北海道大学工学部研究報告
第91号 (昭和53年)

Bulletin of the Faculty of Engineering,
Hokkaido University, No. 91(1978)

Nascent Stage of Cellulose Microfibril in Bacterial Cellulose Biosynthesis

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(Received July 8, 1978)

Abstract

The morphological aspects of biosynthesis of cellulose by *Acetobacter xylinum* were studied by transmission electron microscopy on both freez-etched replicas and sections of cellulose-free cells in suspension culture before and subsequent to the induction of cellulose synthesis. Also examined were freshly synthesized, thoroughly washed, cellulose pellicles. Thin sections of rapidly dividing, glucose-metabolizing cells showed irregular features on the cell surface including small polar invagination which sometimes was contained or was associated with fibrils as fine as 30 Å in diameter of a substance which stains with ellectronmicroscopic negative-stains. Cellulose microfibrils in thin sections of freshly synthesized pellicles were coated with a surface material which also stained with the same negative-stains. The effect of air-drying on freshly synthesized cellulose was striking. When examined by freeze-etching, and thoroughly washed, never air-dried pellicles showed a nascent form of cellulose fibril which consisted of a central, dense core surrounded by a sheath of amorphous gel. This sheath may be up to 1000 Å in width. When the pellicle was air-dried and rehydrated before freeze-etching, the amorphous sheath was scarce and shrunken but ordinary microfibrils of usual dimentions were visible. The sheath and core are sometimes closely associated with the envelope of the cells. These observations can be interpreted in the cortex of recent advances in cellulose biosynthesis by assuming that chains of an initial, highly hydrated, intermediate polyglucosan are released from the cell and that such chains associate to form a nascent fibril external to the cell that are associated with the cell evelope. Air-drying of nascent fibrils converts them to familiar microfibrils and this conversinon is considered here in molecular terms.

1. Introduction

Cellulose is the most abundant biological polymer but its molecular structure has

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only been deduced recently[1,2], the chemical pathway of biosynthesis is just beginning to be understood, and almost nothing is known about the physics of formation of the basic morphological unit, the micro-fibril. Research, a decade ago, established that the primary, biochemical precursor for cellulose biosyntheis was nucleotides containing glucose.[3] About the same time, a soluble, transient polymer of glucose was postulated as an intermediate in the formation of cellulose,[4-6] followed by association and crystalliization of these chains to form the microfibril. There were serious conceptual and observational objections to this scheme and not enough early experimental evidence to support it conclusively. Difficulties of solubility, orientation and linkage of postulated polyglucosan chains led to consideration of an alternate mechanism which involved nearly simultaneous polymerization and crystallization of the sugar residue, either glucose or cellubiose.[7] However, recenly experimantal evidence has confirmed definitively the presence of a soluble, transient, intermediate polymer of glucose in cellulose biosynthesis by *Acetobacter xylinum*.[8-67] The purpose of the present work is to show how this intermediate polymer is released into the extracellular space, to suggest how the polyglucosan chains may associate to form a nascent, hydrated, plastic, cellulose microfibril and to indicate how the nascent microfibril changes on dehydration to form the familiar dense microfibril.

2. Materials and Methods

Cellulose-free, cell suspensions of *Acetobacter xylinum* (NRC 17005)were prepared as described by Hestrin and Schramm[13] to give a final concentration of about 5×10^9 viable cells per milliliter. For any incubation series run with the cellulose-free cell suspensions as starting material, one volume of suspension was added to an equal volume of 0.01% glucose in 0.01M phosphate 0.003M citrate buffer, pH 6, and the mixture was incubated at 35℃. This incubation results in rapid production of cellulose microfibrils and, initially, in a rapid doubling of biomass.[13]

For observation by freeze-etching, the initially cellulose-free cells were incubated in the dilute glucose solution as described above for 0, 10, or 20 min, and then droplets (about 0.8 mm in diameter) were frozen by plunging them into liquid Freon 12 cooled by liquid nitrogen. In addition, macroscopic cellulose pellicles were grown on the surface of inoculated, static medium of Hestrin and Schramm.[13] The pellicles were examined after 17 hr of growth at 28℃. At this time they were washed exhaustively with distilled water to remove all soluble consituents before small portions (1-mm cubes) were frozen as above. The freeze-etching for both cell suspensions and films was done in a Balzers BA 360 M apparatus, as described by Moor.[14] Replicas of the surface were made with platinum-carbon and backed with carbon. Then they were cleaned by 70% H_2SO_4, followed by distilled water.

