Single Crystals of a Soluble Polyglucosan from *Acetobacter xylinum*

—Resemblance of Their Internal Structure to that of Cellulose Hydrate II—

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Abstract

Hexahedral, lamellar, single crystals are formed in water-methanol solutions at room temperature from a extracellular, water-soluble polyglucosan which is produced by *Acetobacter xylinum*, a cellulose-synthesizing bacterium. The crystals are very thin plates whose thickness is usually too small to estimate accurate shadowing. Electron diffraction patterns of these plates show a typical, hexagonal symmetry. The observed reflections may be indexed on the basis of a hexagonal, two-dimensional unit cell with $a=b=5.18$ Å, $\gamma=120°$. The third dimension of the unit cell can be obtained by 0-level Buerger precession X-ray photography which shows $c=20.0$ Å, $\alpha=\beta=90°$. Systematic absences of (0001) reflections were observed with $1=2n+1$. In general, X-ray diffraction patterns of the crystals are very similar to, if not identical with, the electron diffraction patterns. Both lead to the conclusion that the structure of the crystals is similar to that of Cellulose Hydrate II along the fiber axis. The chain axes lie parallel to the surface of the lamellar crystals and form sheets in the (0001) plane (i. e. the lamellar plane). From density data there appears to be 2.5 water molecules to every glucose residue and the water molecules lie in layers between the adjacent (0001) planes of the polyglucosan sheets.

1. Introduction

It is now well established that many linear, synthetic polymers form single crystals in a dilute solution; such crystals are usually thin platelets or lamellae of an order of 100–500 Å in thickness. The axes of the chain molecules are normal to the lamellae and chains are folded sharply through 180 degrees at the surface of the platelet. Stimulated by the results in the synthetic realm, a number of investigators have studied the possibility of similar, folded chain structures in single crystals of native or regenerated cellulose[1~13] and in derivatives of native cellulose such as triacetate[14~19]. The results show definitively that chain folding does take

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place in the crystals of modified celluloses but there is not conclusive evidence for the same type of structure in native cellulose(s).

The question of the presence or absence of chain folding in native celluloses or related substances has become more timely with the recent accumulation of evidence for the existence of water-soluble, transient, intermediate polymers of glucose in bacterial cellulose synthesis[20~24]. One class of water-soluble, branched, $\beta 1 \rightarrow 4$ polyglyucosans in particular is produced by cultures of *Acetobacter xylinum* which are actively synthesizing cellulose[24] and may be involved as an intermediate in cellulose biosynthesis. The purpose of the present paper is to report the crystallization of this polymer(s), the structure of the crystals and the relationship of this structure to that of Cellulose Hydrate II.

2. Materials and Methods

2-1 Polyglucosan

The water-soluble, branched, $\beta 1 \rightarrow 4$ polyglucosan, fraction E60/A80, isolated as described previously[24], was used for crystallization. The intrinsic viscosity of this fraction of the polyglucosan is 4.1 ($K_m = 11.0 \times 10^{-4}$) in water and this parameter corresponds roughly to a degree of polymerization of 200. When stored at room temperature as a solution in aqueous 1% formaldehyde, this fraction slowly releases glucose (period 2-3 weeks) indicating that a part of the polymer is unstable under these conditions.

2-2 Valonia

Single cells of *Valonia macrophysa*, approximately 5-10 mm in diameter, previously stored in formalin for a long period, were placed in aqueous solution of 1% NaOH, by weight. The cells in the solution were allowed to stand at room temperature (approximately 25°C) for several months whereupon a fine, flocculent, crystalline sediment was observed. Portions of this sediment were placed on electron microscope grids, washed and observed as described below.

2-3 Crystallization of the Polyglucosan

The polymer was dissolved in water at room temperature to concentrations varying from 0.01 to 0.02% (w/v). Redistilled methanol was then added dropwise to the aqueous solution until it became very slightly turbid. After adding a drop or two of water to remove the turbidity, the solutions were allowed to stand to evaporate slowly for two to four weeks at 28°C.

To establish the effect, if any, of previous heating on the form and structure of the crystals, aqueous solutions of the polyglucosan were heated for 24 hr at 100°C. The heated solutions were then slowly cooled to room temperature at a rate of 0.5°C/hr and treated as above.

