A Consideration on the Structure of the Nascent Fibril Produced by *Acetobacter xylinum*

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The structure of the nascent fibril produced by *Acetobacter xylinum* was examined by electron microscopy and resistance to alkali. It was found that the inner part of the fibril after production is stained uniformly with sodium tungstophosphate. This suggests that the nascent fibril is in an amorphous state. Moreover, the fact that in the amorphous fibril microscopic fibrils with closely similar widths always appear in the course of time and that the X-ray diffraction diagram of the never-air-dried fibril treated with 11 wt% aqueous NaOH solution show cellulose I not cellulose II diffraction patterns, indicate that the arrangement of cellulose chains in its cross section can transform easily to that of chains in ellulose I crystalline. For such an arrangement, it must be presumed that in the nascent fibril cellulose chains are in the form of sheets of monomolecular layers corresponding to the $(1\bar{1}0)$ plane of cellulose I crystalline.

The structure of the fibril after production by *Acetobacter xylinum (A. xylinum)* is still unknown. Brown *et al.* suggested initially that a fibril (a microfibril) crystallized simultaneously when it is extruded from a synthesizing site in the envelope of a bacterial cell.[1] But lately, they suggested that a nascent microfibril is composed of bundles of chains in the tactoidal state since the inner part of the fibril from an incubated medium with fluorescent brightener was dyed by the brightener.[2,3] Zaar reported that fibril formation from *A. xylinum* is similar to that described by Brown *et al.* He presumed that the crystallization of a fibril (a microfibril) occurred after extrusion from the synthesizing site.[4,5] Colvin *et al.*, from observation of the freeze-etching of incubated suspension of *A. xylinum* reported that a nascent fibril has a dense core surrounded by a sheath of amorphous gel.[6-8] We suggested, from the negative staining behavior of the nascent fibrils, that they are in a homogeneous amorphous state.[9]

It was found that in the course of time microscopic fibrils with almost the same width of 40—50 Å develop in the amorphous fibril.[10,11] Since the same transformation of the amorphous fibril always occurred, we have presumed that it is composed not of random chains but of chains which retain a specific correlation with each other.

In this paper the structure of the fibril produced by *A. xylinum* was examined with respect to its staining behavior toward sodium tungstophosphate and its resistance to alkali.

Experimental

Culture of Cells and Preparations of the Samples for Electron Microscopy. The methods described previously were followed.[10] Two series of samples were prepared for electron microscopy; one of the series is the fibrils from the glucose medium with sodium tungstophosphate and the other is the fibrils, stained by an aqueous solution of sodium tungstophosphate before drying, from the glucose medium without the staining agent.

Ten ml of *A. xylinum*-preserved culture medium (IFO 13693) was added to 100 ml of complex medium (distilled water: 100 ml, D-glucose: 1 g, peptone: 1 g, yeast extract: 0.3 g, sodium chloride: 0.2 g, disodium hydrogenphosphate (anhydrous): 0.14 g, citric acid: 0.035 g, pH adjusted to 6.8 with 1 M† HCl or NaOH aq) and incubated at 28 °C for 72 h. After being thus cultured, 10 ml of the liquid phase of the starter culture was added to 100 ml of the fresh complex medium, and was incubated at 28 °C for 48 h. Separating the cellulose produced in the incubated medium requried filtration through a membrane filter of 5 μm pore size while cooling the filtrate with ice water. The filtrate was immediately filtered through a membrane filter of 0.45 μm pore size, allowing the cells to collect on the membrane filter. Cells were washed with a buffer solution (pH 6.8, disodium hydrogenphosphate: 0.14 wt%, citric acid: 0.035 wt%, 0 °C) to remove the medium components. They were then suspended in 60 ml of the fresh buffer solution. A 4 ml quantity of 0.2 wt% glucose medium (pH 6.8) with 2 wt% sodium tungstophosphate was added to 36 ml of cellulose-free cell suspension. The final concentrations of glucose and sodium tungstohosphate in the medium were 0.02 and 0.2 wt% respectively. These values were determined for the concentrations easily observable by electron microscopy without washing the fibrils produced. A drop of the culture medium was immediately placed on a sheet mesh covered with a collodion membrane and incubated in a 100%RH atmosphere at 28 °C for a given time. After the fibrils so produced had been allowed to settle on the sheet mesh by removing the medium, they were dried over P_2O_5 at room temperature for more than 24 h, and observed through an electron microscope. Preparations of the sample for the fibrils from 0.02 wt% glucose medium without staining agent are the same as described above except for staining with addition of a drop of 0.5 wt% aqueous sodium tungstophosphate solution for 15 min before drying. In the above incubation procedure, the complex medium was sterilized by autoclaving while the glucose medium was sterilized by filtration.

