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Oxidation of Cellulose Microfibril Segments by Alkaline Silver Nitrate and Its Relation to the Fine Structure of Cellulose*

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Synopsis

The oxidation by alkaline silver nitrate of segments (ca 0.1 μ long) of cellulose microfibrils from *Avena* coleoptiles, ramie, bacteria, and *Valonia* has been followed by electron microscopy. For comparison, chitin microfibrils have been oxidized by the same reagent and observed by the same method. Oxidation proceeds equally at both ends of the segments, as judged by the rate of deposition of colloidal silver. As a consequence of oxidation, dissolution of the ordered array of 1→4β-polyglucosan chains (or poly-*N*-acetylglucosamine chains) takes place rapidly from both ends, due to Coulomb repulsion between ionized carboxyl groups and/or electrostriction of water molecules about the same groups. The observations are consistent with the assumption that the neighboring chains run in opposite directions in the cellulose microfibril (i.e., with the Meyer-Misch antiparallel postulate). They are not in agreement with expectations based upon the parallel arrangement.

INTRODUCTION

In spite of the prodigious amount of effort applied to the problem for more than a generation, the fine structure of native cellulose and especially the question of whether the 1 → 4β-polyglucosan chains are parallel or antiparallel (i.e., whether neighboring chains run in the same or opposite direction in the native cellulose microfibril) is still open to speculation. In contrast to its close relative, chitin, where a definitive answer has been obtained,[1,2] there is not enough information in the diagrams obtained from x-ray diffraction, electron diffraction, or polarized infrared investigations of native cellulose microfibrils to give an unequivocal result.[3]

However, two alternative approaches to the problem appear to be feasible. The first is to determine the distribution of glucose labeled with tritium, by means of autoradiography and electron microscopy, along the length of single microfibrils of bacterial cellulose. Because these microfibrils are known to grow only at their tips,[4] a parallel arrangement of the chains should be reflected in an asymmetrical distribution of isotope, since the two ends of the microfibril would then be different. Conversely, an antiparallel arrangement of the chains should be reflected in a symmetrical

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distribution. Unfortunately, extensive preliminary trials of the method showed that the presently available levels of radioactivity in tritium-labeled glucose (~500 mc./mmole) are too low by a power of ten to give significant numbers of silver grains in the very thin emulsions required.

The second approach, the results of which are reported here, is to try to determine if the two ends of segments of single cellulose microfibrils differ detectably in their rates of oxidation by alkaline silver nitrate.[8] If the fine structure of native cellulose microfibrils is that of a parallel array of 1 →4β-polyglucosan chains then the two ends of a segment of a microfibril will differ in the chemical groups exposed to attack by the reagent and therefore in the rate at which colloidal, metallic silver will be released at each end. On the other hand, an antiparallel array would mean that the chemical groups at both ends of the segment are the same and therefore would reduce silver ions to metallic silver with equal facility. Since the deposition of colloidal silver at the two ends of a cellulose microfibril segment may be compared by electron microscopy, the method provides a means for investigating the fine structure of the cellulose microfibril. Different rates of deposition of metallic silver at the two ends of a segment may be considered evidence for parallel arrangement of the 1→4β-polyglucosan chains in the microfibril. Conversely, equal rates may be presumptive evidence for the antiparallel arrangement.

EXPERIMENTAL

Cellulose Samples

Bacterial Cellulose. Pellicles composed of cells of *Acetobacter xylinum* enmeshed in a felt of extracellular cellulose microfibrils were grown under the conditions described by Hestrin and Schramm.[5] After removal from the medium, the pellicles were macerated in a Waring Blendor for 1 min. and as much as possible of the suspending liquid squeezed by hand from the clotted mass of cells and cellulose. The bacterial cells were then hydrolyzed by heating the dispersed mass for 10 min. at 100°C. in 4% NaOH. The remaining cellulose was centrifuged from the alkaline solution and washed in water until it was neutral. The mass of cellulose was then shredded and dried over $CaCl_2$.