2-4 Electron Microscopy and Electron Diffraction

Drops of a suspension of crystals were placed on carbon films carried by copper grids and evaporated at room temperature, 105° or 145°C. After drying, they

were observed under an electron microscope without shadowing except in those instances where attempts were made to verify the thickness of the crystal. For both imaging and diffraction, a Siemens 101 electron microscope equipped with a point filament was used at 80 kV. Selected area, electron-diffraction patterns were taken at a magnification of 55,000 X using a 50 μm aperture (diameter of the selected area, 1 μm). The camera length was calibrated with gold metal as a standard.

2-5 X-ray Diffraction and Associated Density Measurements

X-ray diffraction patterns were taken of larger, hexahedral crystals (0.05 mm × 0.1 mm) grown in the solution for six weeks, with the direction of the beam perpendicular or in parallel to the surface of the lamellae. Photographs were obtained using a Buerger precession camera by Charles Supper in a Norelco-Philips generator. Nickel-filtered Cu K_α radiation of wavelength 1.542 Å was employed with a specimen to a film distance of 60 mm. The exposures varied from 8–10 hr depending upon the size of the crystal.

Densities of the crystals used for X-ray diffraction were determined by floating the crystals to an equilibrium position in a mixture of bromobenzene and carbon tetrachloride at 25°C. The density of the liquid mixture was then obtained by an Anton Paar Digital Density Meter (Model DMA-02C) with a precision of $\pm 1.5 \times 10^{-6}$ g/ml.

3. Results and Discussion

3-1 Electron Microscopy and Electron Diffraction

Under electron microscopy, single crystals of the polyglucosan were found with the form of hexahedral lamellae, approximately 0.2–0.3 μm in the largest dimension (Fig. 1). The lamellae were too thin to measure accurately by shadowing. As shown in Fig. 1A, they were commonly superimposed randomly, indicating relatively strong, non-specific attraction between the faces of the crystals.

Electron diffraction patterns of the crystals could be recorded although they disappeared within a few seconds after direct exposure to the electron beam. The overall form of the crystals was retained after subsidence of a characteristic but difficult to describe "bubbling" (Fig. 1B) but the original pattern had vanished. A typical, initial, electron-diffraction pattern of the crystals is shown in Fig. 2 and it illustrates the distribution of reflections with hexagonal, close packing. The observed reflections could be indexed on the basis of a hexagonal two-dimentional unit cell with $a=b=5.18$ A, $\gamma=120$. At first, we considered the possibility that the chains of the branched, $\beta 1 \rightarrow 4$ polyglucosan were normal to the plane of the lamellae as in many other polymer single crystals. This arrangement of the chains could be excluded, however, because the calculated densities did not agree with the observed density. In addition, none of the observed cell parameter were consistent with any of the other unit cells of cellulose or cellulose hydrates. One possible arrangement which was consistent with the whole of the data observed

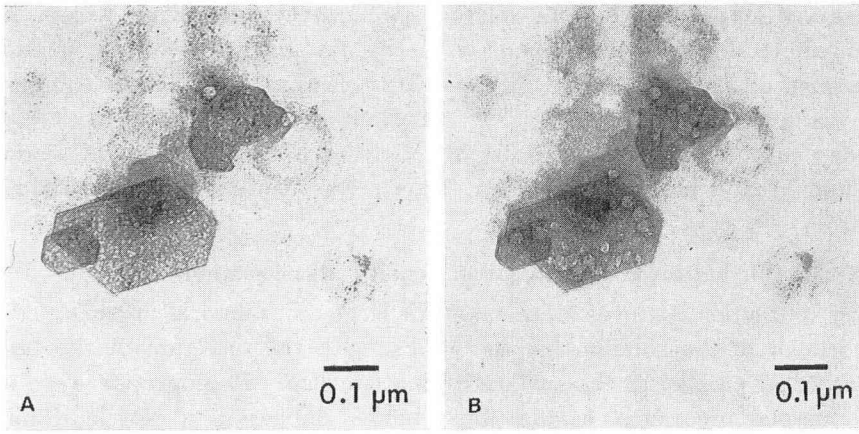

Fig. 1. A Electron micrograph of superimposed crystals of polyglucosan crystallized from methanol-water. Note the hexahedral lamellar shape and the thinness of the crystals.

B Electron micrograph of the same crystal as in A but after exposure for about 5 seconds to the electron-diffraction beam. Note the evidence of beam-induced damage.

