Calcofluor White and Congo Red Inhibit Chitin Microfibril Assembly of *Poterioochromonas*: Evidence for a Gap between Polymerization and Microfibril Formation

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ABSTRACT The influence of the light microscopical stains, Calcofluor white and Congo red, on the process of chitin microfibril formation of the chrysoflagellate alga *Poterioochromonas stipitata* was studied with light and electron microscopy. There is a concentration-dependent inhibition of lorica formation with both dyes. In the presence of the inhibitors malformed loricae are made, which do not show the usual ultrastructure and arrangement of the chitin microfibrils. Instead of long, laterally associated microfibrils, short rods or irregular networks of subelementary (15–25 Å) fibrils are found. Microfibril assembly obviously takes place on the accessible outside of the plasma membrane. There must be a gap between the polymerization and microfibril formation reactions, allowing the stains to bind to the polymerized subunits. Thus, later association of these units to form microfibrils is disturbed. The microfibril-orienting mechanism also depends on normal microfibril formation. A model summarizing these hypotheses is suggested.

The experimental system used, *Poterioochromonas stipitata* (syn. *Ochromonas malhamensis*, see Materials and Methods), is a unicellular chrysoflagellate alga, which may attach to a substratum and secrete a wine-glass-like lorica. This lorica consists of fibrillar material containing chitin (21). Microfibril ultrastructure, kinetics of formation, and the involvement of microtubules in lorica microfibril orientation have already been described (21, 23, 31, 32). It was proposed that the lorica fibrils are formed on the surface of the plasma membrane of the cytoplasmic tail region of *Poterioochromonas*, and oriented parallel to the cortical microtubules of this region (31, 32, for discussion see also references 16 and 17).

It is the classical hypothesis that microfibril formation is caused by simultaneous polymerization and crystallization of the structural polysaccharide (11, 28, 30). There is, however, increasing evidence for a noncrystalline polymer intermediate in microfibril formation (e.g., 1, 9, 26, for opposing view see reference 34), and a gap between polymerization and crystallization (review in reference 6). If such a gap exists, and if microfibril formation takes place on the surface of the plasma membrane, substantial dyes should have access to the newly formed structural polysaccharide polymer, and microfibril formation should be affected. In this paper I demonstrate that Calcofluor white and Congo red, two substances with a high affinity to structural polysaccharides (2, 10, 15, 29), indeed inhibit microfibril assembly.

MATERIALS AND METHODS

Poterioochromonas stipitata Scherffel, syn. *Ochromonas malhamensis* Pringsheim, syn. *Poterioochromonas malhamensis* Peterfi (for discussion of this confusion in nomenclature see reference 33), strain no. 933-1a from the Algal Culture Collection of the University of Göttingen, W. Germany, was cultivated as described (21).

Calcofluor white ST (American Cyanamid Co., Wayne, N. J., USA) and Congo red (Serva, Heidelberg, W. Germany) were applied in the usual culture medium. Concentrations from 0.001–2 mg/ml were tested. Controls under the same conditions and from the same culture flask were run in parallel.

The algae were allowed to attach to glass coverslips or pioloform-coated grids for electron microscopy and to secrete loricae. After 2 h the incubation was stopped by washing off the algae with distilled water. Light microscopical examination was with epifluorescence, Zeiss inverse microscope IM 35, and UV excitation (filter combination G 365, FT 395, LP 420). Calcofluor white-stained material was viewed directly, the Congo red-stained material was again washed with distilled water and then stained with Calcofluor white (0.5 mg/ml water, 5 min at 20°C). For electron microscopy, the material attached to the grid was negatively stained with 1% wt/vol uranyl acetate and examined with the Philips 400 electron microscope.

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RESULTS

Light Microscopical Observations

Control algae form numerous complete, wine-glass-like loricae within the 2 h of incubation (Fig. 1 *a*). Calcofluor white does not poison the cells. They still swim, form cytoplasmic tails, and attach to the coverslip even at the highest concentrations applied. But with addition of 2 mg Calcofluor white/ml, lorica formation is inhibited. The cells attach to the coverslip and secrete a cloud of Calcofluor-positive material.

With a lower concentration of Calcofluor white (1 mg/ml), thick lorica stalks without the mouth parts of the loricae are formed (Fig. 1 *b*). With 0.5 mg/ml, the loricae formed are complete (Fig. 1 *c*), but the lorica stalk is too substantial and too short in comparison with the control loricae (compare Fig. 1 *a* and *c*). At 0.25 mg/ml the loricae still look too massive (Fig. 1 *d*), possibly because the chitin is less condensed and binds more stain.

Congo red application also does not poison the cells. Lorica formation is totally inhibited at a concentration of 0.5 mg Congo red/ml. After incubation, the cells are attached to the coverslip, but after washing them off it is difficult to find any signs of attachment. The attachment material is easily washed away by the water wash before Calcofluor white staining. At 0.05 mg Congo red/ml, the algae form patches of Calcofluor-positive material (Fig. 2 *a*). At 0.02 mg Congo red/ml, these patches get more numerous and even a few complete loricae are found. At 0.001 mg/ml, an almost normal aspect of loricae is observed (Fig. 2 *b*).

