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The Mechanism of Formation of Cellulose-Like Microfibrils in a Cell-Free System from *Acetobacter xylinum**

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Abstract. The mechanism of formation of cellulose-like microfibrils by a non-soluble, particulate enzyme and uridine diphosphoglucose (UDPG) in a cell-free system from *Acetobacter xylinum* was studied by transmission electron microscopy and X-ray diffraction. The suspension of particles to which the enzyme is adsorbed is composed of whole, dense ovoids, 50–250 nm long when wet, of fragments of the ovoids, and amorphous substance. There is a typical unit membrane around each ovoid but initially there is no trace of fibrillar material in the suspension. When the suspension of particles is incubated with UDPG, linear wisps of fibrils are produced which associate rapidly to form longer and wider threads, especially in 0.01 M NaCl. There is no visible attachment of the wisps to the particles. After 20 min incubation, threads with the typical morphology of cellulose microfibrils are formed that later tend to become entangled in clumps. The microfibrils are insoluble in hot, aqueous, alkaline solutions and resistant to the action of trypsin, but may be degraded by glusulase. After treatment with 1 M NaOH at 100° C or with cold 18% NaOH they show an X-ray diffraction pattern which resembles that of Cellulose II from mercerized, authentic bacterial cellulose. Incorporation of radioactive glucose into the insoluble residue is enhanced by drying of the cellulose microfibrils before alkaline digestion and especially by the addition of a gross excess of carrier cellulose after incubation. In this system there is no evidence for participation of linear, axial, synthesizing sites on the cell wall of the bacterium or for ordered, organized granules in the assembly of the microfibrils. That is, cellulose-like microfibrils may be formed in a cell-free system without the action of any of the previously suggested cell organelles. In addition, these observations are consistent with a previously described notion of a transient, hydrated, nascent, bacterial cellulose microfibril. The possibility that cellulose microfibrils of green plants may be formed in the same way is considered.

Key words: *Acetobacter* – Cellulose synthesis – Microfibrils (Cellulose).

Introduction

For nearly a century it has been known that *Acetobacter xylinum* produces cellulose under suitable conditions (for a review, see Buchanan and Gibbons 1974, p. 277). Because this form of cellulose is typical of many others (see Bikales and Segal 1971) and because its formation is convenient for experimental study (see the review by Colvin 1972) it has been a favoured object of investigation, especially for the study of cellulose microfibril biosynthesis (Colvin and Leppard 1977). However, for detailed investigation of microfibril formation the use of whole cells or close fractions (Brown et al. 1976; Colvin and Leppard 1977; Cooper and Manley 1975a, b, c; Forge 1977a, b; Forge and Preston 1977; Zaar 1977, 1979) has the disadvantages that the cells can easily obscure experimental observations of particular aspects or they may complicate and confuse interpretation. For these reasons, use of a cell-free system is preferable. Glaser (1958) showed that suspensions of particles from *A. xylinum* contained a non-soluble enzyme which, in the presence of uridine diphosphoglucose (UDPG), could produce cellulose in the complete absence of intact cells or cell envelopes. Recently, using a modification of the same system, Kjosbakken and Colvin (1975) have shown that there is a transient, soluble, intermediate polymer of glucose in the overall process of cellulose biosynthesis. The presence of the intermediate polymer has been confirmed directly and

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indirectly in other studies (Leppard et al. 1975; King and Colvin 1976; Colvin et al. 1976; Colvin and Leppard 1977) but up to the present time, there has been no experimental investigation of the physical mechanism of formation of the nascent cellulose microfibrils from UDPG by the enzyme on the particles. The purpose of this paper is to report observations with the electron microscope, supplemented by X-ray diffraction studies, of this mechanism for bacterial cellulose, and to indicate the possible significance of these observations for the process of cellulose biosynthesis in other organisms.

Material and Methods

Preparation of Particulate Enzyme

Cellulose-free cell suspensions of *A. xylinum* (N.R.C. 17005, formerly A.T.C.C. 10245) were prepared as described by Hestrin and Schramm (1954) to give a final concentration of ca. 5×10^9 viable cells/ml. These suspensions were then used to prepare the particulate enzyme preparations by sonication as described by Glaser (1958) except that the final pellet of particles carrying the enzyme was suspended and washed in the buffer twice, rather than once, in order to remove a greater proportion of the phosphatases present. The suspensions of particulate enzyme were frozen and stored in liquid nitrogen until used. Their activity remained unchanged for more than a year under these conditions.

Incubation of Particulate Enzyme with UDPG

Incubation of the particulate suspensions with UDPG was essentially as described by Glaser (1958), with the following modifications. No cellodextrins were used as primers because they would have interfered with observations by electron microscopy and because previous work showed that they were unnecessary (Kjosbakken and Colvin 1975). No carrier cellulose was added to the suspensions at the end of the incubation period unless it was required for the purpose of the experiment. Whenever this was done it is so stated explicitly. Temperature of incubation was 35° C, not 28° C, in order to accelerate the reaction, and the usual length of incubation was 40 min.

For observations on the incorporation of glucose into alkali-insoluble material from radioactively labelled UDPG, the incubation mixture was as follows: 1 ml of buffer of the following composition, 0.05 M tris (hydroxymethyl) aminomethane (Tris), 0.01 M $MgCl_2$ and 0.001 M ethylenediaminetetraacetic acid, pH 8.2; 0.7 ml of a particulate enzyme preparation; 0.5 mg of non-radioactive UDPG (Calbiochem, San Diego, Cal., USA); 20 µl of a solution of radioactive UDPG, the glucose being uniformly labelled with ^{14}C (50.0 µCi in 2.5 ml of ethanol-water, 7/3; New England Nuclear, Lachine, Que., Canada). The mixture was incubated with moderate oscillation (4 cycles/s) in a water bath. The reaction was stopped at appropriate times by adding 0.2 ml of glacial acetic acid and the products were treated with 1 M NaOH, followed by washing, as described by Glaser (1958). The insoluble residue was transferred to a scintillation vial, dried, and its radioactivity estimated by a liquid scintillation counter (Model LS-250, Beckman Instruments; Fullerton, Cal., USA) using 2,5-diphenyl-oxazole, 6 g/l in toluene, as the scintillation fluid.

