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THE CHITINOUS NATURE OF FILAMENTS EJECTED BY *PHAEOCYSTIS* (PRYMNESIOPHYCEAE)[1]

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ABSTRACT

Filaments ejected by Phaeocystis globosa *Scherffel, organized in star-like structures, were observed and analyzed before and after their discharge from cells. Ultrastructural observations obtained after cryofixation and cryosubstitution led to a model for their storage within the cell and for their ejection from the cell. Electron diffraction analysis on the ejected filaments demonstrated their chitinous composition. This technique indicated without ambiguity that each filament was in fact a whisker-like α-chitin crystal, with the axes of the corresponding polymer chains aligned with the filament's axis. X-ray microanalysis of the mats of filaments indicated that the silica content suggested by earlier workers was an artifact resulting from the filtration procedure.*

Key index words: α-chitin; cryotechniques; electron diffraction analysis; Phaeocystis*; Prymnesiophyceae; X-ray microanalysis*

Phaeocystis Lagerheim (Prymnesiophyceae = Haptophyceae) is a marine algal genus found worldwide in many oceanic systems, particularly in the coastal zones and high latitude areas of the oceans (Smith et al. 1991). This alga can be a nuisance because its colonial stage clogs fishing nets (Savage 1930), produces massive foam banks on beaches (Lancelot et al. 1987), and releases noxious chemicals such as dimethyl sulfide (Andreae and Raemdonck 1983) and acrylic acid (Sieburth 1960). As part of its life cycle, *Phaeocystis* also exhibits a non-colonial stage (Parke et al. 1971), consisting of biflagellated cells that possess the unique characteristic of ejecting rigid filaments organized as 5- or 9-branched stars (Parke et al. 1971, Moestrup 1979, Pienaar 1991). The biological function of these filaments remains a mystery, and their composition has not been established despite a recent study indicating the presence of silicon in the filaments (Ramani et al. 1994).

This work deals with the filaments produced by *Phaeocystis globosa*; filaments are demonstrated to consist of α-chitin (a crystalline organization of antiparallel poly-β-[1→4]-*N*-acetyl-D-glucosamine chains). In addition, we give some details on the origin of the filaments, their wound state within protruding vesicles, and their uncoiling during discharge from the biflagellated cells.

MATERIAL AND METHODS

Cultures. Cultures of *Phaeocystis globosa* (strains Rosko A and C) in their motile phase, producing a large number of filaments, were maintained in K medium (Keller et al. 1987) at 15° C under a 12-h light:12-h dark cycle of about 100 μmol photons·m^{-2}·s^{-1}.

Ultrastructural observations. To visualize the filaments and their organization into starlike structures, preparations were made by depositing drops of culture on formvar-coated electron microscope grids. These grids were then fixed with osmium vapors, air dried, rinsed in distilled water, and shadowed with platinum using an Edwards Auto 306 coating unit. Ultrastructural observations of thin sections were made on material prepared by cryofixation and cryosubstitution according to Soyer-Gobillard and Géraud (1992). Culture samples were centrifuged at 4° C for 15 min at 900 × *g*, transferred to a filter paper, and frozen on a block of copper cooled by liquid helium to 4° K on a Reichert cryovacublock. This quench–freeze fixation was followed by freeze substitution in a CryoCool apparatus. The cells were embedded in Epon and thin sections were stained with alcoholic uranyl acetate (for 20 min) (Pottu-Boumendil 1989) and lead citrate (for 3 min) (Reimann et al. 1980). The grids were examined with a Hitachi H 600 transmission electron microscope operated at an accelerating voltage of 75 kV.

Low dose electron microscopy and electron diffraction analysis. Two different series of experiments were performed. The first series was devised to investigate the filaments in their native state, wherein a culture of *Phaeocystis* was fixed with formaldehyde vapors and rinsed with distilled water. Drops of the culture were deposited on carbon-coated grids and allowed to dry. The second series was devised to observe the filaments in a cleansed state. For this series, the supernatant fraction of a *Phaeocystis* culture, which was devoid of cells and debris but contained numerous filaments, was centrifuged and redispersed 4 times in distilled water. The resulting pellet was further cleaned by mixing it with 2% NaOH, boiling the mixture for 2 h, dialyzing the mixture for 24 h with tap water, and then dialyzing the mixture for 24 h with distilled water. Drops of the cleaned suspension were then deposited on carbon-coated grids and allowed to dry.

