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The Formation of Spherulites of Bacterial Cellulose from *Acetobacter xylinum*

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Abstract

Two dimensional spherulites are formed in the pellicle of bacterial cellulose by static cultures of *Acetobacter xylinum*. These spherulites are much larger(sometimes more than 2 cm in diameter)than those usually observed in other natural or synthetic polymers. Evidence from polarizing and phase microscopy as well as scanning electron microscopy indicates that the cellulose microfibrils in the spherulites of bacterial cellulose are oriented tangentially and not radially. Also, the orientation may be limited to only a fraction of the thickness of the pellicle. It is suggested that the tangential deposition may be caused by a gradient of concentration of a weakly soluble inhibitor of cellulose formation around the center.

1. Introduction

The formation of spherulites has been recognized as a characteristic mode of crystallization of many synthetic polymers for more than two decades and the properties of these objects are now understood in a broad outline.[1-3] In natural polymers, however, spherulites seem to have been found only in gutta-percha,[4] natural rubber,[5,6] a fraction of starch,[19] and carboxypeptidase.[7] The size of the aggregates is usually microscopic. The purpose of the present paper is to describe the structure of these entities, as shown by three indepedent techniques, and to suggest, tentatively, the molecular mechanism of their formation.

2. Materials and Methods

Pellicles of bacterial cellulose were grown at 28℃ on the surface of static cultures of *Acetobacter xylinum,* ATCC 10245, as follows. Erlenmeyer flasks containing 15 ml of Hestrin and Schramm's medium[8] were inoculated with approximately 0.5 ml of subcultures and incubated for 56 hr. at 28℃. 0.1 ml of the starter culture was diluted with 10 ml of phosphate buffer solution (0.01M Na_2HPO_4, 0.003M citric acid), and 0.1 ml of the diluted culture was then mixed with 5 liters of the Hestrin medium and the

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suspension was distributed into approximately 60 glass Petri dishes, each 15 cm in diameter. These dishes were supported in a rack at 28℃ so that they could be observed periodically above a fluorescent lamp and between crossed Polaroid films without disturbance. When examined in this manner, birefringent areas in the pellicle appeared as bright regions against a dark background(Fig. 1 A–D).