Electron Microscopy of Glaser's Particles

Preparations of the particles were fixed for 1 h at 0° C in 6% glutaraldehyde in 0.01 M phosphate-citrate buffer, pH 6, and post-fixed in 2% osmium tetroxide in the same buffer. After ethanol dehydration, solvent exchange into propylene oxide, and embedding in Epon 812, (Fisher Scientific Co., Fair Lawn, New Jersey, USA) sections (about 50 nm thick) were cut with a diamond knife and stained for 30 min with uranyl nitrate (Watson 1958) and 3 min with lead citrate (Reynolds 1963).

Electron Microscopy of Microfibril Formation

For observations on the physical mechanism of formation of the cellulose-like microfibrils, the incubation mixture was as follows: 2 ml of the same buffer as described in the preceding paragraph; 20 µl of Glaser's enzyme preparation; 1 mg of non-radioactive UDPG. The mixture was incubated with moderate oscillation (4 cycles/s) in a water bath and aliquots (0.1 ml) withdrawn at chosen intervals by pipette. As in the other series, the reaction was stopped by adding the aliquot to 0.9 ml of 0.1 M acetic acid. Drops of this acetic-acid solution were dialyzed for 24 h on formvar films (Ladd Research Industries, Burlington, Vermont, USA) floating on 1% formaldehyde to remove all dialyzable, water-soluble components. The drops were then mounted on electron microscope grids covered with a carbon film, shadowed with palladium-gold (60/40) and examined in a Siemens (West Berlin, Germany) electron microscope, Model 101.

When the effect of dilute solutions of NaCl on the formation of microfibrils was investigated the method was as follows. The aliquot (0.1 ml) from the reaction mixture was added to 0.9 ml of a solution of NaCl of the appropriate concentration to bring the final molarity to the desired level and the suspensions were immediately frozen and lyophilized. The dried residue was then re-suspended in 1 ml of distilled water, drops of this suspension dialyzed, mounted and examined as above.

For observations by the electron microscope on the effect of trypsin or glusulase on the cellulose-like microfibrils produced by Glaser's enzyme particles in the presence of UDPG, the incubation mixtures were dispersed on formvar-carbon films as described above. After drying, the appropriate side of the grids carrying the dispersed material was placed on the surface of a solution of trypsin (Mann Research Laboratories, New York, N.Y., USA) or glusulase (a mixture of carbohydrases, including cellulase; Endo Laboratories, Garden City, N.Y., USA) for the indicated interval at 30° C. Glusulase was necessary because purified cellobiase or endoglucanases do not attack cellulose. For trypsin solutions the concentration was 1% (w/v) in Tris buffer, pH 8.2, described above. The glusulase was diluted, 1 volume to 9 of distilled water, immediately before use. On completion of digestion, the grids were washed 5 times in distilled water, dried, shadowed, and observed as above.

X-Ray Diffraction

For preparation of samples for X-ray diffraction, the method of Glaser (1958) was used except that the radioactive tracer, the cellodextrins and the carrier cellulose were omitted. Hydrolysis of a sample was with 1 M NaOH at 100° C for 10 min. Mercerization of a sample was by 18% NaOH at room temperature (ca. 25°) overnight. Cellulose II was prepared by dissolving bacterial cellulose in dimethylsulfoxide-paraformaldehyde (Johnson et al. 1976) and re-precipitating with water. All samples and residues were thoroughly washed with water before examination.

X-ray diffraction patterns of powdered samples were recorded by a Norelco microcamera (North American Philips Co., New York, N.Y., USA) fitted with flat film in a Hilger and Watts (London, U.K.) generator. Nickel-filtered Cu K α X-radiation of a wavelength 1.54 Å (1 Å = 0.1 nm) was employed with a specimen-to-film distance of 15 mm. This short distance in the microcamera was imposed by the limited quantities of material available. Exposure times varied from 20 to 50 h, depending upon the mass of the sample.

Table 1. Repetition of Glaser's experiment on the incorporation of radioactivity into insoluble cellulose residues in *Acetobacter xylinum*

Time of incubation (min)	Radioactivity incorporated (cpm)
0	36
10	268
20	434
40	727

Results

Activity of Enzyme Preparations

To verify that the non-soluble enzyme preparations were active for the synthesis of cellulose, preliminary trials of the rate of incorporation of radioactive glucose into the alkali-insoluble product were held for each preparation under the conditions of Glaser (1958) including addition of cellodextrins and carrier cellulose. A typical result is shown in Table 1. Over a period of 40 min ca 1% of the total radioactivity was incorporated into alkali-insoluble products, which is about twice the rate for Glaser's (1958) preparations.

Microstructure of Glaser's Particles

As a preliminary phase to this study, the microstructure of undried Glaser's enzyme particles and their micromorphology when dried on a film were determined. Fig. 1 is a typical micrograph of a thin section of the particles, fixed when wet. The suspension is composed of globules or ovoids (50–250 nm long) which are surrounded by a unit membrane and of fragments which appear to be derived from the ovoids; that is, short segments of unit membranes, as well as amorphous material. The interior of the ovids contains a structureless matrix which stains darkly with lead and uranium. Fig. 2 is a photograph of a suspension of Glaser's particles which were dried on a film after incubating in only buffer (without UDPG) for 40 min. During the incubation period, the ovoids tend to clump as does the amorphous, smaller material. In these dried preparations the particles were approximately one half of the above dimensions and there was no evidence of fibrillar material of any size, of ordered organelles or of mature cellulose microfibrils.