***Valonia* Cellulose.** Segments of the cell wall of cells of *Valonia macrophysa* which had been preserved in formaldehyde were dispersed by sonication in a 10 Kcycle Raytheon magnetostriction apparatus. This preliminary treatment was necessary because trial experiments showed that sectioning of the intact whole cell wall by an ultramicrotome was difficult. After sonication, the dispersed material was washed with water, dehydrated and embedded in methacrylate.

Ramie. The ramie phloem fibers were ground in a Wiley mill to pass a 1 mm. screen, extracted with 2:1 benzene-ethanol and then dried under vacuum. Preliminary trials showed that, as with *Valonia*, the small pellets of ground, extracted, broken fibers were difficult to section. Accordingly,

this material too was dispersed by sonication in the same manner as with *Valonia* before embedding.

***Avena* Coleoptile Cellulose.** Samples of some of the pectinase extracted coleoptiles which had been prepared at an earlier date[6] were also used for this investigation. These samples contained only cellulose because the commercial so-called pectinase also contained hemicellulases.

Standard methods were used for dehydration, embedding and sectioning of the dispersed microfibrils in methacrylate.[7] After cutting of the sections, they were collected on carbon films deposited upon stainless steel grids. The polymerized methacrylate was removed by two extractions with ethylene dichloride. This treatment removes all noncellulosic supporting material from the microfibril segments which consequently fall and adhere to the carbon film in a random fashion. Good orientation of the microfibrils was only obtained with the pectinase-treated coleoptiles so the segments from this tissue were usually shortest and most uniform in length.

Chitin

This sample was kindly supplied by Dr. D. R. Whitaker of these laboratories and had been prepared by him from the pen of the Atlantic squid (*Illex illecobrosus*, Lesueur).

The analysis for this sample was as follows: C, 47.03%; H, 6.36%; N, 6.75%.

On the basis of fully acetylated poly-*N*-acetylglucosamine the calculated composition was: C, 47.29%; H, 6.45%; N, 6.89%.

Method for Alkaline Silver Nitrate Oxidation of Cellulose Microfibril Segments

The method used in this study is an adaptation of standard methods employed for detection of reducing compounds in paper chromatography.[8] The grids carrying the films with the cellulose microfibril segments were dipped in acetone-aqueous silver nitrate solution, immediately withdrawn, and air dried. After drying, which took only a few seconds at 25–30°C., the grids were dipped in the alkaline aqueous acetone solution and held there for the required period. Upon removal from the alkaline solution the grids were dipped at once in a solution of nearly saturated sodium thiosulfate to stop the reaction by removing any residual silver nitrate. The grids were then washed by dipping them five times in distilled water. Following the wash, the grids were air dried and examined in the electron microscope without further treatment.

Initial periods of oxidation were 5 min. or longer, but experience showed that this was much too drastic. The optimum period of oxidation varied with the source of the cellulose but was usually 1 min. or less.

Examination of the specimens was at 50 K.v., which was the lowest available.

RESULTS

The effect of oxidation of segments of *Avena* coleoptile microfibrils by alkaline silver nitrate is shown in Fig. 1. Segments which were not treated with silver nitrate (Fig. 1*a*) of course show uniform scattering along the length. Those which were treated while still embedded in the sections of methacrylate (Fig. 1*b*) are also uniform in electron scattering capacity along their length and show no preferential deposition of electron dense material at the tips. There is therefore no detectable tendency for non-specific physical adsorption of silver ions or atoms by the cut ends of the segments. However, oxidation of the segments for 1 min. by alkaline silver nitrate results in rapid deposition of colloidal silver, particularly at both tips of individual cut segments (Fig. 1*c*). This equal deposition of silver at both ends of the segments gives them a characteristic "dumbbell" shape (Fig. 1*d*) especially at high magnification. No tendency for deposition of silver at only one end of the cut segments was observed. Despite the increase in total mass, the edges of the segments became diffuse and vague while the diameter seemed to increase. For oxidation times beyond 1 min., blurring of the images of the segments became pronounced and after 2 min. oxidation the characteristic symmetrical deposition of silver at the tips was obscured. After 5 min. in cold alkaline silver nitrate, outlines of individual segments became indistinct, and only the files of microfibril segments from the layered cell wall structure[6] could be seen. The same result was obtained by oxidation in hot ammoniacal silver nitrate for 15 sec. (Fig. 1*e*).

