# SYNTHESIS OF CELLULOSE BY *ACETOBACTER XYLINUM*

## V. Ultrastructure of Polymer

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ABSTRACT

Appearance of cellulose microfibrils in the medium of a suspension of cells of *Acetobacter xylinum* in buffered glucose solution was preceded by a stage during which the cellulose in the medium was amorphous within the available resolution. The size of the vertical axis of the microfibrils of the bacterial cellulose was found on the basis of measurement of shadow length to be only about 16 A. In good agreement with findings of earlier workers, the size of the lateral axis ("width") of the image of the *metal-shadowed* cellulose microfibrils was found to be 11 mμ. After correcting for a large part probably contributed by deposited metal in the observed width of the microfibrils, the real width is estimated roughly to be in the neighborhood of 3 mμ. To account for the occurrence of diverse morphological elements in the fields and for the fact that the cellulose fibrils are free entities rather than physical appendages of the cell, it is suggested that individual cellulose molecules are released at the cell surface and diffuse into the medium, wherein they finally enter into crystalline patterns.

Cellulose fiber from many different sources consists of elementary threads ("microfibrils"). The *width* (lateral axis) of cellulose microfibrils in metal-shadowed preparations has been reported variously to be between 70 and 300 A (*e.g.* Frey-Wyssling, 2; Balashow and Preston, 3; *cf.* also *e.g.* Mühlethaler, 4; Rånby, 5, 6; Jayme and Koburg, 7; Gezelius, 8). Early investigators appear to have assumed that the image width corresponds closely to the real width of the cellulose microfibril. However, consideration should be given to the possibility that deposited metal may have represented a considerable portion of the observed width of the fibril images. Morehead (10) estimated the *thickness* (vertical axis) of green-plant cellulose micelles (produced by partial acid hydrolysis) on the basis of measurements of shadow length (*cf.* 9) to be in the range 30 to 40 A. Preston (41) concluded that the width of microfibrils of *Valonia* cellulose ranges from about 100 to about 350 A and that the thickness to width ratio of the microfibril lies somewhere between 1:3 and 1:7. In the present study, use has been made of shadow measurements to estimate the size of the vertical axis of the microfibril of native bacterial cellulose. In addition, with the aid of considerations based on analogy, an attempt has also been made roughly to estimate the probable real size of the lateral axis of this microfibril.

An exploratory investigation suggested that cellulose product formed in a buffered glucose solution by washed non-proliferating *Acetobacter xylinum* cells consists of threads within an "amorphous" phase (Hestrin and Schramm, 11), thus resembling the product formed in a complex medium by multiplying cells in an early stage of growth (Frey-Wyssling and Mühlethaler, 12; Mühlethaler, 4). A purified preparation of bacterial cellulose examined by Rånby (5) consisted of threads only. Colvin, Bayley, and Beer (13,

14) concluded that cellulosic product formed by the washed cells consists of threads which grow only in their long axis with time. Ultrastructure, probable dimensions, and the sequence of appearance of cellulosic elements in the extracellular phase of a washed suspension of *A. xylinum* cells are further described in the present contribution.

## MATERIALS AND METHODS

### *Apparatus and Miscellaneous Materials*

An RCA EMU 3B electron microscope was used at 50 and 100 kv. Collodion film was prepared from celloxylin (Siegfried Co., Zoffingen, Switzerland) dissolved at 0.3 per cent concentration in technical grade amylacetate. Partially dehydrated isotonic agar gel for "agar filtration" was prepared as described by Kellenberger and Arber (15).

### *Production of Cells*

Wild type *A. xylinum* (Schramm and Hestrin, 16) was inoculated into liquid medium (0.5 per cent yeast extract (Difco), 0.5 per cent Bactopeptone (Difco), 0.1 per cent $K_2HPO_4$ (B.D.H.), and 2 per cent glucose (Difco); pH 7) which had been cleared by filtration through an asbestos pad and sterilized by autoclaving. Fluid from the liquid phase of a 20 hour culture served as inoculum, added in the proportion 1:10. Mass cultures were in Roux flasks which were quiescently incubated at 30°C and harvested after 36 hours, cells being separated from pellicles and washed as described by Schramm, Gromet, and Hestrin (17). Cells were used either directly or after storage in a frozen state. They formed cellulose from glucose at a rate of 1.0 μmole anhydroglucose units per 10 mg dry weight of cells per hour in a standard assay system consisting of cells (1.0 mg dry weight/ml) and 0.01 M glucose in buffer pH 6.4 (0.02 M phosphate, 0.005 M citrate, $Na^+$) at 30°C under $O_2$ with shaking.

The cells were made absolutely dependent for cellulose synthesis on exogenous supply of substrate by a depletion treatment consisting of incubation in buffer at 30°C under $O_2$ for 2 hours with gentle shaking. Minute agglutination bodies appeared in the suspension during this time and were dispersed by forcing the suspension through a pipette and then passing it through a layer (5 cm thick) of glass wool to remove any formed fiber. Suspensions of the depleted cells remained stable on standing in the absence of glucose at 30°C during 1 hour under $O_2$. The depleted unbroken cells were sedimented from suspension by centrifuging at low speed (5000 *g* for 5 minutes), again washed, and finally resuspended in buffer.

Macroscopic agglutination bodies appeared in a suspension of depleted cells under the standard assay conditions after an interval which varied with cell batch and was not a simple function of the cellulose-synthesizing activity. Several preparations which gave prompt agglutination in glucose buffer (response time less than 5 minutes) were converted on contact with glucose and subsequent passage through several layers of gauze into cells which gave delayed agglutination (response time 8 to 30 minutes). Such a preparation was used for experiments in which an early stage in fiber deposition was resolved.

### *Preparation of Pellicles*

Pellicles were taken from the surface of suspensions of cells (0.9 mg dry weight/ml) in 0.01 M glucose in buffer pH 6.4 at room temperature. In short term experiments (10 minutes), the reaction vessel was a Pasteur pipette into which a copper grid (400 mesh) had been fitted. At the end of the incubation time, the sealed bottom neck of the pipette was opened and its contained fluid was drawn off through the grid insert by allowing the bottom orifice of the tube to form contact with a piece of filter paper. Pellicle retained on the grid was then washed in a slow stream of distilled water. In long term experiments (10 to 20 hours), the reaction vessel was a covered Petri dish, and the pellicle was washed by passage through several changes of distilled water. To remove alkali-soluble components, pellicles were held for 10 minutes in 1.0 N NaOH at 98°C and then rinsed again in water. The dried specimens were mounted on a copper grid (400 mesh), shadowed with platinum, and thinly coated with carbon.

### *Synthesis in a Vibration Field*

To obtain specimens of lower density than that encountered in pellicles, synthesis was allowed to occur in standard assay conditions but at a lowered cell concentration (80 μg dry weight/ml) in a vibrating test tube (50 cycles/second in a Mickle apparatus). Polymerization proceeded in the vibration field at the same rate as in standard assay conditions at the same cell concentration. Pellicle production, however, was efficiently suppressed by the vibration and the polymer was produced in a state of relatively high dispersion. Such material could be pipetted. On standing, it tended to aggregate into loose clumps, but these could be dispersed quite readily. Synthesis reaction was terminated by addition of cyanide to 0.01 M. The material was freed from alkali-soluble components, after washing in water, by exposure to 1.0 N alkali at room temperature for 14 days, and then rinsed in water. Specimens (before and after alkali treatment) were

transferred by spraying (atomizer, orifice 1.0 mm diameter, operated at 0.1 atmosphere $O_2$; droplet size *ca.* 0.5 mm) onto collodion film over a block of partially dehydrated agar gel, and freed from low molecular constituents by filtration and drying, as described by Kellenberger and Arber (15). The collodion film with its mounted specimen was separated from its agar support by flotation over distilled water, then transferred to a copper grid (200 or 400 mesh), dried for 8 hours over calcium chloride, and finally shadowed with platinum.