All low dose electron microscopy and electron diffraction analysis was achieved with a Philips CM200 CRYO transmission electron microscope equipped with low dose software and a Lhesa image intensifier. The images and electron diffraction patterns were recorded at an accelerating voltage of 200 kV, under electron doses ranging from 200 to 500 elec/nm^2 on previously unexposed regions of the specimens. The diffraction diagrams were recorded on specimen diameters ranging from 0.5–1.0 μm. Cal-

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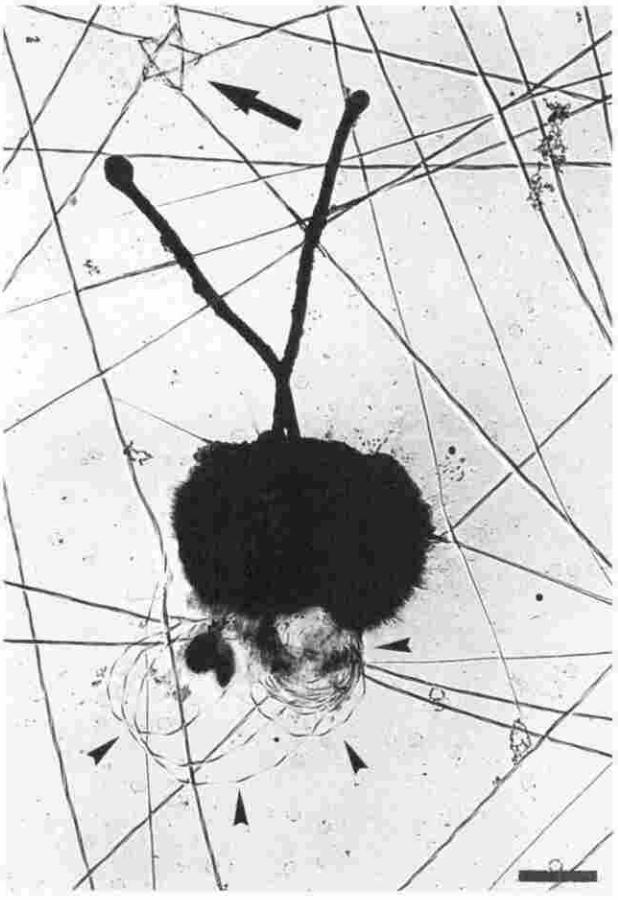

FIG. 1. Filaments and the star–like structure in *Phaeocystis*. Shadowcast of a biflagellated cell ejecting a partly coiled set of filaments (arrowheads) and surrounded by fully extended filaments, some of them forming a typical 5-rayed star (arrow). Scale bar = 1 μm.

ibration of the electron diffraction patterns was performed by comparing the electron diffraction diagrams of the specimens with those of a gold sample recorded under the same electron microscope setting and lens currents. After calibration and indexing of the *d*-spacings, the unit cell parameters of the crystal were refined with a least squares refinement program.

X-ray microanalysis. Experiments were performed on Nuclepore polycarbonate membranes with a pore size of 0.2 μm (Costar Corporation) before and after the filtration of a culture sample, as well as on nylon membranes (with a pore size of 0.2 μm; Magna, MSI) for comparison. Elemental analysis was carried out on a Hitachi S 520 scanning electron microscope equipped with a Tracor Northern 5 500 analyzer.

RESULTS

From the preparations of the specimens in the native state, we observed biflagellated cells in the process of ejecting coils of filaments and surrounded by a number of fully extended filaments (Fig. 1). The organization of the filaments as the arms of delicate and nearly perfect 5-branched stars is a unique characteristic of *Phaeocystis globosa* Scherffel and *P. pouchetii* (Hariot) Lagerheim (Parke et al. 1971, Vaulot et al. 1994). When probed with low dose transmission electron microscopy using electron diffraction conditions, filaments from the both the initial cultures and those taken after the purification of the filaments yielded a series of sharp electron diffraction micrographs (Fig. 2). Despite the fact that the preparations from the initial cultures were not as clean as those of the purified filaments, identical electron diffraction patterns were recorded from the filaments of both preparations. All patterns had in common the same row of spots oriented in the direction of the filament axis. They were related with respect to one another by the rotation around this common row. In fact, full diffraction data sets could be recorded from a single filament just by moving the selected area for the diffraction aperture along the length of the filament. Each filament was therefore a long twisted whisker like monocrystalline microfibril, a few tens of nanometers in width and several tens of micrometers in length. Insets A and B in Figure 2c are 2 examples of the most characteristic diagrams.

