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MOLECULAR-SIZE DETERMINATION OF XANTHAN POLYSACCHARIDE

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ABSTRACT

Hydrodynamic chromatographic separations of xanthan polysaccharide of ultrahigh molecular weight have been obtained by using columns packed with 30-μm, non-porous spheres. From calibration curves of the elution volume versus particle size for spherical, polystyrene latexes, it was found that xanthan is eluted at the same volume as a 0.153-μm diameter sphere. Extremely dilute samples of xanthan (70 p.p.m.) were injected to preclude self-association and aggregate formation. Detection at these low concentrations was accomplished by tagging the xanthan with a fluorescein derivative and using a flow-through fluorometer detector. Flow rates of 1 mL/min yielded run times of ~7 min. Comparison of the accepted molecular conformation of xanthan—a rigid rod-like molecule—with the apparent molecular volume from the spherical-latex calibration indicates that the xanthan molecules are substantially oriented by the flow field in the chromatography column.

INTRODUCTION

Xanthan is an extracellular, microbial polysaccharide possessing a cellulose chain [(1→4)-β-D-glucan] as its backbone structure. A β-(1→3)-linked trisaccharide chain [4,6-*O*-(1-carboxyethylidene)-β-D-mannopyranosyl-(1→4)-β-D-(glucopyranosyluronic acid)-(1→2)-6-*O*-acetyl-β-D-mannopyranosyl] is linked to every other glucosyl residue[1]. The structure of the repeating unit is given in Fig. 1.

Xanthan is widely used as a viscosifier in the food and paint industries, and recent work suggesting its usefuless in tertiary oil-recovery has resulted in a projected rise in the demand for xanthan[2]. The interesting rheological properties of xanthan solutions arise from its ordered helical structure, which produces a rigid rod-like molecule. The exact nature of the helical structure is uncertain as X-ray scattering studies of xanthan fibers suggest a 5-fold single helix[3], whereas electron micrographs suggest a multistranded backbone arranged in a right-handed twist[4]. The backbone structure and high molecular weight of xanthan result in rheological properties that are insensitive to ionic strength in solution as compared with other polyelectrolytes and create a molecule that is resistant to mechanical degradation[5].

There has been considerable interest in determining the molecular weight (M) of xanthan as rheological behavior is strongly influenced by molecular weight, and

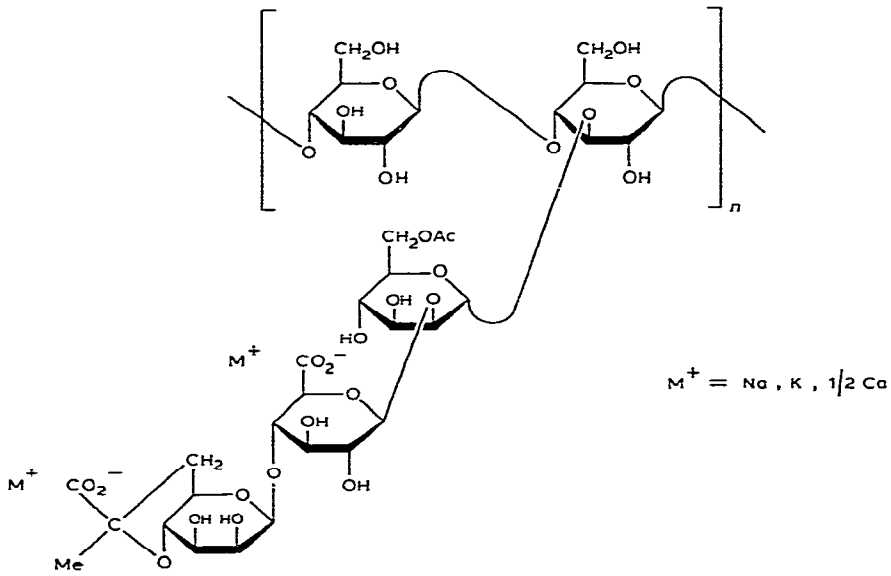

Fig. 1. Repeating unit of xanthan polysaccharide. (From ref. 1)

