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High Resolution Electron Microscopy of Sodium Lignin Sulfonate*

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Synopsis

High resolution electron microscopy of sodium lignin sulfonates revealed a granular microstructure of spherical particles of diameters ranging from 30 to 90 A. The weight-average particle weight, P_w was computed from the dimensions measured on the electron micrographs and the density of lignin. For a fraction of intermediate molecular weight, the particle diameter derived from P_w was 64 A., in fair agreement with a value of 49 A. computed from the sedimentation equilibrium molecular weight. A high molecular weight fraction contained, in addition to the granular moiety, larger structures which appeared to be aggregates of the granules. The particle weight distribution for the high molecular weight fraction was similar to the distribution of sedimentation constants previously found for alkali lignins. The spherical shapes for both the granules and the aggregates was in accord with the compact spherical configuration previously proposed for lignin sulfonates in aqueous solution.

INTRODUCTION

Several reports from this laboratory have dealt with the determination of the molecular weights of soluble lignins.[1–4] Lignin sulfonates,[1,3] alkali lignins,[2] and organosolv lignins[4] have been examined by methods such as osmometry, light scattering, and sedimentation equilibrium. The molecular weights obtained have varied widely. Fractions of lignin sulfonate have shown osmotic number-average molecular weights of less than 4000.[1] Weight-average molecular weights of periodate alkali lignins,[2] as measured by light scattering, were as high as 5×10^7. A sedimentation velocity analysis of alkali periodate lignins showed a wide distribution of molecular weight in any single sample.[5] Polydispersity of lignin sulfonates has also been noted by McCarthy and co-workers.[6,7]

A further characteristic of the lignin macromolecule is its compact configuration in solution. Viscometric and frictional behavior is consistent with a non-freedraining, spherical shape for dioxane lignins[4] and alkali lignins.[2]

A spherical macromolecule of molecular weight 50,000 and density 1.4 g./cm.^{3} will have a diameter of 50 A. Such a particle should be readily

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detectable by the modern techniques of high resolution electron microscopy. Photographs of several types of protein molecules have been published recently by Valentine[8] and Levin.[9,10] The purpose of the work presented herein was to photograph the lignin macromolecule in the electron microscope and to compare the features thus revealed with what is known about the behavior of lignin derivatives in solution. Though several types of soluble lignins have been studied, this report is confined to three fractions of sodium lignin sulfonate. Two of the fractions were prepared from the spent liquor of a laboratory bisulfite cook of spruce wood[11] while the third was obtained by successive sulfonation of a spruce periodate lignin.[12] Attempts to interpret the electron micrographs quantitatively and to compare the particle sizes thus obtained with molecular weights measured by the sedimentation equilibrium technique are described in this report.

EXPERIMENTAL

Lignin Sulfonates

Spruce meal was sulfonated successively[11,12] to give four roughly equal fractions of lignin sulfonate. The acid used for sulfonation contained 1% Na^+ and 6% total SO_2. Sugars, salts, and low molecular weight lignins were removed by dialysis through cellophane bags. The second fraction, 2W, was further fractionated into nine subfractions by dialysis through Millipore membranes of graded pore size. The subfractions selected for electron microscopy were 2W-3 and 2W-7. Fraction 2W-3 was a middle cut of 2W, while fraction 2W-7 was representative of the high molecular weight tail.

The subfractions were obtained in too small a quantity to permit detailed chemical characterization. However, 2W had 14% methoxyl, 6.3% sulfur, and 1.1% carbohydrate and constituted about 20% of the lignin in the wood. Details of the preparation of these samples and the molecular weight determinations by sedimentation equilibrium will be published elsewhere.[11]

Also used was a sample made by successive sulfonation of spruce periodate lignin. This fraction, designated PS-5b, was prepared by ultracentrifugal purification of fraction S-5 as described in a previous paper.[3]

Electron Microscopy

Various techniques for preparing the lignin samples for electron microscopy were tried. These included shadowing with heavy metals, such as a gold–palladium alloy, platinum, and a platinum–carbon mixture. Positive staining of the lignin sulfonate by replacing the sodium cation with cesium was also tried, but the contrast was rather low. Best results were obtained by using the negative staining technique of Brenner and Horne[13] and depositing the sample on a specially prepared "microwindow" carbon film.