The use of the vibration field as described presents manipulative advantages, but suffers from the disadvantage that with this technique the extracellular phase of the specimens tends to become contaminated by cellular debris.

## *Synthesis in a Quiescent Droplet*

The elegant technique described by Colvin Bayley, and Beer (13) was used with a minor modification to obtain specimens whose density as to cellulose was low as desired, in which contamination of the extracellular phase by cellular debris was largely avoided, and in which spatial relationships prevailing during synthesis were still also present in the finally prepared specimen. For short term kinetic experiments (less than 10 minutes), suspensions of the cells (80 μg dry weight/ml) in buffer were reduced to droplet form by spraying onto a collodion film maintained on an agar support. The agar and its adherent film which supported the droplets was then promptly sliced to provide a square (2 × 2 cm) from which the film was separated by flotation over a solution of 0.01 M glucose in buffer at 25°C. After a desired incubation time, the film and its mount were transferred on a rigid net (copper, mesh 40, 3 × 3 cm) to the surface of a formalin-water mixture (1:1, *v/v*). Synthesis was thereby terminated promptly. It may be noted here that formalin stops the cellulose synthesis within less time than does cyanide and thus its use facilitates the observation of the condition which prevails at zero time of the contact with glucose. After 20 minutes over formalin, the film was freed from solutes by flotation over distilled water, then transferred to a copper grid and further processed as described above (see under Synthesis in a Vibration Field).

Experiments in which an incubation time of less than 10 minutes was used were performed in droplets maintained during incubation in a closed Petri dish on a collodion film resting on the surface of a rectangular piece of partially dehydrated agar gel. The composition and concentration of the latter in respect of solutes was the same as that of the overlying droplets. To stop the synthesis, the system was exposed to cyanide vapor. The film was then allowed to remain on the agar block until water and all the low molecular constituents of the droplet had been resorbed into the agar (*cf.* Kellenberger and Arber, 15). Subsequent processing steps were conducted as described in the preceding section (see under Synthesis in a Vibration Field).

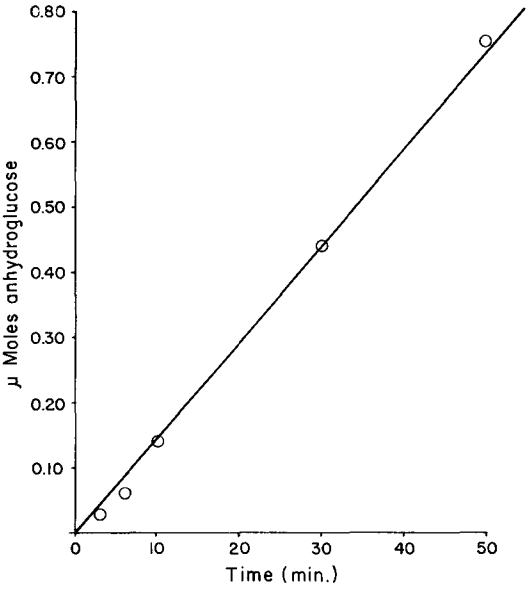



FIGURE 1

Synthesis of cellulose from $C^{14}$-glucose as a function of time. "Delayed agglutination" type cells were used (see Materials and Methods). The reaction mixture consisting of cells (0.3 mg dry weight/ml) and $6 \times 10^{-3}$ M $C^{14}$-glucose (specific activity, 8000 CPM/μmole) in buffer pH 6 was incubated at 30°C under $O_2$. Cellulose was estimated as described under Materials and Methods. Abscissa, time (minutes); ordinate, cellulose (μmoles anhydroglucose/10 mg dry weight cells).

## *Quantitative Estimation of Cellulose*

To estimate the amount of cellulose formed, $C^{14}$-glucose was used as substrate in the standard assay system. The reaction was terminated by 0.01 M cyanide. The polymeric insoluble product was centrifuged at 8000 *g* for 5 minutes in the presence of a sample of dispersed *A. xylinum* cellulose added as carrier (1 mg/ml). The sediment was washed in water until all soluble $C^{14}$ had been removed, then boiled for 10 minutes in 1.0 N NaOH, and finally again washed in water and dried. $C^{14}$ content was measured in a gas-flow counter (Nuclear Chicago Corp., Chicago).

### *Shadowing Technique*

The thickness of the metallic layer deposited during shadowing was kept as constant as possible with the help of a device which will be described by Danon elsewhere. For most measurements of lengths of shadows cast by fine fibers (vertical axis less than 5 mμ), latex spheres (diameter 340 mμ, supplied by Dow Chemical Co.) were sprayed onto the films together with the fiber sample to serve as internal indicators of the "height to shadow" ratio and the shadowing direction (18). Working conditions were as specified by Hall (19), and the height to shadow ratio was held within 1:5 to 1:10.

### *Measurement of Fiber Dimensions*

Lengths of shadow and diameters of images on photographic films (magnification 10,000 to 25,000) were measured with the aid of a binocular microscope (× 25) fitted with a 0.02 mm scale. Estimates of dimensions of an image in the size neighborhood of 1 mμ by this procedure were found to be reproducible within ±30 per cent.

Thickness (vertical axis) of fiber was calculated from the length of shadow cast in a direction perpendicular to the long axis of the fiber, as proposed by Hall (9).

Real width (lateral axis) of fiber was estimated

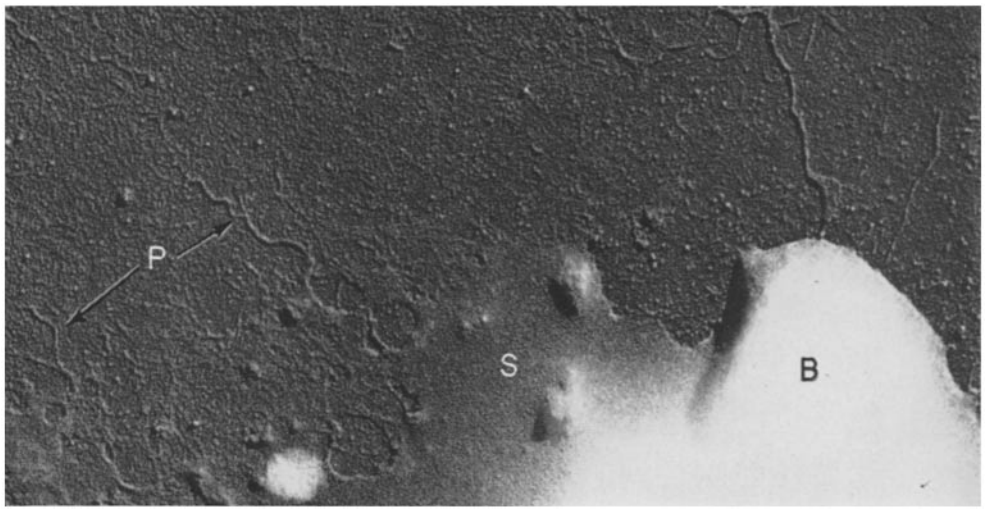



FIGURE 2

Polymer near a cell at an early reaction time.

