The Structure of Cellulose Produced by *Acetobacter xylinum* in the Presence of a Fluorescent Brightener. The Influence of Concentration of a Brightener in the Medium on the Structure of Cellulose

Akira KAI* and Hitoshi KITAMURA†
Department of Industrial Chemistry, Faculty of Technology, Tokyo Metropolitan University, Fukazawa, Setagaya-ku, Tokyo 158
(Received May 13, 1985)

The influence of the concentration of a brightener on the structure of cellulose from an *Acetobacter* culture in the presence of a fluorescent brightener was examined. At a brightener concentration above 0.05 wt%, the X-ray diffraction diagram of cellulose from an incubated medium was observed. Except for the intensity of the diffraction corresponding to the (020) plane of cellulose I, the intensity of diffraction of the (110) plane became very weak and diffraction of the ($1\bar{1}0$) plane disappeared. A new diffraction, not corresponding to cellulose I or II, occurred inside. The position of each diffraction pattern was constant and independent of the concentration of the brightener. As the brightener concentration of the medium became lower, in the X-ray diagram of the dyed cellulose that was produced, the diffraction intensity of both the ($1\bar{1}0$) and (110) planes of cellulose I increased, and a new diffraction pattern gradually disappeared. In the case of an extraction of dyed cellulose from an incubated medium with a brightener (above 0.05 wt%), the same result was obtained. The above results suggest that the monomolecular layer of a brightener is included between the ($1\bar{1}0$) planes of the produced cellulose.

Acetobacter xylinum (A. xylinum) extrudes cellulose in an amorphous state from a cell.[1-3] However, it inevitably continues to form cellulose I crystals. Therefore, it is essential to determine the structure of the nascent cellulose in order to elucidate the biogenesis of a bacterial cellulose microfibril.

Brown *et al.* reported that when *A. xylinum* is cultured in the presence of a fluorescent brightener having a strong affinity toward cellulose, the morphology of the produced cellulose changes.[2] We found that the X-ray diffraction diagram of the specimen obtained from the incubated medium with a fluorescent brightener shows the characteristic diffraction patterns of a cellulose-brightener complex.[4,5] However, when the brightener was extracted from the complex, the diffraction patterns of cellulose I occurred in its X-ray diagram.[4] These facts showed that an examination of the structure of the dyed cellulose produced in the presence of a brightener is very essential in order to elucidate the structure of the nascent cellulose produced by *A. xylinum*.

In this paper, the influence of the brightener concentration of the medium on the structure of cellulose obtained from incubation in the presence of a fluorescent brightener was examined.

Experimental

Cultures of Cells and Preparations of the Samples for X-ray Photographing. Previously described methods were employed.[3]

Culture of A. xylinum (IFO 13693) in the Presence of a Fluorescent Brightener: 60 ml of a cellulose-free cell suspension[3] (pH 7.0) was added to 140 ml of a complex medium with a fluorescent brightener (Nippon Kayaku, Kayaphor FB conc: 4,4′-bis[4-anilino-6-bis(2-hydroxyethyl)amino-1,3,5-triazin-2-ylamino]-2,2′-stilbenedisulfonic acid) of various concentration (ranging from 10^{-4} to 10^{-1} wt%) and was then incubated for 24 h at 28 °C.

Preparations of the Samples: The collected cellulose was washed with a weak aqueous alkali solution several times, and from this the following samples were prepared after further treatment. T-1: the cellulose was washed with a 70 vol% aqueous ethanol solution several times to remove the free brightener at room temperature. T-2: the aqueous ethanol washed cellulose was extracted by boiling in a solution of 70 vol% aqueous ethanol for 18 h (aqueous ethanol was exchanged for fresh one every 3 h). T-3: the ethanol extracted cellulose was refined in a boiling 1 wt% aqueous NaOH solution for 10 h under a N_2 atmosphere.

X-Ray Photographing: Each sample was compressed to an isotropic disk with a diameter of 1.5 mm and a thickness of about 1 mm. X-ray diffraction diagrams of the samples were made with a flat-film camera (with the X-ray beam perpendicular to a flat surface of the disks) with Ni filtered Cu $K\alpha$ radiation (35 kV, 15 mA, time of exposure: about 35 min) at a specimen film distance of 50 mm. A Rigaku Denki made Rota-Unit RU-3v was used for photographing.

Results and Discussion

In the X-ray diffraction diagram of the sample (after a rejection of any free brightener from the incubated medium at a brightener concentration greater than 0.05 wt%) except for the intensity of the diffraction corresponding to the (020) plane of cellulose I, the diffraction intensity of the (110) plane becomes very weak and the diffraction of the ($1\bar{1}0$) plane disappears (Fig. 1b, 1c). A new diffraction which does not correspond to cellulose I or II occurred inside. As the concentration of the brightener in the medium became lower, the diffraction intensity of both the ($1\bar{1}0$) and (110) planes of cellulose I increased. On the contrary, a new diffraction pattern disappeared gradually. In the case of the medium with a brightener of less than about 0.001 wt%, the intensity of each

†Present address: Yakult Honsha Co. Ltd., Higashisinbashi, Minato-ku, Tokyo 105.