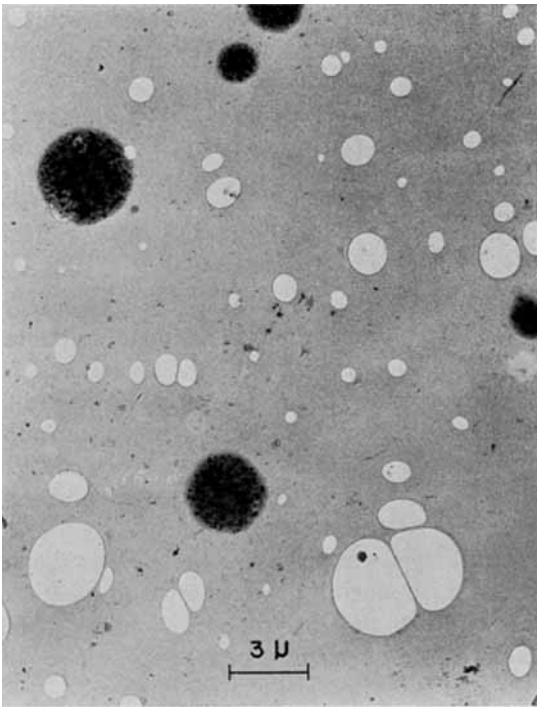

Fig. 1. Low magnification electron micrograph of a specimen preparation. The holes are the microwindows covered with a thin layer of carbon. The black circles are the dried down droplets of the solution of the specimen.

Sjöstrand's[14] method was used to prepare perforated carbon films. A layer of carbon about 100 A. in thickness was evaporated on to a holey Formvar substrate. The laminate was floated on to specimen grids and the Formvar removed with 1,2-dichloroethane. A thin continuous carbon film about 25 A. in thickness was evaporated onto a glass slide which had been pretreated with a trace of sodium metaphosphate to act as a parting agent. The thin film was floated on water and picked up on the grids covered with the perforated film.

The phosphotungstate–lignin solution was sprayed on the microwindow grids by means of a glass Vaponefrin nebulizer.[15,16] By trial and error it was found that best results were obtained if the lignin sulfonate concentration was between 0.05% and 0.1% and the phosphotungstate was 0.5%. Both the sulfonate and the phosphotungstate were in the form of their sodium salts, and the final pH of the mixed solution was approximately 6.5. A typical specimen preparation is shown in Figure 1. The circular black areas are dried droplets of lignin phosphotungstate. Note the numerous holes in the carbon film. That these holes are covered with a thin film may be seen from the small fragments of debris which are supported over the holes. The best areas for electron microscopy occur where the sample drop partially covers a microwindow. For example, the largest drop shown

in Figure 1 lies over two microwindows. The high resolution photographs shown in the next section were all taken at the edge of a droplet through the microwindows.

The electron microscope was a JEM 6A. The filament voltage was 80 kv. and the plate magnification was between ×45,000 and ×80,000. A through focus series was usually taken, and the plate with the best image was selected for photographic enlargement.

RESULTS

High resolution electron micrographs of the lignin sulphonate samples are shown in Figures 2–7. The drawings in Figures 3, 5, and 7 were made by placing tracing paper over the photographs and blacking in the white spots which corresponded to areas of high transparency of the phosphotungstate layer. These tracings are not meant to be exact reproductions but should be regarded as visual aids which contribute to the understanding of the electron micrographs.

The high resolution photograph of fraction PS-5b shown in Figures 2 and 3 was the clearest obtained by the negative staining technique. The lignin sulfonate is shown to have a granular structure of small, spherical particles, clustered together to give a porous appearance. The size of the

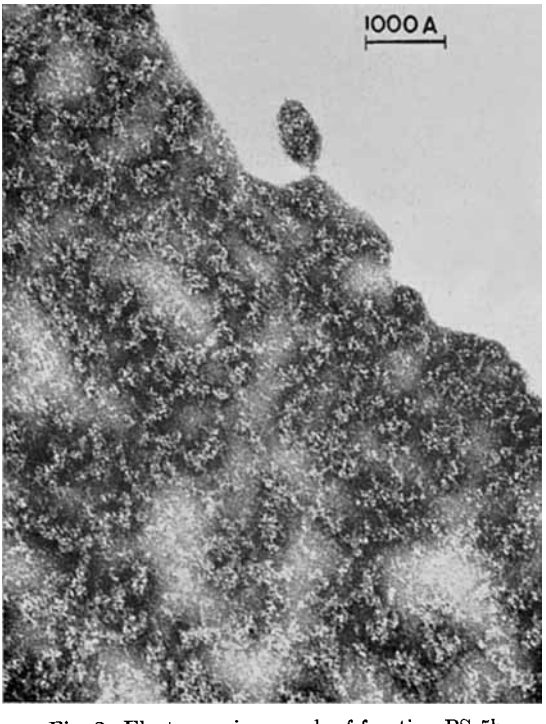

Fig. 2. Electron micrograph of fraction PS-5b.

