CONSTITUTION, SUBMICROSCOPIC STRUCTURE AND DEGREE OF CRYSTALLINITY OF THE CELL WALL OF *HALICYSTIS OSTERHOUTII*

by

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INTRODUCTION

In 1936 VAN ITERSON[3] studied the cell wall of a green unicellular marine alga, *Halicystis Osterhoutii*, obtained from the Marine Biological station at Bermuda. He stated that it consisted mainly of "amyloid", since it coloured blue with a not too dilute solution of IKI, even after prolonged treatment with cuprammonium. True cellulose and polyuronides were practically absent. In swollen sections up to 60 lamellae were observed.

Two years later SISSON[6] revealed that the X-ray ring-diagrams of three *Halicystis*-species resembled that of regenerated cellulose (cell. II). This "cellulose" was oriented at random, except for the 101 crystal plane, which was preferentially oriented parallel to the surface of the cell wall. This is not unusual with films of regenerated or native cellulose. However, in addition there was a non-cellulose interference from a 12.5 A interplanar spacing likewise oriented tangentially. Recently cellulose II or a derivative of it has been found in the cell wall of several other algae[5].

Native cellulose (cell. I) is more crystalline than cellulose II. The former invariably consists of microfibrils of rather uniform thickness, whereas regenerated cellulose is non-fibrillar, although fibril-like threads of varying thickness may be produced by mechanical or ultrasonic disintegration of rayon fibres.

We wondered whether these differences were due to the different way of formation, or were inherent to the different crystallographic forms. In the first case the *Halicystis*-cellulose might be expected to be as highly crystalline as native cellulose and moreover fibrillar; in the second case, moderately crystalline and perhaps non-fibrillar. Therefore we decided to determine the degree of crystallinity with the X-ray method of HERMANS AND WEIDINGER[2] and to prepare electron micrographs.

We found a moderate crystallinity and a fibrillar structure, but chemical analysis compelled us to assume that the main constituent of the cell wall is of hemicellulose nature. Therefore, the question which induced our investigation remained unanswered.

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MATERIAL

We had at our disposal only about 100 mg dry material and some cells preserved with methanal, both being kindly furnished by Prof. VAN ITERSON. This lack of material seriously hampered our work, especially the chemical analysis. In vain we tried to obtain additional material from Naples or Bermuda.

MICROSCOPIC OBSERVATIONS

Some cross-sections of about 5 μ thickness were embedded in methyl benzoate and studied between crossed nicols. As demonstrated by Fig. 1, there were three optically different layers. The higher refractive index is radial in the thick central layer and tangential in the thin outer ones.

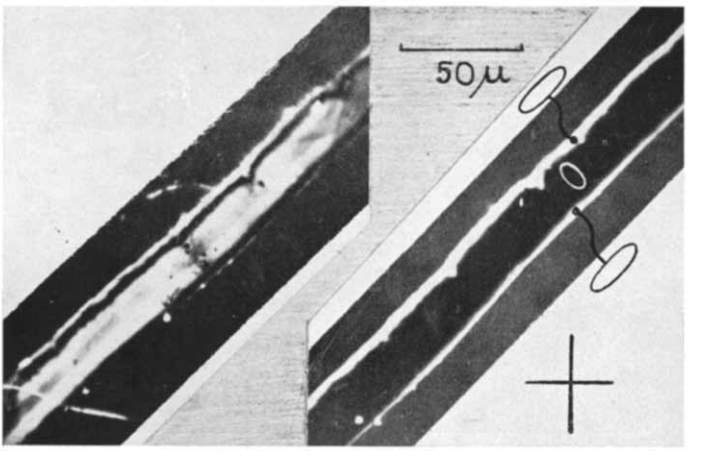

Fig. 1. Section of dry *Halicystis* cell wall, embedded in methyl benzoate and observed with crossed nicols, without (left) and with (right) compensation of the double refraction in the central layer. Vibration planes and direction of n_γ indicated.

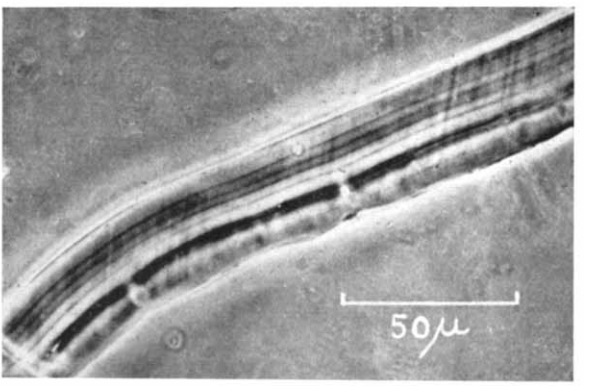

Fig. 2. Section of *Halicystis* cell wall, mounted in water and observed with a phase contrast microscope.

