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Microfibril assembly by granules of chitin synthetase

(chitin biosynthesis/fungal wall/*Mucor rouxii*)

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ABSTRACT **Purified preparations of chitin synthetase (EC 2.4.1.16; UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase), capable of forming microfibrils *in vitro*, were isolated from yeast cells of *Mucor rouxii*. Chitin synthetase was obtained either by substrate-induced liberation of bound enzyme (54,000 × *g* pellet) or by isolation of unbound enzyme present in the 54,000 × *g* supernatant of a cell-free extract. Both preparations contained ellipsoidal granules from about 350 to 1000 Å diameter. Many granules exhibited a marked depression. No typical unit membrane profiles appeared in thin sections of glutaraldehyde/OsO_4-fixed samples. Upon incubation with substrate and activators, chitin microfibrils were produced. The microfibrils were often found intimately associated with granules. The most common configurations were: a microfibril with a granule at one end, or two microfibrils "arising" from the same granule. These findings lend support to the granule hypothesis for the elaboration of cell wall microfibrils by end-synthesis.**

The mechanism of formation of microfibrillar cell walls of plants and fungi has been the subject of various investigations. This topic has been recently reviewed (1, 2). Most studies have been concerned with the formation of cellulose and chitin. It is generally agreed that the nonfibrillar, amorphous components of the wall pose fewer spatial problems of biosynthesis. As to microfibrils, their shape, size, and orientation present a number of spatial demands for their elaboration. Among the organelles commonly suggested as the site of elaboration of microfibrillar polysaccharides are: plasmalemma (3, 4), Golgi apparatus (5, 6), and granules present between wall and plasmalemma (1, 2, 7).

We have recently shown (8) that chitin microfibrils can be assembled *in vitro* by a "solubilized" form of chitin synthetase (EC 2.4.1.16; UDP-2-acetamido-2-deoxy-D-glucose:chitin 4-β-acetamidodeoxyglucosyltransferase) prepared from yeast cells of *Mucor rouxii*. The enzyme was "solubilized" from a crude membrane-rich preparation (35,000 × *g* pellet) by incubation with the enzyme substrate, uridine diphosphate *N*-acetyl-D-glucosamine (UDP-GlcNAc), and activator, *N*-acetyl-D-glucosamine (GlcNAc), at 0°C. The present communication examines the electron microscopic morphology of chitin synthetase isolated by this technique and also of unbound enzyme separated from a crude cell-free extract of the yeast form of *M. rouxii*.

MATERIALS AND METHODS

Cultivation Techniques. Spores (1.5×10^8) of *Mucor rouxii*, IM-80, were inoculated into 0.5 liter of liquid YPG (yeast extract/peptone/glucose) medium (9) in 2-liter Erlenmeyer flasks and incubated in a reciprocating shaker at 28°C for 12 hr. A mixed gas stream (30% CO_2 + 70% N_2) was bubbled through the medium during the entire incubation period.

Cell-Free Extract-Preparation. Yeast cells from 1.5 liters of medium were harvested on sintered-glass filters, washed twice with 200 ml of ice-cold 0.05 M phosphate buffer, pH 6.0, containing 10 mM $MgCl_2$, resuspended in 20 ml of buffer, mixed with 20 ml of glass beads (0.45 mm diameter), and shaken for 30 sec in a Braun model MSK cell homogenizer. Cell-free extracts were centrifuged at 1000 × *g* for 5 min to remove whole cells and cell walls; the supernatant was recentrifuged at 54,000 × *g* (R_{av}) for 45 min. The soluble supernatant and the pellet (mixed membrane fraction) were separated, and the mixed membrane fraction was resuspended and washed by centrifugation as above.