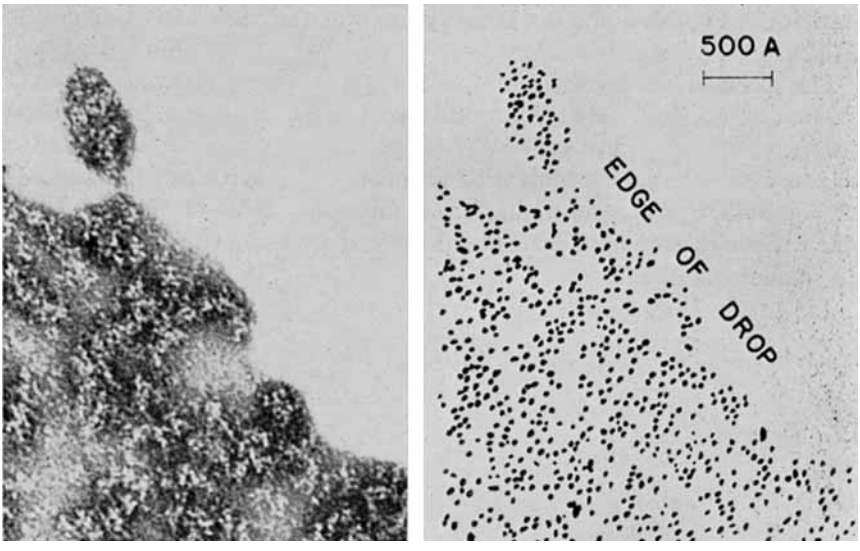

Fig. 3. Detail of Fig. 2 for fraction PS-5b. The tracing shows in black the areas of high transparency in the droplet.

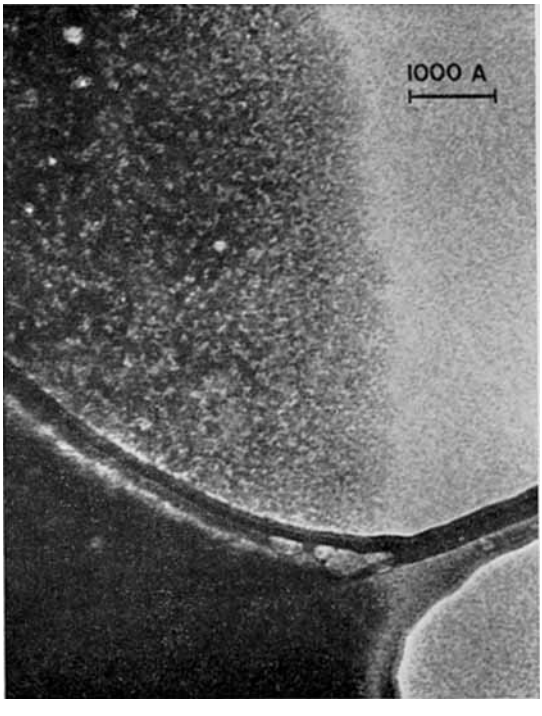

Fig. 4. Electron micrograph of fraction 2W-3 showing edge of drop and microwindow.

granules in Figures 2 and 3 was fairly uniform and varied from diameters of 30 A. to 65 A.

The electron micrographs of fraction 2W-3 were similar to those of fraction PS-5b, except that the granule size was slightly larger, as illustrated in Figures 4 and 5. Fraction 2W-7 (Figs. 6 and 7), however, was found to possess a substantial concentration of much larger particles superimposed on a granular background similar to those of PS-5b or 2W-3. These larger particles were of varying diameters and appeared to be aggregates of the smaller granules.