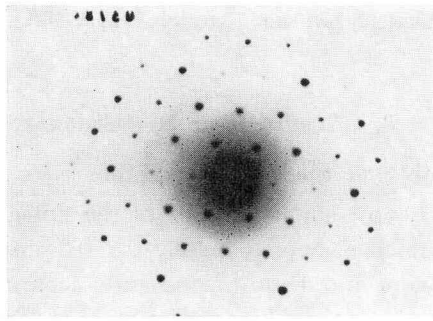

Fig. 2. Electron diffraction pattern of a crystal of polyglucosan showing typical hexagonal symmetry. Beam perpendicular to the plane of the lamellae.

was the structure where the chains of the polymer are parallel to the plane of the lamellae. Making this assumption, the observed unit cell is in accordance with that of Cellulose Hydrate II[25,26], projected along the fiber axis. As shown in the next section, calculated and observed crystal densities agree when this assumption is made. The observed *d*-spacings and those calculated from the above assumption are listed in Table 1. Projections of the proposed structure onto the plane of the lamellae and onto a plane perpendicular to the chain axes are shown in Fig. 3 and Fig. 4 respectively. They demonstrate that the suggested structure for the crystals of the polygucosan is in agreement with the structure of Cellulose Hydrate II postulated long ago by Sakurada and Huchino and later by Hermans and Weidinger[25,26]. Fig. 3 also illustrates clearly how the proposed structure would generate the typical, observed, hexagonal pattern.

TABLE 1. Observed and Calculated *d*-Spacings and Relative Intensities

d-Spacing (Å) obs.	*d*-Spacing (Å) calc.	Reflecting Plane	Relative Intensity	Valonia obs. (Å)
4.49	4.49	$(1\bar{1}00)$ $(10\bar{1}0)$ $(01\bar{1}0)$	v. s.	4.52
2.59	2.59	$(1\bar{2}10)$ $(2\bar{1}\bar{1}0)$ $(11\bar{2}0)$	s.	2.62
2.24	2.25	$(2\bar{2}00)$ $(20\bar{2}0)$ $(02\bar{2}0)$	m.	2.26
1.69	1.70	$(2\bar{3}10)$ $(3\bar{2}\bar{1}0)$ $(3\bar{1}\bar{2}0)$ $(21\bar{3}0)$ $(12\bar{3}0)$ $(\bar{1}3\bar{2}0)$	s.	1.71
1.49	1.50	$(3\bar{3}00)$ $(30\bar{3}0)$ $(03\bar{3}0)$	v. s.	1.51
1.30	1.30	$(2\bar{4}20)$ $(4\bar{2}\bar{2}0)$ $(22\bar{4}0)$	s.	1.30
1.25	1.24	$(3\bar{4}10)$ $(4\bar{3}\bar{1}0)$ $(4\bar{1}\bar{3}0)$ $(31\bar{4}0)$ $(13\bar{4}0)$ $(\bar{1}4\bar{3}0)$	s.	1.25
1.12	1.12	$(4\bar{4}00)$ $(40\bar{4}0)$ $(04\bar{4}0)$	m.	1.13
1.04	1.03	$(5\bar{2}\bar{3}0)$ $(32\bar{5}0)$ $(23\bar{5}0)$ $(\bar{2}5\bar{3}0)$ $(\bar{5}320)$	s.	1.04
0.98	0.98	$(5\bar{1}\bar{4}0)$ $(41\bar{5}0)$ $(14\bar{5}0)$ $(\bar{1}5\bar{4}0)$ $(\bar{5}410)$	m.	0.99
0.90	0.90	$(50\bar{5}0)$ $(05\bar{5}0)$	m.	0.91
0.87	0.86	$(33\bar{6}0)$	w.	0.87
0.85	0.85	$(6\bar{2}\bar{4}0)$ $(42\bar{6}0)$ $(24\bar{6}0)$ $(\bar{6}330)$	w.	0.87
0.81	0.81	$(6\bar{1}\bar{5}0)$ $(5\bar{6}10)$ $(15\bar{6}0)$	w.	0.80
0.75	0.75	$(60\bar{6}0)$	w.	0.75
0.71	0.71	$(7\bar{2}\bar{5}0)$ $(25\bar{7}0)$	w.	0.72

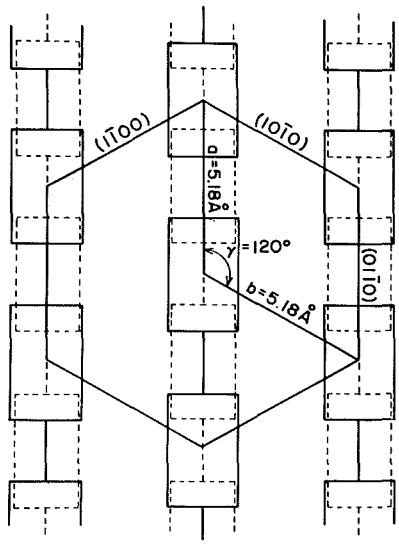

Fig. 3. Projection of the proposed structure for the polyglucosan, viewed along the *c*-axis. The small rectangles symbolize glucose residues. Dotted rectangles indicate the position of molecules in the layer beneath.