Procedure of Colvin *et al.* (13). (See section Synthesis in a Quiescent Droplet under Materials and Methods.) Concentration of cells in droplet, 80 μg dry weight/ml. Reaction time, 2 minutes. Platinum shadow; height to shadow ratio, 1:5. × 50,000.

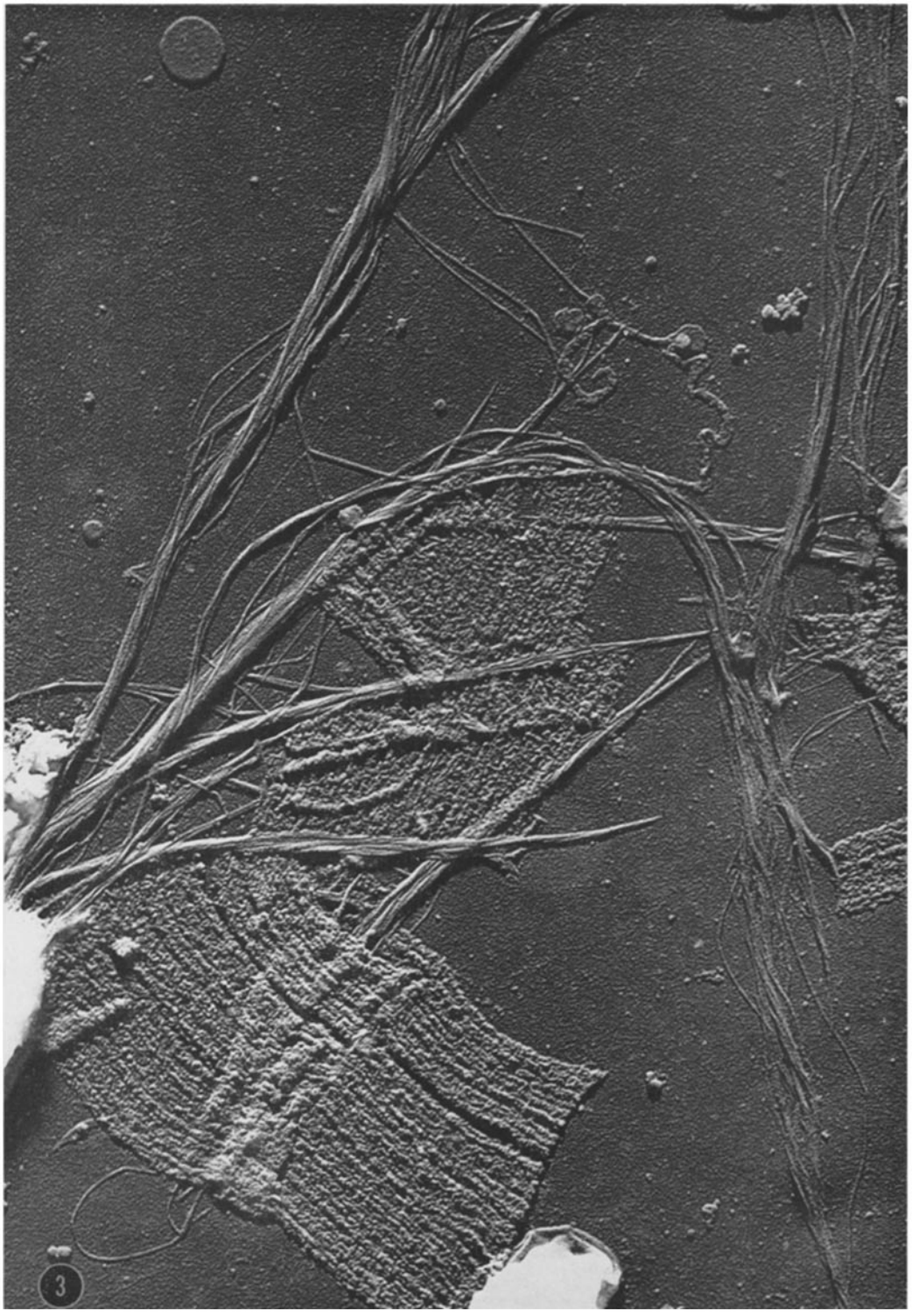
Filamentous branched processes (*P*) extend from a slime zone (*S*) around bacteria (*B*). Compare with picture taken at zero time (Fig. 12).

FIGURE 3

Morphological elements in extracellular polymer at a late reaction time.

For methods, see section Synthesis in a Quiescent Droplet under Materials and Methods. Droplet with 0.02 M glucose and cells (60 μg dry weight/ml) was incubated at 30°C. Reaction time, 2 hours. Platinum shadow; height to shadow ratio, 1:5. × 25,000.

The fibers (ropes, cables, and ribbons) are composite and consist of intertwisted microfibrils. Fiber ends are tapered or frayed. Microfibrils are also in isolated dispositions. Mats partly cover some of the composite fibers. Slime zones are not found at this reaction time. The morphology of the product obtained by synthesis conducted in a vibration field was similar.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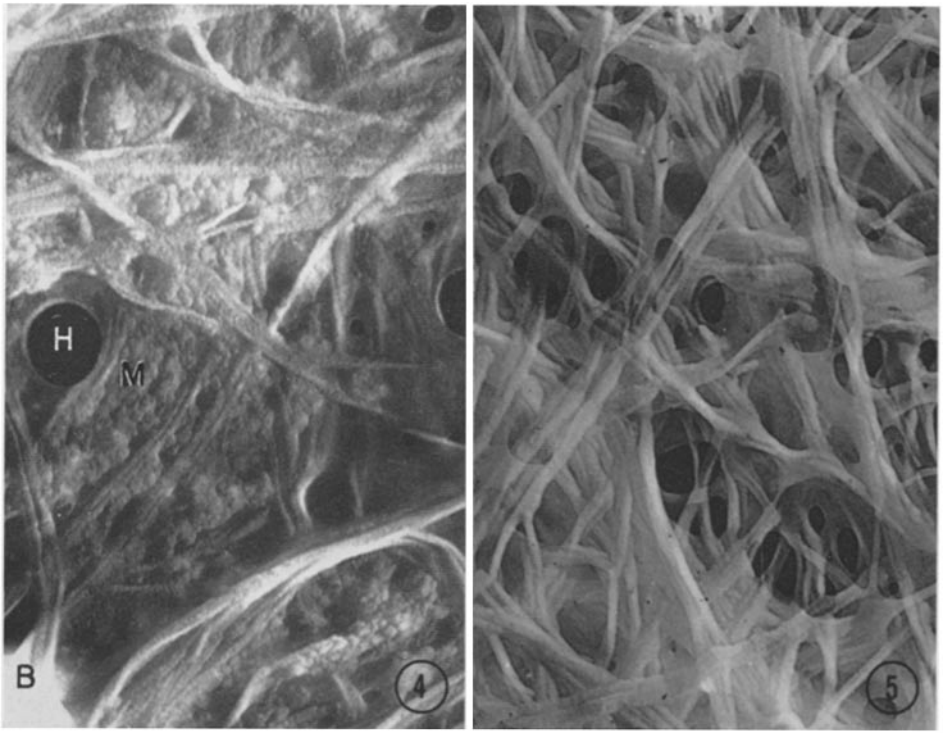


FIGURE 4

Cellulose pellicles before alkali treatment.