Solubilization of Bound Chitin Synthetase. Chitin synthetase was liberated from mixed membrane fractions by exposure to substrate (8). The mixed membrane fraction was first resuspended in 4 ml of buffer and treated with 500 μg of acid protease from *Rhizopus chinensis* (Miles Laboratories, Elkhart, Ind.) at 22°C. [This treatment was necessary to activate chitin synthetase zymogen (8); it had no detectable effect on the subsequent "solubilization".] After 30 min, 15 ml of ice-cold buffer (0.05 M phosphate, 10 mM $MgCl_2$, pH 6.0) were added and the samples were centrifuged at 54,000 × *g* (R_{av}) for 45 min and washed once with buffer by centrifugation. The pellet was resuspended in buffer and incubated at 0°C for 30 min with 2 mM UDP-GlcNAc, 20 mM GlcNAc, and 0.2 mM ATP. After this treatment, samples were centrifuged at 80,000 × *g* (R_{av}) for 1 hr to give transparent supernatants of "solubilized" enzyme. Portions of these supernatants were then centrifuged on sucrose (5–20%) gradients. Large gradients (34 ml) were centrifuged at 81,500 × *g* (R_{av}) for 3 hr in a Beckman SW-27 rotor; small gradients (12 ml) were centrifuged at 150,000 × *g* (R_{av}) for 3 hr in a Beckman SW-41Ti rotor. Gradients were fractionated with an ISCO, model 183, density gradient fractionator. Fractions of 1 ml were collected, and their chitin synthetase activity was assayed on 0.1-ml samples without addition of protease.

Isolation of Unbound Chitin Synthetase from Cell-Free Extracts. The soluble fraction (54,000 × *g* supernatant) of the crude cell-free extract was subjected to gel filtration in a Sepharose 6B (Pharmacia A. B., Uppsala, Sweden) column (2.1 × 35 cm). Samples of 3 ml were collected. Chitin synthetase activity was assayed in 0.1-ml samples. Active fractions were pooled and treated with 80 μg/ml of bovine pancreas ribonuclease (Calbiochem, La Jolla, Calif.) for 30 min at 30°C. There was no loss in chitin synthetase activity by

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this treatment designed to eliminate ribosome contamination. The turbid suspension was centrifuged for 20 min at 10,000 × *g*, and the clear supernatant was layered and fractionated on a large sucrose gradient as described for the solubilized enzyme.

Chitin Synthetase Assay. Reaction mixtures contained in a final volume of 0.125 ml: 0.5 mM UDP-[^{14}C]GlcNAc (0.2 μCi/μmol), 20 mM GlcNAc, 0.2 mM ATP, 50 mM $KNaHPO_4$, pH 6.0, and 10 mM $MgCl_2$. Unless otherwise stated, 10 μg of acid protease was included in the incubation mixtures. After 1 hr, glacial acetic acid (20 μl) was added to stop the reaction. The radioactivity incorporated into chitin was measured by paper chromatography as described (10) or by filtration of the entire incubation mixture through Whatman GF/C glass fiber filters and washing with about 30 ml of a mixture of 1 M acetic acid and 95% ethanol (80:20 by volume). Filters were dried at 70°C and the radioactivity was determined by liquid scintillation in a Packard Tricarb Spectrometer.

Electron Microscopy. Samples (1 ml) of purified "solubilized" enzyme were taken from the most active fractions of a 34-ml sucrose density gradient and incubated with 1.7 mM UDP-GlcNAc, 14 mM GlcNAc, and 0.14 mM ATP in phosphate/magnesium buffer, pH 6.0 (final volume 1.4 ml) for 3 hr at 22°C. Single drops were applied onto Formvar-coated electron microscope grids and allowed to dry at room temperature. The specimens were washed 8 to 10 times by adding a drop of water and removing it with a filter paper. The grids were shadow-cast unidirectionally with Pd at a 19° angle.

Samples (0.8 ml) of unbound chitin synthetase recovered from the sucrose density gradient were incubated with 25 μg of acid protease for 2 min at 22°C. Then UDP-GlcNAc and GlcNAc were added to a final concentration of 1.2 mM and 20 mM, respectively. Samples of 150 μl were removed and fixed with 20 μl of 8% HCHO. Single drops were applied to microscope grids and allowed to stand for 10 min; excess liquid was removed with filter paper. The grids were washed three times with water and shadow-cast as described above.