These descriptions for different oxidation times refer only to the average appearance of the segments. As illustrated by Figure 1, in any particular field the appearance of the segments may vary from apparently untouched lengths to heavily oxidized dispersed material.

The same general pattern is shown in Figure 2 for oxidation of segments of ramie microfibrils. However, because the microfibrils could not be oriented before embedding (in contrast to the coleoptiles) individual segments are not as straight or as uniform in length. The microfibrils of ramie are also somewhat larger in diameter than those of *Avena* coleoptile. Individual segments of microfibrils show equal deposition of silver at both tips after 5–10 sec. of oxidation (Fig. 2*c*) followed by a rapid dispersal of material after 30 sec. Aside from the larger size of the segments, the major differences from coleoptile segments are the more rapid attack of the reagent and some evidence of uneven staining along the length of the oxidized segment (Fig. 2*b*). This uneven staining is similar to that for *Valonia* (Fig. 4). Since the microfibrils for both samples were dispersed sonically prior to embedding, this effect may be attributed to the rupture of a few chains on the surface of the microfibril by cavitation without breaking the whole microfibril. These small "lesions" on the periphery of the microfibril would then be points for attack by the reagent. Not all segments would show this effect nor would it be of equal intensity among different segments.

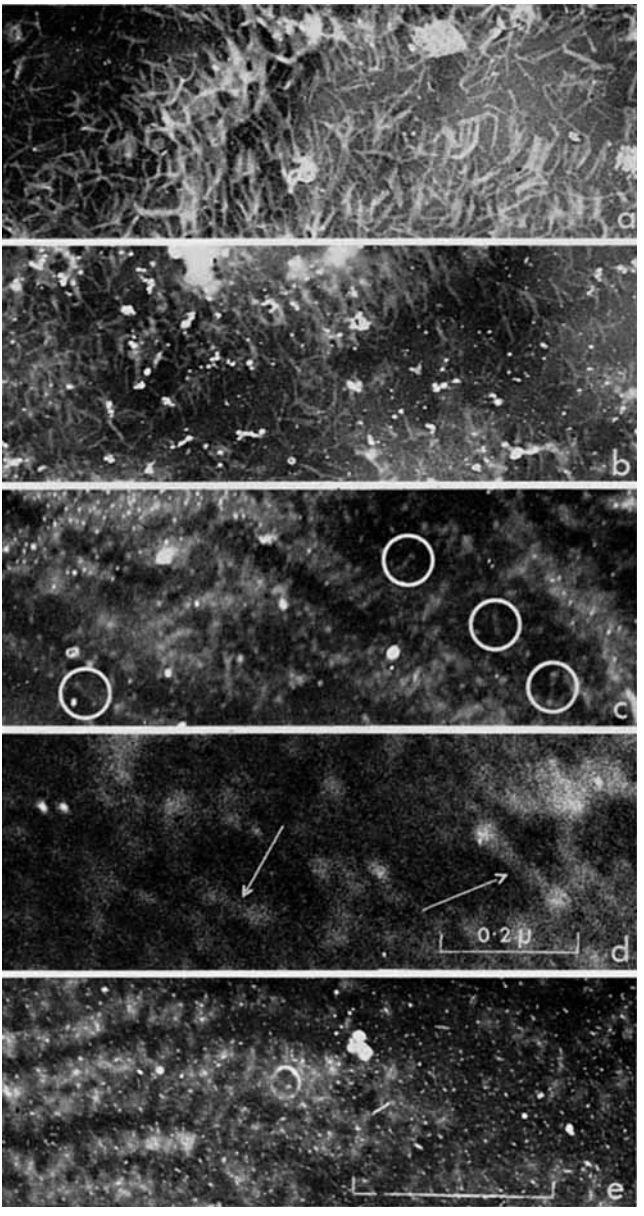

Fig. 1. Segments of microfibrils of cellulose from *Avena* coleoptiles which have been treated or oxidized by alkaline silver nitrate as follows: (*a*) unstained and unoxidized; (*b*) stained with aqueous silver nitrate before removal of methacrylate; (*c*) stained and oxidized with alkaline silver nitrate for 1 min.; (*d*) higher magnification of portion of *c;* (*e*) stained and oxidized for 15 sec. in hot ammoniacal silver nitrate. In this and subsequent figures, all scale lines represent 1 μ unless otherwise stated.