Holzwarth has shown that the molecular weight of xanthan is a function of fermentation conditions[6]. Techniques that have been used to study the molecular size of xanthan include electron microscopy, light scattering, viscometry, and ultracentrifugation. Electron micrographs show[4] native xanthan to have contour lengths of 2–10 μm. Using classical light-scattering methods, Dintzis *et al.*[7] found $M = 2 \times 10^6$ for solutions heated for 3 h at 90° in 4M urea and $M = 13 \times 10^6$ and 50×10^6 for two native samples that were not heated.

Dynamic light-scattering studies by Southwick *et al.*[8] at concentrations <0.01 % gave diffusion coefficients of $D_t^o = 2.4\text{–}2.7 \times 10^{-8}$ cm^2/s for freshly filtered xanthan in deionized water and for xanthan in 4M urea. Upon aging for one week, the diffusion coefficient in water alone decreased to $D_t^o = 1.1 \times 10^{-8}$ cm^2/s but did not decrease with time in urea. This difference was interpreted as being due to aggregation caused by hydrogen bonding and inhibited by the addition of urea. Using the value of the diffusion coefficient for xanthan in urea and a measured value of the intrinsic viscosity, $[\eta] = 2120$ mL/g, they calculated a molecular weight of $M = 2.16 \times 10^6$ from the Flory–Mandelkern equation. Holzwarth[6] used band sedimentation of fluorescently tagged xanthan in 70mM sodium chloride and 4mM sodium phosphate at xanthan concentrations from 0.01–10 μg/mL. From sedimentation and intrinsic-viscosity measurements, Holzwarth obtained weight-average molecular weights of $M_w = 14.8 \times 10^6$ for native, commercial xanthan and $M_w = 62 \times 10^6$ for a native culture-broth. The respective zero shear-rate, intrinsic viscosities were $[\eta] = 12{,}300$ and $[\eta] = 35{,}000$ mL/g. Whitcomb and Macosko[9] modeled xanthan as a prolate ellipsoid having a minor-axis diameter of 2.0 nm. Fitting the calculated intrinsic viscosity of a

solution of ellipsoids (at non-zero shear rates) to intrinsic-viscosity data, they estimated the length of the ellipsoids to be 1.5 μm.

The difficulties associated with obtaining viscosity extrapolations to zero concentration and zero shear-rate for molecules as large and rigid as xanthan are considerable[6]. In addition, Holzwarth found xanthan to be quite polydisperse, with M_w/M_n = 2.4–2.8. There is clearly a need for additional analytical techniques for measuring the molecular weight and the molecular-weight distribution of xanthan. Chromatographic separations of xanthan are attractive, because they yield information on both molecular weight and molecular-weight distribution. Conventional size-exclusion chromatography (or gel-permeation chromatography) is not possible with xanthan because xanthan is totally excluded from porous packings or gels of even the largest available pore-sizes[10].

In this study, we show that hydrodynamic chromatography (h.d.c.) may be used successfully for separation of xanthan according to molecular size. Hydrodynamic chromatography was initially developed by Small[11] in 1974 to determine the sizes of latex spheres from emulsion-polymerization reactors. It has been used[11–14] successfully for study of spherical particles in the range 0.05–1 μm. It has not been used hitherto for study of non-spherical particles or biological polymers. The mechanism of separation in hydrodynamic chromatography is different from that of conventional size-exclusion chromatography. Size-exclusion chromatography employs diffusion of the polymer molecule into and out of pores in a porous-packing particle to effect separation. However, in hydrodynamic chromatography, the column packings are impenetrable solid spheres, and the separation occurs as particles of different sizes encounter different regions of the non-uniform velocity field. The current model of the hydrodynamics of separation considers the porous medium to be a bundle of parallel tubes[12]. A theoretical treatment by Brenner and Gaydos[15] of the flow of a sphere inside a small capillary is then used to determine the average