For thin-section studies, the samples of unbound chitin synthetase incubated with substrate for 1 hr were fixed at 0°C with 2% glutaraldehyde, in 0.1 M potassium phosphate buffer, pH 7.2, for 1 hr, and centrifuged at 94,000 × *g* (R_{av}) for 30 min. The pellet was washed with buffer, fixed with buffered 1% OsO_4 for 2 hr, pre-embedded in agar, dehydrated in acetone, embedded in Luft's Epon, and sectioned. Sections were stained with lead citrate.

RESULTS

Distribution of Chitin Synthetase in the Cytoplasm. Two types of chitin synthetase were separated from the cytoplasm of yeast cells of *Mucor rouxii*. One, referred to as bound enzyme, sedimented at 54,000 × *g*; the other, termed unbound enzyme, remained in the supernatant. Nearly 90% of the total chitin synthetase was in the bound form. Both bound and unbound enzymes were present mainly (86–94%) in a "zymogenic" state and required protease treatment for activation (ref. 8; Ruiz-Herrera and Bartnicki-Garcia, unpublished data).

Chitin Synthetase Liberated by Exposure to Substrate and Activator. Part of the chitin synthetase present in the mixed membrane fraction was liberated ("solubilized") by exposure to the enzyme substrate (UDP-GlcNAc) and the enzyme activator (GlcNAc) at 0°C as described (8). The solubilized enzyme was further purified by sucrose density gradient centrifugation; a single peak of activity resulted (Fig. 1).

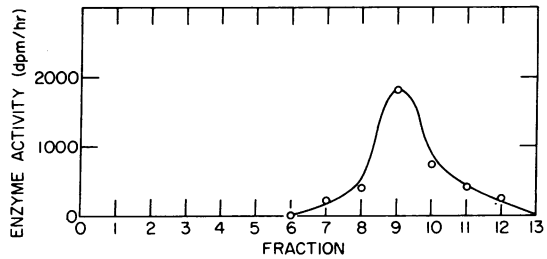


FIG. 1. Sucrose density gradient separation of "solubilized" chitin synthetase. The sample was layered on a 12-ml gradient (5–20%) and centrifuged at 150,000 × *g* for 3 hr. Fractions of 1 ml were collected from the top and assayed for chitin synthetase.

Electron microscopy of enzyme incubated with substrate and activator revealed mainly networks of microfibrils associated with large granules (Fig. 2). The granules were roughly ellipsoidal in shape, measured about 350–1000 Å in diameter, and frequently exhibited a marked single depression. There were also individual microfibrils 120–180 Å wide and up to 2 μm long, scattered between the networks. These were usually associated with a single terminal granule of variable size. The most common images were a long microfibril with a granule at one end (Figs. 4, 6, and 7) and two microfibrils arising from a single granule (Figs. 3 and 5). The microfibrillar product was earlier isolated in larger amounts and determined to be α-chitin by x-ray crystallography (8).