For electron microscopy, all preparations were examined with a JEOL JEM-100U electron microscope operating at 80 kV.

Measurement of the Linear Growth Rate of a Cellulose Fibril.

The linear growth rate of a cellulose fibril at a given temperature was obtained from the relation between the time of incubation and the mean length of ten fibrils after glucose incubation for a given time at a given temperature. The linear growth rate was constant within the range of the measurable length of a fibril.

Resistance of the Never-air-dried Fibrils to Alkali. Samples: 40 ml of 3 wt% glucose medium (pH 6.8) was added to 200 ml

† 1 M=1 mol dm^{-3}.

of cellulose-free cell suspension and incubated at 28°C for a given time. Cellulose gels in the incubated mediums were the samples for alkali-treatment.

Alkali-treatment of the Never-air-dried Cellulose Gels: To the incubated medium an equal volume of aqueous NaOH was added dropwise at twice the given concentration. It was kept at 20°C for 1 h and then 30 vol% aqueous acetic acid solution was added to neutralize. The solution was dialyzed through Cellulose Dialyzer Tubing (Nakarai Chemicals Ltd make, through M.W. cut-off: 8000, diameter: 90 mm, thickness: 0.09 mm) until sodium acetic acid free, and then boiled to collect cellulose. The cellulose collected, before drying, was again boiled in 1 wt% aqueous NaOH solution for 10 h under a nitrogen atmosphere, then neutralized and washed. The wet cellulose was compressed to a disk with the diameter of 1.5 mm and a thickness of about 1 mm for X-ray diffraction photography.

The X-ray diffraction diagram of the cellulose was taken with a flat-film camera with Ni filtered Cu $K\alpha$ radiation (35 kV, 15 mA, time of exposure: about 35 min) at specimen film distance of 50 mm. A Rigaku Denki make, Rota-Unit RU-3v X-ray camera was used.

Results and Discussion

The structure of the cellulose fibril after production by *A. xylinum* may be mainly determined by the mechanism of biogenesis of the fibril. However, it is supposed that if the structure of a fibril after extrusion from a cell is in an amorphous state, it may also be affected by the physicochemical environment, that is, temperature, the elapsed time of an amorphous fibril, the pH of medium and medium components *etc.* Therefore, first, the relation between the linear growth rate of a fibril produced by *A. xylinum* empolyed in this study and the temperature of incubation was examined.

The linear growth rate of a microfibril was maximum at about 28°C as is evident from Fig. 1. As shown in Fig. 2a, the fibril, with its inner part stained uniformly, was obtained from a medium incubated with sodium tungstophosphate for 30 min at the most suitable temperature, 28°C for the biogenesis of fibril. Moreover, it was found that the inner part of the fibril obtained from the incubated medium without staining agent at 28°C for 5 min was uniformly stainable,

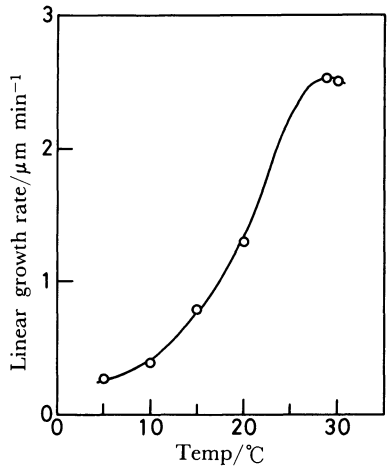

Fig. 1. Influence of temperature on the growth rate of the microfibril in glucose incubation.
Concentration of glucose: 1 wt%, pH of culture medium: 6.8.