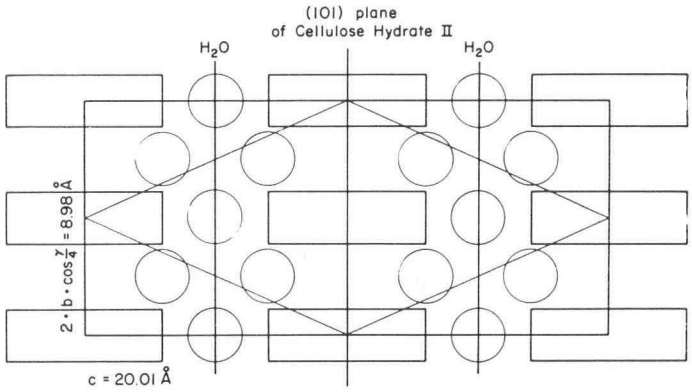

Fig. 4. Projection of the proposed structure for the polygulcosan onto a plane parallel to the *c*-axis and normal to the *a*-axis. The circles suggest the position of water molecules between planes.

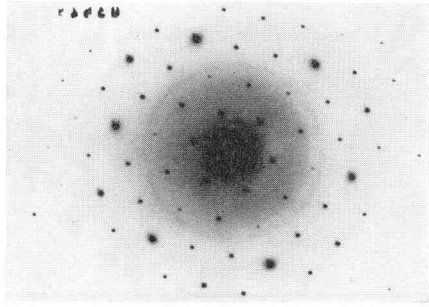

Fig. 5. Electron diffraction pattern of cell-wall fragment of *Valonia*. Note the resemblence to that Fig. 2 for the polyglucosan.

Electron diffraction patterns of small sheets of fibrous material from *Valonia* also gave support to the idea that the chains of the polymer are parallel to the surface of the lamellae. When fragments of *Valonia* cell wall, previously soaked for several months in 1% NaOH, were examined by electron diffraction, they gave a pattern which is very similar to that of the crystals of the polyglucosan (Fig. 5). Since it is known that the cellulose chains within the microfibrils of the *Valonia* cell wall have parallel polarity[27] and also that the microfibrils themselves are generally parallel to the plane of the cell wall[28], the simillarity of the two patterns supports the conclusion that chains of the polyglucosan are parallel to the plane of the lamellae.

The characteristic "bubbling" or raipd release of a certain volatile substance from the hexagonal crystals of the polyglucosan is also to be expected from the composition of a Cellulose Hydrate. The Joule heating by the electron beam releases water from the hydrate, destroys the close periodicity of the structure but does not alter the macroscopic shape of the crystal (Fig. 1).

3-2 X-ray Diffraction

The X-ray diffraction pattern of the crystals of the polyglucosan with the

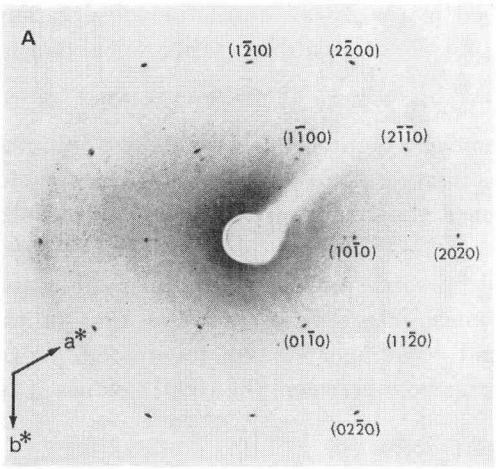

Fig. 6 A. X-ray diffraction pattern of the crystals of the polyglucosan; beam perpendicular to the lamellae of the crystal. Note the similarity to Fig. 2 and Fig. 5.