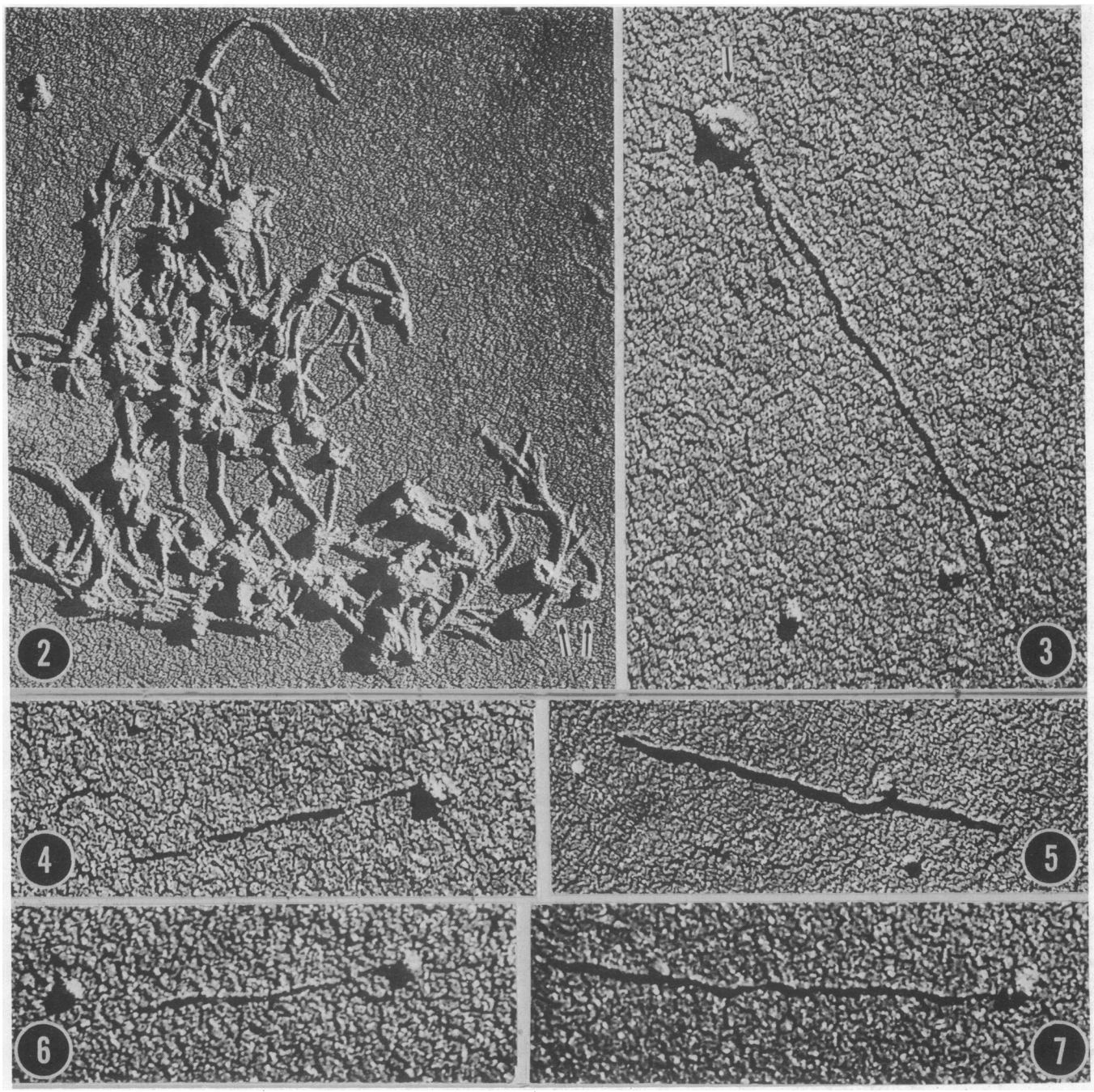
Unbound Chitin Synthetase in the Cell-Free Extract. Unbound chitin synthetase present in the 54,000 × *g* supernatant was purified by passage through Sepharose 6B. The enzyme appeared in the exclusion volume and showed a higher specific activity. Upon subsequent centrifugation in a sucrose gradient, a discrete peak of chitin synthetase activity was obtained. There was full recovery of initial chitin synthetase activity and a 300-fold increase in overall specific activity.

Shadow-cast specimens of purified chitin synthetase showed, under the electron microscope, populations of granules similar to those described for the "solubilized" enzyme (Fig. 8). Upon incubation with substrate and activators, numerous microfibrils were produced. The amount of microfibrils increased greatly with incubation time. At 10 min, short microfibril lengths were seen amidst the clusters of enzyme granules (Fig. 9). At 30 min, extensive networks of microfibrils had been formed (Fig. 10) and the enzyme granules were difficult to discern, except in the periphery of the networks (Fig. 11). Microfibril ends were often seen associated with a single granule.

In thin sections of specimens of unbound chitin synthetase (incubated with substrate), agglomerations of globular elements of variable diameter and diffuse outline were observed (Fig. 12). There were no profiles of typical unit membranes.