Electron Microscopy

The patches secreted by *Poterioochromonas* in the presence of a high concentration of Calcofluor white (1 mg/ml) do not show any similarity with loricae (Fig. 3 *a*). At medium concentration of Calcofluor white (0.5 mg/ml), the secreted products consist of a distinct foot part, a more or less conical stalk, and a reduced mouth part, or a vase-shaped region directly attached to the foot part (Fig. 3 *b*). The cells are able to form a cytoplasmic tail and do not look malformed under the influence of this Calcofluor white concentration (Fig. 3 *d*). At low concentration of Calcofluor white, the loricae formed are too much compressed in the longitudinal axis (Fig. 3 *c*), but consist of a distinct foot region, the stalk, and the mouth region as usual.

The central material of the patches is not microfibrillar, but at very high magnification turns out to consist of a meshwork of extremely thin and short subelementary fibrils (Fig. 4 *a*). Often, laterally aggregated rodlike elements (Fig. 4 *b* and *c*) are found in the patches. The background consists of short and thin rods (Fig. 4 *b*).

In the malformed loricae produced at medium concentration of Calcofluor white, the abundance of long subelementary fibrils with a tendency to lateral aggregation is increased (Fig. 4 *d* and *e*), but these fibrillar aggregates still are very irregular and seem to consist of aggregated subelements. They do not appear as long and smooth as the control lorica subelementary fibrils (Fig. 4 *f*) which are laterally associated to ribbonlike microfibrils.

At low concentration of Calcofluor white (0.25 mg/ml), there is an increase in fibril size towards the upper mouth region (not shown in detail here). A comparison with the fibrillar arrangement in the mouth region of a control lorica, however, clearly reveals that the base of the mouth region, where one can usually easily recognize that the helically arranged stalk microfibrils fray out (Fig. 5 *b*), does not show any sign of microfibril orientation in the Calcofluor white-inhibited sample (Fig. 5 *a*). This loss of orientation of the microfibrils is even more obvious in the stalk region (compare Fig. 5 *c* and *d*): The Calcofluor white-treated sample has a stalk consisting only of an irregular arrangement of intermeshed subelementary fibrils, with a slight predominance of longitudinal orientation.

As calibrated with a grating replica, the width of the extremely thin short elements, as well as the subunits of the somewhat irregular microfibrillar aggregates formed in the presence of Calcofluor white is in the range of 15–25 Å. This is a value also measured for individual subunits of the control lorica microfibrils (Figs. 4 *f* and 5 *d*; compare reference 21).

Congo red application yields ultrastructural effects very similar to Calcofluor white: At high concentration of Congo red (0.05 mg/ml) the patches formed consist of short, often somewhat bent, rods (Fig. 6 *a*) and short segments with a subelementary fibril aspect. At medium concentration (0.02 mg/ml) the patches have a distinct foot region with toes, and a more fibrillar aspect at the periphery (Fig. 6 *b*). At higher magnification a few long subelementary fibrils are found (Fig. 6 *c*). I have not observed such aggregates of laterally associated short rods as with Calcofluor white (Fig. 4 *b* and *c*). In the loricae formed at low concentration of Congo red, the stalk often does not consist of well-oriented microfibrillar ribbons, but of a network of fibrils (Fig. 6 *e*) with only some tendency to helical orientation. The dimensions of the subelementary fibrils with Congo red application are in the same range as with Calcofluor white. Microfibril formation is about 10 times more sensitive to Congo red than to Calcofluor white.

DISCUSSION

The benzidine derivative Congo red is a substantial dye for structural polysaccharides, which because of its size and dipole character binds to fibrous cellulose in an oriented way, enhancing the birefringence (10, 12). The stilbene derivative Calcofluor white also binds oriented with respect to the longitudinal axis of structural polysaccharides, leading to polarized fluorescence of the structures (W. Herth, unpublished observations). Both dyes thus seem to be capable of a paracrystalline incorporation into the chain lattice and to form hydrogen bonds to the polymer chains (2, 10, 12). As described in Results, obviously both dyes do not only bind to crystalline structural polysaccharides but also to the nascent polymer chains. The mere fact that this causes effects on fibril formation shows that the site of microfibril formation must be on the surface or outside the plasma membrane. The dyes do not inhibit the formation of the short rods and extremely thin subelementary fibrils but the lateral association to microfibrillar ribbons of greater widths, as well as the formation of longer microfibrils. The dyes seem to block the development of further hydrogen bonds by blocking the surface of the nascent polysaccharide chains. The subelementary fibrils observed after application of the dyes have the same dimensions as the thinnest individual fibrils in the lorica mouth region and as the subunits of the laterally associated and fasciated microfibrillar ribbons in the lorica stalk region. I conclude from these findings that the fasciated chitin microfibrils of *Poterioochromonas* are not made by simultaneous polymerization and crystallization, but that the first reaction product is the individual polymer chain or a few polymer chains aggregated to short rods or subelementary fibrils in the range of 15- to 20-Å width. The long and fasciated microfibrils seem to be made by physical forces leading to the