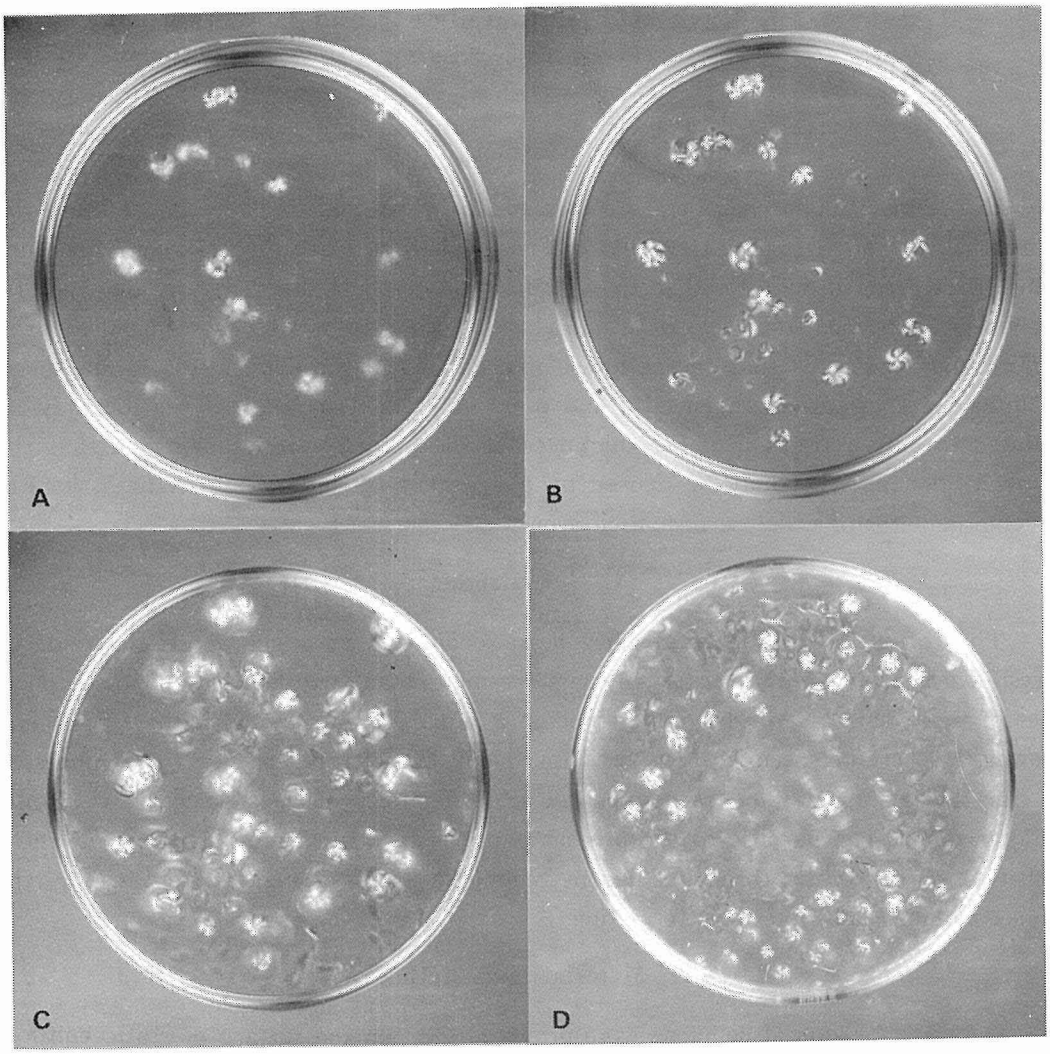

Fig. 1 Development of spherulites in Petri dishes, photographed between cross Polaroid films. The spherulites were grown and observed in each dish ; (A) incubated for 56 hr, (B) 59 hr, (C) 76 hr, and (D) 100 hr at 28℃, respectively.

When spherulites were observed in a pellicle, they were allowed to grow until they were 2–4 mm in diameter. This stage of development usually required about 30 hr after pouring of the inoculated medium into the Petri dishes. The spherulites were cut out gently from the surounding pellicle by scissors, immersed for several hours in 1%

formaldehyde, and then washed overnight in distilled water. Finally, they were washed twice in glass distilled water. All washings were done as gently as possible to avoid mechancal distortion of the samples. The washed samples were laid upon standard specimen stubs, frozen by placing the stubs on solid cabon dioxide, and then freeze -dried.

The dried samples were removed from the stubs and placed in a 5 ml beaker of molten wax(Tissuemat). The beaker containing the sample was then placed for a short time(1-2 min)under partial vacuum to remove trapped air bubbles. Atomospheric pressure was restored just before the wax hardened. To improve infiltration of the wax and the orientation of the spherulite, the Tissuemat was remelted and the sample was gently pushed to about 2 mm from the bottom of the beaker with its plane parallel to the bottom and the wax was allowed to solidify again. After appropriate timming, the spherulite was mounted and sectioned serially in a plane parallel to the plane of the pellicle with a thickness of 40-50 micron per section.

For observation of the serial sections by phase microscopy, polarized microscopy, and scanning electron microscopy in succession the following procedure was adopted. The sections were placed on glass slides and warmed to about 65℃ to remove the bulk of the wax. The sections on the slides were then extracted twice with each of the following liquids: xylene, 75% xylene-25% ethanol, 50% xylene-50% ethanol, 25% xylene-75% ethanol, ethanol, 25% ethanol-75% water. At this point, the water was replaced with 1% aqueous formaldehyde to prevent microbial growth and the sections were photographed first by a phase microscope and then the polarizing microsope using crossed Polaroid films at 10° from maximum extinction. After optical photography of the sections the formaldehyde solution was replaced with water, and the sections were frozen and lyophylized. The dried sections was then rotation-shadowed with gold and observed in a Cambrige Stereoscan MK2 scanning electron microscope at 20 KV.

3. Results and Discussion

A series of sections from the top through to the bottom of a typical pellicle which contained a spherulite showed that the birefringence in the sections and therefore the orientation of the cellulose microfibrils is not uniform across the pellicle(Fig. 2A-F). The section from the upper surface of the pellicle(Fi. 2A)shows only a diffuse birefringence, that immediately below shows two recognizable arms of a Maltese cross (Fig. 2B), and the next section a fully developed Maltase cross(Fig. 2C). The suceeding secctions reflect less and less birefringence until the bottom of the pellicle is reached(Fig. 2D-F). Evidently the tendency of the cellulose microfibrils to form a spherulite was greatest in the center plane of the pellicle.