DISCUSSION

Our present findings indicate that chitin microfibrils are synthesized *in vitro*, by granules of chitin synthetase measuring from about 350–1000 Å in diameter (shadow-cast measurements). Presumably, the granules are made of a variable number of subunits. The behavior of chitin synthetase in Sepharose 6B, where the enzyme was excluded, and



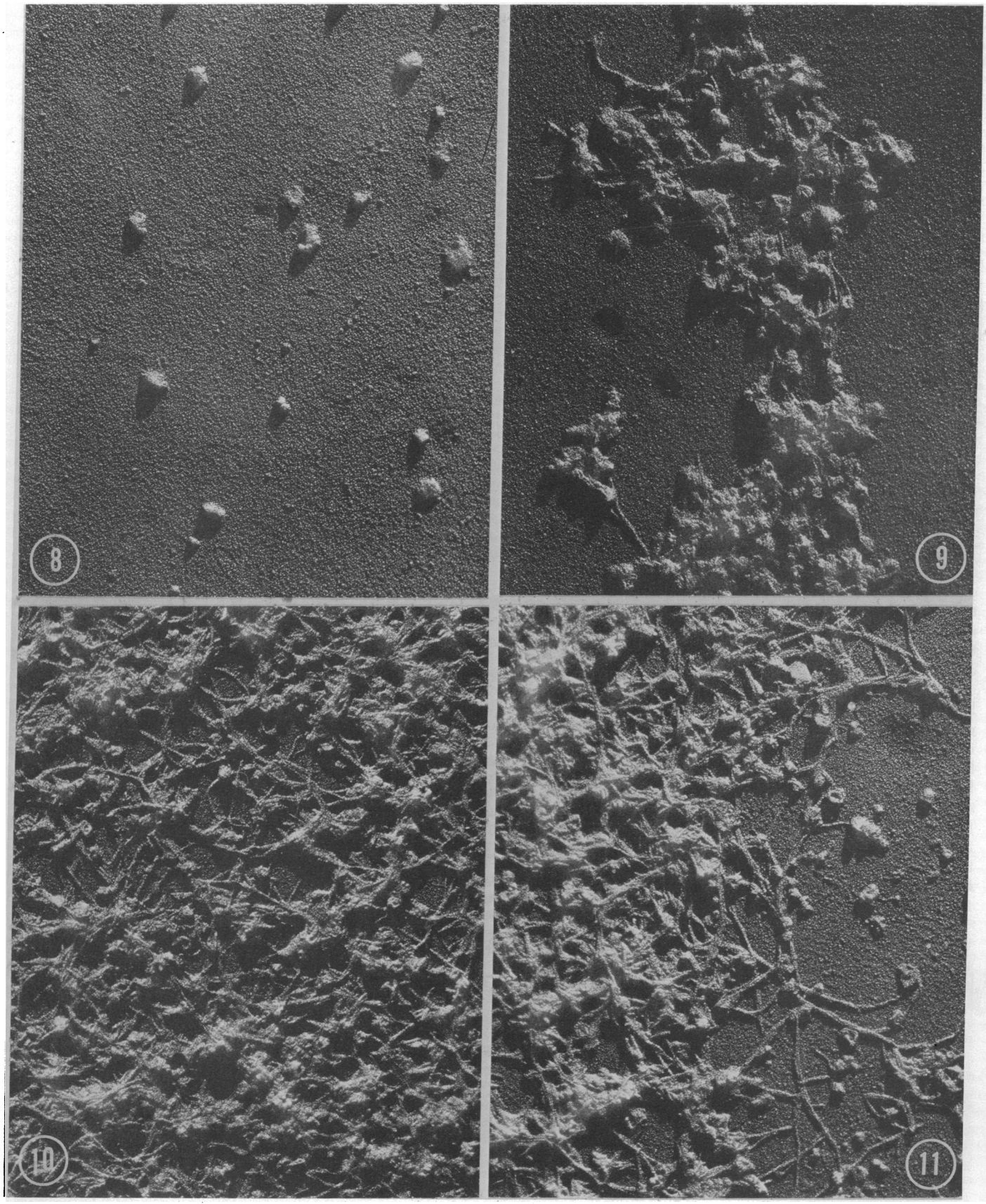
FIGS. 2–7. Electron microscopy of "solubilized" chitin synthetase incubated with substrate showing intimate association of granules with microfibril ends. Granules with a depression are common (arrows). Note that some granules are associated with two microfibrils (Figs. 3 and 5). In Fig. 3, the two microfibrils appear wrapped around each other. Magnifications: Fig. 2, ×60,000; Fig. 3, ×102,000; Figs. 4, 6, and 7, ×93,000; Fig. 5, ×73,000.