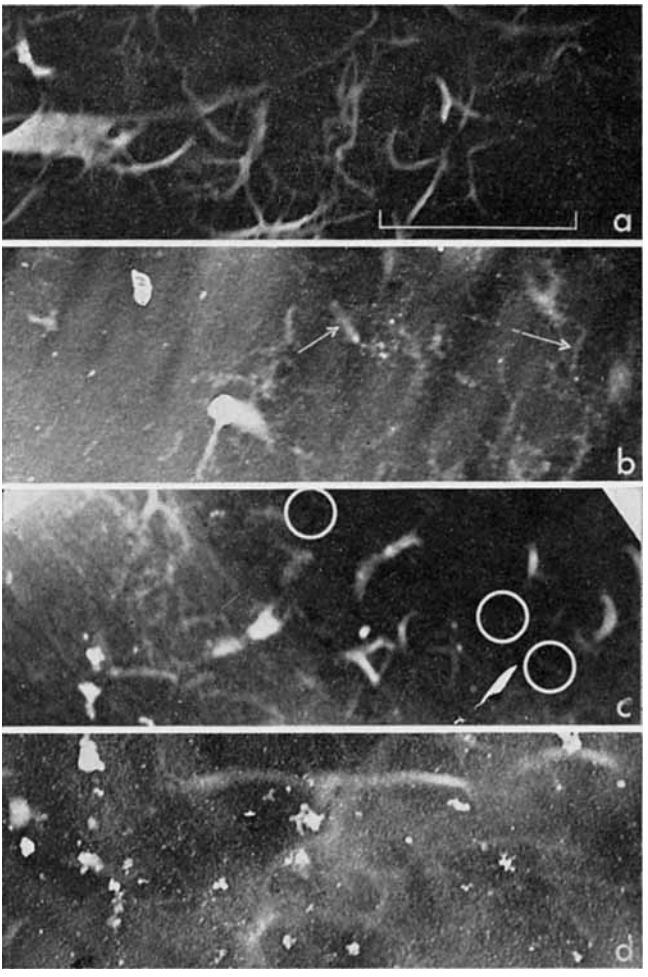

Fig. 2. Segments of sonicated ramie microfibrils treated as follows: (*a*) unstained and unoxidized; (*b*) stained and oxidized for 5 sec. (note the uneven staining along the length of two segments); (*c*) stained and oxidized for 10 sec.; (*d*) stained and oxidized for 30 sec.

Figure 3 shows the results of a sequence of oxidations of segments of bacterial cellulose microfibrils by alkaline silver nitrate. In contrast to coleoptile and ramie segments, localization of the silver at both tips is poor and indistinct. However, a restricted deposition of colloidal silver can be observed in this material and confirms the previously described pattern. After 30 sec. oxidation, swelling and dispersal of the component polyglucosan chains of the segments was extensive, and no pattern of deposition was observed (Fig. 3*d*).

Valonia microfibril segments, on the other hand, show the same pattern clearly (Fig. 4), perhaps because the cross section of the segment is larger. No deposition of silver is detectable at the tips of unoxidized segments or at