After the calibration and indexing of the diffraction micrographs, it was established that the filaments were crystallized in an orthorhombic unit cell, with the refined-cell dimensions of $a = 0.474 \pm 0.002$ nm, $b = 1.902 \pm 0.002$ nm, and $c = 1.032 \pm 0.001$ nm and a $P2_12_12_1$ spacing group. These parameters and the comparison of our patterns with reported X-ray and electron diffraction diagrams of chitin (Blackwell 1969, Minke and Blackwell 1978, Atkins et al. 1979, Gaill et al. 1992, Saito et al. 1995) indicated that each filament consisted of crystalline chitin with the polymer chain's axis oriented along the axis of the filament. A pattern such as the inset A in Figure 2c could correspond to either an electron diffraction diagram of β-chitin (Revol et al. 1988, Gaill et al. 1992, Saito et al. 1997) or of α-chitin (Atkins et al. 1979, Saito et al. 1995). The other patterns in Figure 2a, b and inset B, in Figure 2c displayed strong 013 spots, calibrated at 0.338 nm, together with a *b* unit cell parameter of 1.902 nm. These features indicated unambiguously that the *Phaeocystis* filaments consisted of α-chitin and not the β allomorph.

Based on an X-ray microanalysis of filaments collected on 0.2 μm Nuclepore membranes, a recent report by Ramani et al. (1994) has suggested that the filaments of *Phaeocystis* contained silicon, probably in the form of silica. We have duplicated their experiment and collected *Phaeocystis* filaments on Nuclepore as well as nylon membranes. Typical mats of filaments (Fig. 3) gave a strong silicon signal for the filaments on Nuclepore membranes (Fig. 4a), but no signal for those on nylon membranes (Fig. 4b). Thus, it appears that the occurrence of silicon was in some way connected to the filtration of the seawater sample containing the filaments onto the Nuclepore material and was not intrinsic to the *Phaeocystis* filaments. A control using the membranes themselves found no evidence of silicon (Fig. 4c).

Before their ejection, the filaments are formed within vesicles protruding from the cell (Figs. 5a, b,