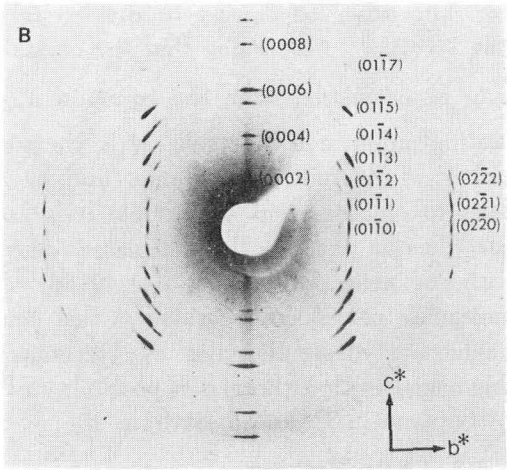

Fig. 6 B. O-level, Buerger, precession photograph of the crystals; beam parallel to the lamellae of the crystal, and an oscillation angle of 20°.

beam perpendicular to the surface of the lamellae, is shown in Fig. 6A. Evidently, the pattern is hexagonal and is closely similar to, if not identical with, the pattern from electron diffraction of the same crystals (Fig. 2). Three hexagonally symmetrical spacings can be recognized at 4.56 Å, 2.62 Å and 2.28 Å which coincide with those of Cellulose Hydrate II. As with electron diffraction, this agreement suggests that the axes of the polyglucosan chains lie parallel to the lamellar surface, namely the (0001) plane.

Zero-level, Buerger, precession photographs of the crystals with the beam parallel to the platelet surface, and an oscillation angle of 20°, were taken to obtain the third dimension of the unit cell (Fig. 6B). Four apacings on the c axis were observed at 10.00 Å [(0002)], 5.00 Å [(0004)], 3.34 Å [(0006)], and 2.50 Å [(0008)] with systematic absences of odd reflections. The observations established the *c* dimension as 20.0 Å. There is therefore a 2 Å difference in the *c* dimension of these crystals compared with that of Cellulose Hydrate II obtained by Sakurada and Huchino[25] and by Hermans and Weidinger[26]. However, the (0001) planes are ordinarily those most densely packed with hydroxyl groups, which correspond to the (101) planes of Cellulose Hydrate II. These are also the planes where the lattice constant and the sharpness of diffraction lines are always first affected, when cellulose is exposed either to intracrystalline sewlling reagents such as sodium hydroxide, potassium acetate, and alkyl amines, or to chemical substitution reactions in fiber form such as acetylation and nitration. Shift of position, line broadening, or decrease in total intensity of the (101) plane of cellulose almost invariably precedes changes in the (10$\bar{1}$) or (002) planes. Therefore, these considerations suggest that a spreading of the (0001) spacing is possible in the crystals of the polyglucosan. Such a spreading is also consistent with the results of density measurements, as outlined below.

The observed density of the crystal used in the X-ray diffraction observations was 1.49166. Assumuing that the crystal is a cellulose hydrate, a theoretical density may be calculated from the equation $d_{\text{calc.}}=\frac{ZM}{AV}$ where Z is the number of repeating units in a unit cell, M is the molecular weight, A is Avogadro's number and V is the unit cell volume, and by assuming a series of ratio of water molecules to glucose residues. When it is assumed that there are 2.5 water molecules per glucose residue, the calculated density is 1.489, which is in good agreement with the observed density of 1.49166. At the same time, this ratio of 2.5 water molecules per glucose residue is just one more water molecule than that of the Cellulose Hydrate II found by Hermans and Weidinger[26]. As mentioned above, this suggests that there is a possibility of spreading between the (0001) planes (i. e., (101) plane in Cellulose Hydrate II).

3-3 Chain Packing

For an understanding of the formation (genesis) of the single crystal, the chain arrangement in the three dimensions of the unit cell should be known. If, as indicated above, the chains lie parallel to the lamellae and occupy the a or b axis, then adjacent chains are separated by 4.49 Å and are also shifted 2.59 Å with respect to each other in the chain direction, i. e. quarterstagger shift (Fig. 3). These polyglucosan chains then form sheets in the (0001) planes (Fig. 4). This structure would be fully consistent with the observed hexagonal patterns. Furthermore, the water may be easily removed from the structure of this hydrate, as is observed.

