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Preparation of Polysaccharide Solutions for Electron Microscope Investigations of Supermolecular Morphologic Formations of the Solid Component

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Synopsis

A new preparation process, which preserves the original morphological state of polysaccharides in solution during removal of the solvent and so makes possible direct electron microscope investigations, has been developed. The process consists of lyophylization of the sample (in very low concentration of water solution) by freeze-drying, modified for the purpose of electron-microscopic examination. Water-soluble mixture of the polysaccharides of hemicelluloses isolated from the wood tissues of beech and the water solution of the mannan from *Saccharomyces cerevisieae* treated by means of the new preparation process (preparation with freeze-drying) and, for comparison, these same samples treated without the application of freeze-drying (by means of drying at laboratory temperature and atmospheric pressure, standard preparation process) are introduced as instance from many polysaccharides electron microscopic examined after this preparation process. This method allows one to make direct electron microscope investigations of the water-soluble polysaccharides in no way stained or otherwise shadowed with good resolution of details in the supermolecular range.

Introduction

Hitherto the usual laboratory equipment has excluded the possibility of direct electron microscope observations of solid substances dissolved in solution. The preparation processes for removal of the solvent in electron microscope preparations have caused secondary agglomerations of the solid component, so that the original morphological arrangement of the solid in solution was not well preserved, and the data thus obtained were of but little importance.

Attempts at fixating the solid component in the solution for electron microscope studies have been made by Ruscher and Zuchold,[1] Audsley and Fursey,[2] Claesson and his co-workers,[3] Thürkauf and his co-workers,[4] and others. Essentially they have involved freeze-drying of the sample. This is the well-known principle of lyophilization used in various other fields of science. The method prevents, among other things, agglomeration of the sample's solid component upon removal of the liquid and improves the electron microscope resolution of supermolecular morphological details. Particular ways of realizing this method are sometimes very different.

The differences arise in the specific requirements which in this case, for instance, differ considerably from those in the preparation of larger samples.

Some workers use, as part of the process, atomization for dispersion of the sample into small volumes. In this treatment, however, the isolated small volumes then contain a rather small amount of the solid to be observed. Moreover, the unfavorable effect of surface tension can become perceptible under certain circumstances. Surface forces in the liquid effect an irregular distribution of the solid substance, the distribution being different on the surface and in the center of a small drop of the liquid in cases of surface-active substances and solutions of very low concentration. Lyophilization for the electron microscope usually involves very low concentrations of the substance in solution, and when the solution is freely atomized, the dissolved substance is apt to be concentrated in the centers of the isolated drops and so be in a condition favorable for agglomeration. Thus the substance is not uniformly dispersed in the solution.

The method for dispersing the solution of a solid substance before employing lyophilization seems, therefore, to be one of several main factors in the correct results. To decide whether the picture obtained by lyophilization is correct, it is necessary to take into account all the circumstances and conditions of preparation of the solution and, above all, to compare the lyophilized preparation with an unlyophilized one. Indeed, it is not possible to define a uniform morphological design for lyophilized different organic compounds in the domain of their supermolecular formations. It is, for instance, impossible to assert that in general fibrous appearance is the correct pattern of the preparation or that it is, on the contrary, an artifact.

We assume that the following ensure good fixation of the macromolecules in the morphological state in which they were before removal of the solvent: (*1*) proper spreading of the solution into a thin layer, limiting the effect of surface forces in the solvent on the distribution of the solid substance in the solution and (*2*) quick freezing of the thin layer and subsequent, almost instantaneous, evacuation of the external atmosphere around the sample.

It is advantageous to stabilize the lyophilized preparation by metal shadowing and carbon coating after removing the solvent and before moving the preparation into a normal laboratory atmosphere but, according to our experimental experience, this seems to be unnecessary. A special stabilization of the preparation, however, is indispensable for the protection of objects sensitive to electron beams.