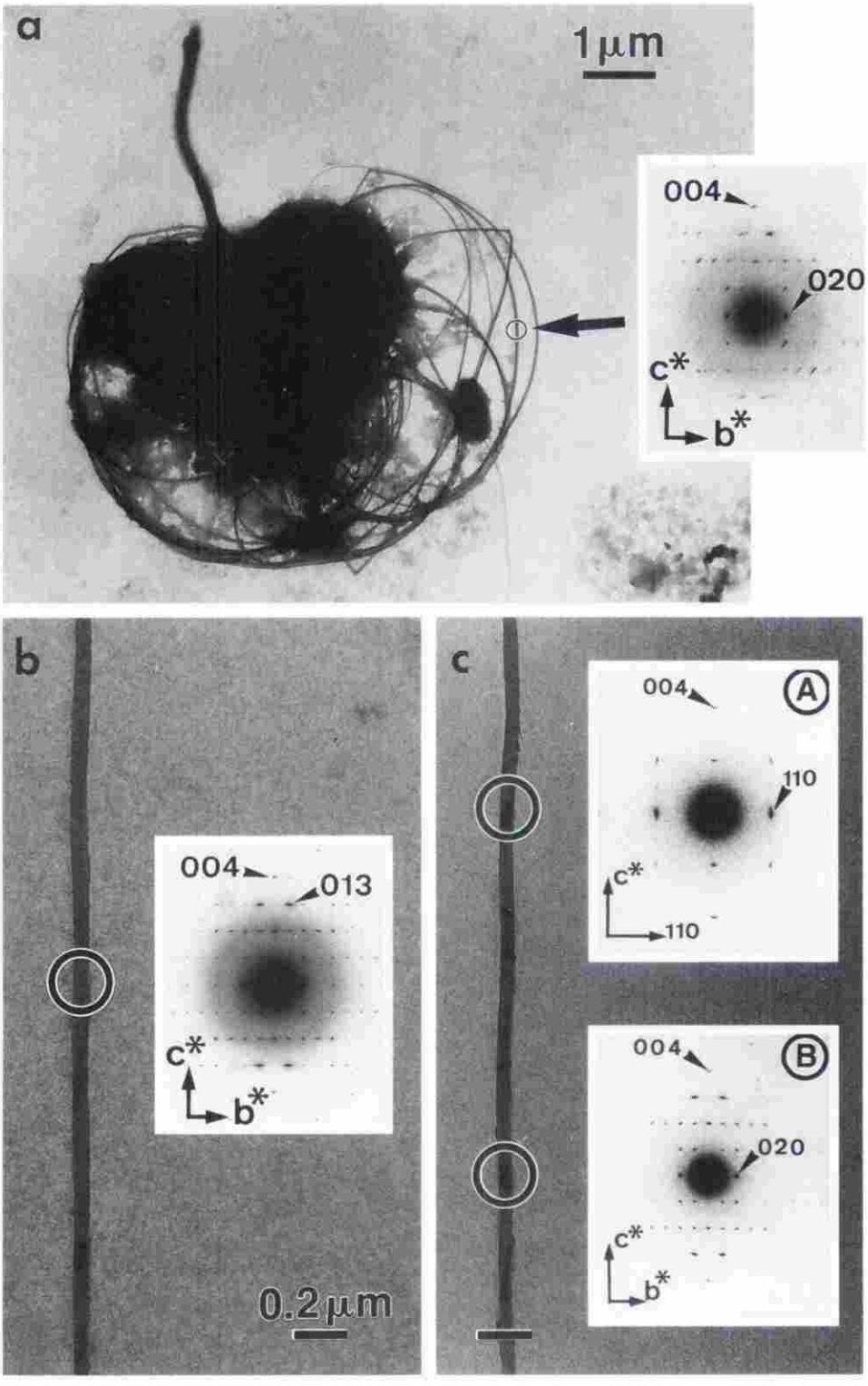

FIG. 2. Electron diffraction analysis. a. Low dose electron diffraction micrograph of an unstained and unshadowed *Phaeocystis* biflagellated cell ejecting a partly coiled set of filaments. Scale bar = 1 µm. Inset: typical electron diffraction pattern corresponding to the circled area and showing the b^*c^* electron diffraction diagram of α-chitin. This diagram is slightly distorted as the result of the curvature of the partly uncoiled filament. b. A typical straight filament occurring in the initial cultures of *Phaeocystis*. Scale bar = 0.2 µm. Inset: electron diffraction pattern of the circled area showing the clear b^*c^* pattern of α-chitin. c. Image as in 2b but of a filament after cleaning by boiling in dilute NaOH. Scale bar = 0.2 µm. Inset A is the most commonly observed pattern. It corresponds to a (110) zone axis of the reciprocal net of α-chitin with the c/c^* axis aligned with the filament axis. The pattern in inset B, which occurs periodically as the filament twists, is indexed as a b^*c^* projection of α-chitin.

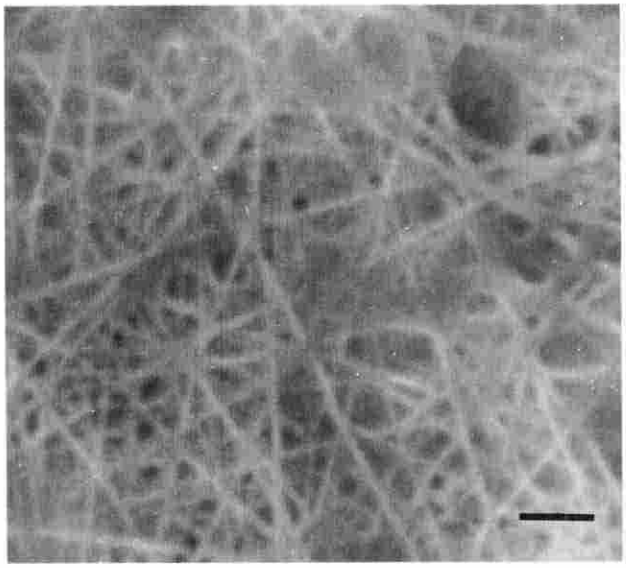

FIG. 3. Scanning electron micrograph of a mat of filaments collected on a Nuclepore filter for elementary analysis. Scale bar = 1 μm.