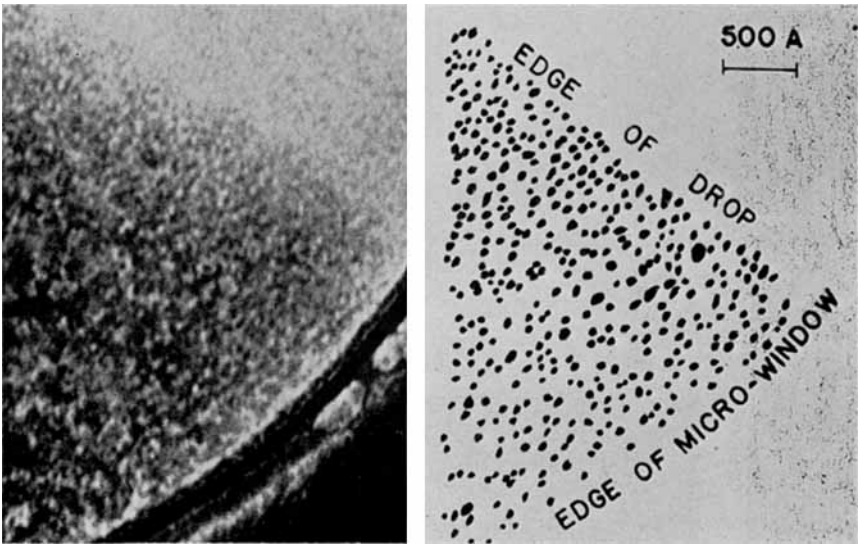

Fig. 5. Detail of Fig. 4 for fraction 2W-3.

The dimensions of the granules were measured from suitable photographs and reduced to true diameters by means of the known magnification factor. The granules were assumed to be approximately spherical, and a particle weight P, corresponding to a given radius r, was calculated from

$$P = 1.33\pi r^3 N\rho \tag{1}$$

in which N is Avogadro's number and ρ is the density of dry lignin sulfonate, which was assumed to be 1.4 g./cm.^{3}.

The diameters of 100 granules chosen at random were measured and the corresponding particle weights calculated from

$$P_n = \Sigma n_i P_i / \Sigma n_i \tag{2}$$

$$P_w = \Sigma n_i P_i^2 / \Sigma n_i P_i \tag{3}$$

in which n_i was the number of particles of weight P_i, and P_n and P_w the number-average and weight-average particle weights, respectively.

TABLE I
Comparison of Particle Weights from the Electron Microscope and Molecular Weights from Sedimentation Equilibrium

Fraction	P_n	P_w	P_w/P_n	M_w	$(D)P_w$ A.	$(D)M_w$, A.
2W-3, granules	98,000	114,000	1.16	52,000	64	49
2W-7, granules	144,000	176,000	1.22	—	73	—
2W-7, aggregates	14×10^6	41×10^6	2.9	—	450	—
2W-7, granules + aggregates	212,000	13.6×10^6	64	650,000	320	110

Values of P_n and P_w for fraction 2W-3 and the granular moiety of fraction 2W-7 are given in Table I. Also shown are average diameters, $(D)_{P_w}$ and $(D)_{M_w}$ computed from

$$(D)_{P_w} = (6P_w/\pi N\rho)^{1/3} \tag{4}$$

and

$$(D)_{M_w} = (6M_w/\pi N\rho)^{1/3} \tag{5}$$

in which M_w is the weight-average molecular weight as determined by the sedimentation equilibrium method.

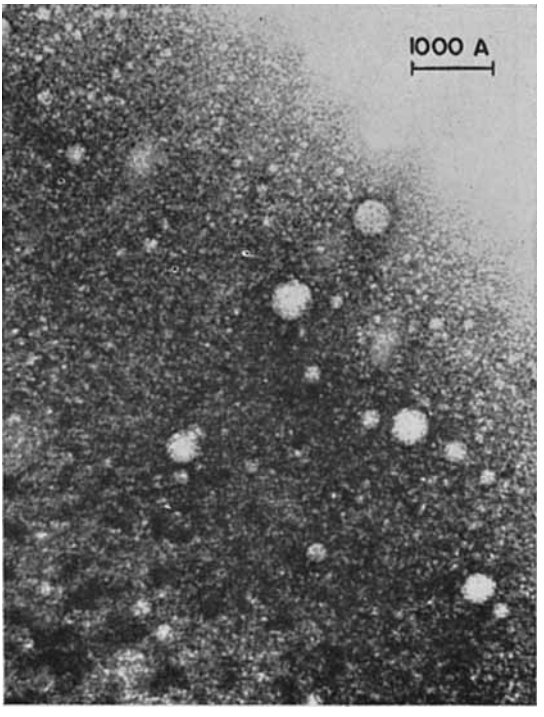

Fig. 6. Electron micrograph of fraction 2W-7. Large aggregates seem to be superimposed on a granular background similar to that shown in Figs. 2 and 4.