Procedure as under Materials and Methods. Reaction time, 20 hours. × 70,000.

Twisted composite fibers are seen within a granular matrix (*M*). An opaque cell border (*B*) is at the bottom left; a large circular hole (*H*) in the pellicle is seen at middle left. The product presented essentially the same morphology as at 10 minutes.

FIGURE 5

Cellulose pellicles after alkali treatment. Conditions as in Fig. 4. × 70,000.

within a rough order of magnitude as $z = y - x$, *i.e.* by deducting from the total measured width ($y$) of the fiber image a value ($x$) which was expected to be roughly equal to the portion of the added metal in the lateral axis of the image. All measurements of $y$ were made on fibrils whose long axis was perpendicular to the direction of shadowing. A tentative value for $x$ was derived from findings on tobacco mosaic virus (TMV) rods. The width of the latter is known to be 150 A (40). For the standard working conditions used in the course of the experiments here reported, the value of $x$ in images of TMV rods was found to be 80 ± 10 A where the direction of the shadowing was perpendicular to the long axis of the rod (experiments to be published in full elsewhere; *cf.* also 19, 39). Since surfaces of the cellulose microfibril are probably essentially flat (41), the application to this case of a value of $x$ that has been observed in a larger object of circular cross-section, such as the TMV particle, is problematic. However, Hall observed that when metal was deposited on a TMV rod from the direction of the latter's long axis, the depth of metal cap added to the flat end of the rod was about 90 A (19). In this connection it is pertinent, furthermore, to record that, even if the cross-section of the cellulose microfibril is actually assumed to be circular, only a slight adjustment in the value assigned to $x$ (*i.e.* allowing $x$ to be 90 rather than 80 A) would afford for $z$ a value similar to that which would be given by a calculation on the basis

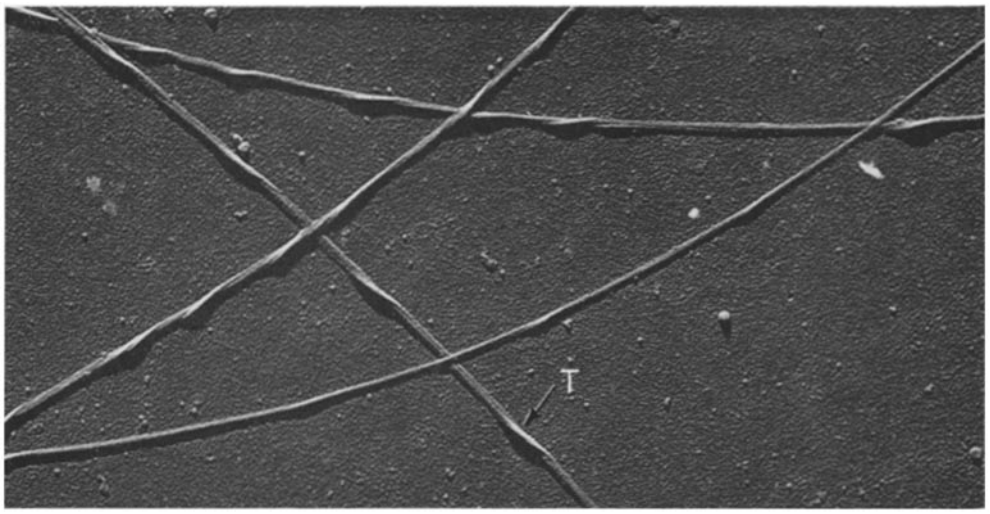


FIGURE 6

Twisting in composite fibers.
Procedure as in Fig. 3. × 25,000.
Broadening of the fiber image at points of twisting (*T*) suggests that these structures are ribbons.

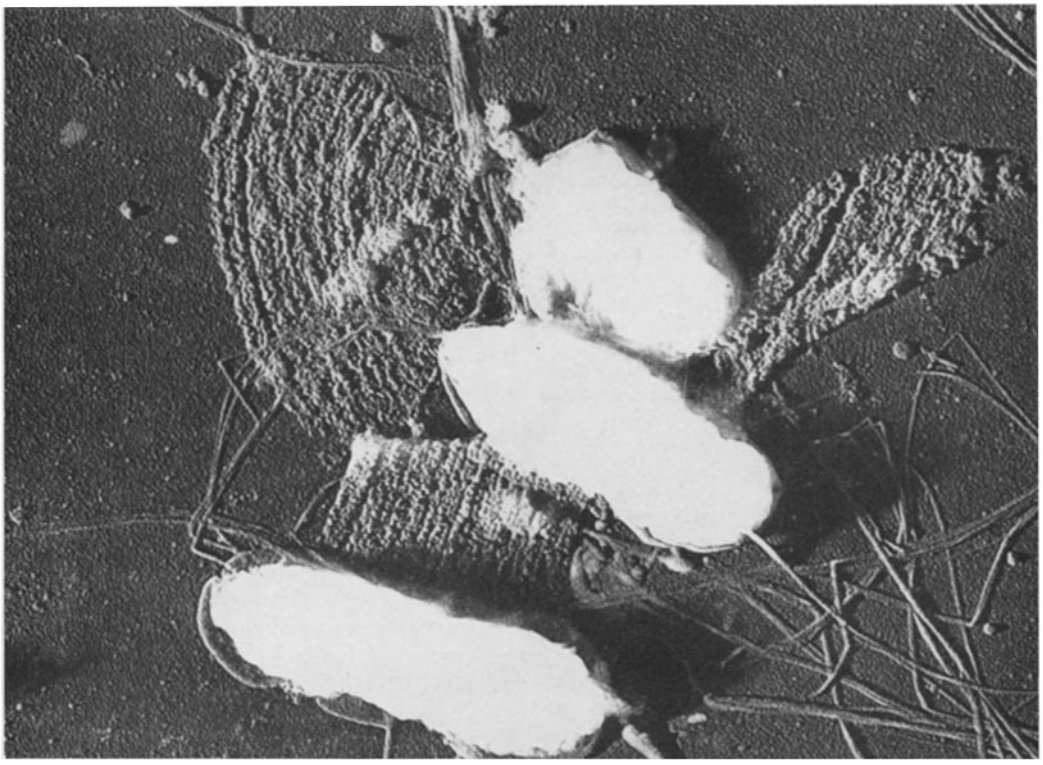

FIGURE 7

Mats and composite fibers at late reaction time. Procedure as in Fig. 3. × 25,000.

of the observed shadow length. The use of the proposed correction also involves the assumption that $x$ remains essentially constant at values of $y$ between 100 and 300 A. Nevertheless, it seems reasonable to suppose that the use of this correction yields a value which is closer to the truth than is an uncorrected raw measurement of the total width.