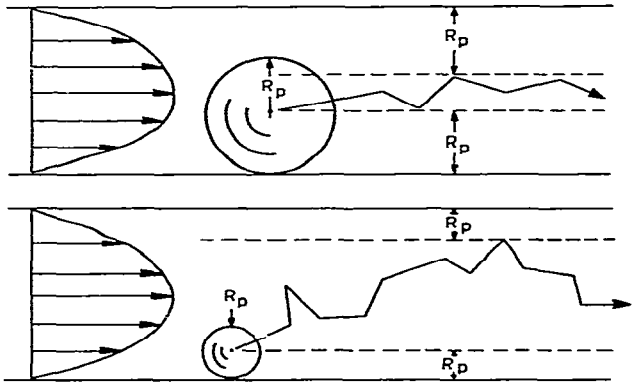

Fig. 2. Model of the hydrodynamic chromatography separation. The larger particle (top), with its center confined to the center of the tube where the velocity is higher, moves more rapidly than the smaller particle (bottom).

particle-velocity for particles of different sizes. Because of steric, electrical, and hydrodynamic exclusion of larger particles from the wall region, where the fluid velocity is small, larger particles are confined to the region of faster-moving fluid in the center of the tube. Smaller particles, which can encounter both the slower-moving velocity field near the wall and the faster-moving fluid in the center of the tube, move on average more slowly than the larger particles. This effect is shown in Fig. 2. Silebi and McHugh[12] found quite good agreement between experimentally determined retention-times for spherical latex particles and calculated retention-times using *a priori* estimates of the model parameters that characterized their system.

We have extended the applicability of hydrodynamic chromatography to non-spherical, ultra-high molecular weight, rigid, biological polymers. The technique has been successfully demonstrated for both xanthan polysaccharides and a variety of DNA's. However, with solutions of DNA, the effect of shear degradation during flow through the column is unknown, but it is quite probably significant. The DNA work will be reported in more detail elsewhere.

EXPERIMENTAL

Equipment. — The chromatography apparatus, schematically shown in Fig. 3, was similar to that used for size-exclusion chromatography, except for the column packing. The pump (LDC Model 711 Simplex) had an integral pulse-dampener and pressure gauge. The injection valve (Rheodyne Model 70–10) had a 20-μL injection loop. Two stainless-steel columns, 4.6 mm (i.d.) × 1.0 m, were dry-packed with different bed-materials. One contained DuPont Zipax ® spherical silica beads, shown by electron micrographs to have diameters of 27 $\pm$6 μm. The second column was packed with fractionated glass beads having a mean size of 30 μm and a slightly broader size-distribution than the Zipax ® beads. The glass beads were fractionated in our laboratory from batches of commercial glass beads (Potters Inc.) having a very broad size-distribution. The fractionation was performed with a three-column, glass elutriator built for us by Hercules Chemical Company. The glass beads were succes-

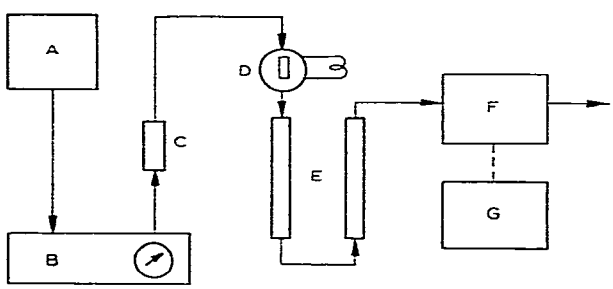

Fig. 3. Hydrodynamic chromatography apparatus. A, solvent reservoir; B, high-pressure liquid chromatography pump; C, column prefilter; D, loop sample-injection valve; E, columns (4.6 mm i.d. × 1 m); F, fluorescence detector; G, strip-chart recorder.

sively washed in 0.1M hydrochloric acid, methanol, and acetone, dried in a vacuum at 110°, and then screened through a 212-μm wire mesh to break up aggregates. The columns had 20-μm stainless-steel frits at both ends. The Zipax * column had a higher resolving-power than the glass-bead packed column, as determined by measurements of the height of an equivalent theoretical plate (h.e.t.p.) with D_2O tracers at flow rates of 1.0 mL/min.