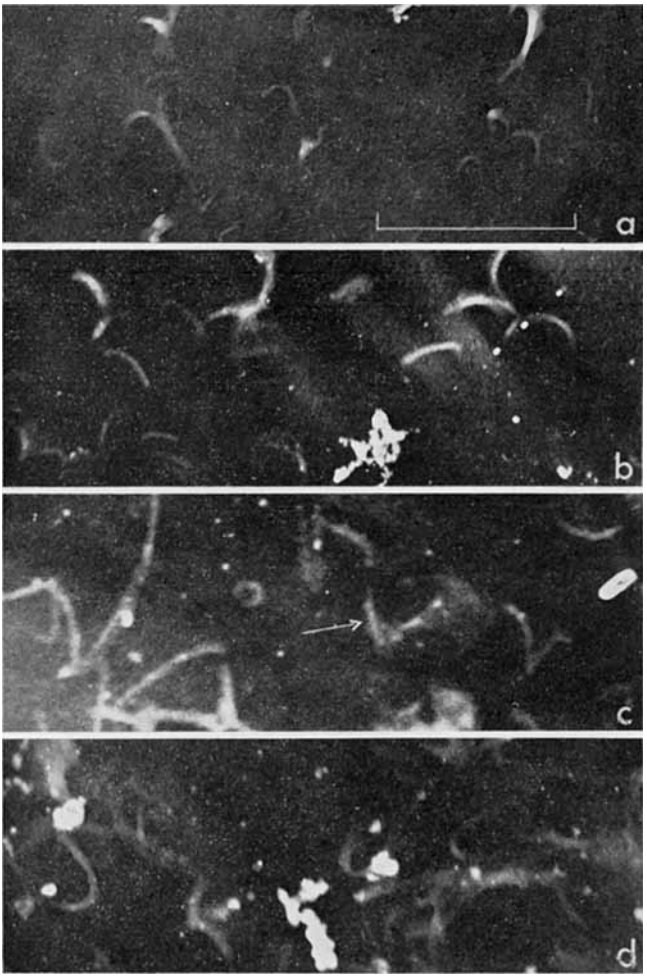

Fig. 3. Segments of bacterial cellulose microfibrils treated as follows: (*a*) unstained and unoxidized; (*b*) stained and oxidized for 5 sec.; (*c*) stained and oxidized for 10 sec.; (*d*) stained and oxidized for 30 sec.

the tips of segments which have been oxidized for only 15 sec. For segments oxidized for 1 min. equal symmetrical deposition of electron dense material, presumably silver, can be observed at both ends of cut segments. As noted above, these segments show uneven staining clearly (Fig. 4*e*).

Chitin is closely related to cellulose in composition and in morphology and has been shown to possess the antiparallel structure unequivocally by x-ray diffraction.[1,2] Oxidation of cut segments of chitin microfibrils therefore provides a useful check upon the results of oxidation of cellulose microfibril segments. Figure 5 shows the results of a time sequence for the oxidation of chitin segments and illustrates that when chitin microfibril segments are oxidized with alkaline silver nitrate, an equal deposit of colloidal

silver occurs at both ends of the segment. This symmetrical deposition is consistent with the known structure of chitin. An unexpected result, however, was the rapidity of the oxidation of chitin compared to that of cellu-