and 6a), as described previously (Parke et al. 1971, Pienaar 1991, Davidson and Marchant 1992). Just after their release, some filaments were still partially coiled (Figs. 1, 2a, and 5c), but later on they became completely uncoiled, thus leading to the spreading of the 2-dimensional star structure (Figs. 1, 5d, and 7c). Although absent in conventional preparations (Parke et al. 1971, Pienaar 1991), filaments still in the vesicle were clearly visible by electron microscopy in thin sections of cells after cryofixation and cryosubstitution. We observed a cell that held 2 micron-sized vesicles poised for release (Fig. 5a). This has also been observed by Pienaar (1991) using light microscopy on a Natal (South Africa) isolate (Fig. 6a). Each vesicle was flat in the center and swollen on each side (arrows) where the coiled filaments were located (Fig. 5a, b). Cross sections of these filaments appeared as unstained dots, from 2 to 50 nm in diameter. Ultrastructural observations of structures appearing as small white rods, that is, those not taking up electron microscopy stains, are indicative of a neutral polymer in a crystalline array (Neville 1975). This has been demonstrated classically for chitin in arthropod cuticles (Rudall 1965, Giraud-Guille et al. 1990) and in vestimentiferan tubes (Gaill et al. 1992). In the case of *Phaeocystis*, crystallographic identification of α-chitin was the answer to the interpretation of these white dots. Up to 26 filament sections were found in each vesicle. Because there are 5 filaments in each star, this means that each filament was wound on itself approximately 5 to 6 times. The first winding corresponds to the initial attachment of the 5 filaments (Fig. 5c) at a distance such that the final shape of the star is fixed (Fig. 5d).