Experimental Methods and Results

We used in our experiments the lyophilization process, carried out with the equipment shown in Figure 1. A small amount (about 1.10^{-4} cm.^{3}) of the solution of the sample was pipetted on the carbon supporting film on the grid. The solution was spread out by laying a thin, perfectly pure glass plate on the drop. Then the preparation (grid with supporting film, thin layer of solution, and glass plate) was contact-exposed to the instantaneous

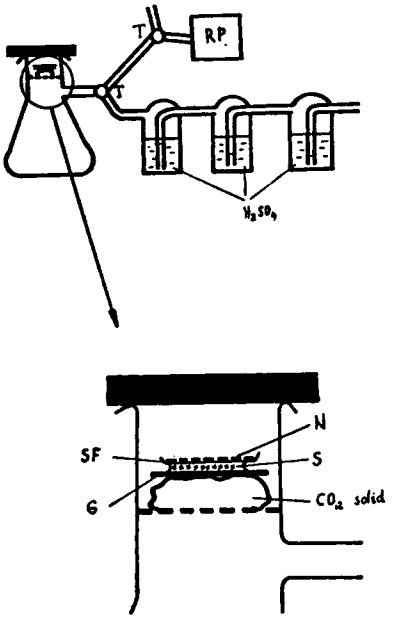

Fig. 1. Apparatus used for preparation of polysaccharide solutions for electron microscope observation: RP, rotary pump; N, grid; S, sample; G, glass template; SF, supporting film.

effect of low temperature (−78.5°C.) and of a vacuum of 10^{-2} torr, which was reached within 30 sec. After removal of the solvent by freeze-sublimation under uninterrupted vacuum 10^{-2} torr the sample was brought to laboratory temperature within 25–30 min. without interruption of the vacuum and then the vacuum was interrupted and the sample was slowly transferred to normal atmospheric pressure and laboratory moisture with partly predried air. Next the preparation was examined under an optical microscope for integrity of the supporting film and absence of larger impurities. The preparations for most of the observations were in no way contrasted (e.g., by means of metal shadowing). Sporadic groups of preparations which we subjected to gold–palladium shadowing have not shown any essential elucidation of the morphological details of the samples. Our practice has shown also that this method of fixation does not require "reinforcement" of the lyophilized condition of the sample. There is a certain disadvantage in that part of the preparation is mechanically damaged upon separation of the glass plate from the supporting film, between which the sample is placed. Any impurities or formations of artificial origin in the supporting carbon film before lyophilization may be distinguished from the object by, for example, the method of Audsley and Fursey.[2] This method consists of double-shadowing of the preparation in two mutually perpendicular directions before freeze-drying and after it; the impurities or artificial formations in the supporting film may be dis-

tinguished from the sample by their double shadow, the sample casting only one shadow.

One of many test objects of our experiments was a mixture of polysaccharides of hemicelluloses isolated from the wood tissues of beech. The polysaccharides were dissolved in distilled water at increasing temperatures

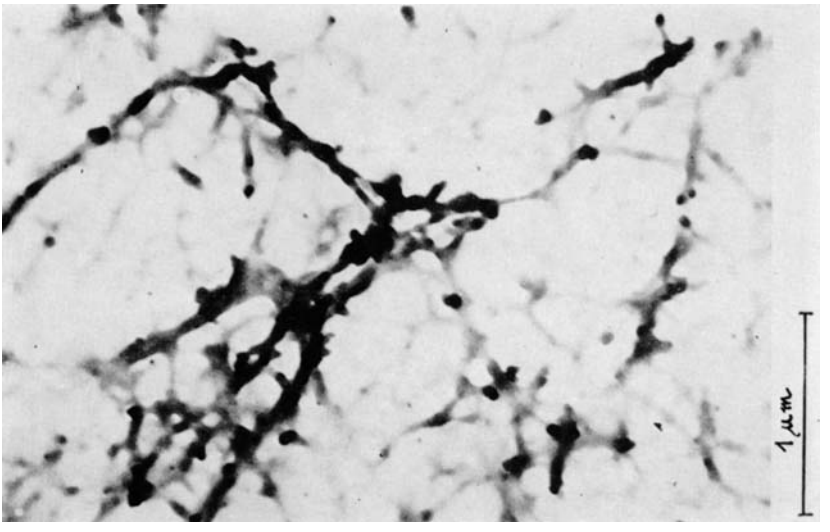

Fig. 2. Electron micrograph of polysaccharide mixtures from beech hemicelluloses treated by freeze-drying according to the new method. In no way was the sample stained or otherwise shadowed.