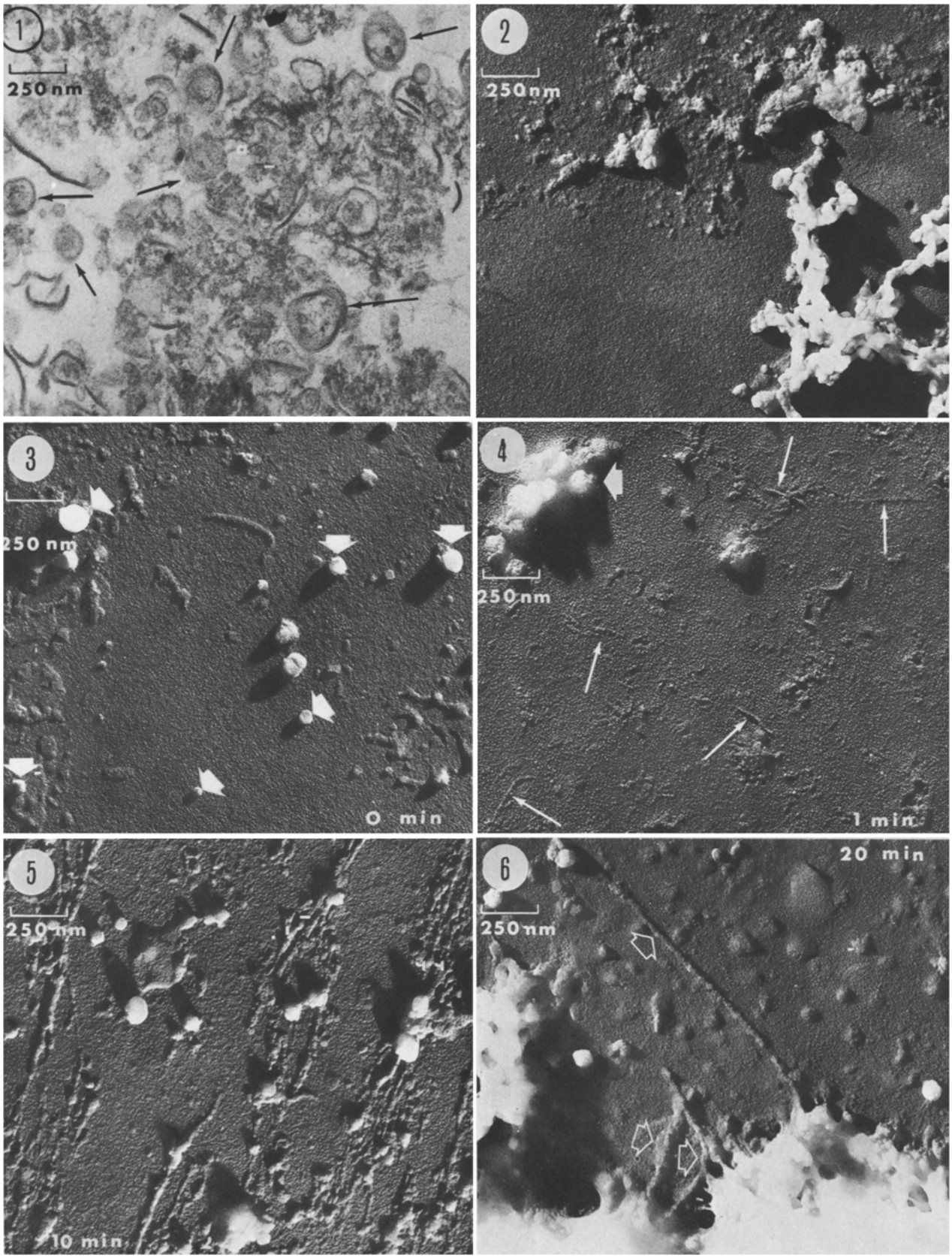
Formation of Cellulose-Like Microfibrils

When incubated in the presence of UDPG, the particles are initially isolated and dispersed (Fig. 3), without fibrillar material, among the fragments of the suspension. After 1 min incubation with the sugar nucleotide, they tend to become aggregated into small clumps. Wisps of a fine, linear, fibrillar material, which were not detectable before, become visible between the clumps at that time (Fig. 4). These wisps are not visible by negative staining with phosphotungstic acid because they adsorb the heavy ion. After 4 min incubation, the wisps are longer and wider and the aggregated, fibrillar material begins to resemble nascent cellulose microfibrils (Leppard et al. 1975). By 10 min, the process of nascent microfibril formation is well advanced (Fig. 5), with some strands more than 30 μm long and with cellulose microfibrils of normal width interspersed among smaller, shorter wisps. There is no evidence of terminal attachment of the Glaser's enzyme particles to the wisps or to the ends of the nascent microfibrils, other than because of chance superposition (Fig. 5). It should be noted also that in local areas the microfibrils tend to lie parallel to each other as if there were a general, orienting influence (Fig. 5). After more than 10 min of incubation, the microfibrils become enmeshed (with themselves and the Glaser's enzyme particles) in large irregular clumps from which only an occasional tip extends (Fig. 6). These tips have the typical appearance of mature, cellulose microfibrils (Colvin 1972). After 40 min incubation, areas which contain non-entangled microfibrils are relatively rare but the morphology of the strands in these areas is similar to that observed for samples incubated for only 10 min (Fig. 5). After this time, the average width of the microfibrils does not increase.

Digestion of Microfibrils by Hot Alkali, Trypsin and Glusulase

The foregoing observations demonstrated that when particles carrying Glaser's enzyme were incubated in the presence of UDPG a fibrillar substance is produced after 40 min which is indistinguishable in morphology from cellulose microfibrils. Although there is no evidence that cultures of *A. xylinum* or their derivatives produce any similar, non-soluble, fibrillar product other than cellulose, it was necessary and prudent to determine the effect of hot, aqueous alkali, of trypsin and of glusulase on this fibrillar substance. Fig. 7 is a photograph of Glaser's enzyme particles which were incubated with UDPG for 40 min, dried and then treated with 1 M NaOH at 100° C for 10 min. The particles which carry the enzyme were degraded by the hot, aqueous alkali to amorphous material but the microfibrils enmeshed in this substance resisted the treatment (Fig. 7, arrows).

Digestion with trypsin for 30 min had no effect on the microfibrils formed during 40 min incubation,


1
250 nm
2
250 nm
3
250 nm
0 min
4
250 nm
1 min
5
250 nm
10 min
6
250 nm
20 min

other than to accentuate their appearance (Fig. 8), presumably by removal of encrusting proteins.

On the other hand, incubation of the microfibrils with glusulase rapidly degraded them, first to short segments and later to amorphous material (Fig. 9). It is instructive to compare Fig. 6 with Fig. 9 because the original samples were both incubated for 20 min. Digestion with trypsin followed by glusulase removed all the fibrillar material except a small number of short, thin rods (Fig. 10). These short, thin rods may be the residue of crystallites which are more resistant to enzymatic action (see Bikales and Segal, 1971).

Repeated attempts failed to detect any evidence for an activating effect of trypsin digestion on cellulose microfibril formation, similar to that observed for chitin (Ruiz-Herrara et al. 1977; Duran and Cabib 1978).

The preceding observations on the morphology of the microfibrils produced by the enzyme particles and UDPG led, by chance, to the detection of an unexpected effect of salt concentration upon the formation of microfibrils. Fig. 11 is a photograph of cellulose-like microfibrils in the product of incubation which was dried from 0.01 M NaCl. The same product of incubation dried from water or from 0.02 M NaCl did not show microfibrils. This specific effect of salt is easily reproducible and may be related to an effect of buffer concentration on whole-cell fractions (Forge 1977a).