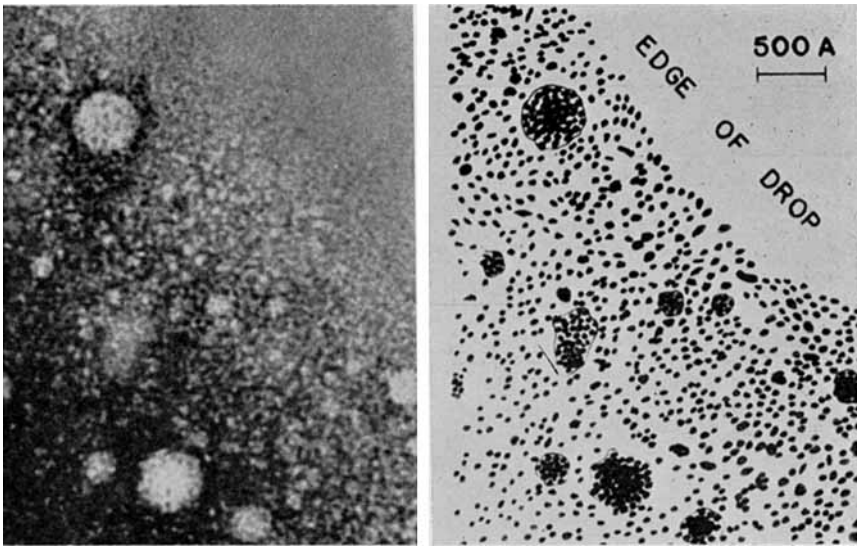

Fig. 7. Detail of Fig. 6 for fraction 2W-7.

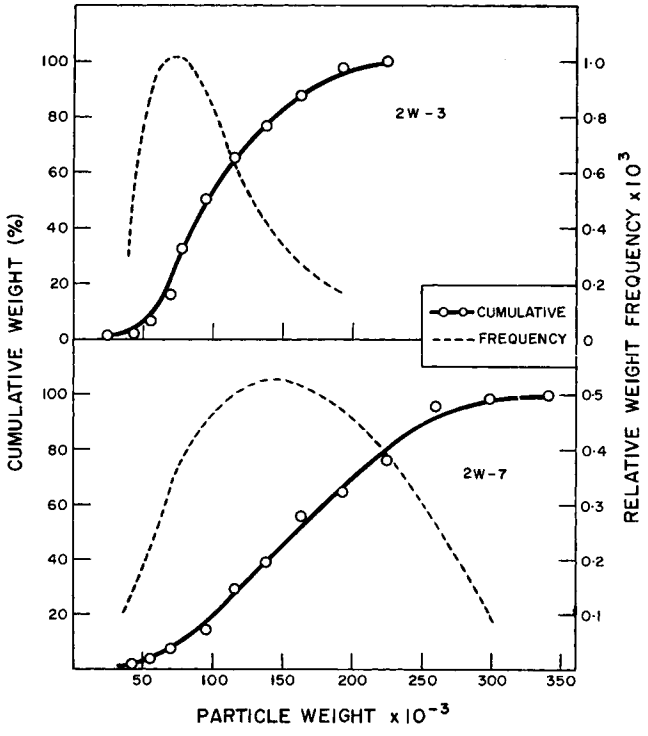

Fig. 8. Distribution of particle weight for fraction 2W-3 and the granular moiety of fraction 2W-7.