For observations of thin sections by transmission electron microscopy, cells were incubated for 0, 2, 4, and 8 min in the dilute glucose solution, fixed immediately for 1

hr at 0℃ in 6% glutaraldehyde in phosphate-citrate buffer, pH 6, and postfixed in buffered 2% osmium tetroxide. After dehydration, solvent exchange into propylene oxide and embedding in Epon, sections(about 700 Å thick)were cut with a diamond knife and stained for 30 min with uranyl nitrate[15] and 3 min with lead citrate.[16]

For observations of never-air-dried, pellicle fibrils by electron microscopy, young pellicles(17-hr incubation at 28℃)were rinsed thoroughly with distilled water until free of the medium. A small piece of a rinsed pellicle was frozen on a grid in liquid Freon 12 cooled by liquid nitrogen and then deep-etched. Replicate grids carring small pieces were then examined without further treatment. For air-dried fibrils, the rinsed pellicle was allowed to dry overnight above anhydrous $CaCl_2$ under vacuum in a desiccator and then rewetted. The rewetted material was prepared and mounted for observation exactly as for the never-air-dried.

Photographs were made with an AEI-6B and a Simens 101 electron microscope.

3. Results and Discussion

3. 1 Fine Fibrils

Upon stimulation of cellulose production, the cell envelope developed distinctive, small, terminal invaginations when examined in thin section. Low magnification studies indicate that typically 1 to 10% of cells in given 700 Å sections show such invaginations. These distinctive invaginations were seen only in thin sections of cells

Fig. 1 Thin section of a cell of *A. xylinum* chemically fixed initially in glutaraldehyde after glucose incubation for 4 min, then dehydrated by solvent exchange, embedded, and nagative-stained with uranyl and lead ions. The extended, electmon-opaque, fine fibrils appear to be associated with a surface irregularity at the end of the cell and appear to be dispersing in the external environment.
50,000×(The scale represents 0.2 μm).

after being subjected to 4 min or more of incubation with dilute glucose. Transmission electron microscopy of the sections shows that fine filaments are released from the cells into extra-cellular space by polar pores in the bacterial cell wall. These fine filaments are not completed cellulose microfibrils because of their breadth(less than 30 Å)and because they absord heavy metal ions such as those of uranium and lead (Fig. 1). However, because cellulose is the only extracellular, fibrillar substance produced by the bacteria, it is reasonable to assume that the extruded filaments are agrregates of discovered polymer on the way to cellulose.[8–12]

3. 2 Nascent(Thick)Fibrils and Their Transformation by Desiccation

The thick fibrils of the thoroughly washed, glucose-free, bacterial cellulose which has not been air-dried(Fig. 2)have a markedly different appearance from those which have been air-dried at least once(Fig. 3)when observed by freeze-etching. Instead of the usualy compact thread with a diameter of about 100–120 Å, the nascent(never-air-