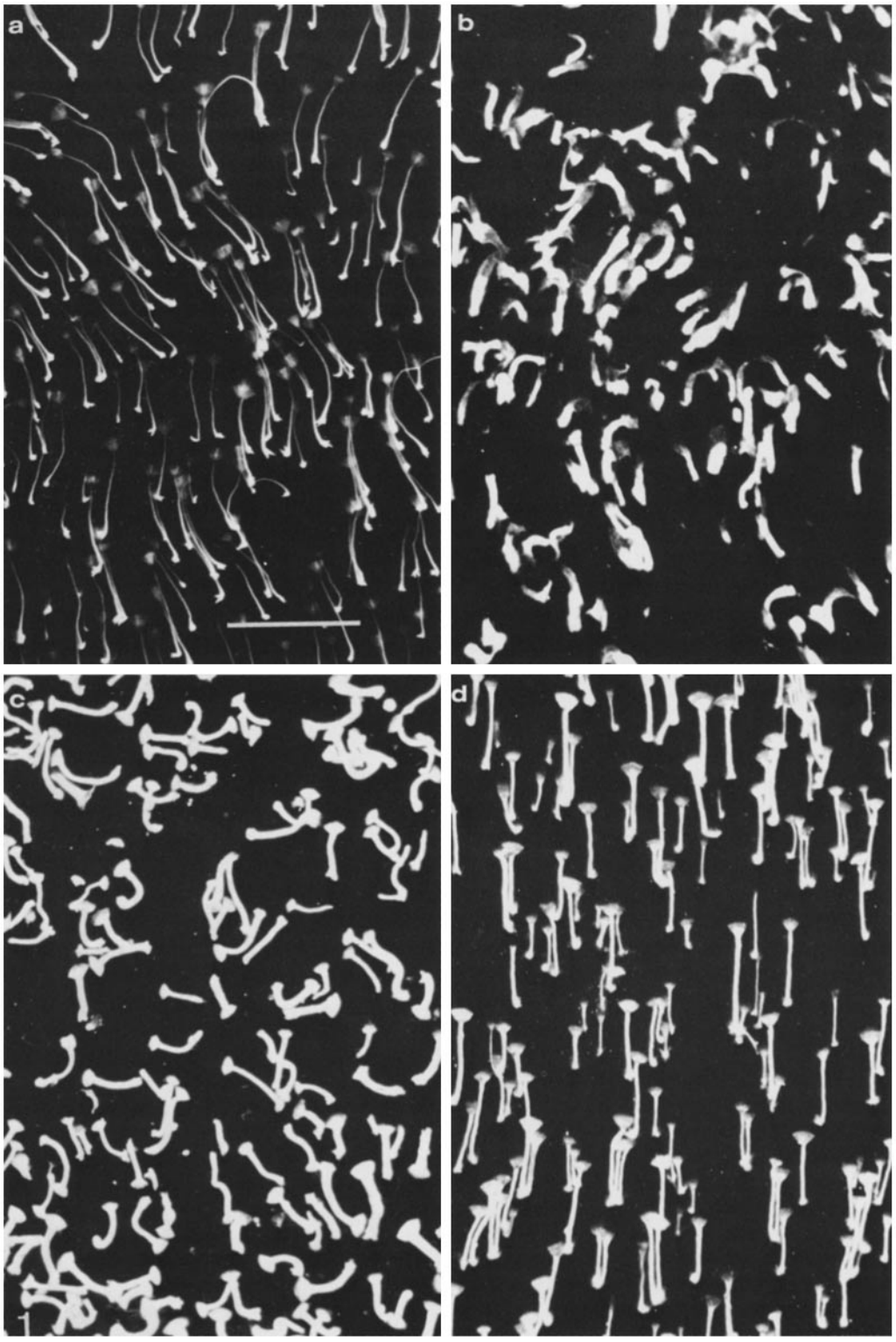

FIGURE 1 Calcofluor white experiments, light microscopy. (*a*) Control with numerous complete loricae after 2 h of incubation without Calcofluor white, stained for 5 min with 0.5% aqueous Calcofluor white. (*b*) Incomplete loricae consisting mainly of stalks formed in the presence of 1 mg Calcofluor white/ml. (*c*) Complete, but too short and massive loricae formed in the presence of 0.5 mg Calcofluor white/ml. (*d*) The loricae are still too massive with 0.25 mg Calcofluor white/ml. Bar, 50 μm. × 400.

formation of more and more hydrogen bonds and aggregation of the subelements.

These results agree well with the influence of Calcofluor white on cellulose microfibril formation in *Acetobacter xylinum* (14). A gap between polymerization and crystallization might therefore be a general phenomenon in the formation of both chitin and cellulose microfibrils. Such a gap has already been postulated earlier (see Introduction).

The experimental evidence for "swollen hydrated nascent cellulose microfibrils" by Leppard et al. (26) now is disputed