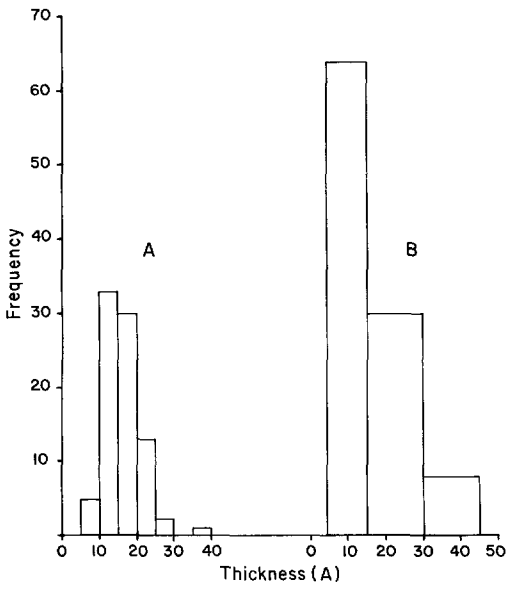


FIGURE 8

Histograms of the thickness of randomly selected samples of microfibrils and rods. A, microfibrils from Fig. 3 and four other electron micrographs. B, rods in early product from Fig. 10. For methods see section Measurement of Fiber Dimensions under Materials and Methods. Abscissa, thickness (A); ordinate, frequency (number of the measurements whose value fell in the specified thickness class). Dimensions of thickness other than those recorded in the figure were not encountered in the sample.

TABLE I

*Average Dimensions (A) and Standard Deviations of Microfibrils and Rods*

Calculations relate to data of Figs. 8 and 9.

| Dimension | Average value* | S.D.‡ |
|---|---|---|
| Width of microfibril image | 108 | 27 |
| Thickness of microfibril | 16 | 5 |
| Thickness of rods in early product | 15 | 8 |

* $\sum Xifi/\sum fi$. $Xi$, class average; $fi$, class frequency.

‡ $\sqrt{\sum d^2/\sum fi}$. $d$, deviation from average value.

## *Light Microscopy*

Droplets consisting of cells (100 to 200 $\mu$g dry weight/ml) and 0.01 M glucose in buffer were incubated at room temperature on glass slides. The optical equipment consisted of a Reichert phase contrast microscope.

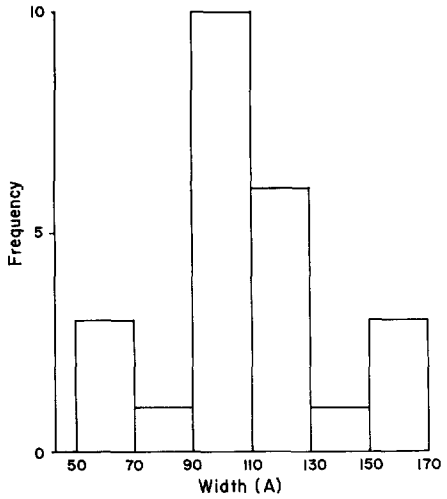


FIGURE 9

Histogram of average width of microfibril images. Widths of images of a total of 24 microfibrils were measured at five points (500 to 1000 A apart) in each microfibril. Five micrographs were used. For methods see section Measurement of Fiber Dimensions under Materials and Methods. Abscissa, image width (A); ordinate, frequency (number of measurements whose value fell in the specified width class). The variation of the image width along the microfibrils was found to be within 3 to 11 per cent (S.D. of the average image width). Dimensions of image width other than those recorded in the figure were not encountered in the sample.

## RESULTS

### *Identification and General Morphology of Extracellular Polymer*

The apparent amount of the polymer seen in the extracellular phase of cells suspended in buffered glucose solution increased with time. The reaction was absolutely dependent on the presence of glucose in the synthesis system and proceeded at a constant rate (Fig. 1).

The product found at relatively short incubation times (less than 6 minutes) consisted largely either of fine rods and granules or of a system of branched processes extending from amorphous zones (Fig.

2). *Composite fibers* became predominant in the fields at all times after 10 minutes. They were in the form of threads and ribbons with sharply defined boundaries, and consisted of a filamentous subelement, the *microfibril*. The latter also occurred in the fields not infrequently in isolated disposition, often with at least one end visibly free in the medium and unconnected to any cell. In addition, material the organization of which resembled that of a "mat," was distinguished. All the main morphological elements are represented in Fig. 3.

It has been shown that the extracellular product formed from glucose by these washed cells is a typical native cellulose (Hestrin and Schramm, 11). In agreement with this result, both mat and fiber seen in the extracellular phase of the droplets were found to be insoluble in hot aqueous alkali. Alkali treatment of cells at zero time afforded preparations which were fiber free but did comprise granular material sometimes in patterns resembling the mat. Therefore it should not be concluded that *all* material in mats was cellulose formed in the course of an experiment.

In both young and old pellicles, composite fibers appeared to be lodged within an encompassing "amorphous" matrix (Fig. 4). The matrix had a granular texture, and the morphology in this respect was reminiscent of that of the mat. Alkali treatment removed cells from the pellicle and caused swelling of the cellulosic elements, but still left both fiber and matrix in a resolvable state (Fig. 5). The apparent sparsity or absence of amorphous matrix in the droplet material raises the possibility that at relatively high concentration of cells as in the pellicle the conditions favor accumulation of product in an "amorphous phase," whereas conditions in the droplet facilitate organization of product as fiber.

## *Morphology of Individual Structural Elements*

Composite fibers ranged widely in width (image width 20 to 300 m$\mu$) and were more than 3 $\mu$ long (Figs. 3 and 7). Twisting occurred at intervals (1 $\mu \pm 0.3$) in the long axis (Fig. 6). Ends frequently showed fraying or tapering. Ropes, consisting of interwoven composite fibers, constituted a major portion of the fibrous material.

Microfibrils were readily discernible both in mid-regions and frayed ends of composite fibers (Figs. 3 and 7). It was possible to resolve from three to as many as fourteen microfibrils within an individual composite fiber. They presented parallel lateral arrangements within ribbons and showed intertwining in cables. The thickness of the microfibrils was found to be fairly uniform, 16 A (S.D. 5) (Fig. 8; Table I). The *image* width (*i.e.* width of the sum of filament plus metal) was 108 A (S.D. 27) (Fig. 9; Table I). Fluctuations of thickness and width within any microfibril were small. Within the limits of the available resolution, the typical microfibril ran in an essentially straight unbranched line. Its length ranged widely (1 to 3 $\mu$). Continuous stretches of a microfibril *within* a composite fiber were sometimes at least 2.5 $\mu$ long (Fig. 3), *i.e.* a value which corresponds to 5000 anhydroglucose units and is about twice the length of an average molecule of bacterial cellulose (Rånby, 5).

Mats consisted of granules and rods in parallel linear arrays. The width of the granule images was about 30 m$\mu$. Edges of the mat were straight and sharp, and often bounded by a composite fiber or by a cell wall (Figs. 3 and 7). The possibility can be considered that the mats consist of a framework of microfibrils upon which granular material has been deposited, and that they thus have a structure which corresponds to that of the pellicle.

At short reaction time, the product of polymerization comprised contorted and frequently branched processes extending radially from the periphery of slime zones which were generally in the vicinity of a cell (Fig. 2). In addition, granular elements (spheres, ellipsoids, and short rods) were seen at such times in intercellular regions (Fig. 10). The thickness of the short rods was 15 A (S.D. 8) (Fig. 8; Table I).

Polymeric product from a fairly early stage in synthesis (reaction time, 10 minutes) passed in part through a collodion membrane of pore size 0.45 $\mu$, though it did not diffuse through the collodion support of the electron microscopic specimen (Fig. 11). The filtrable fraction contained material which underwent swelling but was not totally dissolved after 10 minutes in hot 1.0 N sodium hydroxide.