Powder X-Ray Diffraction Studies

The molecular order of the microfibrils was studied by powder X-ray diffraction of the dried product of incubation of Glaser's enzyme particles with UDPG, of the alkali-insoluble residue from this product (Hyd.) and of the residue from mercerization of the product (Mer.). The appropriate patterns are shown in Fig. 12, A and B, together with patterns of Cellulose I and Cellulose II. The untreated dried product of incubation showed no detectable evidence of crystallinity, even after long expsoure (Fig. 12A). The alkali-insoluble residue and the residue from the mercerization of the product showed at least five discrete reflections which were the same (Fig. 12A) and which resembled those of Cellulose II (Fig. 12B).

The parameters of the reflections from Fig. 12 are listed in Table 2. The spacings for Cellulose I agree with those reported by Forge (1977a). All the spacings for Cellulose II are consistent with those observed by Hunter and Dweltz (1979) for fibre diagrams of regenerated cellulose. The chief differences between the patterns for Cellulose I and Cellulose II are the relative intensities of the reflections for the 0.60- and 0.52-nm spacings, which are much weaker for Cellulose II, and the nearly complete loss of the 0.25-nm reflection in Cellulose II. Taking into account the variability of the intensities of individual reflections in Cellulose II (Hunter and Dweltz 1979), the patterns and the spacings for the hydrolyzed and the mercerized residues resemble those of Cellulose II more than those of Cellulose I.

Effect of Drying

The preceding observations and their implications for the physical mechanism of bacterial cellulose microfibril formation indicated that there should be a detectable effect of drying upon the degree of incorporation of radioactive glucose from UDPG into the insoluble, fibrillar residues because drying would promote the formation of irreversible, inter-chain, hydrogen bonds. In addition, there should be a substantial effect of addition of carrier cellulose to the mixture after incubation upon the degree of incorporation of radioactivity, because the intermediate, 1→4-β-glucans would be strongly and irreversibly adsorbed to the microfibrils of the carrier cellulose. They therefore would be less likely to be removed by subsequent alkaline treatment.

Fig. 1. Cross-sections of fixed Glaser's enzyme particles from *Acetobacter xylinum* (arrows) and their fragments. Note the structureless matrix within intact particles, the unit membrane which surrounds them and the segments of broken membranes. ×40,000

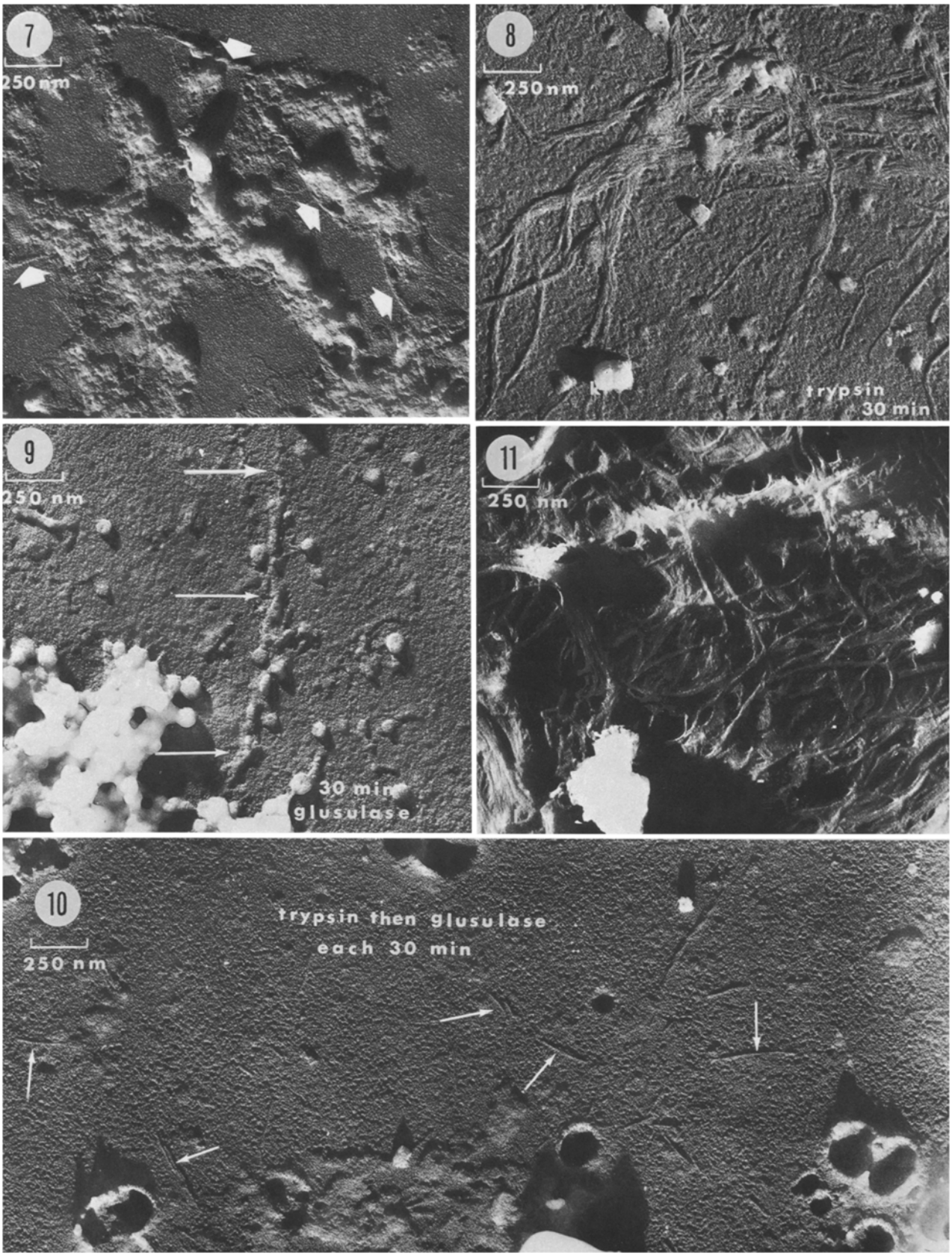
Fig. 2. Clumps of dried Glaser's enzyme particles and their fragments, after 40 min incubation without UDPG. Note the complete absence of any fibrillar material and the tendency of the ovoids to aggregate. It may be necessary to emphasize that there is no trace of cellulose microfibrils in these preparations. ×40,000