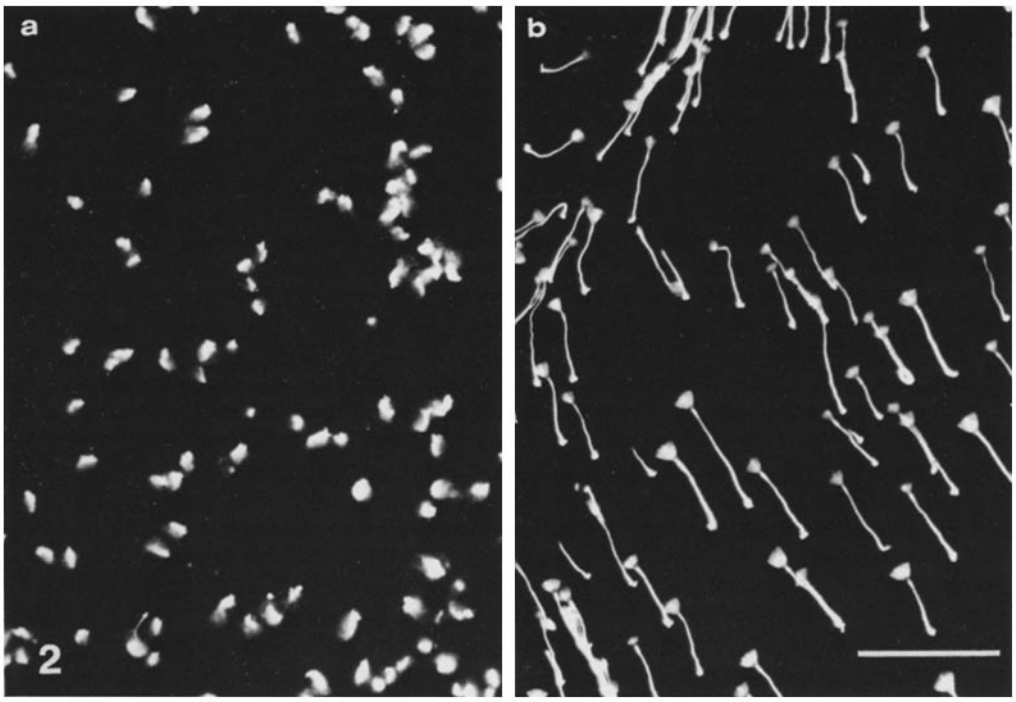

FIGURE 2 Congo red experiments, light microscopy. (*a*) Patches of Calcofluor white-stainable material secreted in the presence of 0.05 mg Congo red/ml. (*b*) Almost normal loricae formed in the presence of 0.01 mg Congo red/ml. Bar, 50 μm. × 380.

to be caused by technical artifacts consisting of replicating carbon (34). The discrepancy that the impressions of microfibrils in fractured plasma membranes have greater cross-sectional dimensions than the microfibrils themselves is interpreted to be a result of the relationship between the microfibrils and the plasma membrane (34). The primary wall cellulose microfibrils terminating in globules and the cellulose microfibrillar ribbon of *Acetobacter xylinum* do not look swollen up to their point of origin (34). On the other hand, irregular curled structures are described for *Acetobacter xylinum* between the bacterial surface and the cellulose ribbon (35), and ribbon assembly is affected by Calcofluor white (14). Therefore, in my opinion, the concept of a hydrated nascent polysaccharide chain as a transient phase in the biosynthesis of structural polysaccharides is not devaluated by the controversial experimental evidence available now. It is not to be excluded that in different organisms microfibril assembly is accomplished by different methods. This might especially be true for the very crystalline celluloses, e.g., *Valonia* and cotton secondary wall cellulose (10, 12, 30), and the highly crystalline β-chitin fibrils of diatoms (3, 22, 24, 25). However, there again are some cytological details that might suggest that the fibrils are formed by lateral association of preformed polymer chains (22, 25). The high crystallinity in these cases would be caused by the almost exclusive availability of homopolymers as the nearest neighbors with which to form hydrogen bonds. In those cases where (as in seed slimes such as quince slime) acidic polysaccharides, or (as in primary walls) xyloglucans are secreted at the same time, we would have a similar situation as with the addition of the substantial dyes: the contact with these heteropolymers would also lead to inhibition of lateral association. That is exactly what has been suggested earlier to explain the existence of elementary fibrils (11, 28) and is in agreement with the x-ray diffraction patterns of such thin fibrillar structural polysaccharides indicating lateral disorder (7, 8, 18–21). It is difficult to explain from this model alone the rather uniform 35-Å dimension of the elementary fibrils (for further discussion of factors involved see references 11 and 28).

If one tries to relate these thoughts to the published freeze-etch data on the formation of structural polysaccharides (for summary see e.g., references 4, 13, and 27), there is another interesting aspect: In the cases with formation of thin fibrils, there is a rather scattered distribution of particles or particle arrangements thought to be involved in fibril formation, whereas in cases with formation of highly crystalline, ribbon-like structural polysaccharides there are densely packed particle aggregates. This has been postulated to reflect the size of the fibrillar ribbon to be formed (13). It is obvious that the frequency of corresponding neighbor polymer chains available for lateral aggregation and hydrogen bonding is higher when they are all polymerized in a restricted area.

Microfibril orientation in *Poterioochromonas* is parallel to cortical microtubules of the cytoplasmic tail region (32). In my experiments with Calcofluor white and Congo red, the cytoplasmic tail and the cortical microtubules are arranged as usual (W. Herth, unpublished observations), but microfibril orientation is absolutely suppressed when normal fibril formation is inhibited. The orienting influence of the microtubules seems not to act on the polymerization reaction, but on the formation of the microfibrils (this is also in agreement with the observed effects of colchicine, 17, 31, 32). This leads to question whether really, as suggested repeatedly in the literature (review in reference 16), contractile structures associated with the microtubules move the postulated plasmalemma-localized synthetases. The kinetic force of the microfibril formation reaction seems also to be necessary. This force must be rather high, as in *Acetobacter xylinum* it is sufficiently strong to move the whole bacterium (5). I suggest that the kinetic force of fibril formation might be capable of moving the synthetases still attached via the noncrystalline polymer chains (Fig. 7). The