The whole section stained with chlor-zinc-iodide and with congo red. The dichroism confirmed a tangential orientation of the stained cell wall constituent(s) in the outer

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and a radial orientation in the central layer. Radial orientation is very unusual in cell walls. Protein reactions were negative and ruthenium red stained faintly.

The lamellar structure, already described by VAN ITERSON, was beautifully revealed in a Leitz phase contrast microscope, even without swelling, but the number of visible lamellae was very much increased after swelling in diluted cuprammonium. See Figs. 2 and 3. In concentrated cuprammonium the whole section disappeared as also pieces of cell wall do.

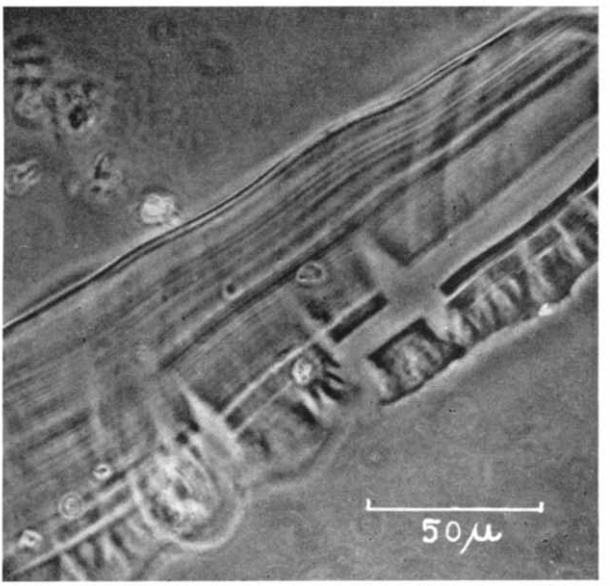

Fig. 3. Section of *Halicystis* cell wall, mounted in diluted cuprammonium and observed with a phase contrast microscope.

ELECTRON MICROGRAPHS

The methanal-preserved material was used for this part of the investigation. The cell wall of *Halicystis* is too thick for direct examination. By tearing it, either before or after treatment with hot alkali or chromic acid, or by scraping a piece of cell wall, previously glued onto a slide, we in vain tried to obtain lamellae thin enough for direct observation.

Therefore we resorted to a replica technique, which could show details of less than 100 A[1]. To prevent an interchange of the inside and the outside of the cell wall, special measures were taken (cotton thread drawn through the cell before dissection). After washing, pieces of the cell were left to dry on a glass slide and the edges were fastened with small strips of Scotch tape.

After shadowing with Pt the specimen was coated with a "carbon" skin, formed by polymerised fission products of an aromatic hydrocarbon, *e.g.* toluene in a glow discharge[4]. For that purpose the specimen on the slide was placed in a vacuum jar on a metal support acting as a positive electrode. At a distance of some cm above the specimen, the other electrode was mounted. The vacuum jar was filled with toluene

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vapour of a pressure of 0.01 mm Hg. Next, a tension of 2 kV was put on the electrodes for about 30 sec, the current density being 0.5 mA/sq. cm. In this way a film of about 600 A was formed on the specimen.

In the glow discharge the toluene molecules are disrupted, forming small molecules, ions and radicals such as CH, C_2, C, C^+, H and H_2. These fragments recombine and form a film on the anode and on the objects lying on it. Such a film is insoluble in all known solvents; the transparency for electrons is very high.

The thus created triple layer of cell wall, platinum and "carbon", was cut into portions of the size of a specimen grid and brought upon the surface of a cuprammonium solution, the "cellulose" side downwards. The cell wall material dissolved and the replica

Fig. 4. Electron micrograph of a replica of the exterior of the untreated *Halicystis* cell wall.