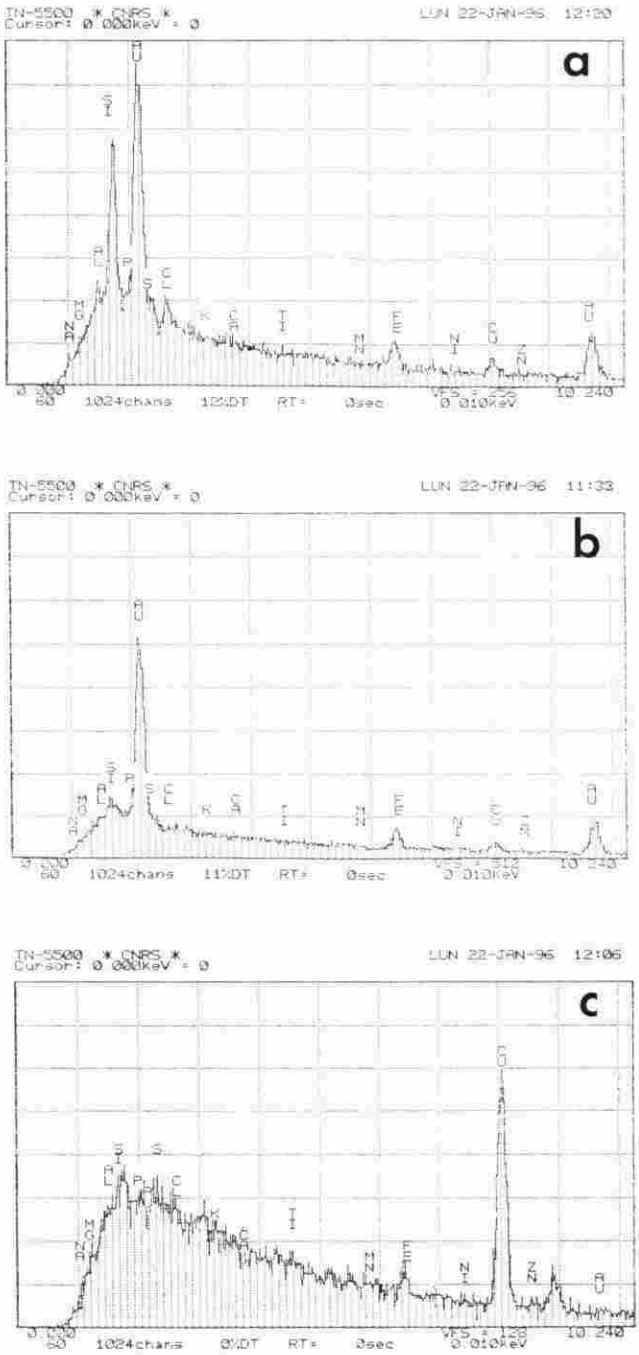

FIG. 4. X-ray microanalysis of membranes. a. Filaments on a Nuclepore membrane as in Figure 3, showing a peak of silicon (Si). The presence of gold (Au) is because of the coating of the filter. b. Filaments on a nylon membrane in similar conditions: note the absence of silicon. c. Analysis of an uncoated Nuclepore membrane: the copper peak (Cu) is attributed to a piece of copper stuck to the membrane.

DISCUSSION

Distribution, synthesis, and deposition of chitin in protistan organisms has been addressed in a review by Mulisch (1993). Although rare, chitin has been reported to occur within certain types of algae. For example, extracellular spines of diatoms are made