## *Increase in Dimensions of Morphological Elements as a Function of Time*

The composite fibers were mostly more than 20 $\mu$ long at a reaction time of 10 minutes, but less than 10 $\mu$ long at 2 minutes. The observed

increase in length could be ascribed entirely or in part to intertwining between composite elements (Figs. 12 to 15). Microfibrils revealed no apparent tendency to grow in thickness or width over the incubation period examined. Whether their average length increased as a function of time within the intervals investigated could not be determined.

### *Light Microscopy*

Bacteria suspended in buffered glucose solution became immobilized in an invisible zoogloeal phase within an incubation time of $\leq 5$ minutes. Fibrillar elements were not discernible in moist zoogloea by ordinary light microscopy.

## DISCUSSION

On the basis of the electron microscopic evidence, it can be suggested that the formation of typical cellulose fiber in a suspension of cells of *A. xylinum* is preceded by an interval during which the state of the extracellular molecules of the polymer is relatively amorphous (*cf.* also Mühlethaler, 4). It should be noted that such recognition of stages in the process of fiber formation is meaningful only in the morphological manifestation. Chemically, the course of polymer release from the cells remained uniformly linear in time over the entire interval of these observations.

Cellulose could be seen at a distance of *ca.* 5 $\mu$ from the nearest cell after a reaction interval of 2 minutes. The average distance which a randomly coiled individual cellulose molecule is likely to traverse by diffusion in water at 20°C may be calculated on the basis of equation (1):

$$\bar{\sigma} = \sqrt{2Dt} \qquad (1)$$

where $\bar{\sigma}$ is the average distance in centimeters, $t$ is time in seconds, and $D$ is the diffusion coefficient (20). For a molecular weight of about $0.5 \times 10^6$ (Rånby, 5), a value of $10^{-7}$ cm$^2$/sec. can reasonably be assumed for $D$.[1] On this basis, one obtains for $\bar{\sigma}$ a value of 4 $\mu$/sec. Thus the observed occurrence of polymeric product at a distance from a cell can be comprehended in terms of thermal diffusion of the molecules.

Solvated individual cellulose molecules in water

[1] $D$ for cellulose of $0.3 \times 10^6$ mol. wt. in a dilute aqueous cupriethylenediamine solution of low concentration (0.08 M) has been found to be $10^{-7}$. The intrinsic viscosity of this preparation was 10.3 (Vink, 21).

FIGURE 10

Short rods and granules in the extracellular phase of a cell suspension.
Procedure as in Fig. 3. Reaction time 10 minutes. × 70,000.

FIGURE 11

Filtrable fraction of polymer formed from glucose.

Reaction mixture contained a relatively high cell concentration (2 mg dry weight/ml). The suspension was partially cleared by centrifuging at 5000 *g* for 5 minutes and passed through a collodion membrane (millipore filter, 0.45 $\mu$ pore diameter, Millipore Filter Corp., Bedford, Massachusetts) which had been mounted directly on a stainless steel grid. A specimen of the filtrate was sprayed onto collodion film over isotonic agar and purified by agar-gel filtration according to Kellenberger and Arber (15), as described under Materials and Methods. The film with its mount was placed on a copper grid (200 mesh) and shadowed with platinum; height to shadow ratio, 1:6. × 70,000.

FIGURE 12

Cells in glucose solution at zero time.
Procedure as in Fig. 2. × 8000.

Bacteria hurled upon the collodion film by a spray gun operated at excessive pressure may have caused the folding seen in the film at the points of impact. The extracellular phase is practically devoid of resolvable structures.


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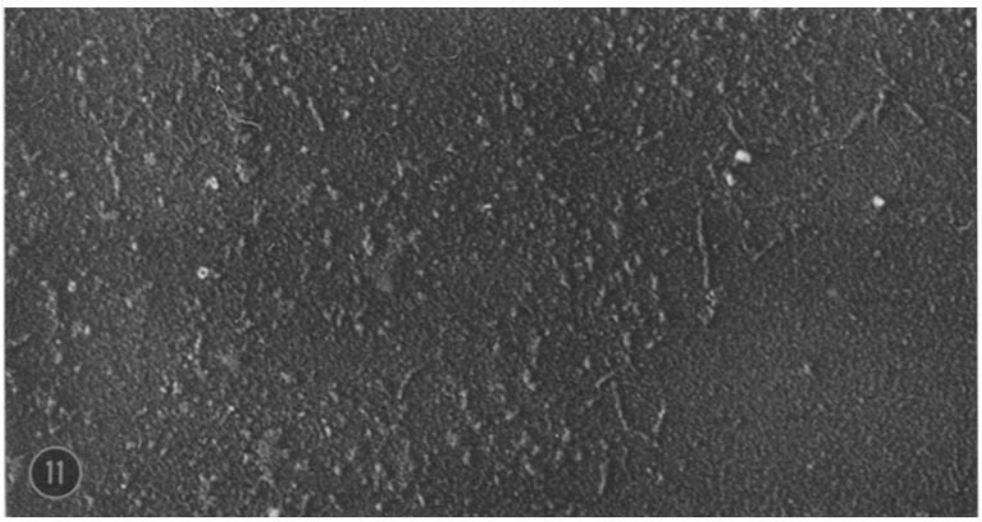
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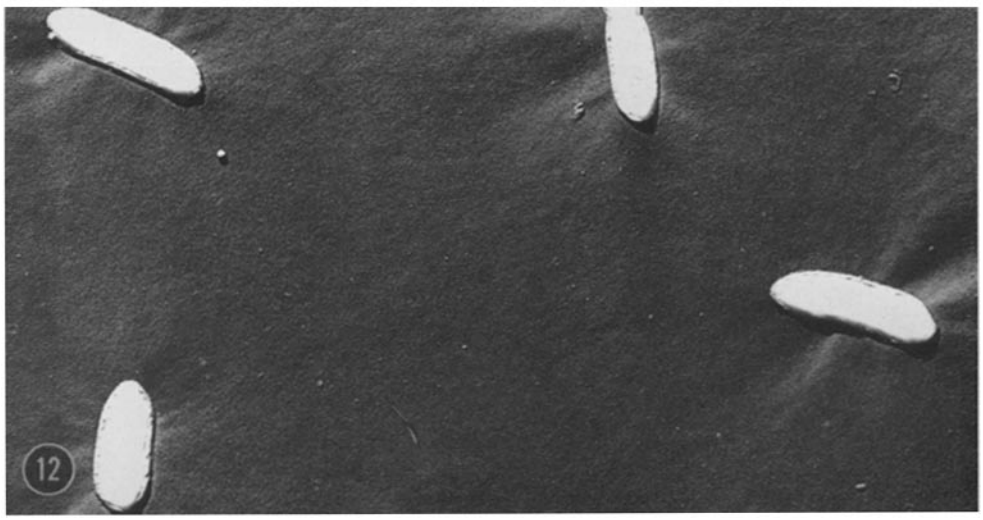
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would be expected to be prone to assume a random coil configuration. According to Benoit (22), mean square distance ($\bar{R}^2$) between the ends of a randomly coiled cellulose chain is equal to 114 $p$ (in A), where $p$ is the polymerization degree. The average radius ($\bar{S}$) of the particle conforms to equation (2) (23):

$$\sqrt{\bar{S}^2} = \sqrt{\bar{R}^2/6} \qquad (2)$$

For the bacterial cellulose molecule with average polymerization degree 2600 (Rånby, 5), one obtains for $\bar{S}$ from the above formulation the value 220 A. Particles of such a magnitude can be expected to be readily resolved by electron microscopy. It is noteworthy that the images of the smallest cellulosic granules which are resolved in our shadow-cast preparations of *A. xylinum* presented radii in the range of 100 A. Allowing 35 A in the radius for added metal, as estimated by Hall (19), the true radii of these cellulosic particles can be inferred to have been about 65 A. If such granules were indeed individual molecules, the polymerization degree of the latter could have been about 220.