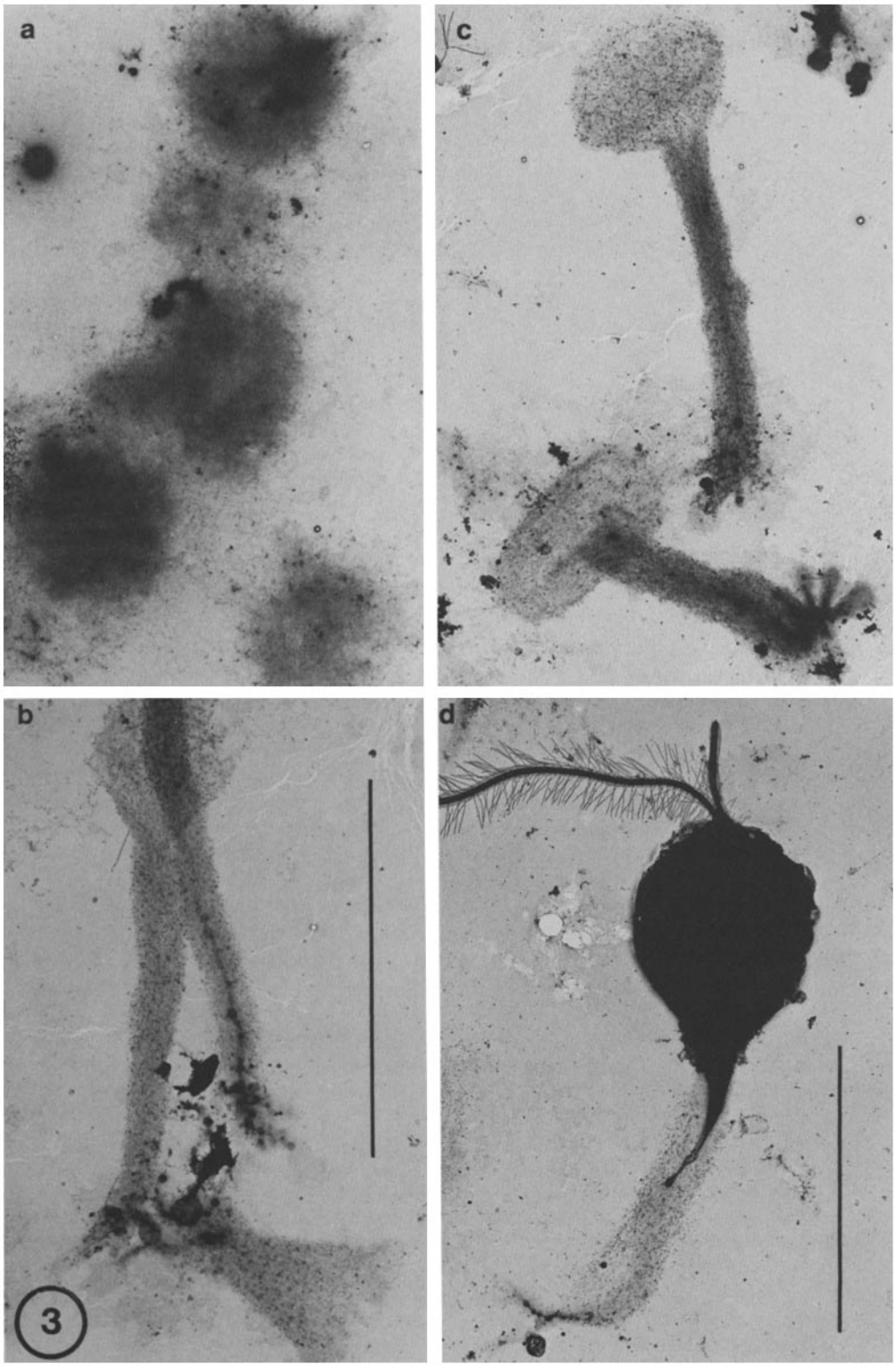

FIGURE 3 Calcofluor white inhibition, electron microscopy. (*a*) Patches at high Calcofluor white concentration (1 mg Calcofluor white/ml). (*b*) Stalk with reduced mouth region and stalkless lorica at medium Calcofluor white concentration (0.5 mg Calcofluor white/ml). (*c*) Short massive loricae at lower concentration (0.25 mg Calcofluor white/ml). (*d*) *Poterioochromonas* with typical cell form and cytoplasmic tail attached to incomplete lorica stalk at 0.5 mg Calcofluor white/ml. Bars, 10 μm. (*a* and *b*) × 5,600; (*c* and *d*) × 4,200.

