1968</td>
<td>3.91</td>
<td>0.61</td>
<td>0.58</td>
<td>110</td>
</tr>
<tr>
<td>6</td>
<td>Weak to medium</td>
<td>25.5</td>
<td>0.2207</td>
<td>3.49</td>
<td>0.53</td>
<td>0.53</td>
<td>103</td>
</tr>
<tr>
<td>7</td>
<td>Weak</td>
<td>30.3</td>
<td>0.2614</td>
<td>2.94</td>
<td>0.46</td>
<td>0.47</td>
<td>004</td>
</tr>
<tr>
<td>8</td>
<td>Medium</td>
<td>34.1</td>
<td>0.2932</td>
<td>2.62</td>
<td>0.41</td>
<td>0.43</td>
<td>104</td>
</tr>
<tr>
<td>9</td>
<td>Strong</td>
<td>40.2</td>
<td>0.3437</td>
<td>2.24</td>
<td>0.35</td>
<td>0.35</td>
<td>204</td>
</tr>
</tbody>
</table>

*λ(Cu-Kα) = 1.539 A. Bragg equations: \( d = \frac{\lambda}{2 \sin \theta} \); \( \sin^2 \theta = \frac{\lambda^2}{4} \left( \frac{h^2 + k^2 + l^2}{a^2} \right) + \frac{1^2}{c^2} \)

(average 76 Å) in measurements on four separate and differently magnified (400,000 to 700,000 X) micrographs.

Individual crystal-like elements were not easily seen in phosphotungstate impregnations (Fig. 20). High magnification of selected areas faintly revealed a hexagonally perforate pattern of darkly stained centers. The comparable dimensions of the pattern in these elements and in the characteristic exosporium fragments, together with the evidence of association and derivation, leads to a conclusion that the crystal-like elements represent structural elements in the basal membrane of exosporium.

**Discussion**

Figure 21 is a tentative model in cross section of the fine structure of exosporium, deduced from the foregoing observations. Exosporium was interpreted as a two-layered structure, with a lead-stainable, hirsute covering over a laminated basal membrane. Warth et al. (1963) reported that the exosporium of terminalis spores consists of a dense membrane, approxi-
FIG. 18-19.

1785
mately 45 Å thick, between two faint layers, each approximately 150 Å thick and embedded with granules. We also saw an inner faint layer along sectors of sectioned exosporium, but considered it an artifact of oblique sectioning. Giesbrecht (1964) noted hairlike structures at the wall surface of sectioned spores of B. subtilis and also on spores known to have a prominent exosporium; he described a pattern "like the surface of the brain" on negatively stained spores. The hirsute layer may correspond to a capsule-like zone around the exosporium of B. cereus strains as seen with a phase microscope after mounting in India ink and treatment with homologous spore antiserum (Tomesik, Bouille, and Baumann-Grace, 1959). As observations on spores developed, somewhat similar ones were reported for the outer envelope of Lampropedia hyalina (Chapman and Salton, 1962; Chapman, Murray, and Salton, 1963; Murray, 1963b). The envelope of this unusual bacterium is two-layered, the surface layer having a regular pattern of hexagonally punctate spines.

A hypothetical model was also constructed (Fig. 22) of the surface ultrastructure of the exosporium basal membrane, in an attempt to correlate the dimensions of the perforate pattern of dark centers, observed with the electron microscope after phosphotungstate staining, and the dimensions of the unit cell determined from the X-ray powder pattern. Since both the stained pattern and the diffraction pattern were hexagonal, it seemed simplest to assume that the dark centers represent hexagonal holes in a main fabric comprised of hexagonal units. Each of these units in turn was assumed to be made up of 48 parallelepipedal lattice cells, one of which is drawn enlarged and three-dimensionally in Fig. 22 to show the three axes and the relationship to a hexagonal space lattice. Since the unit cell measured 7.5 to 7.8 Å along the c axis, the periodicity of the perforate pattern in the electron micrographs should be some regular multiple of this measurement. It is apparent from the model that this multiple should be 12; the total interspace distance should be 12 × (7.5 to 7.8) = 90 to 94 Å, which correlates fairly well with the (68 to 86 Å) interspace distance in the electron micrograph. Similarly, 4 × (11.8 to 12.0) = 47
FIG. 22. Diagrammatic interpretation of the surface ultrastructure of exosporium basal membrane, deduced from the X-ray diffraction patterns and from electron micrographs of phosphotungstate-stained fragments. The hexagonally perforate pattern of dark centers, assumed to be hexagonal holes in a fabric, measured 68 to 86 Å from center to center in electron micrographs. Each of the hexagonal units, which are assumed to comprise the fabric, is made up of 48 parallelepipedal unit cells, which measured 7.5 to 7.8 Å along the a axis and 11.8 to 12.0 Å along the c axis by X-ray diffraction analysis. One such unit cell is enlarged separately (lower right) in a three-dimensional diagram, which shows the three axes and the geometric packing of three unit cells into a hexagonal space lattice.

to 48 Å seems to correlate with the estimated 40 to 50 Å thickness of the crystal-like elements. It remains to be seen whether the above correlations can be confirmed by low-angle X-ray scattering. Previous applications of diffraction analysis with bacteria (Hurst, 1952; Grossbard and Preston, 1957, 1958) resulted in relatively few and diffuse diffraction rings and were not associated with crystal-like structure observable by electron microscopy.

The ordered structure found by means of the electron microscope in the basal layer of exosporium has frequently observed counterparts, especially among cell walls of gram-negative bacteria (Salton, 1964). Apparently, the inner perforate layer in the Lampropedia envelope, for example, is a honeycomb network of hexagonally distributed holes with a repeat spacing of 145 Å; this structure, coarser than in terminalis exosporium, also fragments hexagonally. Robinow (1960) provided early evidence of finely textured structure in exosporium, surprisingly seen in a direct electron micrograph at comparatively low magnification.

Permeability measurements on intact, exosporium-covered spores indicated that the effective surface is heteroporous, with randomly sized openings allowing penetration of molecules
up to 180 A in effective diffusion diameter (Gerhardt and Black, 1961). This does not agree with the above observations of an orderly perforate basal layer. Also, if the dark centers seen in phosphotungstate-stained fragments represent holes in the fabric, their size is considerably less than predicted from permeability experiments.

Berlin, Curran, and Pallansch (1963) used gas-adsorption techniques to determine the porosity of washed spores, including a strain of \textit{B. cereus} (720) that is believed to have an exosporium. Extreme conditions of dryness and cold (−195 °C) were used. Their calculations indicated that less than 5% of the total spore volume is occupied by pores penetrable by gaseous nitrogen, and that these pores have an average diameter of 470 A and a maximum twice that. Openings so few and enormous were probably cracks or rents caused by extreme conditions of handling, rather than natural features of the spore surface structure. This may also explain the discrepancy between conclusions from the permeability measurements of Gerhardt and Black (1961) and the above observations on exosporium structure. Handling and washing procedures, or even natural spore development and release, may break the delicate exosporium so that the effective diffusion barrier is the underlying coat.

Acknowledgments

A number of individuals collaborated in this study, and their respective contributions are identified in the figure legends. In addition, E. A. Bannan accomplished the initial work on fractionation procedures, and L. Matz demonstrated the comminution of exosporium into crystal-like elements.

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