An attempt was made to allow for the aggregates in fraction 2W-7 shown in Figures 6 and 7. From a single large electron micrograph all the aggregates with diameters greater than 100 A. were measured. They numbered 37. The number of granules in the same electron micrograph was estimated to be 7300 from counts over several small areas. The particle weight of an aggregate was calculated by substituting the radius of the aggregate for r in eq. (1). Values P_n and P_w could then be calculated for the aggregate alone and also for the mixture of granules and aggregates which, of course, represents the fraction 2W-7. These results are given in Table I along with the weight-average molecular weight, M_w obtained by the sedimentation equilibrium technique.[11]

The distribution of particle weights for fractions 2W-3 and the granular moiety of fraction 2W-7 is shown in Figure 8. A polynomial was derived by computer to give the best fit for the cumulative experimental points. The frequency curve was then obtained by differentiation.

DISCUSSION

There is always the danger in high resolution electron microscopy that the features recorded in the electron micrograph are not related to the material under examination. Granular artifacts are often produced by metallic shadowing, and considerable effort has been made to reduce grain by proper choice of materials and conditions.[17] In this respect, the negative staining technique with phosphotungstate is particularly useful. To the author's knowledge, anomalous granular structures have never been reported for phosphotungstate staining, in spite of the widespread use of this technique for work at high resolution.

Though not included in the results, several other soluble lignins showed granular structures at high resolution. The electron micrographs in Figures 2 and 4 were similar to the earlier photographs of negatively stained lignin sulfonates.* A dioxane lignin negatively stained with phosphotungstic acid in dioxane gave a clear pattern of spherical particles approximately 15 A. in diameter.* Spruce periodate alkali lignin, when shadowed with a gold–palladium alloy, was observed as an array of small spherical particles about 105 A. in diameter which was quite distinct from the background of metal grain on areas of the grid not covered by the specimen.* A cesium lignin sulfonate was deposited from an aqueous solution containing no other staining agent. Though low in contrast, the electron micrographs showed the characteristic "fish-egg" appearance found for the other soluble lignins. Therefore, it can be stated with some degree of certainty that the small, approximately spherical particles shown in Figures 2–7 were indicative of the microstructure of the lignin sulfonate fractions studied. In passing, it is interesting to note that Sachs, Clark, and Pew[18] have observed small spherical structures for lignin on electron microscopy of cell walls

* Photographed by A. W. Agarat Aeon Laboratories, Surrey, England.

from which carbohydrate had been removed by a special hydrofluoric acid treatment.

The question now arises as to whether there is any correlation between particle size measured from the electron micrographs and the weight-average molecular weight determined by sedimentation equilibrium. For fraction 2W-3 in Table I, M_w is smaller than P_w by a factor of 2.2. This factor corresponds to a value of 1.3 when calculated for particle diameters instead of weights. In view of the uncertainty of measurements on the diffuse outlines produced by the negative staining technique[19] and the possibility of flattening of the granules on drying,[20] the correlation between the particle diameters determined by the two methods can be claimed to be fairly good.

Interpretation is more uncertain for the high molecular weight fraction, 2W-7, shown in Figures 6 and 7. Here it appears that the small particles are accompanied by aggregated structures which are seen as larger spherical shapes. The ratio P_w/M_w for fraction 2W-7 is 21, which corresponds to a value 2.9 for $(D)_{P_w}/(D)_{M_w}$. There are several possible reasons for this discrepancy. The larger aggregates seen in the electron micrographs would tend to sediment to the base of the ultracentrifuge cell and therefore would not contribute to the sedimentation equilibrium molecular weight. Also they may be disklike rather than spherical in shape, which would cause P_w computed for spheres to be erroneously high. Significantly, the ratio $(D)_{P_w}/(D)_{M_w}$was 1.15 when P_w was calculated on the assumption that the aggregates were disks, one granule thick.

Whatever the source of the larger particles, it seemed likely that these structures were responsible for the significant difference in the molecular weights of fractions 2W-3 and 2W-7. The particle size analysis in Figure 8 showed fraction 2W-3 to have a narrow distribution with $P_w/P_n = 1.16$ (Table I). Fraction 2W-7 was considerably more polydisperse, as might have been expected. The value of P_w/P_n for the unaggregated moiety of 2W-7 was 1.22, only slightly greater than for 2W-3. The wide spread of particle size in 2W-7 was produced by the polydisperse aggregates which comprised 32% of the weight of the fraction. The particle size distribution in fraction 2W-7 was similar to the distribution of sedimentation coefficients previously reported for spruce periodate alkali lignins.[5] In both a fairly monodisperse, low molecular weight component is combined with a widely polydisperse, high molecular weight tail.