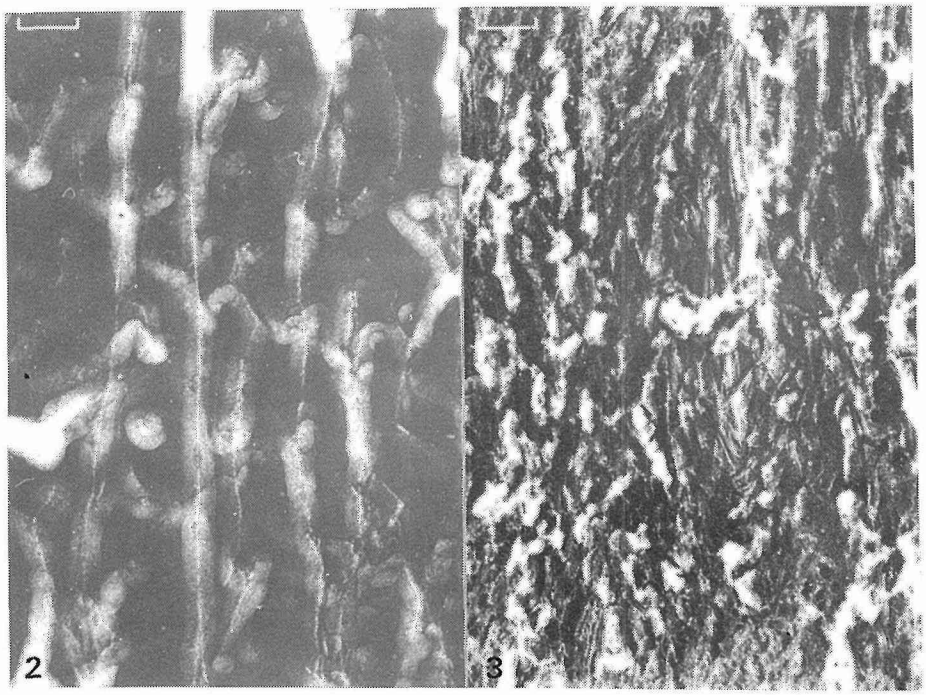

Fig. 2 Replica of a physically fixed, thoroughly washed, glucose-free, freeze-etched pellicle of *A. xylinum* which had never beeη air-dried. Note the extended, parallel, thick fibrils about 1000 Å wide composed of a central core surrounded by bn amorphous coating or sheath. 40,000×(The scale represents 0.2 μm).

Fig. 3 Replica of a physically fixed, thoroughly washed, glucose-free, freeze-etched pellicle of *A. xylinum* which was air-dried and rewetted before freezing and fracturing. The left or interlaced mass of typical, compact microfibrils (~100 Å in diameter) is quite evident. Note the contrast in appearance and size of the microfibrils in this photograph with those in Fig. 2. 40.000×(The scale represents 0.2μm).

dried)fibril consists of a core of consolidated material surrounded by a sheath of less dense, apparently amorphous substance. The sheath may have a diameter of up to 1000 Å. The core itself usually has a diameter near the resolution limit for freeze-etch replicas and is distinctly smaller than apparently similar, entire, classical-type microfibrils described for *Acetobacter xylinum* in the past and those which are generated by air-drying a nascent cellulose gel. It has been shown that when a nascent fibril is freeze-fractured at right angles, the end of the core may project from the end of the sheath and there may be a distinct separation between the material of the sheath and the surrounding vitrified water matrix. As indicated in Fig. 2, the nascent fibrils often have a tendency to be approximately in parallel. Dessication(by either air-drying or solvent exchange)of a freshly synthesized cellulose gel alters the form of its fibrous component(Fig. 2)and the resulting 100 Å to 120 Å microfibrils(Fig. 3)show a form which has been familiar and recognized for at least 25 years.[7] The interpretation of this change of breadth due to dessication is that the polymers of glucose extruded from the cell first associate longitudinally to form a highly hydrated strand in the aqueous environment of the medium or pellicle. When the pellicle is air-dried the layers of water between polymers are removed and the polyglucosan chains come to