A vertical as well as horizontal variation in the direction and degree of orientation of the microfibrils in the portion of a pellicle which includes a spherulite may be observed by scanning electron microscopy following oblique sectioning(Fig. 3). The

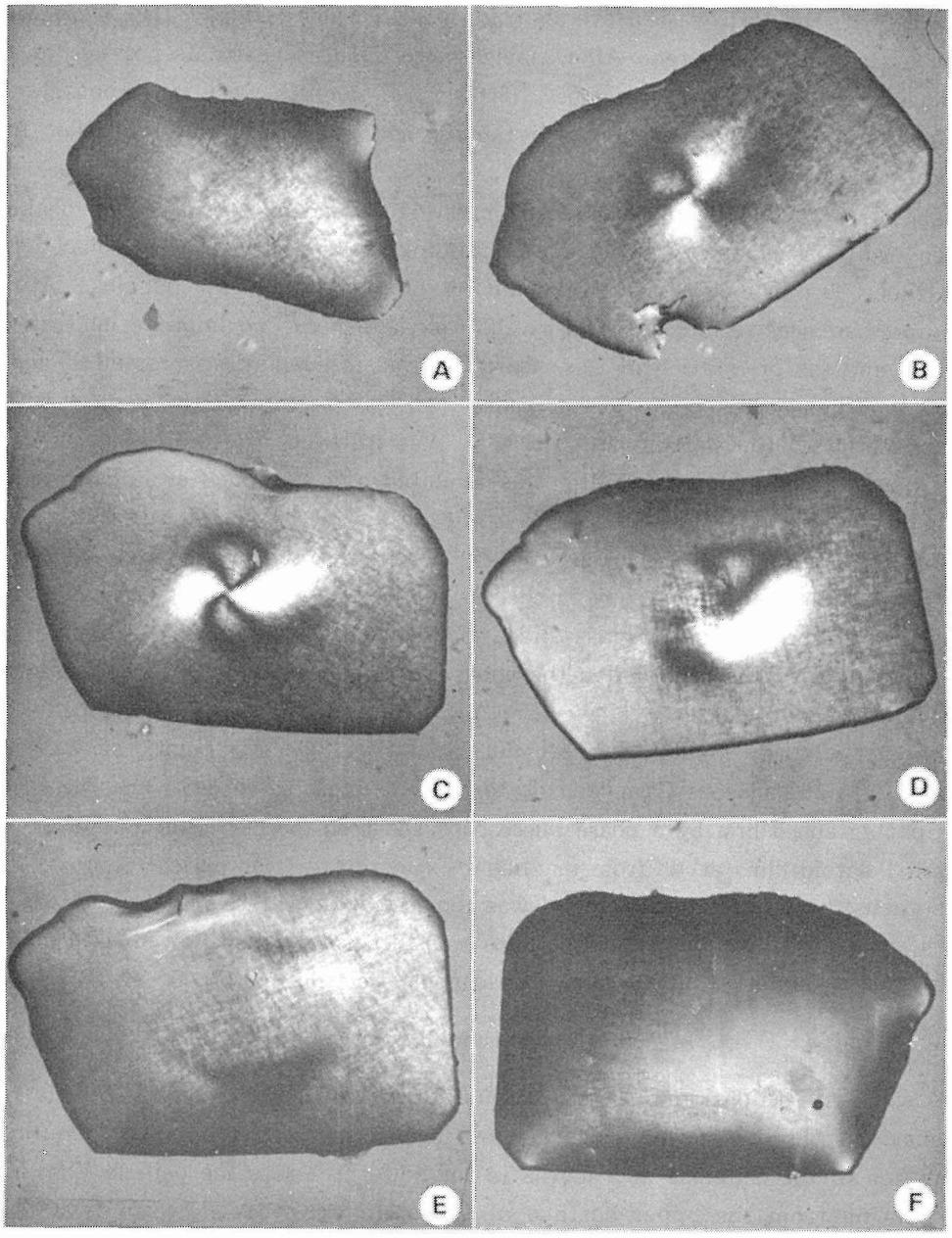

Fig. 2 Sections from the top of a pellicle of bacterial cellulose which contained a two-dimentional spherulite, photographed botween cross Polaroid films. Note the general diffuse birefringence with no evidence for a spherulite in top (A) and bottom (F) sections. The middle sections (C and D) show the maximum development of a Maltese cross. 11X

microfibrils in the layers at the top of the pellicle have a different average direction from those in the center portion which in turn have a different direction from those in the bottom layers.