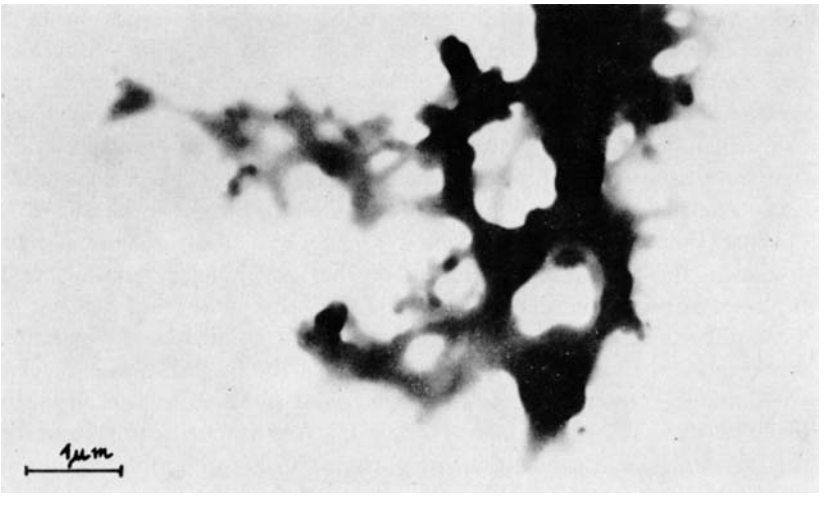

Fig. 3. Electron micrograph of same material prepared under same conditions as in Fig. 2, except that freeze-drying was not applied; sample was dried at laboratory temperature and atmospheric pressure only. In no way was the sample stained or otherwise shadowed.

of 75–80°C. The hemicelluloses were isolated by means of 5% NaOH at laboratory temperature from sap that had been extracted from beech sawdust with a 1:1 mixture of alcohol and benzene. The filtrate was separated on fritted glass by being washed with hot water; it was then

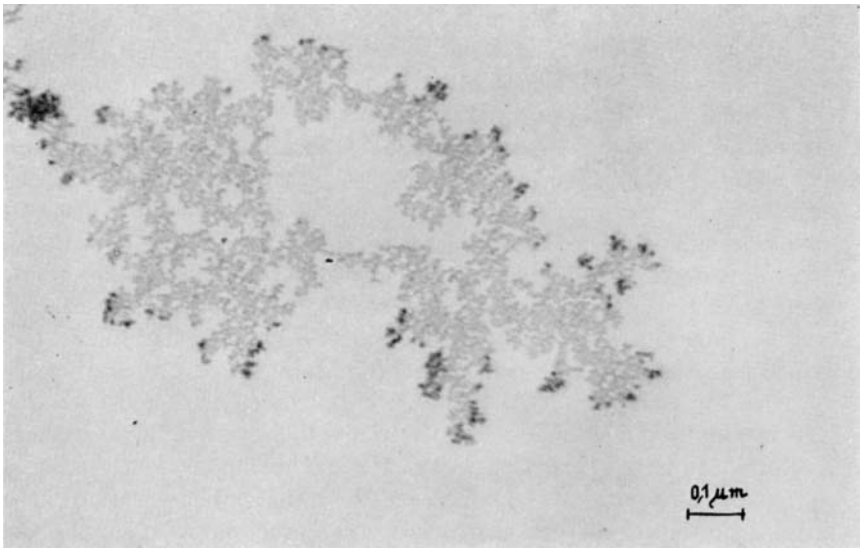

Fig. 4. Electron micrograph of mannan from *Saccharomyces cerevisieae,* treated by freeze-drying according to the new method. In no way was the sample stained or otherwise shadowed.