A DuPont differential refractometer was used with latex spheres for evaluation of column performance and optimum mobile-phase composition. However, at very low concentrations, which must be used to prevent xanthan self-association and column plugging, neither r.i. nor u.v. detectors, were sensitive enough to detect xanthan. We therefore used a fluorescent-tagging technique described next and a Kratos Model 970 flow-through fluorometer with a deuterium lamp and 5-μL sample cell. A deuterium line at 480 nm provided excitation, and fluorescence was detected above 500 nm. It was found that latex calibration-spheres (Duke Scientific Co.) fluoresce weakly at an excitation wavelength of 450 nm. Therefore the fluorescence detector may be used both to calibrate the column with latex standards and to conduct xanthan separations.

Hydrodynamic chromatography of xanthan. — Xanthan samples, previously purified according to the procedure described by Holzwarth[6], were kindly provided by Dr. Donald Siano of Exxon Central Laboratories, Linden, N.J. The xanthan had been purified from commercial xanthan broth (Pfizer Inc., Flocon 4800) by successive filtration, precipitation, and freeze-drying. The fluorescent-tagging procedure of Holzwarth was followed. First 5–25 mg of xanthan was dissolved in 100 mL of 2mM sodium chloride. To this solution dimethyl sulfoxide (30 mL) and water (70 mL) were added. Then 30 μL of acetaldehyde (MCB Co.), 30 μL of cyclohexyl isocyanide (Aldrich Chemical Company), and 3 mg of 5-aminofluorescein (Sigma Chemical Company), which had been dissolved in 3 mL of dimethyl sulfoxide, were added. The solution was allowed to react for 3 h at room temperature. The tagged xanthan was precipitated from solution by using 3 parts of ethanol to 2 parts of reaction mixture, filtered, and redissolved in 50 mL of 0.5 wt% sodium chloride. This operation was repeated twice. The wet xanthan precipitate was finally redissolved in a mobile phase consisting of 0.05M sodium sulfate, 0.2% sodium dodecyl sulfate, and 2mM sodium azide (added as a bactericide). The xanthan was dialyzed for 3 days with the solution being changed each day. After the dialysis, samples were stored in a refrigerator.

The mobile phase just mentioned gave maximal resolution for latex spheres. The flow rate for all experiments was 1.0 mL/min, not necessarily because this led to the best separation, but because it resulted in rapid run times, on the order of 7 min. Studies of the effect of flow rate on the separation are still in progress. The effect of flow rate is expected to be significant, because the degree of orientation of the xanthan depends on the strength of the flow. At 1.0 mL/min, column pressures were 700 lb.in.$^{-2}$ across one column and 1200 lb.in.$^{-2}$ across two columns. Sample volumes of 20 μL were injected, with xanthan concentrations of $\sim$70 p.p.m. (Southwick, *et al.*[8] reported no self-association below 200 p.p.m.) A small amount of fluorescein was added to

the xanthan solution to act as a marker from which the difference in elution volume between the xanthan peak and marker could be determined. The peaks eluted were recorded on an integrating strip-chart recorder (Houston Instruments Inc.).