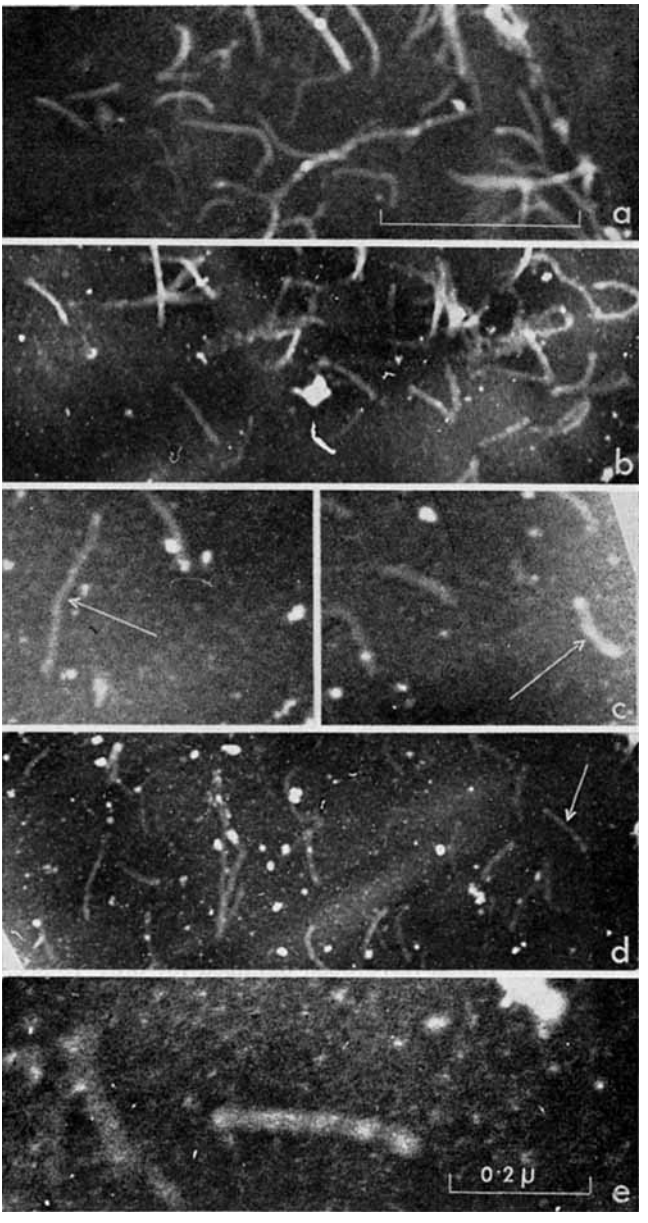

Fig. 4. Segments of microfibrils of *Valonia* treated as follows: (*a*) unstained and unoxidized; (*b*) stained and oxidized for 15 sec.; (*c*) stained and oxidized for 1 min.; (*d*) stained and oxidized for 1 min.; (*e*) higher magnification of portion of *d*.

lose. After 1 min. oxidation, under the same conditions as for cellulose, the outline of the microfibril segments had disappeared almost completely (Fig. 5*e*).

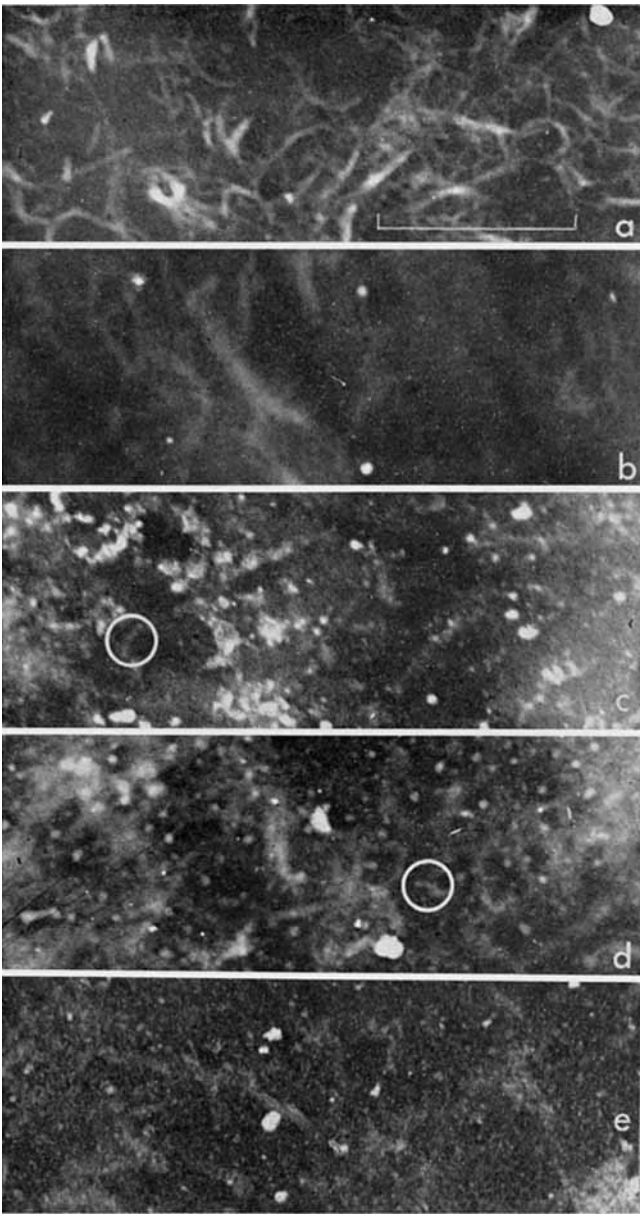

Fig. 5. Segments of chitin microfibrils treated as follows: (*a*) unoxidized and unstained; (*b*) stained and oxidized for 5 sec.; (*c*) stained and oxidized for 10 sec.; (*d*) stained and oxidized for 15 sec.; (*e*) stained and oxidized for 1 min.