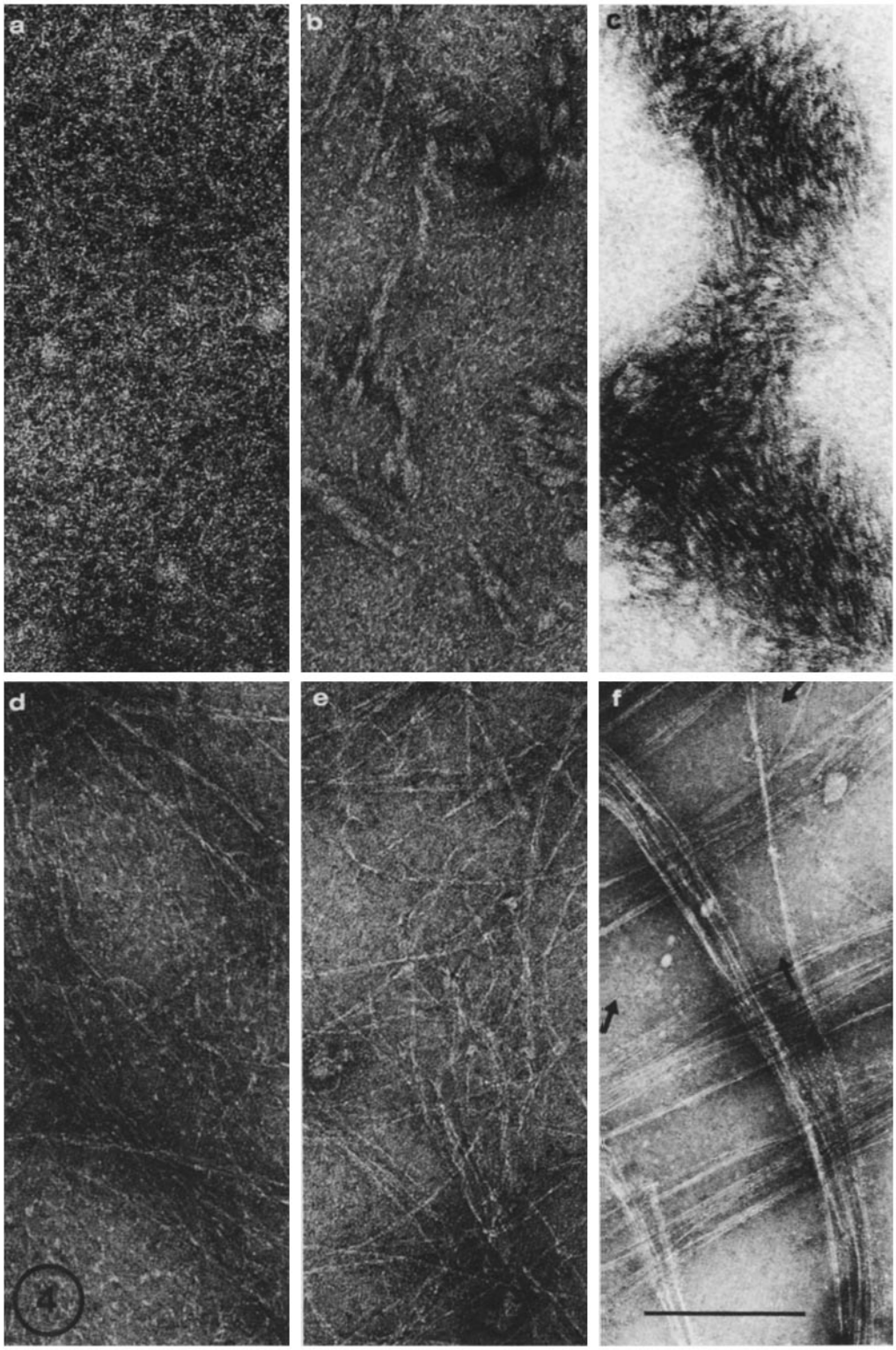

FIGURE 4 High magnification of negatively stained lorica material. (*a–e*) Calcofluor white experiments, (*f*) control. (*a*) Meshwork of short rods and short subelementary fibril pieces (1 mg Calcofluor white/ml). (*b* and *c*) Aggregates of short rods (1 mg Calcofluor white/ml). (*d*) Subelementary fibrils partially aggregated to microfibrils and short rods (0.5 mg Calcofluor white/ml). (*e*) Irregularly aggregated microfibrils (0.5 mg Calcofluor white/ml) from the peripheral region of a patch. (*f*) Control microfibrils consisting of laterally associated subelementary fibrils and a few individual subelementary fibrils (arrows) in the range of 15-Å width. Bar, 0.1 μm. × 240,000.