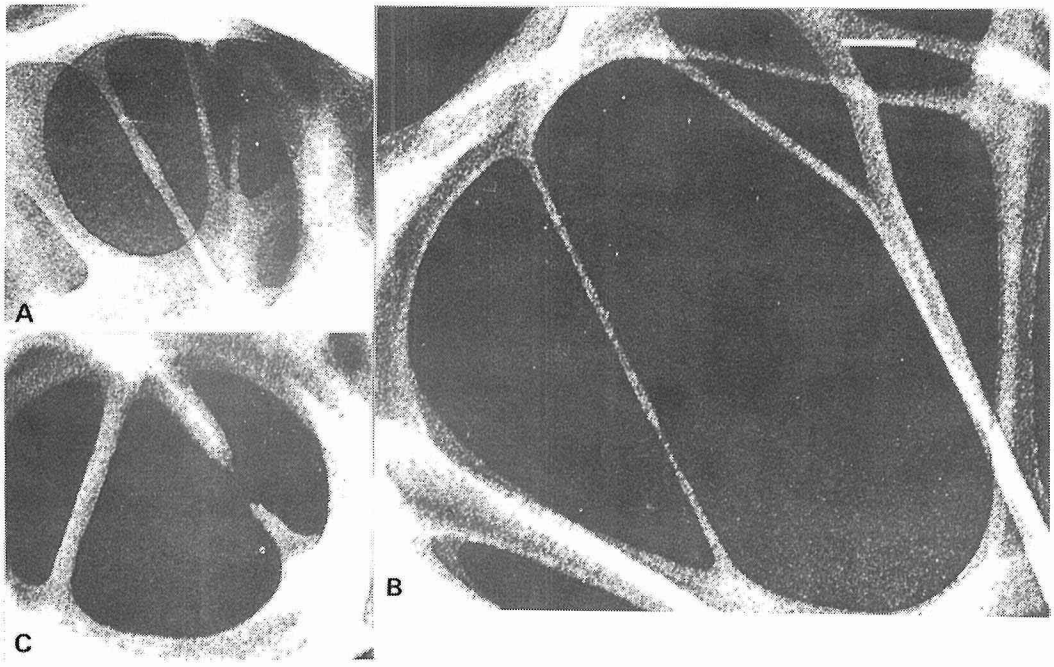

Fig. 4 Appearance of thick fibrils and freshly broken tips of thick fibrils of never-air-dried. The scale represents 0.2 μm.

A. Never-air-dried fibrils ; the variation in diameter of the fibril along its length suggetst plastic extention before loss of water by severe deep-ething.

B. Necking of the never-air-dried fibril before loss of water.

C. The tips of newly broken fibril of never-air-dried cellulose. Note the tendency to round up, as if plastic flow had occurred.

interact directly and irreversibly to form the usualy, dense, cellulose microfibril.

Transmission electron microscopy of a freshly synthesized pellicle subjected to extremely deep etching gave further evidence for an unconsolidated, nascent, developing form of the cellulose fibril(Fig. 4). The fibrils showed evidence of having been susceptible to drawing or extension(Fig. 4A)before loss of water. The diameter of the fibrils varied from point to point as if the strand has been stretched while plastic and extensible(Fig. 4A). Occasionally, the fibrils showed evidence of necking(Fig 4B). In addition, when these never-air-dried fibrils broke under tension either before, during, or after the loss of water, the newly formed tips tended to be rounded up as if they were plastic(Fig. 4C). In contrast, the tips of the newly broken but previously air-dried fibrils remain angular showing a slight tendency of the substance in the tips to flow. All three observations are consistent with the conclusion that(never-air-dried)nascent fibrils are much more deformable before they are dried than after.

In addition, transmission electron microscopy of wisps of bacterial cellulose microfibrils without shadowing with heavy metals(Fig. 5A, B)showed that individual microfibrils sometimes occluded electron-dense particles. These particles were held within the microfibril in such a way that they must have been trapped during the association and in the form of an intermediate, polymeric substance. The occluded particles are therefore further evidence for a transient, intermediate polymer and also, indirectly, for the existence of a nascent hydrated microfibril.

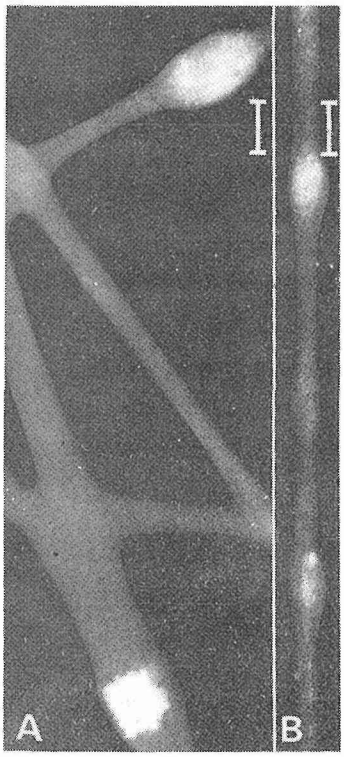

Fig. 5 A. Microfibrils of bacterial cellulose which has occluded particles or granules. 75,000X
B. A microfibril which has occluded three granules of varying size during the process of its formation. 75,000×(The scale represents 0.1 μm). Note the swelling of the microfibril as a granule, as for the unshadowed specimens in A and B.