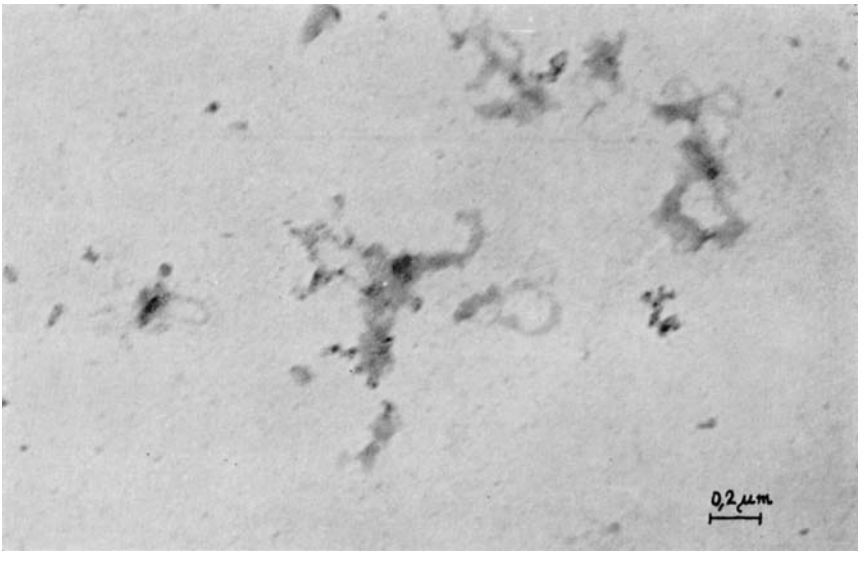

Fig. 5. Electron micrograph of same material prepared under same conditions as in Fig. 4, except that freeze-drying was not applied; sample was dried at laboratory temperature and atmospheric pressure only. In no way was the sample stained or otherwise shadowed.

neutralized and acidified with glacial acetic acid to pH 6. Precipitation was carried out with a fivefold volume excess of ethanol (96%), and the precipitate thus formed was separated on fritted glass, washed with ether, and dried under vacuum at about 45°C. According to qualitative chromatographic analysis of the acid hydrolysate of a portion of the sample, the hemicelluloses contained mainly xylose. The other monosaccharides usually found in beech hemicelluloses were present in small amounts, including 4-*O*-methyl-D-glucuronic acid. The whole mixture of polysaccharides without fractionation was used for the experiments. Figure 2 characterizes the morphological pattern of this mixture. The picture is one of more than a hundred electron micrographs of similar appearance that were obtained from several preparations of the same material. The initial concentration of the sample in water was $5 \times 10^{-1}\%$. When freeze-drying of the solvent was substituted for evaporation of the solvent at laboratory temperature and atmospheric pressure, a different picture, of considerably agglomerated particles of the solid substance, was obtained; see Figure 3. This pattern of the final state of the preparation was very much the same whether the mixture of polysaccharides had been dissolved in a hydrophilic liquid or dispersed in a hydrophobic liquid, such as chloroform, the initial concentrations of the solid component in the liquid being equal in all cases. It seems that agglomeration or conservation of the original condition of dispersion depends on the method of removing the liquid from the sample. If the sample is not freeze-dried, the morphological pattern of the solid component after removal of the liquid is not essentially influenced by the lyophilic or lyophobic nature of the solid substance with regard to the liquid.

A further example of the new method's use is shown in Figure 4. The same material processed under the same conditions, except that freeze-drying was not applied, is shown in Figure 5. In both cases (Figs. 4 and 5) the polysaccharide is mannan. The mannan was isolated from cell walls of *Saccharomyces cerevisieae* yeast cells with a 0.5 *M* solution of NaCl in an autoclave at 140°C. The cellular membranes had been removed by centrifugation. The polysaccharides were precipitated with 96% ethanol. Further separation and isolation was carried out according to Haworth and his coworkers.[5] The isolated mannan was dissolved in water ($5 \times 10^{-3}\%$ solution). Figure 4 is one of 155 similar electron micrographs of several preparations of the same polysaccharide. Structural analysis indicates (Šikl and Masler[6]) that the polysaccharide is considerably branched and has at each unit of the main chain lateral branchings with several links. In spite of the limited resolving power of the electron microscope (limit of resolution, 25 A.) such supermolecular details in which probably only associates of the polysaccharide macromolecules participate can be seen in Figure 4.

Separation of the individual macromolecules from one another depends on the type of solvent, the initial concentration of the solution, and on other factors; this, however, is not the purpose of this paper.

The treatment of the preparation shown in Figure 4 proves that the method offers the possibility of obtaining an electron microscope preparation the details of which are resolved without any staining or shadowing of the sample and which is suitable for supermacromolecular morphological studies.

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