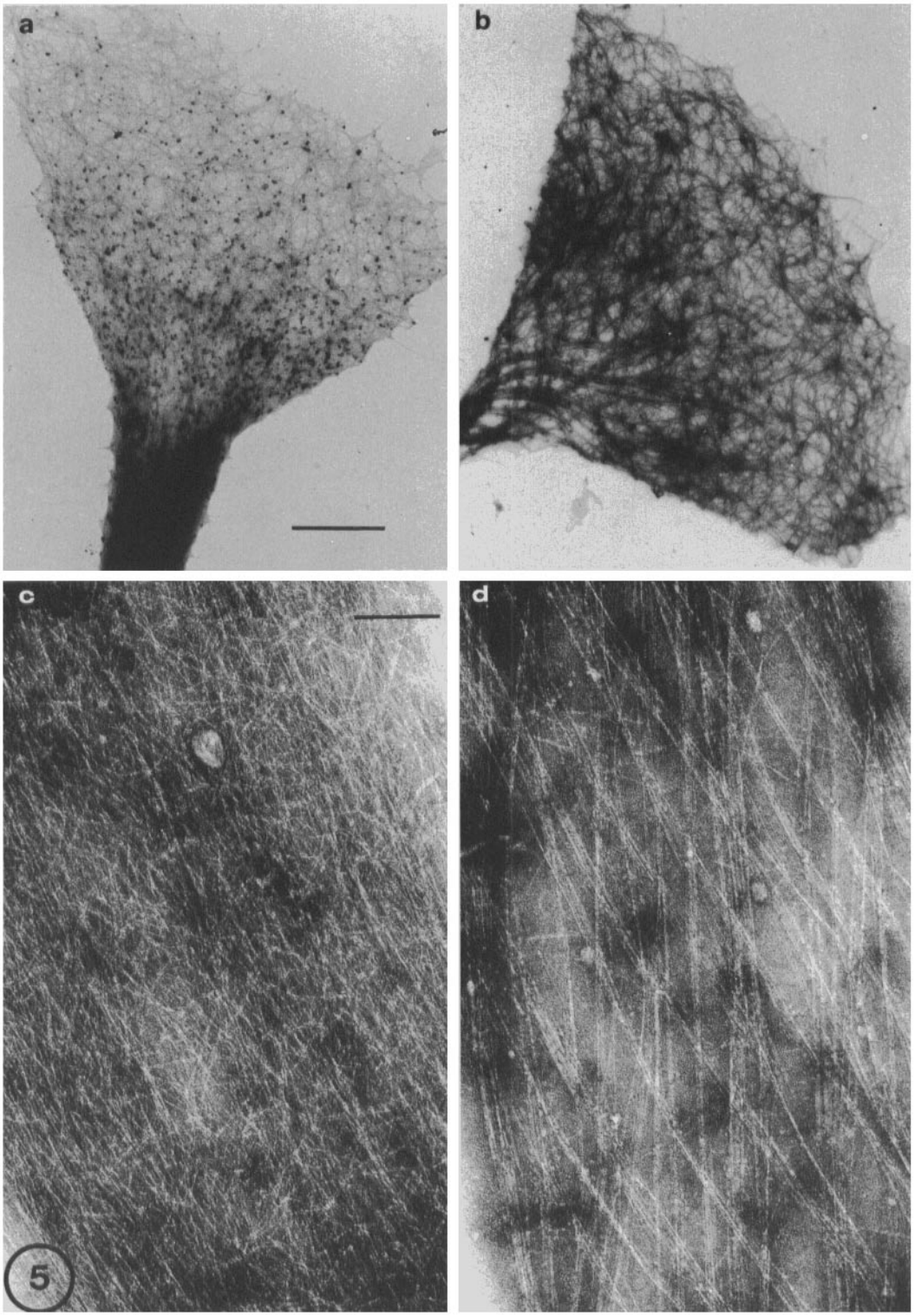

FIGURE 5 Loss of microfibril orientation with Calcofluor white inhibition. (*a*) Lorica mouth region at 0.25 mg Calcofluor white/ml. (*b*) Control. Note absence of helical microfibril arrangement at the base of the lorica mouth in *a*. (*c*) Lorica stalk region, same Calcofluor white concentration, with irregular fibril arrangement. (*d*) Control lorica stalk with parallel arrangement of microfibrillar ribbons consisting of laterally associated parallel subelementary fibrils. Bars, (*a* and *b*) 1 μm; (*c* and *d*) 0.1 μm. (*a* and *b*) × 14,000; (*c* and *d*) × 130,000.