Fig. 3. Dispersion of Glaser's enzyme particles in the presence of UDPG after a very short time of incubation (0–10 s). Note that the whole particles are generally dense, single, isolated and heterogeneous in size (arrows). The fragments of broken particles and membranes can be seen on the left hand side. The sickle-shaped membrane fragment was the only one observed. ×40,000

Fig. 4. Wisps of fine, linear, fibrillar material (small arrows) after 1 min of incubation of the Glaser's enzyme particles with UDPG. Note also that the particles or ovoids have begun to aggregate (large arrow). Substitution of Pt shadowing for Pd-Au did not improve resolution of these wisps, which are aggregates of 1→4-β-glucans

Fig. 5. Microfibrils of bacterial cellulose of typical morphology formed after 10 min of incubation. Note that there is no evidence of special attachment of the ovoid particles to the microfibrils. Note also that fine, globular material, which may be protein, is adsorbed to the microfibrils. ×40,000

Fig. 6. Edge of a large clump of ovoid particles and microfibrils tangled together after 20 min incubation. Several ends of microfibrils extend from the clump (arrows). ×40,000


7
250 nm
8
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trypsin
30 min
9
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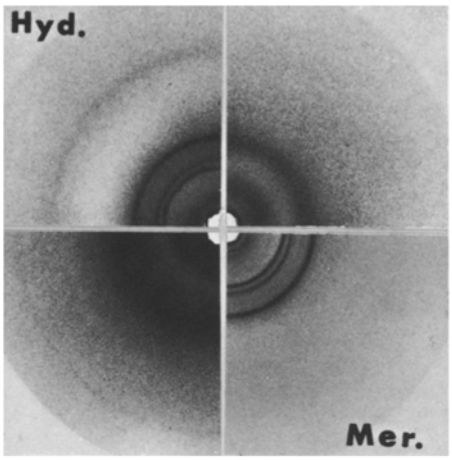
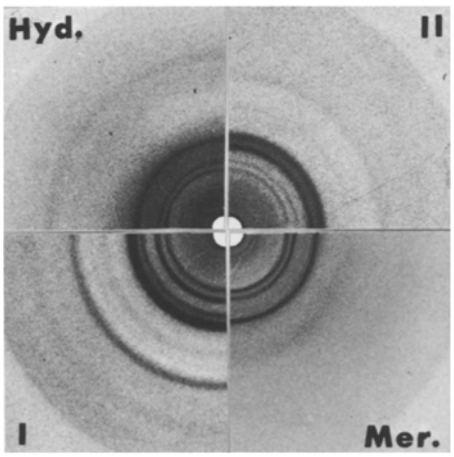
30 min
glusulase
11
250 nm
10
250 nm
trypsin then glusulase
each 30 min



A



B

Fig. 12. A Powder X-ray diffraction patterns of (a) the alkali-insoluble residue from the product of incubation of Glaser's enzyme particles from *Acetobacter xylinum* with UDPG (Hyd); (b) the mercerized product (Mer); (c) the dried product at two degrees of exposure. Note that there is only amorphous scattering from the dried product and that the patterns of the hydrolyzed and mercerized residues are the same. **B** Powder X-ray diffraction patterns of (a) the alkali-insoluble residue from the product of incubation of Glaser's enzyme particles from *A. xylinum* with UDPG (Hyd); (b) the mercerized product (Mer); (c) of Cellulose I; (d) of Cellulose II. Note that the patterns of the hydrolyzed and mercerized residues resemble that of Cellulose II. The intensity of the 4.5-Å spacing in both patterns varies from weak to strong, depending upon the sample. Such variability of specific intensities has been attributed to varying disorder along the chains (Hunter and Dweltz, 1979). The sample chosen here shows a weak intensity for that reflection. Although a spacing at 7.5 Å is sometimes considered diagnostic for Cellulose II, note that this reflection is absent in the pattern from an authentic sample of bacterial Cellulose II prepared by precipitation from dimethyl sulfoxide-paraformaldehyde. Obviously, the presence or absence of this spacing is less reliable than supposed

These expectations were tested by incubating four series of samples of particulate enzyme plus radioactive UDPG, each series containing four replicates. Each series was treated in an identical fashion except as below: the first series was centrifuged and treated with hot 1N NaOH immediately after stopping the reaction; the second series was centrifuged and taken to dryness before being heated with 1N NaOH; 10 mg of carrier cellulose was added to the mixtures of the third series immediately *after* the reaction was stopped and then the contents of the tubes were centrifuged and heated with 1N NaOH; the contents of the tubes in the fourth series were taken to dryness immediately after stopping the reaction, then 10 mg of carrier cellulose was added, the whole resuspended in water, centrifuged, and hydrolyzed. The results of one such experiment, which are typical of four others, are shown in Table 3. Clearly, drying of the mixture prior to treatment with hot alkali tends to promote incorporation of radioactivity (approximate increase, 10%) into the final, insoluble product, probably because it accelerates formation, irreversibly, of interchain hydrogen bonds (Bikales and Segal 1971). However, the effect of adding a massive excess of carrier cellulose is ca. 4 times greater, probably because the initial glucan chains and their aggregates tend to be

Fig. 7. Microfibrils of bacterial cellulose, from a preparation from *Acetobacter xylinum* which was incubated for 40 min, then dried and hydrolyzed (arrows). The masses of amorphous substance are presumably the insoluble remainder of the ovoid particles. × 40,000

Fig. 8. A clump of bacterial cellulose microfibrils from a preparation which was incubated for 40 min, then digested with trypsin for 30 min. The trypsin has removed much of the encrusting material, presumably protein, revealing the microfibrils. × 40,000

Fig. 9. The residue of microfibrils which was produced after incubation of a preparation for 20 min, followed by digestion with glusulase (30 min). Note that the glusulase has begun to degrade the microfibrils (arrows) but not the ovoid, Glaser's enzyme particles. × 40,000

Fig. 10. Residue from bacterial cellulose microfibrils which were formed by incubation of UDPG and Glaser's enzyme particles for 60 min, followed by digestion with trypsin and glusulase (each for 30 min). Note that the microfibrils have been degraded to short, straight threads (crystallites?). × 40,000