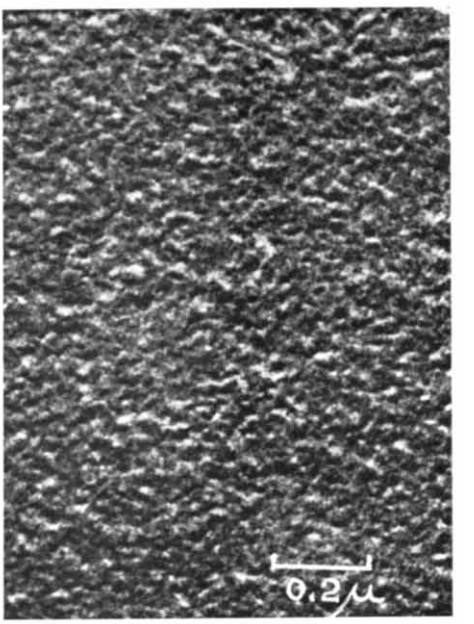

Fig. 5. Electron micrograph of a replica of the interior of the untreated *Halicystis* cell wall.

floated on the solution. After rinsing with distilled water for half an hour, it was mounted on a specimen grid, ready for use in the electron microscope.

Because of the much higher contrasting effect of the heavy atoms of the platinum with regard to the light carbon atoms of the "carbon" film, the image formed in the microscope may be considered to be caused by the platinum layer only. Thus a positive replica is obtained, giving details as occurring on the original specimen.

The electron micrograph of Fig. 4 shows that the exterior of the untreated cell wall has a granular surface without special structure. Probably some kind of incrusting material is present.

As apparent in Fig. 5, the inner side shows a network of microfibrils, oriented at random. The fibrils have a diameter of about 80 A.

On the (wrong) assumption that the cell wall consisted mainly of cellulose II, and wanting to obtain electron micrographs of cleaned cell walls, a cell was cut in two and

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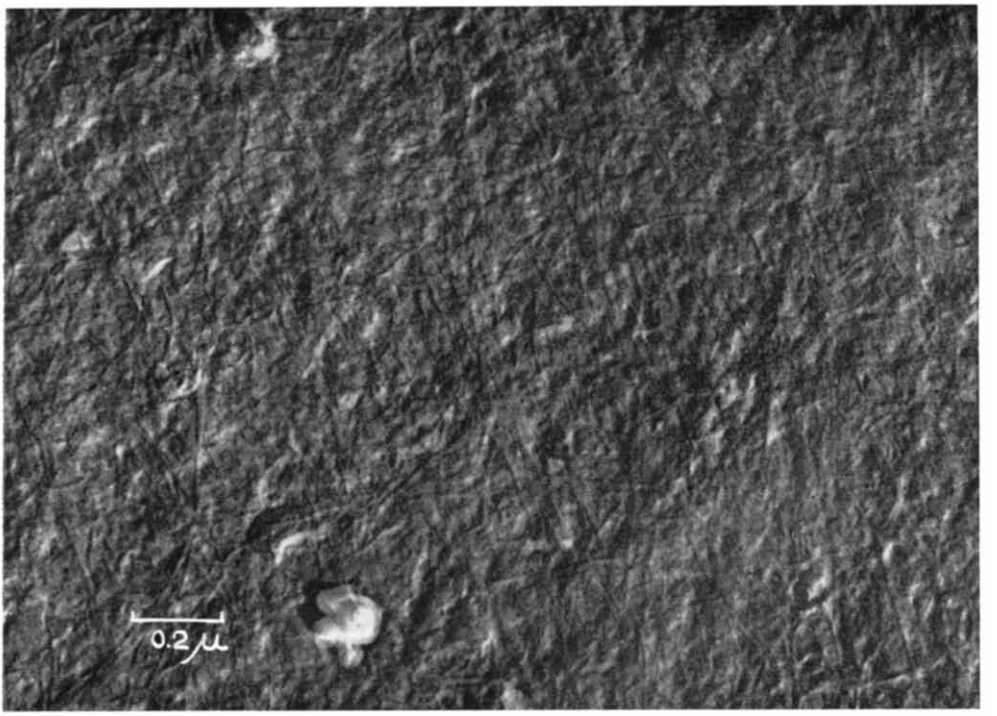

Fig. 6. Electron micrograph of a replica of the exterior of *Halicystis* cell wall which had been treated with hot dilute alkali.