DISCUSSION

The foregoing photographs establish clearly that alkaline silver nitrate will oxidize segments of cellulose microfibrils rapidly (i.e., within 1–5 min.). Since the visual effect of this reagent on chromatographic paper is well known, the above conclusion is not surprising, but it is significant that the oxidation seems to take place equally at both ends of a given cellulose microfibril segment. Within the limits of observation for four different kinds of cellulose, equal amounts of colloidal silver are deposited at both tips. Occasionally a segment may appear to have more electron scattering material at one end than at the other (Fig. 2*b*), but because these systems contain much free colloidal silver, the rare exceptions to the general rule may be attributed to chance superposition of an end and a silver aggregate.

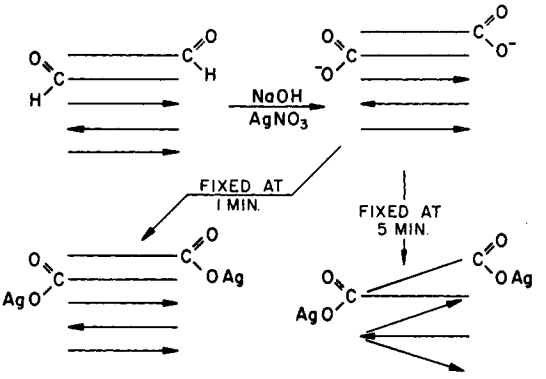

Fig. 6. A diagrammatic sketch of a possible mechanism of dispersal of the polyglucosan chains of cellulose microfibril segments when oxidized with alkaline silver nitrate. For simplicity, the hemiacetal groups are represented as free aldehydes. The above sketch does not include the colloidal silver aggregates which result from oxidation of the potential aldehyde groups and also contribute to electron scattering at the tips of the segments.

Accordingly, if both ends of a segment are oxidized at equal rates, then both ends are presumably similar in chemical composition. For straight chain orientation in the microfibril, only the antiparallel structure is consistent with this similarity in chemical properties of both ends and therefore these observations are further independent evidence for the Meyer-Misch postulate. This conclusion is confirmed by the similarity of the effects observed for both chitin and cellulose.

The preceding photographs show that during the course of the oxidation the outlines of the microfibril segment become indistinct, blurred, and finally disappear. The characteristic dumbbell shape of the partially oxidized segment is obscured leaving a nearly amorphous mass of electron dense material. An interpretation on the molecular level of these observations is given in Figure 6. The hemiacetal groups at the ends of the chains at the tip of the segment are quickly oxidized to carboxyl groups by the reagent. Since the sodium gegenions will not screen the charges com-

pletely, these closely packed ionized carboxyl groups will repel each other by Coulomb forces and, if given sufficient time, will disperse the previously associated polyglucose chains. In addition, formation of the carboxyl groups will increase hydration of the chains by electrostriction of water about these groups and this effect will also tend to separate the polyglucosans. Since the number of covalent crosslinks between such chains is small at most, the process of separation should go to completion quickly, leaving only an amorphous mass. In chitin, this dispersal process seems to take place even more rapidly than in cellulose, which may mean that the crosslinks between chains in chitin are weaker than in cellulose. During the dispersal process, no evidence for discrete, submicrofibrillar filaments was observed. If they exist in cellulose microfibrils, the more highly crystalline sub-filaments disperse at the same rate at the *para*-crystalline material, which seems rather improbable.