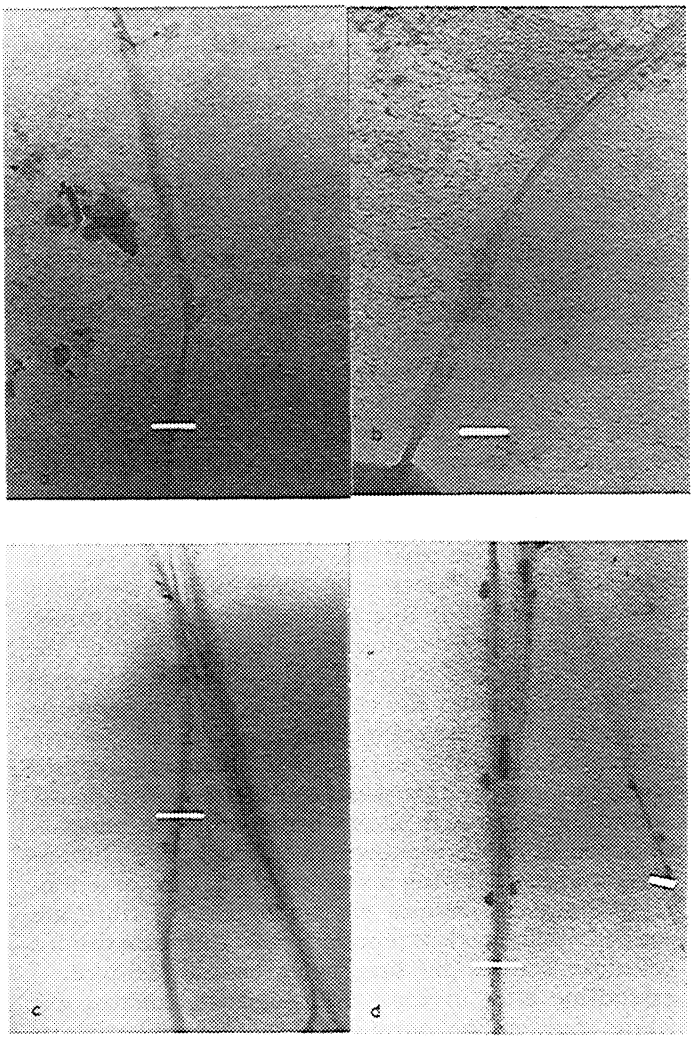

Fig. 2. Electron micrographs of the fibril obtained from 0.02 wt% glucose incubation included 0.2 wt% sodium tungstophosphate (pH 6.8) (a, c, and d) and the fibril obtained from 0.02 wt% glucose incubation (pH 6.8) (b) for a given time at 28°C. The fibril obtained from the medium without staining agent before drying, was stained with 0.25 wt% aqueous sodium tungstophosphate solution for 15 min at room temperature. Time of incubation; a: 30 min, b: 5 min, c: 1 h, d: 5 h.
Scale bars=0.1 μm.

similar to the fibril obtained from the incubated medium with staining agent (Fig. 2b). The higher electron opacity of the twisted, thickened portions of these fibrils seemed to be the cause of the uniform staining of their inner part. In these fibrils the dense core, as reported by Colvin *et al.*, could not be found. According to the staining behavior of a nascent fibril toward sodium tungstophosphate, the fibril after production seemed to retain an amorphous state for about 30 min. Subsequently unevenness occurred perpendicular to the long axis of the fibril (some striations occurred parallel to the fibril axis) (Fig. 2c). Later microscopic fibrils with similar widths of 40—50 Å appeared simultaneously within the amorphous fibril about 5 h after production (Fig. 2d). Since the same transformation of the amorphous fibril as shown

in Fig. 2 always occurred, we suggested that a nascent fibril is composed not of random chains but of chains keeping specific correlation with each other.

The microscopic fibrils found in this study correspond to the elementary fibril formerly regarded as the fundamental structure of bacterial cellulose.[12-14] Recently, Brown *et al.*[1,2] and Zaar[4,5] pointed out that the synthesizing sites exist in the envelope of the cell and microfibrils (which correspond to the microscopic fibrils named in our paper) with the width of 30 Å are extruded from the synthesizing sites. Their illustrations allow us to understand the reproducibility of the microscopic fibrils in the amorphous fibril. Furthermore, the so-called "microfibril" of the earlier workers are assemblies of microscopic fibrils as shown in Fig. 2d. However, it is not always clear whether the crystallization of microscopic fibrils occurs before assembly or after.

It is considered that the staining agent, sodium tungstophophate does not have affinity towards polysaccharides. If this is true, it is to be assumed that sodium tungstophosphate does not change the structure of the nascent fibril, but is absorbed in it when it is extruded from the cell and is rejected from the stained fibril in the course of crystallization. Indeed, the reproducibility of the width of a microscopic fibril seems to support this process. On the other hand, according to Brown *et al.*[2,3] the cellulose material obtained from the incubated medium with a fluorescent brightener having a vital stain[15] is also in a non-crystalline state because the inner parts of fibril are stained. They also reported that this amorphous cellulose stained by brightener became cellulose I crystalline after drying. Although the microscopic fibrils could not be found in the cellulose material; its crystallinity was reduced, and the width of a crystallite changed from 30 to 28 Å. However, it is noteworthy that the influence of both sodium tungstophosphate and the brightener on the width of a crystallite after drying seems to be little different, although the influences of both on the morphology of the product are different because of their different affinity. This fact suggested that the fibril after production is not a mere bundle of chains but is restorable to the original width of the crystallite even if the product has absorbed the staining agent. For such a structure to be restorable, it must be assumed that in the nascent fibril cellulose chains are in the form of sheets of a monomolecular layer corresponding to the $(1\bar{1}0)$ plane of cellulose I. If the fibril after production has such a structure, the results observed both by Brown *et al.* and in this study can be satisfactorily explained.