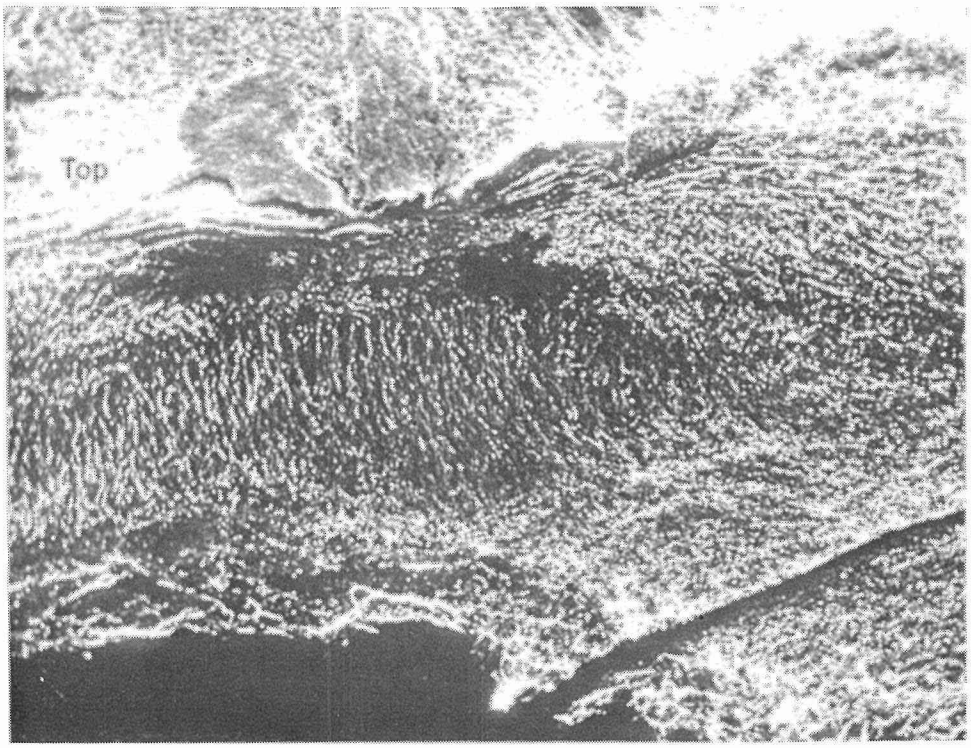

Fig. 3 Photograph from the scanning electron microscope of a portion of a pellicle which contained a spherulite and which was sectioned obliquely. The upper portion of the photograph shows the top surface of the pellicle. Note the difference between the orientation of the bundles of microfibrils in the central lens-shaped part and those of the bundles in the top and bottom layers. 300X

The sections which were observed by the polarizing microscope were also examined by phase microscopy and then scanning electron microscopy. Figure 4 shows the appearance of the same section as in Fig. 2C when examined by phase microscopy. This technique indicates that there is a cavity or at least a paucity of microfibrils at the center of the spherulite and that the more refractive elements in the section form short spindles of fibrils which appear to form a vortex or swirl about the center(note the difference from Fig. 5 which is of the same section as in Fig. 2A). As Fig. 4 is printed, the vortex has the appearance of a counter-clockwise spiral towards the center. Clearly, however, not all the elements or spindles adhere closely to the " stream-lines " of this vortex.

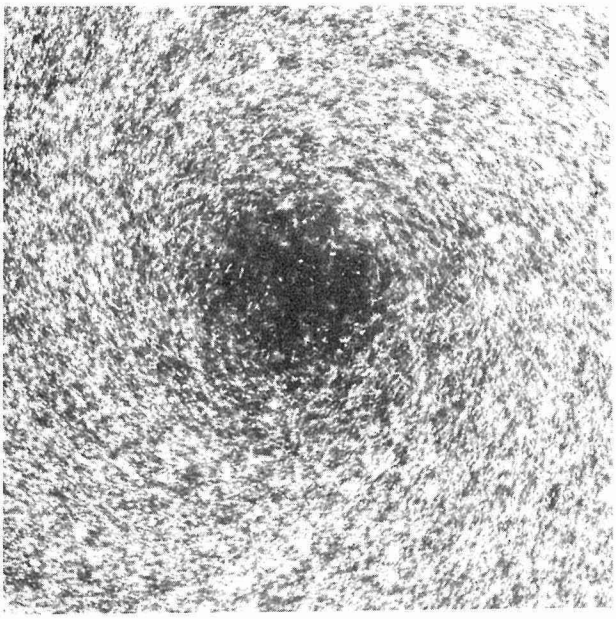

Fig. 4 The same section as in Fig. 2C but photographed under phase microscopy. The black spot with few refractive fibrils corresponds to the center of the spherulite. Note that the fibrillar elements from a spiral about this center. 160X