The foregoing observations demonstrate an unexpected difference in the rates of oxidation of microfibril segments from different species by alkaline silver nitrate at room temperature. Qualitatively, the rate of oxidation decreases in the following order: chitin, ramie, bacterial cellulose, *Avena* coleoptile, *Valonia*. Since the rate might be expected to be inversely correlated with the degree of crystallinity, the relative ease of oxidation of ramie segments is anomalous. At the present time, no interpretation of this anomaly is evident.

In addition to the differences between species, the present observations demonstrate a difference between individual segments of microfibrils from one species in the rate at which they are oxidized. Whether this difference is due to variations in the degree of crystallinity along the length of individual microfibrils,[9] to variations in the degree of crosslinking, or to the effect of the cut ends upon the properties of shorter microfibril segments is not known.

One criticism of the above analysis may be obviated in advance. During cutting of microfibril segments, it is not known whether individual polyglucosan chains are broken at the glucosidic bond or across the pyranose ring. If chains rupture only at the glucosidic link, then the groups exposed at the ends of the microfibril segment will be only the expected hemiacetals or hydroxyls. However, if some or all of the polyglucosan chains break across the ring, highly reactive fragments of glucose molecules may be exposed at both ends of the microfibril segment which would be capable of reducing silver ions. However, these fragments cannot be responsible for the overall observed symmetrical deposition because, as shown, oxidation of the segments (and therefore of the polyglucosan chains) is progressive along their length from both ends. Consequently, any easily oxidized fragments are quickly removed, leaving only the ends of the polyglucosan chains with their original structure to be affected by the reagent. For this reason, the equal rates of reaction at both ends of a microfibril segment must reflect an intrinsic similarity in their chemical properties in addition to a possible transient similarity imposed by the conditions of cutting.

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Résumé

L'oxydation par le nitrate d'argent alcalin de segments (environ 0.1 μ de long) de microfibrilles de cellulose provenant de coleoptiles *Avena*, de ramie, de bactéries et de *Valonées* a été suivie par une étude au microscope électronique. A des fins de comparaison des microfibrilles chitines ont été oxydées par le même réactif et étudiées de la même façon. L'oxydation se produit également aux deux extrémités des segments comme on peut en juger par le taux de déposition de l'argent colloidal. Comme conséquence de l'oxydation la dissolution de la série ordonnée des chaînes 1→4β de polyglucosane (ou des chaînes poly *N*-acetyl de glucosamine) a lieu rapidement aux deux extrémités par suite de la répulsion Coulomb entre les groupes carboxyles ionisés et/ou l'électrostriction des molécules d'eau aux abords des mêmes groupes. Les observations concordent avec l'hypothèse que les chaînes avoisinantes vont dans des directions opposées dans la microfibrille de cellulose (par exemple, avec le postulat antiparallèle Meyer-Misch). Elles ne concordent pas, cependant, avec les hypothèses fondées sur la disposition parallèle.

Zusammenfassung

Die Oxydation von ca. 0,1 μ langen Abschnitten von Zellulosemikrofasern aus *Avena* Coleoptrilen, Ramie, Bakterien und *Valonia* durch alkalisches Silbernitrat wurde mittels Übermikroskopie verfolgt. Zum Vergleich wurden Chitinmikrofasern durch dasselbe Reagens oxydiert und mittels desselben Verfahrens beobachtet. Nach der Abscheidungsgeschwindigkeit des kolloidalen Silbers zu urteilen, ist der Verlauf der Oxydation an den beiden Enden eines Abschnitts gleich. Als Folge der Oxydation kommt es zur raschen Auflösung der geordneten Reihen von 1→4β-Polyglukosanketten (oder Poly-*N*-Acetylglukosaminketten) von beiden Enden aus, was auf die elektrostatische Abstonung zwischen ionisierten Carboxylgruppen und/oder auf Elektrostriktion von Wassermolekülen um dieselben Gruppen herum zurückzuführen ist. Diese Beobachtungen stimmen mit der Annahme überein, dass benachbarte Ketten in der Zellulosemikrofaser in entgegengesetzten Richtungen verlaufen (d.h. mit der Meyer-Misch Antiparallelbedingung), nicht aber mit den Folgerungen aus einer parallelen Anordnung.