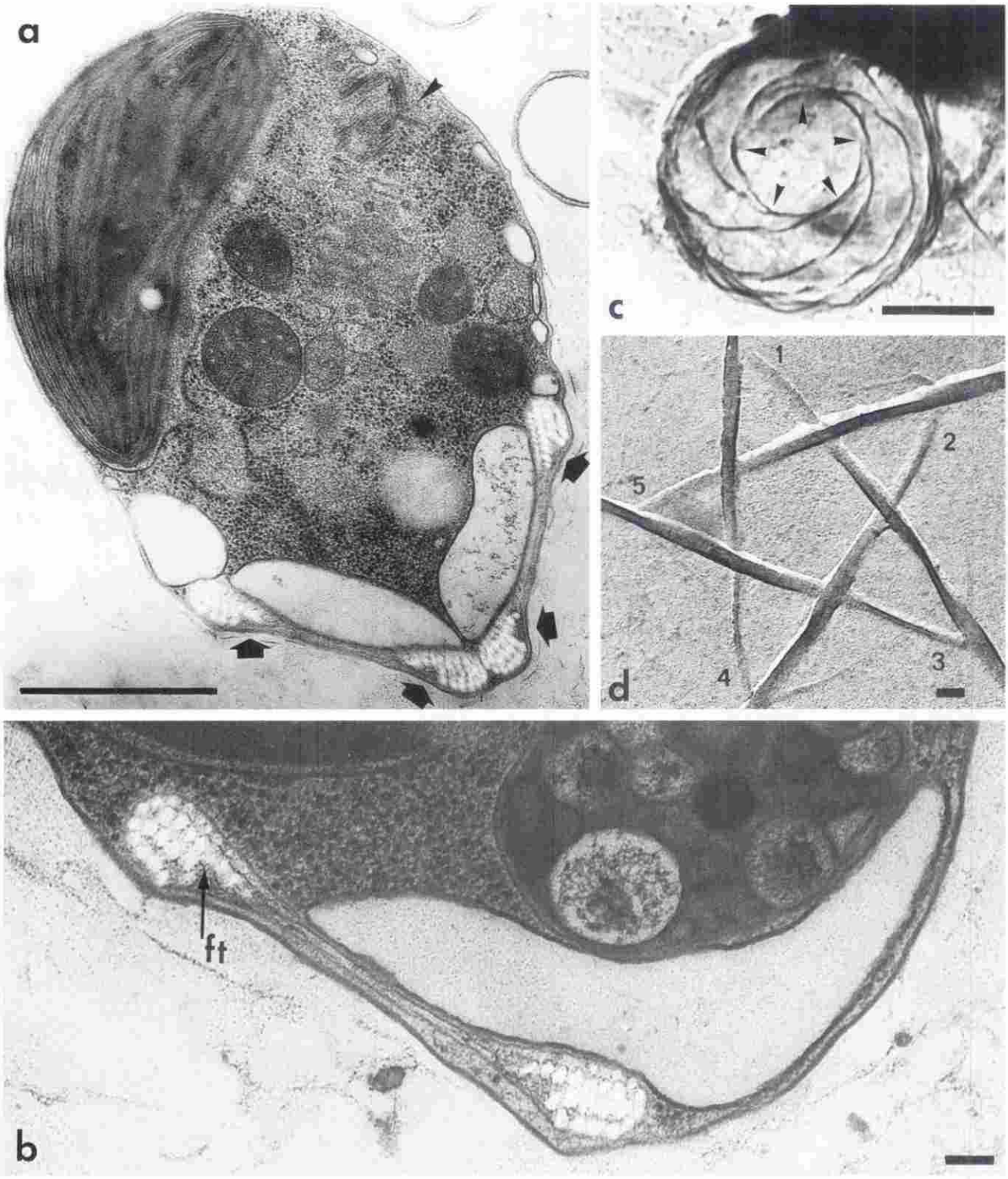

FIG. 5. Transmission electron microscopy of the filaments after cryo-treatment. a. Thin section of a motile cell (flagellar bases indicated by an arrowhead) showing 2 lateral vesicles containing filaments (arrows). Scale bar = 1 μm. b. Detail of a vesicle containing sectioned filaments (white dots, ft). Scale bar = 0.1 μm. c. Ejection of a partly uncoiled set of filaments. Successive insertion of the 5 filaments onto the initial winding is indicated by arrowheads. Scale bar = 1 μm. d. The starlike structure formed after the complete extension of the filaments. Numbering of the filaments indicates that 1 is attached to 4, 2 to 5, 3, to 1, 4 to 2, and 5 to 3. Scale bar = 0.1 μm.

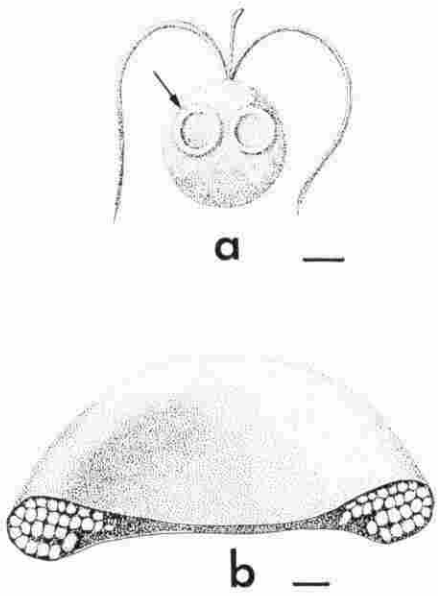

FIG. 6. Schematic illustration of a filament containing vesicles. a. Diagram of a cell with 2 protruding vesicles (drawn from Pienaar 1991). b. A sectioned vesicle showing the filaments as in Figure 5a, b. Scale bars: a =1 μm; b =0.1 μm.

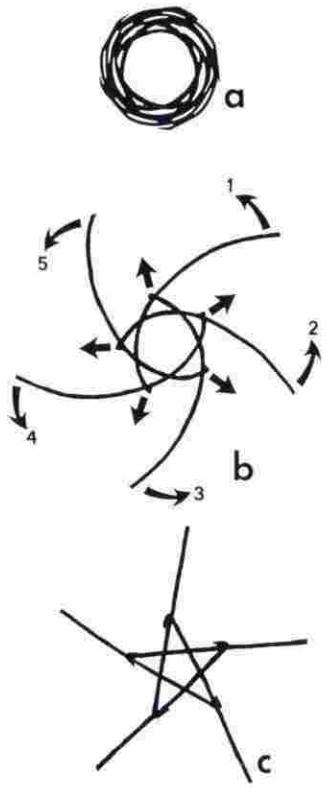

FIG. 7. Schematic diagram of the release of a set of 5 filaments. a. Filaments as they are coiled in their vesicle before release. b. Schematic unfolding of the filaments after release. Their extension is believed to occur in a Catherine wheel manner. c. Central part of a fully extended set of filaments forming a 5-rayed star.