RESULTS AND DISCUSSION

Xanthan molecules were successfully separated by either the single Zipax ®-filled column or, with greater resolution, by both columns in series. The elution volume of the xanthan peak corresponded to that of a 0.153-μm latex sphere. To demonstrate that the separation was based on molecular size, the xanthan samples were degraded by sonicating a 150-mL sample of tagged xanthan solution with an Ultrasonics Model W-370 dismembrator (using a 1-cm diameter probe at a power setting of 6/10). The sample beaker was placed in an ice bath during the ultrasonic degradation. Samples (3 mL) of solution were withdrawn at intervals of 5, 20, and 60 min. Holzwarth[6] and Chen[10] have shown that the molecular weight of xanthan is decreased because of chain scission by this treatment. The results of hydrodynamic chromatography on these samples, with the Zipax ® and glass-bead columns in series, are shown in Fig. 4. As the molecular weight is decreased by sonication, the xanthan peak moves closer to the marker peak, as would be expected. Also shown in Fig. 4 is the retention factor, defined as the ratio of the average velocity of the xanthan peak to the average velocity of the marker peak: $R_F = (V_x)/(V_m)$. This quantity is related to the difference in

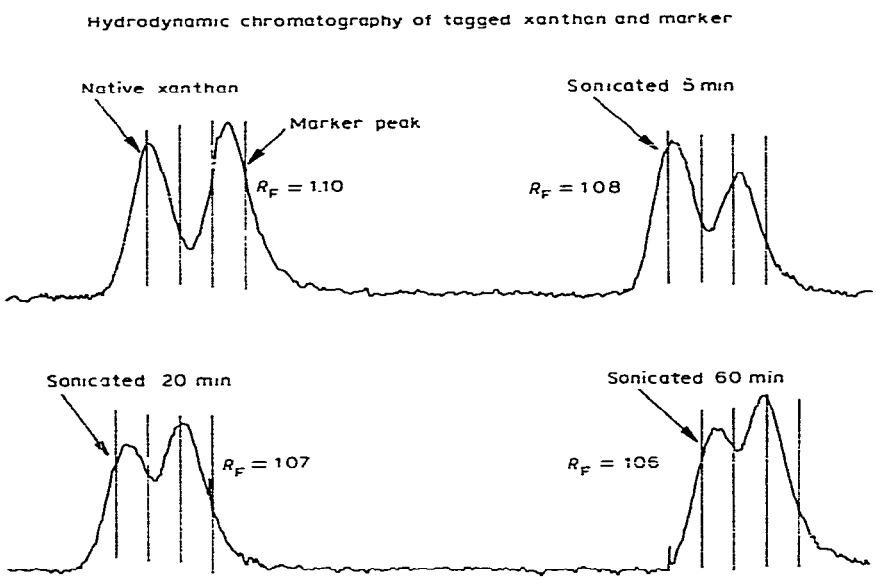

Fig. 4. Hydrodynamic chromatography separations of xanthan polysaccharide. Four runs are shown for tagged xanthan and a fluorescent marker. Increasing duration of sonication leads to shifting of the xanthan peak towards the marker peak, that is, the retention factor (R_F) decreases.

elution volume between the xanthan peak and the marker peak by $\Delta V = V_m(1 - 1/R_F)$.

As hydrodynamic chromatographic separations are based on molecular volume, it is interesting to compare the "apparent molecular volume" of xanthan (as determined from the spherical latex particle having the same retention factor as xanthan) to the molecular size of xanthan as determined by other techniques. The volume swept out in space by a freely rotating, rod-like xanthan molecule, as determined by intrinsic-viscosity measurements[6-8], is of the order of 1×10^8 dL/gmol, whereas the actual volume of a xanthan molecule or aggregate having a contour length[1,6] of ~ 2 μm and radius[2,6,8] of ~ 2 nm is 1.5×10^4 dL/gmol. The volume of a 0.153-μm latex sphere is 1.03×10^7 dL/gmol. As shown in Fig. 5, the "apparent molecular volume" of xanthan lies between that expected if the xanthan molecule were freely tumbling and that observed if the molecule were perfectly oriented by the flow and traveled through the chromatography column with its backbone oriented along a streamline. We consider that this result indicates that the xanthan molecule is substantially oriented by the flow field. The orientation will be strongly influenced by the shear and elongational strains experienced by a xanthan molecule as it flows through the interstitial volume of the bed. Although these spatially dependent strains are difficult to calculate in a complicated bed-geometry, order-of-magnitude calculations yield a characteristic shear-rate of 1100 s^{-1} and a characteristic, elongational strain-rate of 1200 s^{-1}.