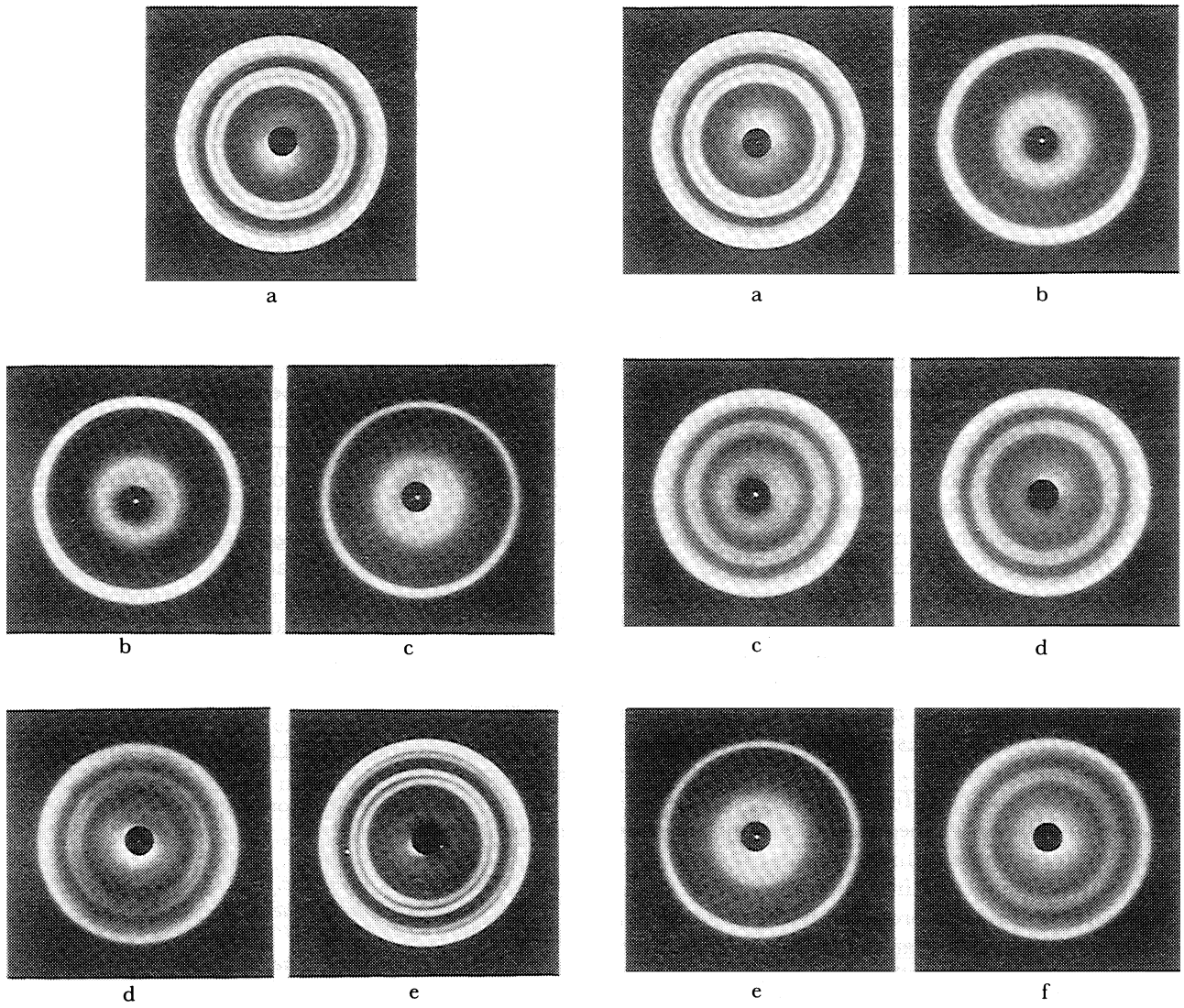

Fig. 1. X-Ray diffraction diagrams of samples, after rejection of the free brightener, obtained from the *Acetobacter* culture in the presence of a fluorescent brightener of various concentrations.
The brightener concentration of the medium (wt%); a: 0 (Cellulose I sample), b: 0.1, c: 0.05, d: 0.01, e: a little under 0.001.