Fig. 11. Product of incubation of Glaser's enzyme particles with UDPG, after drying from 0.01 M NaCl. Note the microfibrils with the typical morphology of cellulose. The same product of incubation did not show microfibrils when dried from water or from 0.02 M NaCl. × 40,000

Table 2. Powder X-ray diffraction parameters of bacterial cellulose I, bacterial cellulose II and of the alkali-insoluble product of incubation of Glaser's enzyme particles *Acetobacter xylinum* with UDPG (m. = medium intensity; s. = strong; w. = weak; v. = very)

Bacterial cellulose I		Bacterial cellulose II		Alkali-insoluble product	
Ring diam.	Spacing	Ring diam.	Spacing	Ring diam.	Spacing
0.73 cm (m.)	0.65 nm	0.80 (v.w.)	0.60 nm	0.76 (w.-m.)	0.63 nm
0.88 (m.)	0.54	0.92 (v.w.)	0.52	0.87 (w.-m.)	0.55
1.08 (w.)	0.45	1.05 (s.)	0.45	1.05 (w.-s.)	0.45
1.21 (s.)	0.41	1.18 (s.)	0.41	1.21 (v.s.)	0.41
1.55 (v.w.)	0.33	1.55 (v.w.)	0.33	–	–
1.95 (v.w.)	0.27	–	0.29	–	–
2.10 (m.)	0.25	2.15 (v.w.)	0.25	2.18 (v.w.)	0.25

Table 3. Effect of drying and of addition of carrier cellulose upon incorporation of radioactivity into insoluble cellulose by Glaser's enzyme particles from *Acetobacter xylinum* acting upon UDPG

Treatment	Radioactivity incorporated (cpm)[a]
No drying	179
Dried	198
No drying and carrier cellulose	263
Dried and carrier cellulose	303

[a] Average of four replicates

adsorbed irreversibly to the microfibrils of carrier cellulose.

Discussion and Conclusions

Bacterial Cellulose Biosynthesis

The foregoing observations show that preparations of Glaser's enzyme particles (1958) are a mixture of globules or ovoids, 50–250 nm long, and their fragments with amorphous material. Whole ovoids are surrounded by a unit membrane which encloses an electron-dense, structureless matrix. Initially there is no trace of fibrillar material. However, when the preparations are incubated with UDPG, within 1 min wisps of amorphous, ill-defined, linear threads are observed in the electron microscope which have a breadth down to the limit of resolution imposed by the shadowing technique. These initial wisps are probably aggregates of the 1→4-β-glucans which are released by active, whole cells (Colvin and Leppard 1977). The wisps associate rapidly (less than 20 min) to form a network of extended microfibrils which have an approximate average width of 10 nm and are indefinitely long. The process of association of the wisps into microfibrils is promoted by 0.01 M NaCl but not by higher concentrations of the salt. When the process is complete (40 min or less) these microfibrils have the characteristic morphology and dimensions of normally-formed bacterial cellulose microfibrils.

The following facts are relevant to the question of the composition of the cellulose-like microfibrils produced in the incubation mixture. Glaser (1958) showed that the product of incubation was degraded by partial acid hydrolysis to cellobiose, cellotriose and cellotetrose. After further enzymatic hydrolysis only glucose was detected. The present study confirmed that the microfibrils in the product of incubation are insoluble in hot, molar alkali and showed that they are not affected by treatment with trypsin. They were digested by glusulase, a mixture of carbohydrases which includes cellulase. Taken together, these results indicate that the cellulose-like microfibrils are not composed of a protein or of a carbohydrate heteropolymer; they indicate that they are composed of a glucan which has, at the least, a high proportion of 1→4-β-linkages. All these results are consistent with the conclusion that the microfibrils are composed of a form of cellulose.

The observations from X-ray diffraction give additional support to the conclusion that the microfibrils are a form of cellulose. The pattern of the dried product of incubation shows only amorphous scattering, indicating that there is little or no crystalline material present initially. However, after treatment with hot, aqueous molar alkali or with mercerizing concentrations of cold alkali, the pattern of the residue of the product of incubation from either treatment shows at least five reflections which are the same as those from athentic bacterial Cellulose II. In each case, the alkaline treatment, followed by washing and drying, has induced a moderate degree of order which was not present in the original, dried product and this degree of order resembles that of the second polymorph of cellulose, i.e. Cellulose II.

From the foregoing, it is reasonable and plausible to conclude that the microfibrils which are produced in the incubation mixture are a nascent form of cellulose. No other product produced by *A. xylinum* is compatible with all the above results and there are no observations which are inconsistent with the con-

clusion. If the conclusion is accepted, the formation of bacterial cellulose microfibrils has been observed in vitro, in contrast to the situation for green-plant cellulose microfibrils (see review by Delmer 1977). Although much detail still has to be determined, the general course of formation of these cellulose or cellulose-like microfibrils can be sketched as follows. Transient, soluble 1→4-β-glucans are synthesized rapidly in an extended form from UDPG by the enzyme which is carried in or on the particles of the suspension. These extended, transient β-glucans associate longitudinally to form non-crystalline, nascent microfibrils (Leppard et al. 1975). In whole-cell systems, the glucans of these nascent microfibrils will gradually and partially crystallize to form the ordered domains of Cellulose I of mature microfibrils (for a review see Colvin 1972). However, in the incubation product of Glaser's enzyme plus UDPG, which is produced under markedly non-physiological conditions, extraneous debris from the preparation must be removed by alkali before the glucans can associate in an orderly fashion. When the glucans do crystallize under the influence of the alkali they come together to form the Cellulose II structure.

If the microfibrillar material which is formed is cellulose, several important conclusions may be drawn. First, because only UDPG and the ovoid particles or their fragments, which carry the enzyme, are initially present in the incubation mixture, the microfibrils of bacterial cellulose are formed (crystallize) from the synthesized 1→4-β-glucans without the intervention of the postulated individual, axial, synthetic sites on the cell wall of the bacterium (Brown et al. 1976; Zaar 1977, 1979) or of ordered, organized, granular, enzymatic, terminal complexes (Robinson 1977). These cell organelles, if they are involved at all in bacterial cellulose synthesis by whole cells, must play an auxiliary role, not a primary one, because they are absent from Glaser's enzyme preparations. Even the involvement of single intramembrane particles (Forge and Preston 1977) is doubtful because there is no evidence that the ovoids or their fragments are attached to the microfibrils. Second, there is no evidence from this study for participation of extended, crystalline, discrete, elementary fibrils in the biosynthesis of bacterial cellulose (Colvin 1972). Finally, there is no evidence for tip growth of cellulose microfibrils, as usually conceived (Colvin 1972). If the threads are cellulose, bacterial cellulose microfibril formation may be regarded as an example of rapid polymer crystallization in a biological milieu (Atalla and Nagel 1974; Petermann and Gluter 1977).