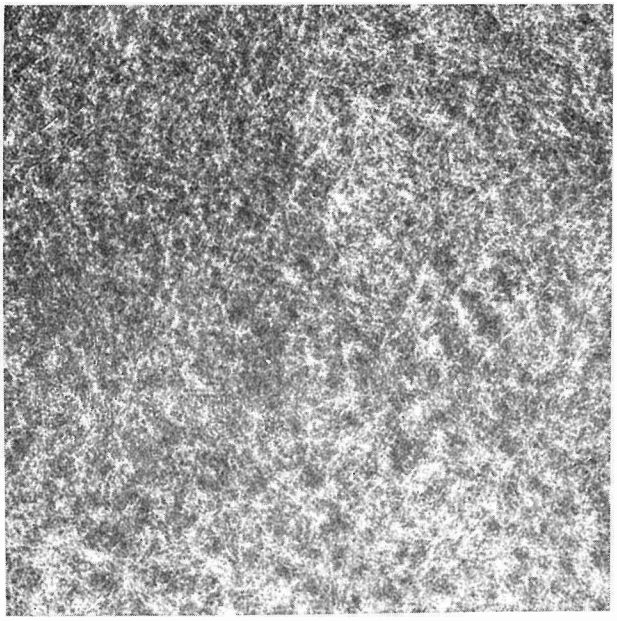

Fig. 5 The same section as in Fig. 2A but photographed under phase microscopy. Note the complete absence of any evidence for orientation of the fibrillar elements. 160X

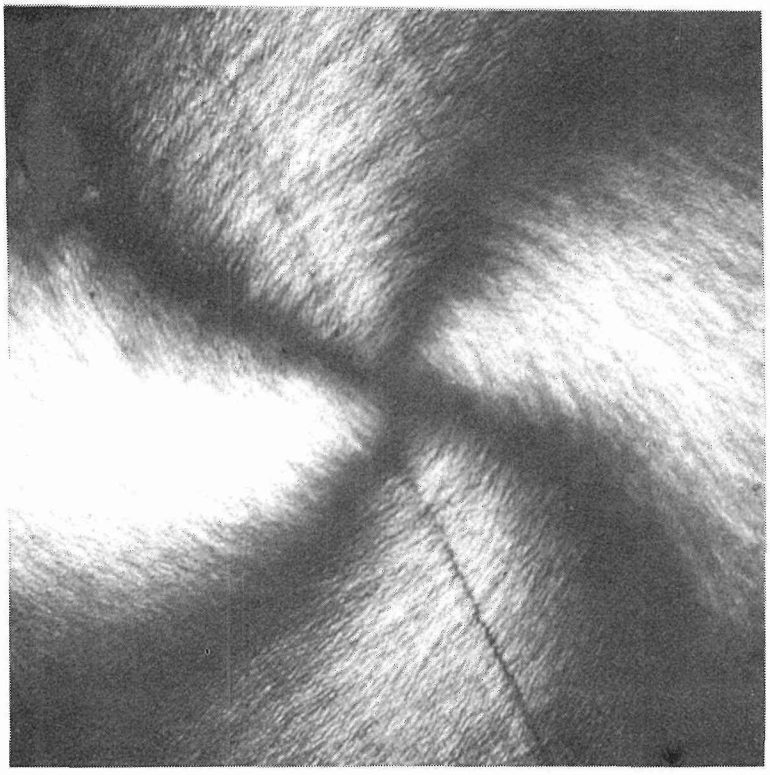

Fig. 6 The same photograp as Fig. 2C but shown at higher magnification to demonstrate the spiral of birefringent fibrillar elements about the center of the spherulites, similar to that shown in Fig. 4 by phase microscopy. 62X

Figure 6 shows the appearance of the same section between crossed Polaroid films but at a higher magnification than in Fig. 2C. At this level the birefringent elements (bundles of microfibrils)are clearly seen to form a counter-clockwise spiral into a dark center, similar to the vortex or spiral which can be shown by phase microscopy.