of chitin, as demonstrated for *Thalassiosira* (McLachlan et al. 1965) or *Cyclotella* (Herth and Zugenmaier 1977). Chitin has also been analyzed by X-ray diffraction in the lorica of *Poterioochromonas* (Herth et al. 1977) and a chitin-like product was also reported in a *Chlorella* species (Kapaun and Reisser 1995). In the diatom spines, which resemble the present filaments, chitin occurs in the β allomorph, that is, crystallized with the chitin chains organized in a parallel fashion (Blackwell 1969, Gardner and Blackwell 1975). In both the *Chlorella* chitin-like product and the *Poterioochromonas* chitin, the diffraction patterns are rather poor, and for this reason, it is not possible to decide whether the diffracting matter is α- or β-chitin. In the present study, the chitin of the filaments of *Phaeocystis* corresponded unambiguously to the α allomorph, which contains 2 antiparallel polymer chains per unit cell (Carlström 1957, Minke and Blackwell 1978). These patterns were similar to those recorded from thin fragments of grasping spines of *Sagitta* (Atkins et al. 1979, Saito et al. 1995), specimens considered to be the most highly crystalline α-chitin available (Rudall 1976, Saito et al. 1995). In the *Sagitta* spines however, the diffracting fragments are much thicker than the *Phaeocystis* filaments in our study. For this reason, the *Sagitta* electron diffraction micrographs exhibit clear double diffraction spots in particular spots with odd indices on the a^*, b^*, and c^* axes (Atkins et al. 1979, Saito et al. 1995). This is not the case for the thin *Phaeocystis* filaments for which the double diffraction feature is either very weak or totally absent. Thus, these filaments appear to be superior α-chitin specimens amenable for an electron crystallography study.

The biological function of the chitin filaments of *Phaeocystis* remains a mystery at present. The winding of the filaments inside the vesicles is probably a consequence of their biosynthesis within a confined space. A probable result of this winding is that the filaments behave as a coiled spring whose stored energy is released once the vesicles are broken and the filaments ejected. Indeed, the molecular conformation of the poly-β-(1→4)-*N*-acetyl-D-glucosamine chains is similar to those of the poly-β-(1→4)-D-glucose chains found in cellulose (Minke and Blackwell 1978) for which the minimum conformational energy corresponds to fully extended chains (Sathyanarayana and Rao 1971). The equilibrium state of chitin whiskers also corresponds to structures made of fully extended chains and, accordingly, it is in the form of straight microfibrils that chitin is found as a reinforcement for a number of biological matrices ranging from arthropod cuticles to fungal cell walls, and a number of other biocomposites (Neville 1993). It is therefore expected that the present chitin filaments will uncoil in a spring-like fashion once they are no longer confined within their original vesicles. Our concept of the winding of the filaments within the vesicles and their unwinding in the man-

ner of a Catherine wheel during their ejection is shown in Figure 7a–c.

Theoretically, the occurrence of α-chitin filaments such as those reported here raises a fundamental question about the biosynthetic mechanism that produces polymer chains crystallized in the antiparallel mode required by the $P2_12_12_1$ symmetry. One can easily understand how an extracellular crystalline β-chitin whisker such as a diatom spine is extruded, such that its contitutent polymer chains are parallel. Indeed, such spines are vizualized as originating from organelles wherein the chitin precursors are assembled and spun in a continuous unidirectional fashion (Herth and Schnepf 1982). With α-chitin whiskers, however, a discontinuity must exist between the time of polymer biosynthesis and filament assembly. We therefore hypothesize that the polymer must exist in a fluid state prior to its extrusion. The experimental evidence of such a fluid state and its generalization to the biogenesis of other α-chitin systems remains to be demonstrated. Further study of the morphogenesis of the fairly large *Phaeocystis* filaments therefore appears to be a good approach to better understanding of the synthesis of poly-β-(1→4)-*N*-acetyl-D-glucosamine chains and the method by which the polymer chains are spun into the antiparallel form of α-chitin.

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