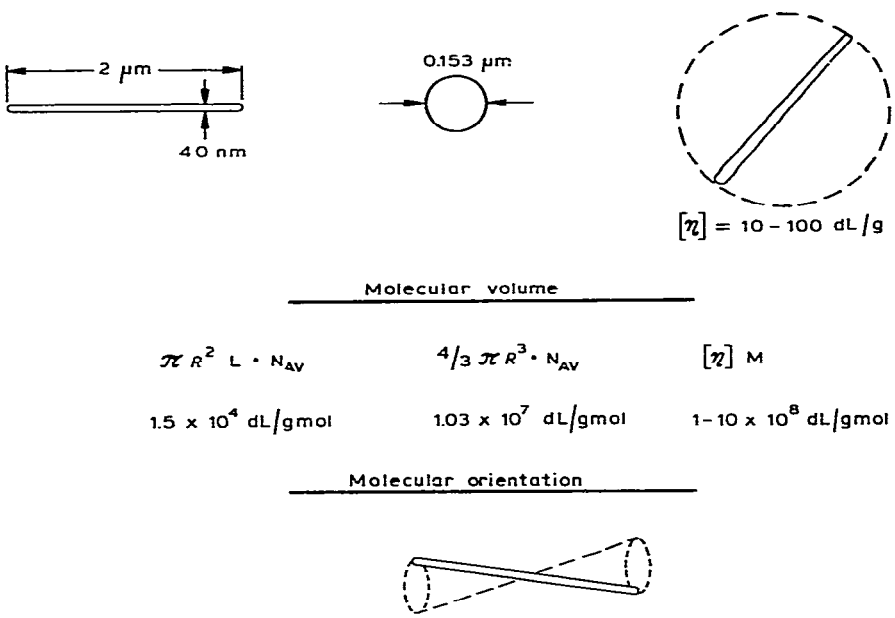

Fig. 5. Comparison of volume occupied by xanthan molecules and spheres. The xanthan is eluted from the column at the same volume as a 0.153-μm sphere. This result indicates significant orientation of the xanthan by flow.

These strain rates, though quite high, are below the values where shear degradation of xanthan occurs. A more complete discussion of the extent of molecular orientation will be reported elsewhere[16]. Recently, researchers using hydrodynamic chromatography have been able to successfully deconvolute chromatograms for latex spheres to determine quantitative particle-size distributions[14]. It seems feasible to do the same for rod-like macromolecules. To perform the deconvolution of the experimental peaks, information is needed on both separation and dispersion of rod-like particles in the hydrodynamic chromatography column. This may be done either empirically, as has been done with latex spheres, from experimental data on monodisperse, rod-like particles, or it may be done by employing a predictive model of the separation and dispersion. Viruses might be candidates for model, uniform-length, rod-like particles. A fundamental predictive model of the hydrodynamic separation must consider the complicated relationship between separation and dispersion of rod-like particles in a complicated bed-geometry. Even the simplest approaches to this problem appear difficult, and an adequate model for the separation has not yet been developed.

CONCLUSION

Chromatographic separations of xanthan polysaccharide macromolecules have been demonstrated. It is found that the elution volume of xanthan falls precisely in the range where hydrodynamic chromatography is most effective. Significant orientation of the long, rod-like molecule appears to occur during flow through the chromatography column. It now remains to optimize packing size, flow rate, and column geometry.

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