in Sepharose 2B or 4B, where the enzyme was included (Ruiz-Herrera and Bartnicki-Garcia, unpublished), was compared with that of cow pea virus (300 Å) and found consistent with the above measurements.

The granules occur freely suspended in the 54,000 × *g* supernatant (unbound enzyme) or can be recovered from the mixed membrane fraction by substrate-induced "solubilization". The manner of association of chitin synthetase to the mixed membrane fraction and its mode of release are not known; one plausible hypothesis was previously offered (8).

The walls of the yeast and mycelial forms of *M. rouxii* (ref 10; Ruiz-Herrera and Bartnicki-Garcia, in preparation) have significant levels of chitin synthetase activity and may conceivably contain similar enzyme granules. Interestingly, in oblique section of the walls of a related fungus, *Gilbertella persicaria*, Bracker and Halderson (11) found globular elements associated with the microfibrils.

The enzymic capacity of the cytoplasmic granules isolated herein was demonstrated by the production of chitin microfibrils upon incubation with substrate and activators. The frequent association of single granules with the terminal ends of microfibrils suggests that the former give rise to the latter. Occasionally, two microfibrils were seen "arising" from the same granule.



FIGS. 8–11. Electron microscopy of unbound chitin synthetase incubated with substrate for various times. At 0 time, there are no microfibrils, only granules (Fig. 8). At 10 min, some microfibrils are visible among tight agglomerations of enzyme granules (Fig. 9). At 30 min, extensive microfibril networks have been formed (Figs. 10 and 11); individual granules are difficult to discern except in the marginal areas (Fig. 11). Magnification, ×71,000.

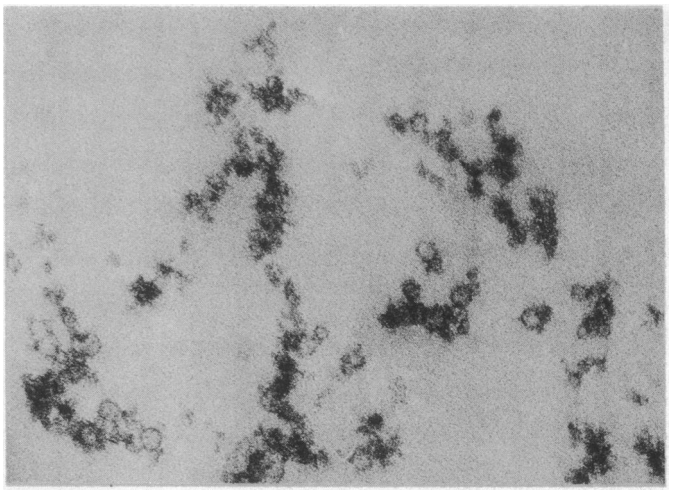


FIG. 12. Thin section of a specimen of purified unbound chitin synthetase incubated with substrate and fixed with glutaraldehyde/OsO_4. Note the agglomeration of globular elements. Magnification, ×90,000.

Our findings lend experimental support to the end-synthesis theory of microfibril elaboration via a terminal enzyme granule. This was originally advocated by Preston (1, 7) for the formation of cellulose microfibrils, but can now be extended to chitin and possibly other microfibrils (1). Accordingly, the molecular chains of chitin are probably not formed independently and then spontaneously crystallized into a microfibril, but are synthesized and assembled collectively into a microfibril by a large multienzyme aggregate or chitin synthetase granule.