At this point, it is useful to summarize and evaluate the conclusions about bacterial cellulose microfibril formation which have been drawn from extensive and heuristic studies on whole cells (Brown et al. 1976; Forge and Preston 1977; Zaar 1977, 1979). Although the significance of some of the observations may be questioned because they were made on thoroughly washed, glucose-free cells which could not have been synthesizing cellulose (Brown et al. 1976), each of these investigations suggested that formation of the cellulose microfibril was necessarily closely associated with the bacterial cell wall and that an organized system of intramembrane particles or pores was involved. The conclusion was consistent with the data from whole cells but the observations on the simpler, more circumscribed, cell-free system demonstrate that the cell walls need not be involved and that the role of organized pores is doubtful.

The foregoing experimental observations and the conclusions drawn from them are fully consistent with the idea of a transient, hydrated, nascent, bacterial cellulose microfibril which has been described earlier (Leppard et al. 1975; King and Colvin 1976; Colvin et al. 1976). Because they are in an aqueous solution, the newly biosynthesized 1→4-β-glucans and their aggregates will be highly hydrated initially and therefore the nascent microfibrils formed from them will be greater in breadth when observed by freeze-etching (Leppard et al. 1975; Colvin and Leppard 1977) than when observed after drying for conventional electron microscopy, as here.

The effect of drying and the much larger effect of adding carrier cellulose on incorporation of radioactivity into the insoluble cellulose-containing residues, noted above, are equally consistent with the idea of the nascent microfibril. During drying, the hydroxyls of the newly synthesized, extended, β-glucan chains will form a number of irreversible hydrogen bonds with each other as the water molecules are removed. A proportion of these dried aggregates will therefore no longer be soluble in hot alkali and consequently the apparent degree of incorporation of insoluble, radioactive, glucose residues will be increased. To an even greater extent, the addition of a gross amount of carrier cellulose will permit formation of irreversible bonds between the hydroxyl groups of the synthesized β-glucans and the hydroxyls of the microfibrils of carrier cellulose, thereby increasing incorporation of the radio-isotope into the insoluble residues.

Possible Extrapolation to Biosynthesis of Cellulose in Green Plants

The foregoing facts and the conclusions drawn from them lead to a picture of bacterial cellulose biosynthesis which is clearly different from that generally as-

sumed hitherto. The question arises whether this scheme of microfibril formation is limited to bacteria or whether it may be extended to green plant biosynthesis. Up to now, the most generally accepted hypothesis of cellulose microfibril formation is that originally proposed as a speculation by Preston (1964) and supported by subsequent observations. A recent summary of the concept and supporting observations is given by Preston (1974, pp. 425–456). In brief, the concept postulates an ordered array of organized, synthetic granules on the outside surface of the plasmalemma of plant cells, each granule being capable of spinning a microfibril from glucose residues which are supplied by nucleotide glucose donors. The preformed part of the microfibril either moves away from the organized granule or the granule moves on the surface of the plasmalemma. A superb exposition of this notion has been used to interpret observations on green plant cells in each of two recent papers (Giddings et al. 1980; Mueller and Brown 1980). The idea has been markedly heuristic but in spite of its intrinsic appeal, complete proof of the notion is still lacking (Preston 1974). Moreover, there is at the present time some reason to doubt that this mechanism may operate in any green plants. As stated by Robinson (1977), "If plasmalemma particles are involved in the synthesis of cellulose then it is disturbing that they have been so infrequently and imperfectly demonstrated with the freeze-etch method." This statement is still applicable to the conclusions of the two careful, painstaking studies mentioned above. In each of them, bodies which are assumed to be terminal complexes are seen on the *inside* of the outer leaflet of the plasmalemma and are assumed to be in direct connection with the ends of impressions of microfibrils which are on the *outside* of the leaflet. In each study, there is no direct, unequivocal demonstration of terminal complexes at the ends of cellulose microfibrils in place. Furthermore, there are serious dangers of over-interpretation in this kind of work. As an example, interior aggregations which are identified as terminal complexes by Mueller and Brown (1980, Fig. 2) are identical in size and appearance to internal projections shown by Giddings et al. (1980, Fig. 8) in the *middle* of impressions of microfibrils, where they cannot be synthesizing terminal granules. Each study does not explain how the postulated synthesizing organelle can move the microfibril longitudinally to permit growth of the thread. At present, it is still true that there is no clear, definitive demonstration of attachment of cellulose microfibrils in green plants to synthesizing plasmalemma particles.

In contrast, there is one recent investigation of the mechanism of formation of cellulose microfibrils by tobacco protoplasts which is in complete accord with the mechanism proposed above for bacterial cellulose microfibrils. Grout (1975) has concluded, from an electron-microscopic investigation of regenerating tobacco mesophyll protoplasts, that formation of cellulose microfibrils by these entities is not associated with any structured particles on the surface of or with apparently differentiated regions in the plasmalemma. He concludes that after biosynthesis of the required $1\rightarrow4$-β-glucans the microfibrils of higher-plant cellulose may be constructed without the aid of an enzyme which has a specific function of assembly of the chains. This conclusion for green plants is completely consistent with the above conclusion for bacterial cellulose microfibril formation.

Finally, although conclusions from analogy are often misleading, it is worth noting that the mechanism of chitin microfibril formation is the same in principle as that outlined for bacterial cellulose. The effective catalytic agents for formation of chitin microfibrils of the fungus *Mucor rouxii* are spheroidal globules about 100 nm in diameter surrounded by a membrane (Bracker et al. 1976). These globules have been called chitosomes. When chitosomes and uridine diphospho-N-acetyl-glucosamine are incubated together, coiled polymeric precursors of the chitin microfibril appear within the chitosome. The shell of the chitosome breaks and extended fibrils emerge. On further incubation, slender fibrils associate to form larger ones and finally the normal, mature, extended microfibril of chitin is observed. The resemblance of this process to that for bacterial cellulose is evident and it is probably not accidental.

The reason for the formation of Cellulose II, rather than Cellulose I, in the hydrolysis of the product of incubation of Glaser's enzyme particles with UDPG is presently under study.