It has been shown that the cellulose elements are not a physical appendage of the bacterial cell (present results; *cf.* also Mühlethaler, 4; Colvin, Bayley, and Beer, 13, 14), but rather occur in free forms that are scattered within the medium. This would indeed be their expected condition under the assumption that the morphologically resolvable structure arises by the crystallization of a diffusible entity.

The size of the vertical axis ("thickness") of the microfibril of bacterial cellulose is shown by our measurements of shadow length to be 16 A (standard deviation, 5).

The size of the lateral axis ("width") of the images of the microfibril was found by us to be about 108 A (standard deviation, 27). This value is in agreement with that found by earlier workers. If it is allowed that metal represented 80 ± 10 A in the observed total width of the images (see under Materials and Methods), it can be deduced that the real width of the cellulose microfibril was probably in the neighborhood of 3 mμ (108 (±27) A − 80 (±10) A = 28; standard error, 25 A). Further, it should be noted in this context that "crystallites" in cellulose fiber have been estimated on the basis of x-ray diffraction (24), viscosity behavior, and water-sorbtion properties to be variously from 2 to 7 mμ wide (*cf.* Howsmon and Sisson, 25). Moreover, Mühlethaler (43) has recently found with the use of a negative staining technique that the elementary fibrils of a green-plant cellulose are uniformly 3.5 mμ wide.

The area of the cross-section of a cellulose microfibril may be estimated roughly on the basis of the present electron micrograph measurements to be probably in the neighborhood of 4 or 5 mμ$^2$ (28 A × 16 A = 448 A$^2$). Such a cross-section could accommodate about 8 crystallographic unit cells of cellulose containing each the equivalent of two anhydroglucose rings.[2] If the rings in

[2] This computation is based on the assumption that the area occupied in the cross-section by a cellulose unit cell is sin 84° × 7.9 A ($c'$ axis) by sin 84° × 8.35 A ($a'$ axis) = 64 A$^2$ (Van der Wyk and Meyer, 26).

FIGURE 13

Polymer in extracellular field at early reaction time.

Procedure as in Fig. 2. × 8000.

Short composite fibers ($F$) and isolated microfibrils ($M$) are seen in this field, which also contains several bacteria ($B$) and a bacterial ghost ($BG$).

FIGURE 14

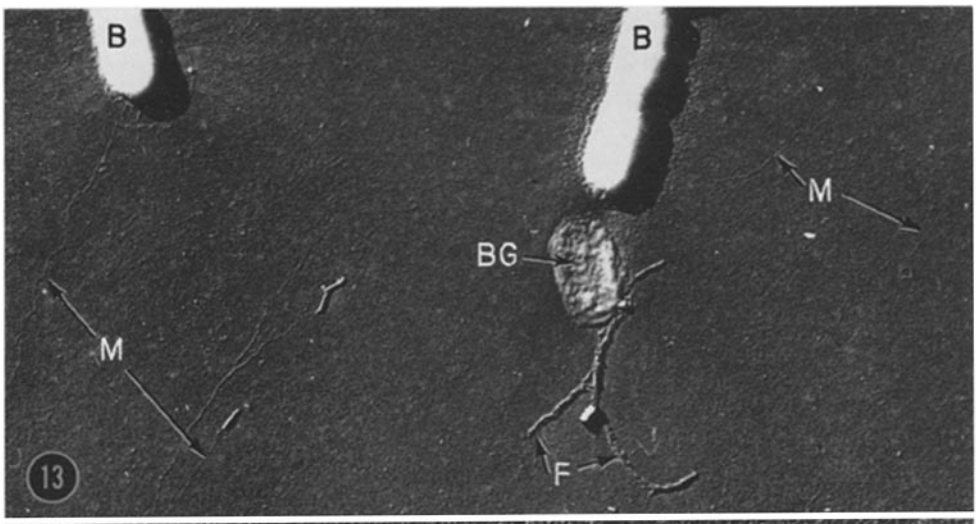
Fine filamentous processes extending from a region of granules in product at an early reaction time.

Procedure as in Fig. 3. Reaction time 3 minutes. × 70,000.

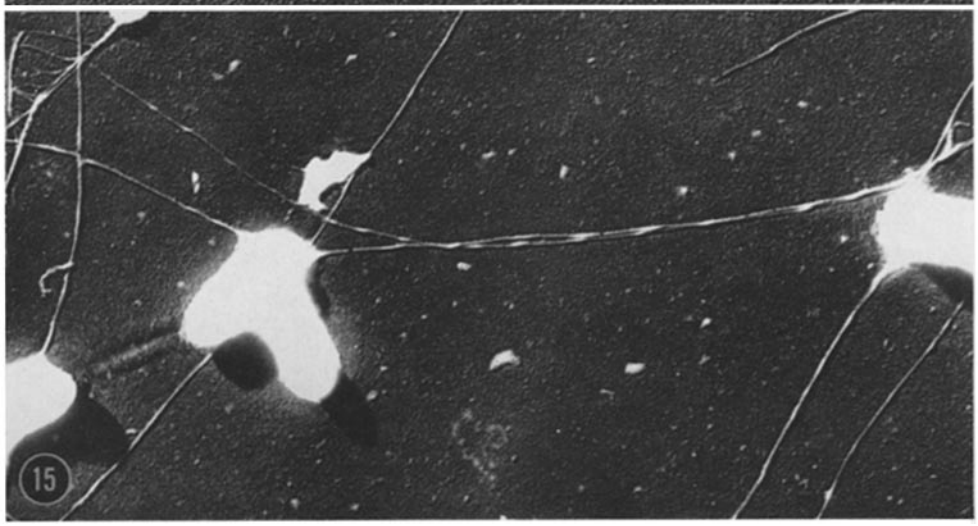
FIGURE 15

Intertwisted composite fibers in extracellular phase at intermediate reaction time.

Procedure as in Fig. 2. Reaction time 10 minutes. × 8000.


B
B
M
BG
M
F
13


14


15

the microfibril stand on their edge in a disposition parallel to the vertical axis, the structure can be regarded as probably bimolecular in height (Fig. 16, B). If the glucose rings in the microfibril are taken as piled face to face in the vertical axis, a structure is obtained which is tetramolecular both in width and in thickness (Fig. 16, A).