It was found that the stainable state of the inner part of a fibril obtained from the incubation at 5 °C was retained for about 3 h (At this temperature the linear growth rate of a fibril is equivalent to about 1/10 of its value at 28 °C. See Fig. 1) as shown in Fig. 3. Although the influence of temperature on the crystallization of the amorphous fibril is unknown, it is apparent that the inner part of a fibril after production is in a stainable state even if the linear growth rate is different, and moreover, the microscopic fibrils after assembly are in an amorphous state.

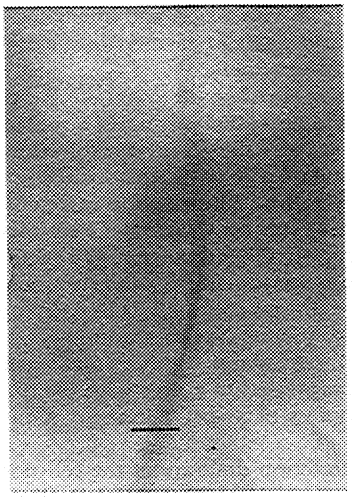

Fig. 3. Electron micrograph of the fibril obtained from 0.02 wt% glucose incubation included 0.2 wt% sodium tungstophosphate (pH 6.8) for 3 h at 5 °C. Scale bar=0.1 μm.

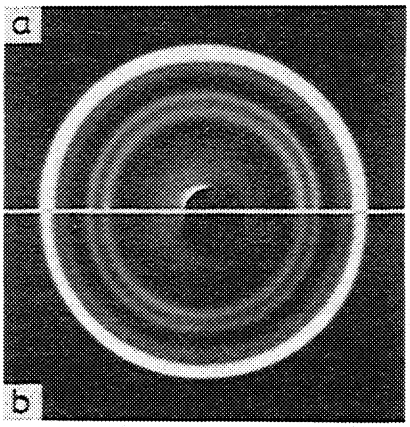

Fig. 4. X-Ray diffraction photograph of the 11 wt% alkali-treated cellulose from the glucose medium incubated for 5 h at 28 °C.
a: Purified and dried cellulose. b: Never-air-dried cellulose was treated in 11 wt% aqueous NaOH solution, then purified and dried.

It is very difficult to determine directly the structure of a fibril after production because the quantities required for usual analytical measurements are not easily obtainable, and even if they can be obtained, the structure of the nascent fibril is very changeable. However, it is supposed that the examination of the resistance of the never-air-dried fibrils to alkali allows us to assume the structure of a fibril after production. From the relation between the yield of cellulose as a function of the incubation time and the progress of the transformation of an amorphous fibril, it is assumed that the never-air-dried cellulose material from the incubated glucose medium at 28 °C for 5 h is composed of 11 wt% of the fibril having the stainable inner part, 60 wt% of the fibrils with staining-unevenness, and 29 wt% of microscopic fibrils.[16] The X-ray diffraction diagram of the never-air-dried cellulose treated with 11 wt% aqueous NaOH solution showed apparently cellulose I as shown in Fig. 4. The diffraction patterns of cellulose II could not be found in the diagram. This result

suggest that not only the microscopic fibrils but also the fibrils with staining unevenness already have the lattice of cellulose I crystalline or a structure able be transform easily to cellulose I crystalline. In spite of the never-air-dried cellulose material containing the amorphous fibrils, the non-occurrence of the diffraction pattern of cellulose II may be due to reject amorphous material by alkali-treatment. But, the weights of the sample treated with 11 wt% alkali and the untreated sample were almost the same. This result shows that the non-occurrence of the diffraction pattern of cellulose II is not because of rejecting the low order region. Accordingly, it is reasonable that the non-occurrence of cellulose II is due to the structure of an amorphous fibril. If an amorphous fibril is composed of some sheets of monomolecular layers corresponding to the $(1\bar{1}0)$ plane of cellulose I as described above, it is supposed that the nascent fibrils may have resistance to 11 wt% alkali, and furthermore, the dehydration of the water contained among the cellulose sheets or the shrinkage of some part of the fibril with alkali-treatment allow the cellulose sheets to bind easily. The structure of the nascent fibril proposed in this paper may also be supported by papers reporting that the swelling of cellulose I with alkali-treatment takes place along the $(1\bar{1}0)$ plane in the first place.[17,18]

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