In discussing the distribution of molecular weights in fractions 2W-3 and 2W-7, it should be remembered that the parent sample, 2W, was the nondialysable portion of the lignin sulfonate from which some low molecular weight material was lost in the process of dialysis. Also, the first of the four fractions produced in the cook was 1W, which was of lower molecular weight than 2W.[11] Therefore, a substantial portion of the lignin made soluble in the sulfonation would be of considerably lower molecular weight than 2W-3. Electron microscopy was attempted on a very low molecular weight fraction but was abandoned because the molecules seemed to be too small to be detected.

In several previous papers, it has been pointed out that the configuration of a soluble lignin is probably that of a compact, approximately spherical macromolecule.[2-4] It is interesting to compare this concept with spherical shape of both the granules and aggregates shown in Figures 2–7. Although no definite relationship need be expected between the ultrastructure of the dry lignin sulfonate and the shape of the macromolecule in solution, the preponderance of a granular habit in the electron micrographs is consistent with the previously proposed spherical configuration of the solvent-swollen macromolecule.

Although the electron micrographs supply no information concerning the nature of the forces which hold the granules together to form aggregates, it is possible to speculate on their origin. It is likely that the sulfonation reaction will favor the exposed surfaces of the lignin as proposed by Marraccini and Kleinert.[21] Fragments of the lignin network may thus acquire an asymmetric distribution of the polar sulfonate group. On solution there may be a tendency for the hydrophobic portions to attract each other to give a type of macromolecular micelle with the charge-bearing hydrophilic surfaces turned towards the aqueous environment. A similar type of hydrophobic bonding has been proposed by Némethy and Scheraga[22] for the association of proteins. Some evidence for micellar behavior in low molecular weight lignin sulfonates has already been presented by Gardon and Mason.[23] Macromolecular micelles of the type suggested above would be similar in several respects to the microgel model previously suggested for high molecular weight lignin sulfonates.[3] A more detailed correlation of the properties of the aggregate with the solution behavior of lignin sulfonates is reserved until further experiments are carried out.

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Résumé

La microscopie électronique à haute résolution de sulphonates sodiques de la lignine révèle une microstructure granulaire des particules sphériques possédant des diamètres de 30 à 90 A. Le poids moléculaire moyen en poids de la particule, P_w, est déterminé à partir des dimensions mesurées sur les micrographies electroniques et à partir de la densité de la lignine. Pour une fraction de poids moléculaire intermédiaire, on a obtenu pour le diamètre de la particule, à partir de P_w, une valeur de 64 A. Ceci est en bon accord avec la valeur de 49 A obtenue à partir du poids moléculaire déterminé par l'équilibre de sédimentation. Une fraction de poids moléculaire élevé contient en plus de la partie granulaire des structures plus grandes qui sont des aggrégats de granules. La distribution particulaire en poids pour la fraction de poids moléculaire élevé était semblable à la distribution des constantes de sédimentation trouvées auparavant pour des lignines alcalines. La forme sphérique pour les granules et les aggrégats est en accord avec la configuration sphérique compacte proposée antérieurement pour les sulphonates de lignine en solution aqueuse.

Zusammenfassung

An Natrium-Ligninsulfonaten wurde mittels hochauflösender Elektronenmikroskopie eine aus körnigen Teilchen von 30-90 Å Durchmesser bestehende granulierte Mikrostruktur nachgewiesen. Das Gewichtsmittel des Teilchengewichts P_w, wurde aus den an den elektronenmikroskopischen Aufnahmen gemessenen Dimensionen und der Dichte von Lignin berechnet. Für eine Fraktion mittleren Molekulargewichtes war der aus P_w ermittelte Teilchendurchmesser 64 Å. Dies stimmt mit dem aus dem Sedimentationsgleichgewichts-Molekulargewicht berechneten Wert von 49 Å gut überein. Eine Fraktion hohen Molekulargewichtes enthielt neben den körnigen Anteilen grössere Strukturen, die offenbar aus Körneraggregaten bestehen. Die Teilchengewichtsverteilung der hochmolekularen Fraktion war der früher für Alkalilignine bestimmten Verteilung der Sedimentationskonstanten ähnlich. Die kugelförmige Gestalt sowohl der Körner als auch der Aggregate steht mit der in früheren Arbeiten für Ligninsulfonate in wässriger Lösung vorgeschlagenen kompakten sphärischen Konfiguration in Einklang.

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