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References

Atalla, R.H., Nagel, S.C. (1974) Cellulose: Its regeneration in the native lattice. Science **185**, 522–523

Bikales, N.M., Segal, L. (1971) Cellulose and cellulose derivatives, pts. IV and V, vol. V, High polymers, 2nd edn. Wiley-Interscience, New York

Bracker, C.E., Ruiz-Herrera, J., Bartnicki-Garcia, S. (1976) Structure and transformation of chitin synthetase particles (chitosomes) during microfibril synthesis in vitro. Proc. Natl. Acad. Sci. USA **73**, 4570–4574

Brown, R.M., Willison, J.H.M., Richardson, C.L. (1976) Cellulose biosynthesis in *Acetobacter xylinum*: Visualization of the site of synthesis and direct measurement of the *in vivo* process. Proc. Natl. Acad. Sci. USA **73**, 4565–4569

Buchanan, R.E., Gibbons, N.E., (1974) Bergey's Manual of determinative bacteriology, 8th edn. Williams & Wilkins, Baltimore

Colvin, J.R. (1972) The structure and biosynthesis of cellulose. Crit. Rev. Macromolec. Sci. **1**, 47–81

Colvin, J.R., Leppard, G.G. (1977) The biosynthesis of cellulose by *Acetobacter xylinum* and *Acetobacter acetigenus*. Can. J. Microbiol. **23**, 701–709

Colvin, J.R., Takai, M., Leppard, G.G. (1976) Granules within cellulose microfibrils. Naturwissenschaften **63**, 297

Cooper, D., Manley, R.S.J. (1975a) Cellulose synthesis by *Acetobacter xylinum*. I. Low molecular weight compounds present in the region of synthesis. Biochim. Biophys. Acta **381**, 78–96

Cooper, D., Manley, R.S.J. (1975b) Cellulose synthesis by *Acetobacter xylinum*. II. Investigation into the relation between cellulose synthesis and cell envelope components. Biochim. Biophys. Acta **381**, 97–108

Cooper, D., Manley, R.S.J. (1975c) Cellulose synthesis by *Acetobacter xylinum*. III. Matrix, primer, and lipid requirements and heat stability of the cellulose-forming enzymes. Biochim. Biophys. Acta **381**, 109–119

Delmer, D.P. (1977) The biosynthesis of cellulose and other plant cell wall polysaccharides. Rec. Adv. Phytochem. **11**, 45–77

Duran, A., Cabib, E. (1978) Solubilization and partial purification of yeast chitin synthetase. J. Biol. Chem. **253**, 4419–4425

Forge, A. (1977a) The synthesis of cellulose microfibrils by particulate fractions released from *Acetobacter xylinum* by washing. Ann. Bot. **41**, 447–454

Forge, A. (1977b) Electron microscopy of a non-pellicle-forming strain of *Acetobacter xylinum*. Ann. Bot. **41**, 455–460

Forge, A., Preston, R.D. (1977) An electron microscope examination of *Acetobacter xylinum* showing the ultrastructure of the cells and the association of cellulose microfibrils. Ann. Bot. **41**, 437–446

Giddings, T.H., Brown, D.L., Staehelin, L.A. (1980) Visualization of particle complexes in the plasma membrane of *Micrasterias dentriculata* associated with the formation of cellulose fibrils in primary and secondary cell walls. J. Cell Biol. **84**, 327–339

Glaser, L. (1958) The synthesis of cellulose in cell-free extracts of *Acetobacter xylinum*. J. Biol. Chem. **232**, 627–636

Grout, B.W.W. (1975) Cellulose microfibril deposition at the plasmalemma surface of regenerating tobacco mesophyll protoplasts: A *deep*-etch study. Planta **123**, 275–282

Hestrin, S., Schramm, M. (1954) Synthesis of cellulose by *Acetobacter xylinum*. 2. Preparation of freeze-dried cells capable of polymerizing glucose to cellulose. Biochem. J. **58**, 345–352

Hunter, R.E., Dweltz, N.E. (1979) Structure of Cellulose II. J. Appl. Polym. Sci. **23**, 249–259

Johnson, D.C., Nicholson, M.D., Haigh, F.C. (1976) Dimethylsulfoxide-paraformaldehyde: A non-degrading solvent for cellulose. Appl. Polym. Symp. **28**, 931–943

King, G.G.S., Colvin, J.R. (1976) Intermediate polymers of cellulose biosynthesis. Appl. Polym. Symp. **28**, 623–636

Kjosbakken, J., Colvin, J.R. (1975) New evidence for an intermediate polymer of glucose in cellulose biosynthesis by *Acetobacter xylinum*. Can. J. Microbiol. **21**, 111–120

Leppard, G.G., Sowden, L.C., Colvin, J.R. (1975) Nascent stage of cellulose biosynthesis. Science **189**, 1094–1095

Mueller, S.C., Brown, R.M. (1980) Evidence for an intramembrane component associated with a cellulose microfibril-sythesizing complex in higher plants. J. Cell Biol. **84**, 315–326

Petermann, J., Gluter, H. (1977) Electron microscopic observations on the crystallization of row structures in strained melts. Polym. Lett. Edn. **15**, 649–654

Preston, R.D. (1964) Structural and mechanical aspects of plant cell walls with particular reference to synthesis and growth. In: The formation of wood in forest trees, pp. 169–188, Zimmermann, M.H., ed. Academic Press, New York

Preston, R.D. (1974) The physical biology of plant cell walls. Chapman & Hall, London

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J. Cell Biol. **17**, 208–212

Robinson, G.D. (1977) Plant cell wall synthesis. Adv. Bot. Res. **5**, 89–151

Ruiz-Herrera, J., Lopez-Romero, E., Bartnicki-Garcia, S. (1977) Properties of chitin synthetase in isolated chitosomes from yeast cells of *Mucor rouxii*. J. Biol. Chem. **252**, 3338–3343

Watson, M.L. (1958) Staining of tissue sections for electron microscopy with heavy metals. J. Biophys. Biochem. Cytol. **4**, 475–478

Zaar, K. (1977) The biogenesis of cellulose by *Acetobacter xylinum*. Cytobiologie **16**, 1–15

Zaar, K. (1979) Visualization of pores (export sites) correlated with cellulose production in the envelope of the gram-negative bacterum *Acetobacter xylinum*. J. Cell Biol. **80**, 773–777

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