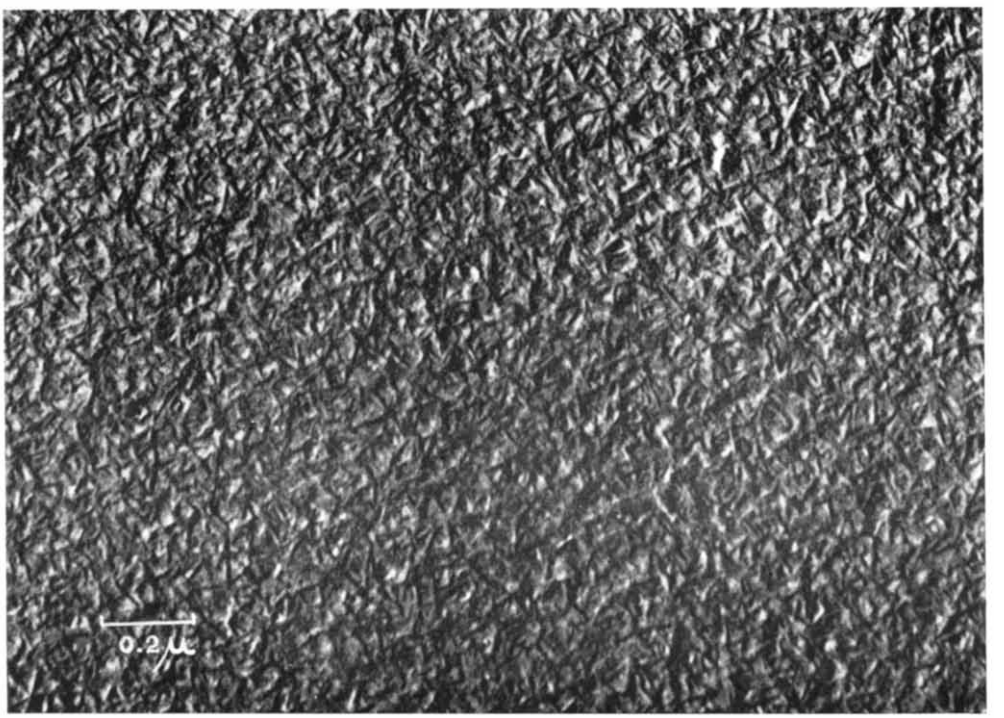

Fig. 7. Electron micrograph of a replica of the interior of *Halicystis* cell wall which had been treated with hot dilute alkali.

treated with 2% NaOH at 100° C for 2 hrs. This treatment reduced the dry weight by 60%.

As visible in Figs. 6 and 7, a fibrillar network now is present both on the outside and on the inside. On the outside the fibrils are longer and they seem to be embedded in non-fibrillar material, which might have been present originally, but might also have been produced by disintegration as a result of the alkali treatment. On the inside the fibrils are short, or may be shortened by the treatment.

X-RAY DIAGRAM AND DEGREE OF CRYSTALLINITY

A. *Character of the diagram.* The ring diagram of the untreated cell wall stuffed into the specimen holder of an Astbury X-ray camera is similar to a rayon-diagram with the exception of the curious interference corresponding to a 13.0 A spacing (see Fig. 8, I and II). The other interferences correspond closely to the 002, $10\bar{1}$- and 101-reflections of rayon. This has been controlled by taking diagrams with monochromatic focussed radiation in the multiple Guinier camera after DE WOLFF. Compared with the cellulose-diagram, however, the intensity of the 101 interference seems somewhat reduced and that of the $10\bar{1}$ interference slightly increased.

The spacing of the 13.0 A interference varied between 12.4 A in sharply dried and 13.8 A in wet cell wall material. It did stay after heating in 2% NaOH at 100° C for 2 h (Fig. 8, III) or after boiling for 24 h with water and treatment for 18 h at 35° C with a pancreatic enzyme solution (Fig. 8, IV). As mentioned, the alkali treatment caused a loss of 60%, but the "cellulose"-part of the diagram cleared up only a little, whereas the 13.0 A interference was markedly intensified (compare Fig. 9, I and II).

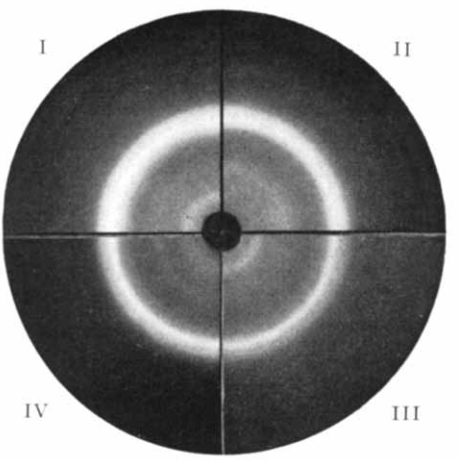

Fig. 8. Powder X-ray diagrams of:
I. Untreated *Halicystis* cell wall.
II. Viscose rayon fibres.
III. *Halicystis* cell wall treated with hot dilute alkali.
IV. *Halicystis* cell wall boiled with water and treated with a solution of pancreatic enzymes.
Quadrants I and II occur on the same film, III and IV on films which have been exposed somewhat shorter. Pinhole 0.5 × 40 mm; distance specimen-film 40 mm; Cu K radiation.