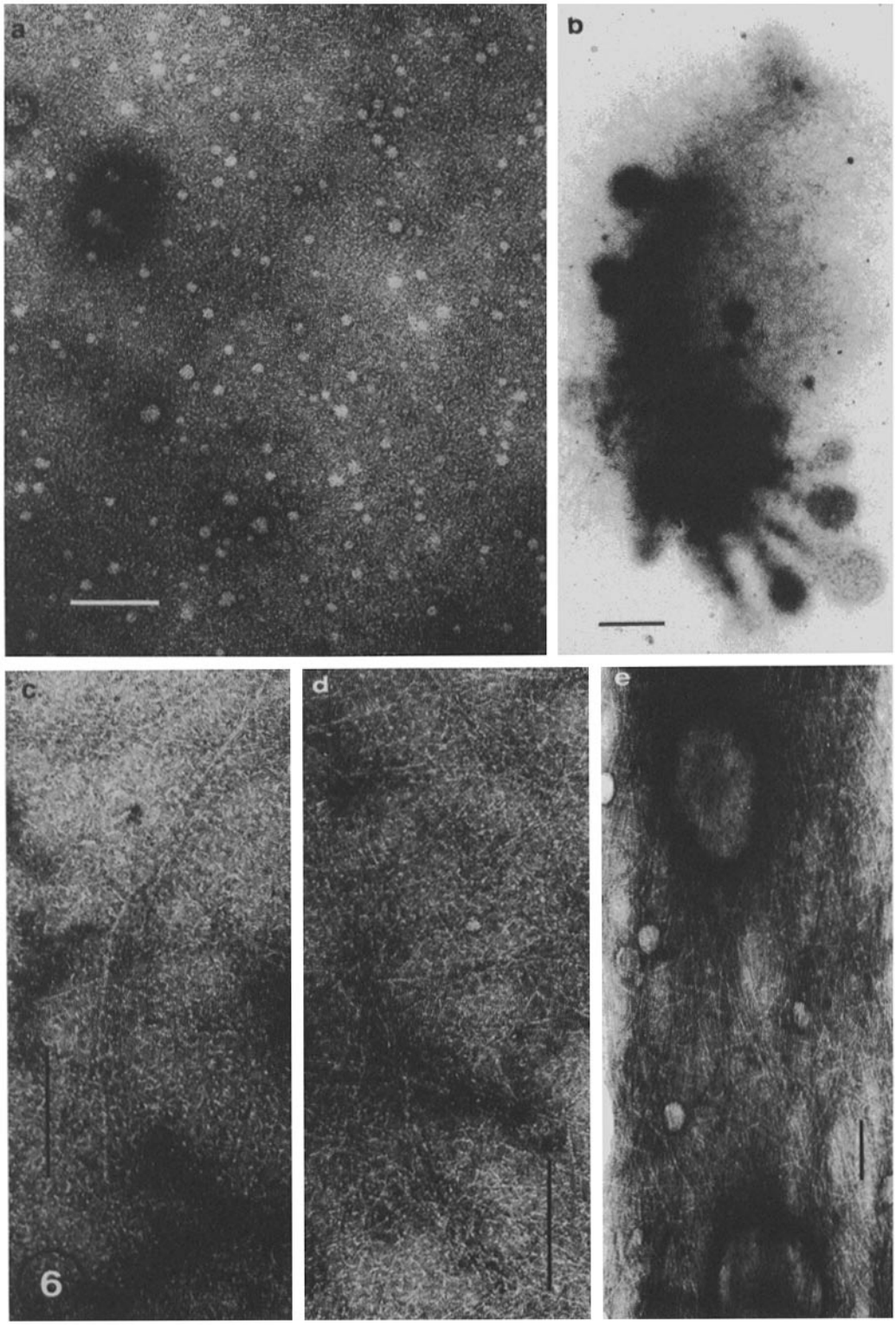

FIGURE 6 Congo red inhibition effects. (*a*) High magnification of the short rods and short subelementary fibril pieces secreted at 0.05 mg Congo red/ml. (*b*) Survey of a malformed "lorica" consisting of foot part and an amorphous cloud of material (0.02 mg Congo red/ml). (*c*) Irregular microfibril and short rods secreted at 0.02 mg Congo red/ml. (*d*) Network of subelementary fibrils, in some places aggregated to microfibrils, at the periphery of a patch secreted at 0.20 mg Congo red/ml. (*e*) Lorica stalk with irregular fibrillar arrangement (0.001 mg Congo red/ml). Bar, (*a*, *c–e*) 0.1 μm; (*b*) 1 μm. (*a*) × 125,000; (*b*) × 10,000; (*c*) × 200,000; (*d*) × 200,000; (*e*) × 90,000.

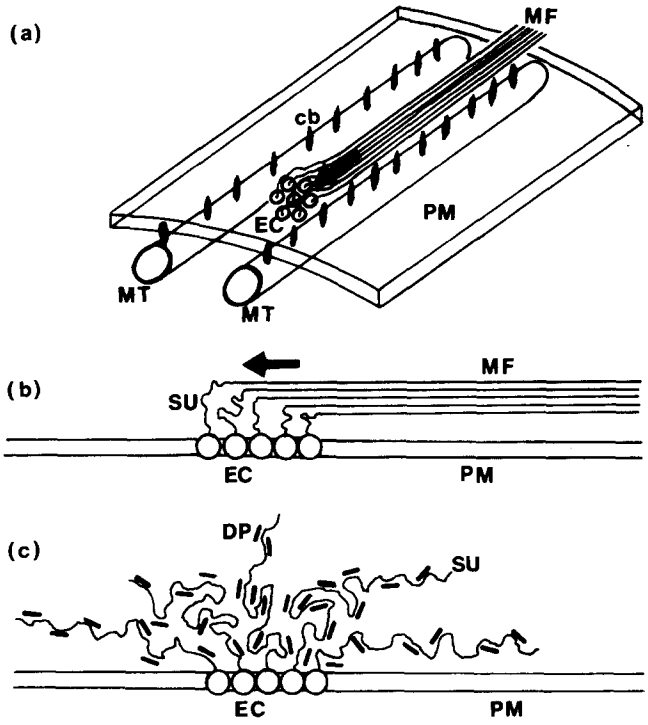

FIGURE 7 Model drawing. (*a* and *b*) The physical aggregation of polymerized subunits to microfibrils leads to a kinetic force (arrow) driving the enzyme complexes in a direction channeled by the cortical microtubules. (*c*) With an intercalating dye like Calcofluor white or Congo red, the subunits are bound by the dye particles and therefore cannot regularly aggregate to microfibrils; the enzyme complexes cannot be moved regularly because of loss of the kinetic force of aggregation. *SU*, polymerized subunits; *DP*, dye particle; *MF*, microfibrillar ribbon; *PM*, plasma membrane; *MT*, microtubule; *cb*, cross bridge; *EC*, enzyme complex.

microtubules and their associated proteins only need to restrict the fluidity of the membrane in such a way that the enzyme movements are channeled parallel to the microtubules (Fig. 7). As an alternative, the microtubules might also bind the newly formed polymer chains via microtubule-associated transmembrane proteins like lectins and thus direct the start orientation of the microfibril, which then would extend automatically from this starting point in the given direction.

Of course this model does not exclude a cooperation with contractile elements which might either lead to oriented exocytosis, or oriented incorporation of synthetase aggregates, or movement of synthetases parallel to microtubules. This has to be elucidated by further experiments.

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