Cellulose-synthesizing activity of a cell in the conditions used has been calculated to be about $1.2 \times 10^6$ anhydroglucose units per minute (*cf.* also Hestrin and Schramm, 11), equivalent to about $5 \times 10^2$ molecules of cellulose (each 2600 glucose residues long). This mass of cellulose could constitute a total of 31 microfibrils (16 cellulose molecules in each cross-section), which would be expected to be grouped in a total of about 6 composite fibers each 1.3 $\mu$ long (length of a cellulose molecule consisting of 2600 glucose residues). At this incidence rate, if composite fibers or individual microfibrils grow out directly from the bacterial surface, it would surely have been easy to find them at the cell surfaces or in their periphery after one or two minutes of reaction with glucose. Yet at this early stage of the reaction the fibers were very rarely seen. Rather, fields abounded at this time only in amorphous aggregations of polymer. The assumption that a polymolecular fiber in this system arises by crystallization from a polymeric extracellular phase is thus further supported by these observations.

Longitudinal growth of a microfibril could conceivably involve (*a*) successive physical additions of diffusing finished polymer molecules to growing ends of the microfibril, or/and (*b*) a chemical interaction in which a diffusible "monomeric" precursor becomes incorporated into ends of growing polymer molecules within the microfibril. Colvin, Bayley, and Beer (13, 14) have interpreted elongation of fibers (estimated width range, 200 to 300 A) on the basis of (*b*). However, their fibers show twisting and could have been composites which comprised several microfibrils. That mechanism (*a*) is operative during microfibril growth is hardly in doubt in the light of present findings. Whether, in addition, mechanism (*b*) is also operative in the extracellular medium remains undecided. It has been indicated on the basis of x-ray diffraction analysis that molecular chains in a cellulose fibril are probably in an antiparallel arrangement (31). It can readily be visualized how such an arrangement might arise where the molecules crystallize from solution. It would be much more difficult, however, to account for this arrangement if the fibril is assumed to elongate by chemical addition of monomer to the proximal tip of a bundle of polymer chains growing out from the bacterial surface.

The question arises whether cellulose fibrils occurring in specimens from different species of organisms or from an individual organism at different stages of growth have a constant dimension. To clarify this aspect, the thickness of fibrils in different celluloses needs to be examined with adequate techniques based on measurements of shadow length.

Forces which determine ordered patterns of fibril deposition in cellulose walls (Roelofsen and Houwink, 27; Steward and Mühlethaler, 28; Wardrop, 29; Setterfield and Bayley, 30; Preston, 31; Frey-Wyssling, 32; Green, 33, 34) and chitin (Aronson and Preston, 35) have been a subject of much discussion. The explanations which have been advanced for growth in wall thickness involve two general alternatives: (*a*) the catalyst of polymer synthesis has a mobile locus which leaves a relatively static trail of polymer in its wake, or (*b*) the catalyst of the synthesis has a fixed locus, and mobility characterizes either molecules or crystals of the formed polymer. Beer and Setterfield (36), Setterfield and Bayley (37), and Bayley *et al.* (38) have adduced evidence which suggests that wall growth in celery collenchyma cells and *Avena* coleoptiles involves the penetration of an existing cellulose wall by a diffusible "precursor" of cellulose. The results of the present study suggest that bacterial cellulose is an example of a system in which the production of the cellulose fiber is mediated by a diffusible cellulose form.

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Experiments presented in this communication are a part of a Ph.D. thesis being submitted by I. Ohad to the Hebrew University, Jerusalem. A preliminary discussion of this work was included in a recent symposium lecture (Hestrin, 1).

For Part IV of this work see Gromet, Schramm, and Hestrin (42).

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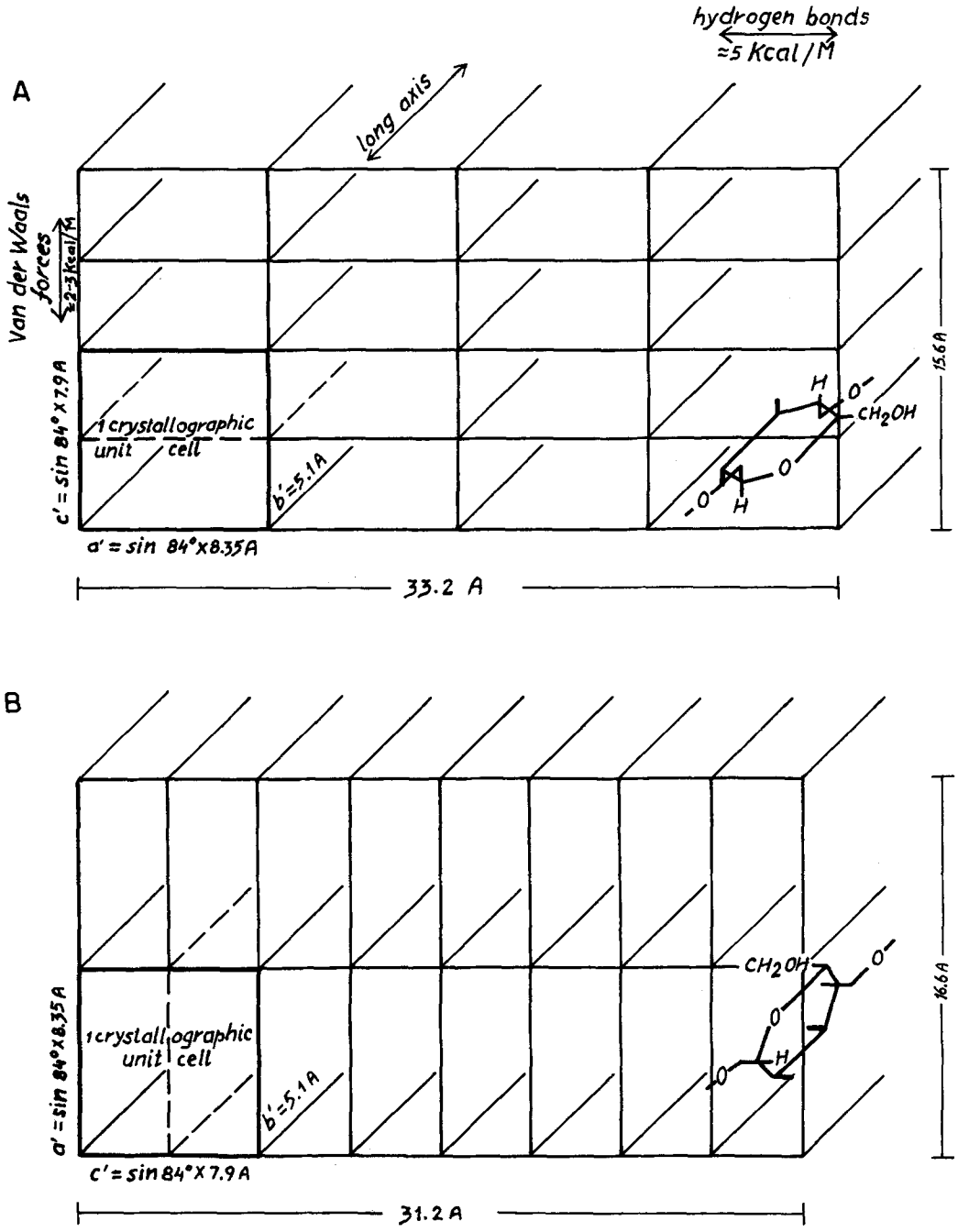


FIGURE 16

Alternative schemes of the probable arrangement of cellulose molecules within a microfibril. The microfibril depicted has a cross-section of 16 × 31 A and comprises 8 crystallographic unit cells of cellulose. A, glucose rings piled face to face in a tetramolecular layer. B, glucose rings standing on their edges in a bimolecular layer.

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