B. *Degree of crystallinity*

For a description of the method used, the reader is referred to HERMANS AND WEIDINGER's publication[2]. To enable the preparation of the 3 mg standard pellets, the cell wall was first cut into "fibres" of 20 μ thickness with a microtome. Four and three determinations were made with enzyme-treated and alkali-treated cell wall material, respectively, with rayon as a reference. One photometer curve of each is shown in Fig. 9.

Assuming a 40% crystallinity in rayon, the degrees of crystallinity of the cell wall samples were calculated and plotted in the nomogram of Fig. 10. In view of the limited reliability of such determinations, the figures must be considered equal and roughly 40%, both *in treated and untreated cell wall material.* The intensification of the 13.0 A interference following the alkali treatment (see Fig. 9, II),

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although marked, appears still to be too small to modify the total degree of crystallinity.

As far as we can see, the disappearance of 60% dry weight during alkali treatment without marked change in the "cellulose"-part of the diagram, nor in the degree of crystallinity, but accompanied by intensification of the 13.0 A interference, can only be explained by assuming that:

1. there is an alkali-resistant non-cellulose minor constituent causing the 13.0 A interference;

2. the "cellulose"-part of the diagram is in all probability due to a major cell wall constituent, which is moderately crystalline like regenerated cellulose and also resembles it in other respects, but gradually dissolves in hot dilute alkali.

Finely ground viscose-rayon loses much less weight during similar alkali treatment and moreover withstands boiling with 2% HCl better than *Halicystis*-cell wall material which rapidly dissolves. This difference in resistance at first was thought to be due to a very low DP of the *Halicystis* cellulose II, but according to the chemical analysis (see next section), it seems to be a hemi-cellulose.

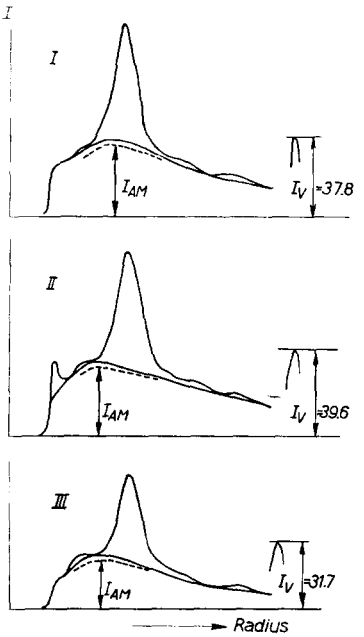

Fig. 9. Photometer curves of powder X-ray diagrams and of reference substance (Iv.).
I. *Halicystis* cell wall boiled with water and treated with pancreatic enzyme solution.
II. *Halicystis* cell wall treated with hot diluted alkali.
III. Viscose rayon fibres.

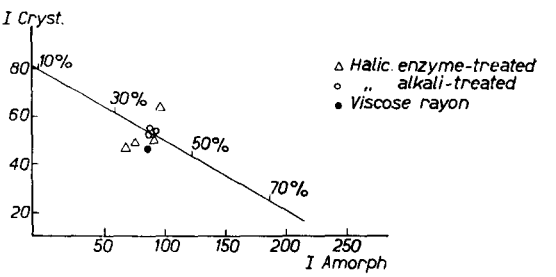

Fig. 10. Approximate degrees of crystallinity in *Halicystis* cell wall as compared with rayon.

If there were not one, but two or more quantitatively important cell wall constituents in addition to the alkali-resistant one, then their alkali-solubilities would very probably be different and consequently some marked change in the "cellulose"-part of the diagram or in the degree of crystallinity would have been caused by the alkali treatment.