All the foregoing results suggest that a reconsideration of the physical mechanism of bacterial cellulose synthesis is necessary. The new facts, when coupled with previous suggestions of an intermediate polymer,[4-6] indicate that the idea of nearly simultaneous polymerization and crystallization of glucose residues during cellulose microfibril formation[7,17] must be replaced by a more general physical mechanism. Clearly, if the most recently described intermediate polymers detected by biochemical means,[8,10] the fine, electron-opaque fibrils described herein, and the sheathed nascent fibrils revealed by freeze-etch replicas are a part of the process of bacterial cellulose microfibril formation, then the notion of simple addition of glucose or cellobiose residues to the tip of the microfibril[7] is not applicable. In the following, a general scheme is outlined which integrates the present, pertinent, known facts of cellulose biosynthesis and is intended to replace the former suggestion.

As an introduction, it should be emphasized that there is no reason to change the present ideas of the internal, biochemical events leading to cellulose synthesis. These are summarized and referned to in Fig. 6. The key precursors in bacterial cellulose biosynthesis are still uridine diphosphate glucose(UDPG). Furthermore, the participation of an intermediate lipid carrier between the nucleotide and an ultimate acceptor of the cellobiose unit remains established. The intermediate lipid-carbohydrate compound functions as shown in Fig. 6.

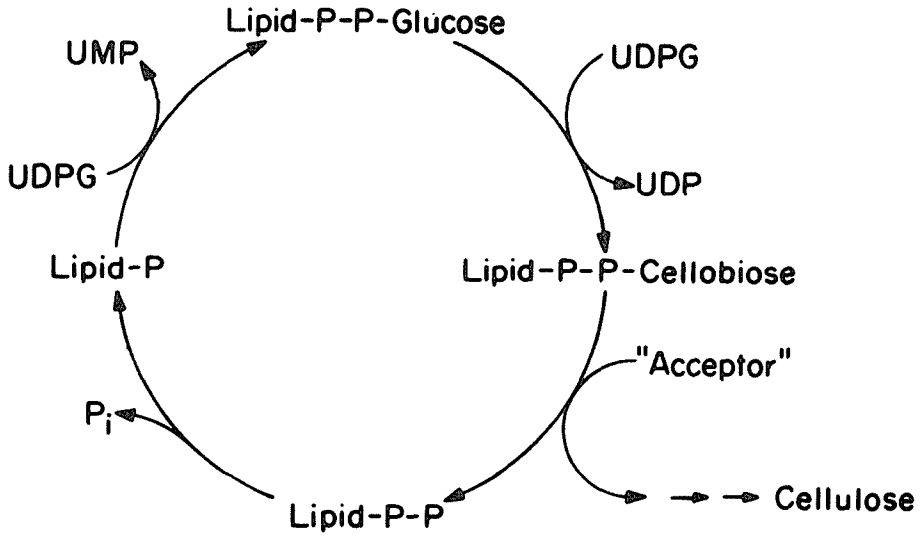

Fig. 6 Suggested role of lipid-phosphate-carbohydrate intermediates in cellulose biosynthesis. It seems certain that for bacterial cellulose the key precursor is uridine diphosphate glucose (UDPG) generated within the cell. In addition, the participation of an intermediate lipid carrier between UDPG and an ultimate acceptor of the cellobiose unit remains established. The intermediate lipid-carbohydrate compound is probably formed on or close to the cytoplasmic membrane.

However, the foregoing observations indicate, in addition, that the acceptor is not necessarily only the tip of previously formed cellulose microfibril but may also be the end of an, as yet undescribed, unconsolidated, soluble polymer of glucose or an association of such polymers. These extruded polymers may associate themselves outside the cell to form the nascent fibril. In this nascent fibril, according to our hypothetical model, the extended glucosan chains, mutually attracted by van der Waal's forces, lie in parallel or nearly in parallel to the axis of the fibril and are held apart by intervening adsorbed water layers, to form a sheath about a central, more consolidated core. This sheath is responsible for the breadth of the nascent fibril. When the nascent fibril is dried(by air-drying or by solvent exchange), with removal of the water layers the hydroxyl groups of the parallel chains can associate irreversibly and the cmmonly recognized, densely packed(crystalline)microfibril is formed. The nascent, intermediate stage was probably not seen earlier because nearly all previous methods of examination involved desiccation of the sample before final preparation.

Finally, it will be evident that the existence of both the intermediate polymers and the nascent, highly hydrated fibril pose many new problems, including solubility of the oligomers, the detailed mechanism of their association, and especially the mechanisms of their mutual attachment to form chains of many thousands of glucose residues. These problems are now under study.

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