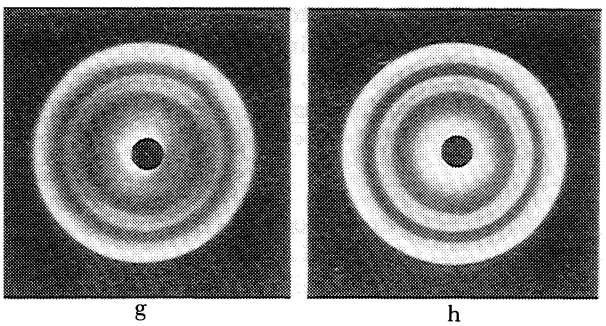

Fig. 2. Change in the X-ray diffraction diagram of the dyed cellulose with extraction.
a: Cellulose I sample, b: T-1-treated sample, which was obtained from the medium with a brightener of 0.1 wt%. c: T-2-treated sample b, d: T-3-treated sample c, e: T-1-treated sample, which was obtained from the medium with a brightener of 0.05 wt%. f: T-3-treated sample e, g: T-1-treated sample, which was obtained from the medium with a brightener of 0.01 wt%. h: T-3-treated sample g.

diffraction of the cellulose was almost the same that of cellulose I (Fig. 1).

Brown *et al.* reported that for concentrations of a brightener greater than 0.1 mM (0.0086 wt%), the brightener disrupts the assembly of crystalline cellulose I microfibrils and their integration into a composite ribbon by a stoichiometric binding to glucose residues of newly-polymerized glucan chains.[6] The minimum concentration, at that the morphological change of cellulose takes place, generally corresponds to the result from the X-ray test on the complex in this study.

On the other hand, in the case of an extraction of the cellulose-brightener complex obtained from an incubated medium with a brightener above 0.05 wt%, the same result was found as in the case of lowering the brightener concentration of the medium. When the complex was treated with methods T-2 and T-3, in

its X-ray diagram the intensity of each diffraction of cellulose I increased as removing a brightener from it. The intensity of a new diffraction pattern gradually became weaker (Fig. 2). However, the intensities of the diffractions of each sample differed with the brightener concentration of the medium.

We reported previously, from a calculation regarding an X-ray diagram of the cellulose-brightener complex obtained from the incubated medium with a brightener of 0.1 wt%, that a new diffraction corresponds to $(1\bar{1}0)$ plane of the complex, and that a brightener is included between the $(1\bar{1}0)$ planes.[5] Therefore, the spacing of the $(1\bar{1}0)$ plane of the complex becomes 7.4 Å broader than that of cellulose I. The spacing of the $(1\bar{1}0)$ plane of the complex was unchanged even if the amount of the brightener in a complex differed. This fact suggests that a monomolecular layer of a brightener is included between the $(1\bar{1}0)$ planes of the complex. In the sample obtained from the incubated medium with a brightener of above 0.05 wt% (as its X-ray diagram was unchanged), the surfaces of cellulose sheets (corresponding to the $(1\bar{1}0)$ plane of the complex) capable of being dyed seemed to be saturated by a brightener. As the given concentration of a brightener in the medium becomes lower, the surfaces of cellulose sheets become dotted with the brightener. Therefore, it is suggested that the intensity of the diffraction of the $(1\bar{1}0)$ plane of cellulose I increased for the possibility of an increased mutual binding of the cellulose sheet.

Brown *et al.* suggested from the observation of the morphology of cellulose produced in the presence of a fluorescent brightener that the cellulose chains are extruded from a bacterial cell to be in the form of sheets corresponding to the (020) plane of cellulose I, and that the cellulose sheet is formed by hydrogen bonding of cellulose chains.[6] Therefore, they suggested that a fluorescent brightener is included between the (020) planes. However, the fact, that both the intensity and the spacing of the diffraction pattern corresponding to the (020) plane of cellulose I are scarcely changed even with the amount of brightener in a sample, suggests that their theory is inadequate.

The fact that the spacing of the $(1\bar{1}0)$ plane of the cellulose produced in the presence of a fluorescent brightener widens with a definite value and that the spacing of the (020) plane is unchanged independent of the concentration of a brightener, indicates that cellulose chains in the form of a monomolecular layer corresponding to the $(1\bar{1}0)$ plane of cellulose I are extruded from a cell. The behavior of the nascent cellulose toward a fluorescent brightener shows that between the cellulose sheets, the medium components are included. This view is equivalent to that obtained from an examination of the behavior of the nascent cellulose toward sodium tungstophosphate.[3] It is well known that the spacing of the $(1\bar{1}0)$ plane widens preferentially when natural cellulose is exposed either to intracrystalline swelling reagents (such as sodium hydroxide and potassium acetate) or to chemical substitution reactions (such as acetylation and nitration).[7] A cellulose sheet corresponding to the $(1\bar{1}0)$ plane may be an elementary unit of natural cellulose.

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