PRELIMINARY CHEMICAL ANALYSIS

Owing to lack of material, only a qualitative determination of the sugars in the main cell wall constituent was possible. About 3 mg of untreated or alkali-treated cell wall material was kept in 1 ml 72% sulfuric acid at 20° C for 3 days. The solution was diluted to 10 ml and boiled for 8 h. After neutralising with $BaCO_3$, filtering and concentrating, paper chromatograms were prepared with butanol-acetic-acid-water as running liquid and aniline phthalate as spray.

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To our astonishment, all showed the presence of *xylose and glucose in about 1:2 proportion, both before and after alkali treatment.* With ninhydrin spray traces of amino acids showed up. Very probably a xyloglucan is the main cell wall constituent, for if these sugars were in separate polymers, some marked change in proportion might be expected after alkali treatment. This is in line with the results of the X-ray work. As is well known, several hemicelluloses stain blue with IKI and dissolve in cuprammonium.

It is hoped that others will be able to check and extend this preliminary analysis.

CONCLUSIONS

The microscopic observations reveal a poly-lamellar cell wall, which readily dissolves in cuprammonium and is stained dichroitically with IKI, chlor-zinc-iodide and congo red. Double refraction and dichroism indicate that the molecules of the stained birefringent constituent(s) are radially oriented in the central layer and tangentially in the outer ones.

The preliminary chemical analysis suggests that the main constituent is not cellulose II, but a xylo-glucan. The fact that the ratio of xylose to glucose does not seem to change after 60% of the cell wall material is dissolved in hot dilute alkali does not suggest the presence of separate xylan and cellulose II, for, usually, pentosan is much more alkali-soluble than cellulose II. However, it is not impossible that some other glucan and a xylan, *both moderately alkali-soluble,* are present.

The X-ray investigations suggest the occurrence of an alkali-insoluble non-cellulose minor cell wall constituent with a characteristic 13.0 A interplanar spacing, which somewhat varies with different moisture content.

In addition, a moderately alkali-soluble main cell wall constituent is present, or there might be more constituents, but in that case these must equal alkali-solubility. This main cell wall constituent (or one of the constituents) produces X-ray interferences which are quite similar to the 101, $10\bar{1}$ and 002 ring-reflections of cellulose II, although there seem to exist differences in relative intensity. The mean degree of crystallinity of the cell wall is of the same magnitude as that of cellulose II.

The E.M.-graphs of the untreated cell wall reveal a non-fibrillar, granular surface on the exterior and a fibrillar network on the inside. After the—rather destructive—alkali treatment, fibrils are to be seen on both sides, but those on the inside are shorter. Comparison with the untreated cell wall suggests the possibility that the fibrils on the inside might have been fragmented by the alkali treatment. The abundance of microfibrils suggests that the main cell wall constituent is fibrillar, but the alkali-resistant constituent might be fibrillar too. The occurrence of two types of fibrils in the treated cell wall is in line with that possibility.

ACKNOWLEDGEMENTS

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SUMMARY

The poly-lamellar cell wall of *Halicystis Osterhoutii* very probably contains a xylo-glucan as major constituent. Its X-ray ring diagram closely resembles that of regenerated cellulose and it also indicates the same moderate degree of crystallinity. In addition, an unknown alkali-resistant non-cellulose minor cell wall constituent with a 13.0 A crystal plane spacing is present. Electron micrographs reveal that the major constituent or both are fibrillar.

RÉSUMÉ

La paroi cellulaire polylamellaire de *Halicystis Osterhoutii* contient très probablement un xyloglucane comme constituent principal. Son diagramme de rayons-X ressemble de près à celui de la cellulose régénérée et il indique le même degré modéré de cristallinité. La paroi contient en plus un constituant secondaire inconnu, résistant à l'alcali et non-cellulosique avec une distance réticulaire de 13.0 A. Des micrographies électroniques font voir que soit le constituant principal, soit les deux constituants sont fibrillaires.

ZUSAMMENFASSUNG

Die polylamellare Zellwand von *Halicystis Osterhoutii* enthält sehr wahrscheinlich als Hauptbestandteil ein Xyloglucan. Das Röntgen-Ringdiagramm ist dem von regenerierter Cellulose sehr ähnlich und zeigt ebenfalls den gleichen mittleren Kristallinität. Ausserdem ist noch ein unbekannter alkaliresistenter, nicht aus Cellulose bestehender Zellwandnebenbestandteil mit einem Kristallnetzebenenabstand von 13.0 A vorhanden. Elektronenmikroskopische Aufnahmen zeigen, dass der Hauptbestandteil oder beide Bestandteile fibrillär sind.

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