The pronounced birefringence suggested that there was a marked orientation of either or both of the solid component of the pellicle. That the bacterial cells were not preferentially oriented in the plane of the pellicle was directly demonstrated by staining the cells in the same sections with crystal violet and examining them microscopically for evidence of a preferred direction. No directional orientation of the cells was detected and this observation was confirmed by electron microscope photographs of replicas of birefringent areas(Fig. 7).

The above results show definitely that the birefringence and therefore the degree of orientation of the birefringent elements(cellulose microfibrils)in a spherulite of bacterial cellulose varies vertically within the pellicle as well as horizontally. The vertical variation is particularly significant because of disc-like form of the spherulite with a ratio of axes of about1 : 10. Since the pellicle increases in thickness by sinking

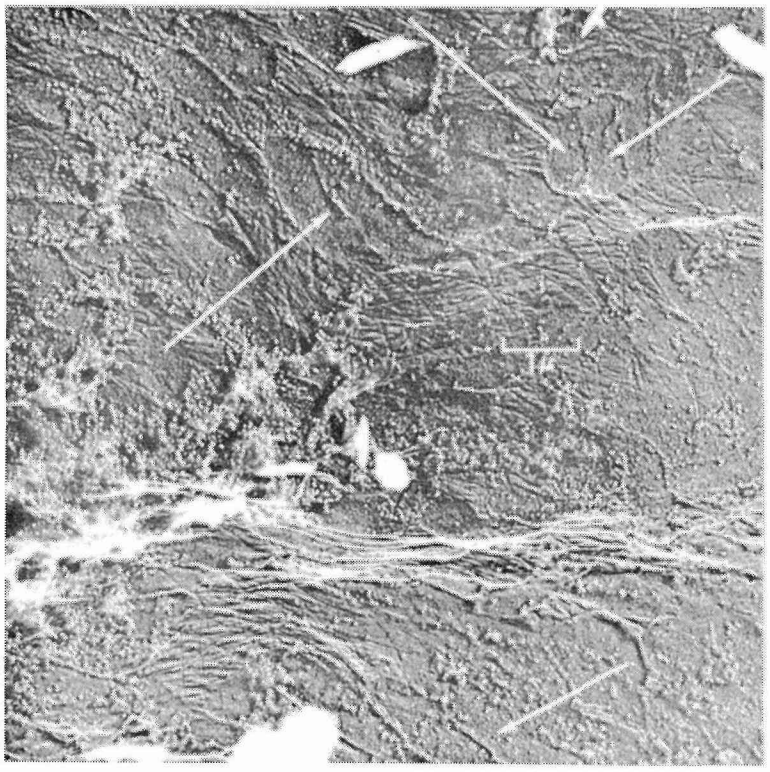

Fig. 7 Electron microscope photofraph of a replica of the surface of a birefringent area in pellicle of *A. xylinum* The clear areas previously occupied by the surfaces of cells are indicated by arrows. Note the preferential direction of the microfibrils and the lack of orientation of the long axes of the cells.

slowly into the medium, the variation in birefringence means that some influence was not operating at first, later induced the formation of a spherulite, and then ceased to cause the orientation of the microfibrils. The nature of this influence is still unknown but it may be a diffusible metabolite.

In addition, the results from phase and polarizing optical microscopy as well as scanning electron microscopy also indicate clearly that the orientation of the fibers within a spherulite is predominantly tangential, not radial.

In spite of the spectacular visual aspect of the spherulites between crossed Polaroid films, it is necessary to emphasize that only a fraction of microfibrils in a spherulite are oriented non-randomly. The brilliant Maltese cross is superimposed on a dark background and its intensity is misleading. Probably only a small proportion of the cellulose microfibls in one layer of the spherulite has a tangential deposition. Since the microfibrils are completely extracellular[8-11] this characteristic form is produced without the direct influence of cytoplasm of the cells. Aside from their role

as a supplier of material, the effect of the cell contents must be secondary and indirect.

The tangential deposition of some of the microfibrils to form a two-dimensional analog of a spherulite may have been induced as follows. If a weakly soluble substance which inhibits or retards the crystallization of cellulose diffused radially from a point on the surface of the medium, then a circular gradient of inhibition of the growth of the microfibrils would be established around this point. Recently, experiemntal evidence has confirmed definitively the presence of a soluble, transient, intermediate polymer of glucose in cellulose biosynthetis by *Acetobacter xylinum*.[12~16] It seems reasonable to assume that the intermediate polymer or an intermediate lipid-carbohydrate compound[17,18] plays an important role in the nucleation of spherulites.

Acknowledments

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