The granule theory of microfibril formation has hitherto been supported by electron micrographs showing arrays of granules on the external surface of plasmalemma or on the inner surface of the cellulosic walls of algae (2, 7, 12, 13) and higher plants (14). In *Apium graveolens*, Roland (15) found long cellulose microfibrils with a small globule (100–300 Å) at one end. Granules, often connected to short microfibrils, have also been found at the plasmalemma/wall interphase in yeast cells (2, 16, 17). In the early stages of wall regeneration by protoplasts of *Polystictus versicolor*, Strunk (18) found globular particles (about 400 Å) associated with the incipient microfibrillar network.

Although our results question the direct involvement of typical membranes in chitin microfibril elaboration, the participation of the plasmalemma (or similar membranes) in the orientation of the enzyme granules and in the movement of precursors cannot be discounted.

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1. Preston, R. D. (1974) *The Physical Biology of Plant Cell Walls* (Chapman and Hall, London).
2. Northcote, D. H. (1974) in *Plant Carbohydrate Biochemistry*, ed. Pridham, J. B. (Academic Press, London), pp. 165–181.
3. Wardrop, A. & Foster, R. C. (1964) "A cytological study of the oat coleoptile," *Aust. J. Bot.* **12**, 135–141.
4. Van Der Woude, W. J., Lembi, C. A., Morré, D. J., Kindinger, J. I. & Ordin, L. (1974) "β-Glucan synthetases of plasma membrane and Golgi apparatus from onion stem," *Plant Physiol.* **54**, 333–340.
5. Ray, P. M., Shininger, T. L. & Ray, M. M. (1969) "Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes," *Proc. Nat. Acad. Sci. USA* **64**, 605–612.
6. Brown, R. M., Franke, W. W., Kleinig, H., Falk, H. & Sitte, P. (1969) Cellulosic wall component produced by the Golgi apparatus of *Pleurochrysis scherffelii*," *Science* **166**, 894–896.
7. Preston, R. D. (1964) in *Formation of Wood in Forest Trees*, ed. Zimmerman, M. H. (Academic Press, London–New York), pp. 169–188.
8. Ruiz-Herrera, J. & Bartnicki-Garcia, S. (1974) "Synthesis of cell wall microfibrils *in vitro* by a "soluble" chitin synthetase from *Mucor rouxii*," *Science* **186**, 357–359.
9. Bartnicki-Garcia, S. & Nickerson, W. J. (1962) "Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of *Mucor rouxii*," *Biochim. Biophys. Acta* **58**, 102–119.
10. McMurrough, I., Flores-Carreon, A. & Bartnicki-Garcia, S. (1971) "Pathway of chitin synthesis and cellular localization of chitin synthetase in *Mucor rouxii*," *J. Biol. Chem.* **246**, 3999–4007.
11. Bracker, C. E. & Halderson, N. K. (1971) "Wall fibrils in germinating sporangiospores of *Gilbertella persicaria (Mucorales)*," *Arch. Mikrobiol.* **77**, 366–376.
12. Staehelin, L. A. (1968). "Ultrastructural changes of the plasmalemma and the cell wall during the life cycle of *Cyanidium caldarium*," *Proc. Roy. Soc. London Ser. B.* **171**, 249–259.
13. Robinson, D. G. & Preston, R. D. (1971). "Fine structure of swarmers of *Cladophora* and *Chaetomorpha*," *J. Cell Sci.* **9**, 581–601.
14. Robards, A. W. (1969). "Particles associated with developing plant cell walls," *Planta* **88**, 376–379.
15. Roland, J. C. (1967) "Sur la sécrétion de la cellulose dans les parois des cellules végétales," *C. R. Hebd. Seances. Acad. Sci. Ser. D*, **264**, 2757–2760.
16. Moor, H. & Muhlethaler, K. (1963) "Fine structure of frozen-etched yeast cells," *J. Cell Biol.* **17**, 609–628.
17. Streiblova, E. (1968) "Surface structure of yeast protoplasts," *J. Bacteriol.* **95**, 700–707.
18. Strunk, C. (1969) "Licht-und elektronenmikroskopische Untersuchungen an regenerierenden Protoplasten von *Polystictus versicolor*," *Z. Allg. Mikrobiol.* **9**, 205–216.