3-4 Twinning

As mentioned above, the polyglucosan crystallizes from dilute solution at room temperature in the form of hexahedral, lamellar single crystals. During this process the phenomenon of twinning is occasionally observed. Figure 7 shows a trapezoid-shaped, twinned crystal of the polyglucosan. The growth mechanism is not clear at present but the twinning habit has been reasonably interpreted from its electron

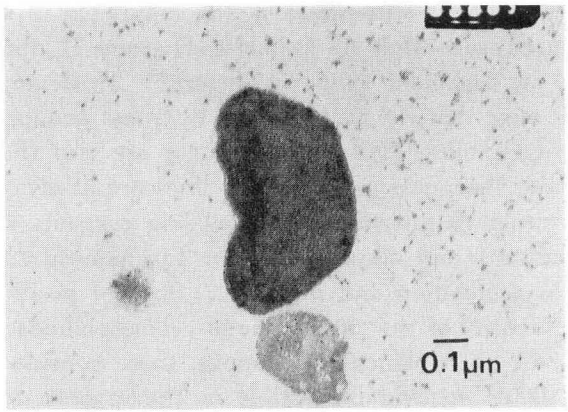

Fig. 7. Electron micrograph of trapezoid-shaped, twinned crystal of the polyglucosan.

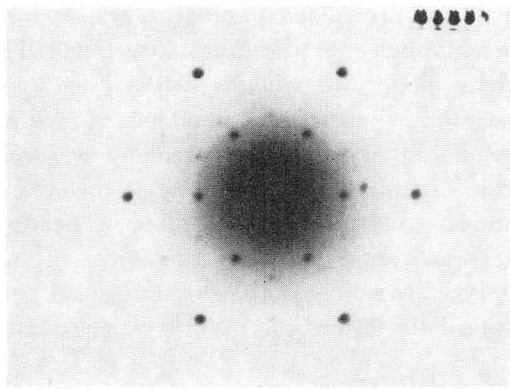

Fig. 8. Electron diffraction pattern of the crystal in Fig. 7. Note the difference between Fig. 2 and this diffraction pattern.

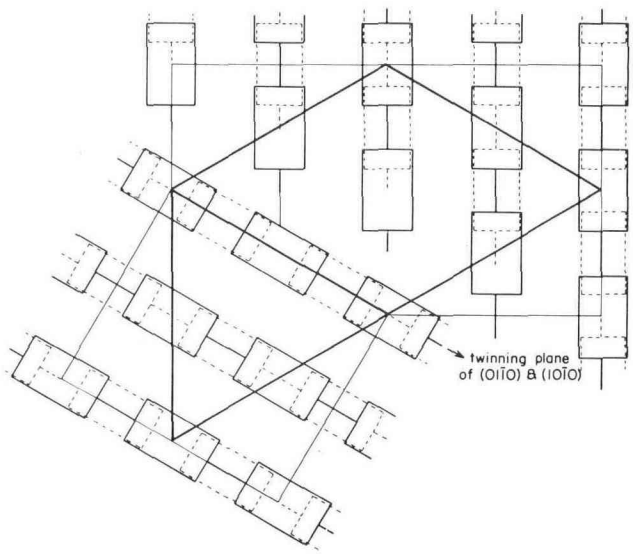

Fig. 9. One possible mechanism of twinning in the crystal. Note the trapezoid-shape in the packing connected with (01$\bar{1}$0) and (10$\bar{1}$0) planes.

diffraction pattern (Fig. 8), as follows. In such diffraction patterns, the longest spacing (4.49 Å) which is characteristic of the usual uncomplicated hexahedral crystals, does not appear (compare Fig. 2 with Fig. 8). On the other hand, two new reflections can be recognized at 3.0 Å and 1.73 Å (Fig. 8). It is impossible to index them in the hexagonal unit cell as established above. However, a refiection at 1.50 Å does coincide with that of the pattern of the non-twinning crystal. When considering the growth mechanism and the twinning habit, the suggested chain packing shown in Fig. 3 is an aid. Twinning may occur of the (01$\bar{1}$0) plane when it is rotated 60° around the *c*-axis (normal to the paper) as shown in Fig. 9.

Bittger[29] has shown that in ribbonlike fibrils (~200 Å diameter) of a mannan

from *tubera salep* the chains are folded parallel to the surface of the ribbon, with the folded chains at right angles to the fibril direction. This structure leads to kinks and cross-striations along the ribbons as well as to fragmentation of the ribbons into small crystallites when ethanol is added. Since neither of these events were observed for the present crystals this possibility was rejected.

Finally, because the structure of the hydrate is unstable with respect to temperature, it was of interest to determine the effect of heating the solution of the polygucosan prior to crystallization and also the effect of heating the crystals to 105° and 145°C after crystallization. Both procedures led to structures which are altered from the one presently described. Work on these structures is continuing.

Acknowledgments

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