Low-Angle Laser Light Scattering–Aqueous Size Exclusion Chromatography of Polysaccharides: Molecular Weight Distribution and Polymer Branching Determination

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Synopsis

A low-angle laser light scattering detector (LALLS) used with size exclusion chromatography (SEC/LALLS) has been applied for the determination of molecular weight, molecular weight distribution (MWD), and degree of branching of polysaccharides in 0.5*N* NaOH aqueous solution. Data from both detectors [differential refractive index (DRI) and LALLS] are used to calculate the absolute molecular weight at each point in a sample chromatogram. The correct average molecular weight and MWD can be obtained without calibration methods used in conventional SEC. As a consequence of this technique, Mark–Houwink coefficients can be predicted from a single broad-distribution, homopolymer without recourse to time-consuming fractionation methods. Moreover, the hydrodynamic volume separation mechanism of SEC can be exploited with the SEC/LALLS method to gain information about polymer branching. In the studies described in this paper, SEC/LALLS has been employed to obtain data about the branching parameters g_v and g_M for samples of amylose, amylopectin, starch, and glycogen. For three homopolymers (amylose, amylopectin, and glycogen), branching frequency (as measured by chemical means), and the branching parameters (g_v and g_M) are inversely related. This trend is consistent with theoretical predictions. For starch, a nonhomogeneous branching distribution is observed as a function of molecular weight.

INTRODUCTION

Polymer characterization, in terms of the type of monomer bond linkages which constitute the polymer, is fundamental to understanding various physical and chemical phenomena of these materials. The specific behavior of a given polymer depends on several measurable parameters including number- and weight-average molecular weights, molecular weight distribution, and the nature of short and long chain branching. The importance of these properties has motivated the development of a number of analytical measurement methods. Size exclusion chromatography (SEC) is undoubtedly the single most powerful analytical tool currently used for collecting this information,[1,2] SEC is widely used for routine characterization of neutral synthetic polymers soluble in organic solvents. Hamielec and Meyer[3] have reviewed the literature on this subject, including branching measurements employing low-angle laser light scattering and viscometry. The application of these same techniques to water-soluble polymers has been more difficult for a number of reasons among

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which are polyelectrolytic effects.[4,5] The literature on this latter subject has recently been reviewed by us[6] and others.[7] Advances in the field of water-soluble polymer characterization in terms of both basic theoretical concepts and the analytical analysis methods are on going.[8,9] These methods can be used to obtain molecular weight information but not polymer branching information. For many applications of water-soluble polymers (particular biopolymers), both molecular weight distribution and branching information are essential. In this paper we report advances in the combined use of SEC with low-angle laser light scattering (LALLS) for branching characterization of polysaccharides.

THEORETICAL BACKGROUND

The LALLS detector serves essentially two purposes: as an internal check on SEC calibration and as a direct means of determining the degree of chain branching of an eluting sample. Both of these measurements are of interest to us as their values are required for subsequent development of appropriate kinetic depolymerization models of linear and branched polysaccharide substrates. The general principles of light scattering for polymer molecular weight measurement and the theoretical concepts employed for polymer branching studies are briefly described.

MW Determination by SEC / LALLS

For a macromolecule in dilute solution, the relationship between the excess Rayleigh factor and the weight-average molecular weight $\bar{M}_w$ is given by fluctuation theory of light scattering[10] as

$$Kc/R(\theta, c) = 1/\bar{M}w \cdot P(\theta) + 2A_2c + 3A_3c^2 + \cdots, \tag{1}$$

where

$$K = (2\pi^2n^2/\lambda^4N)(dn/dc)^2(1 + \cos^2\theta) \tag{2}$$

and c is the solute concentration (g/mL), $R(\theta, c)$ is the excess Rayleigh factor for unpolarized incident radiation at the scattering angle θ, n is the refractive index of the solution, λ is the wavelength in vacuo, N is Avogadro's number, while A_2 and A_3 are the second and third virial coefficients, respectively. The term $P(\theta)$ is the form factor which is a function of the size and shape of the macromolecule in solution and represents the modulation of the scattered radiation intensity due to the finite molecule size and to the polymer's deviation from sphericity. The term dn/dc is the specific refractive index increment and represents the change in solution refractive index as a function of solute concentration. If experiments are conducted in the limit of zero scattering angle, $P(\theta)$ has a value of unity, and, in extremely dilute solution, terms containing c^2 can be neglected. Hence, with LALLS connected in series to a SEC, the molecular weight of a polymeric solute within a given eluting volume element can be calculated from

$$Kc_v/R(\theta, c_v) = 1/M_v + 2A_2c_v, \tag{3}$$

where the subscript v is used to denote the constant elution volume comparison. The M_v values calculated across a chromatogram allow determination of number-, weight-, and Z-average molecular weights from the relations shown below:

$$\overline{M}_n = \sum c_v / \sum (c_v / M_v),$$

$$\overline{M}_w = \sum c_v M_v / \sum c_v,$$

$$\overline{M}_z = \sum c_v M_v^2 / \sum c_v M_v.$$

In actuality, M_v is a weight-average molecular weight $(\overline{M}_w)_v$ of a nonmonodispersed sample within the detector cells due to both the finite resolution characteristics of SEC columns and the mixing effect which occurs in the DRI and LALLS detector cells. This does not affect the accuracy of the determined $\overline{M}_w$ values. The calculated values of $\overline{M}_n$ and $\overline{M}_z$ will be, respectively, somewhat greater and less than their true values due to dispersion in the detector cell.[1]

LALLS detection is also a convenient method for determination of the SEC molecular weight vs. elution volume calibration curve. This is of particular importance for characterization of polymers whose narrow MWD standards are not available or whose SEC separation does not closely follow the universal molecular weight calibration.[9] These situations are often encountered in aqueous SEC methods.

Branching Parameters

The branching parameter g_M was defined by Zimm and Stockmayer[11] as the ratio of the mean-square radius of gyration $\langle R^2 \rangle$ of branched and linear polymer of the same molecular weight

$$g_M = \langle R^2 \rangle_b / \langle R^2 \rangle_l \tag{4}$$

where the subscripts l and b denote linear and branched polymers, respectively. The calculation of g_M is usually done using intrinsic viscosity data. In fact, if it is assumed that the Flory–Fox equation

$$[\eta] = \Phi \langle R^2 \rangle^{3/2} / M \tag{5}$$

is valid, where the Flory constant Φ is a function of the Mark–Houwink exponent,[12] then it immediately follows that the ratio of the intrinsic viscosities of a branched and a linear molecule of the same molecular weight is

$$([\eta]_b / [\eta]_l)_M = g_M^{3/2} \tag{6}$$

Zimm and Kilb[13] derived relationships similar to eq. (6) but having an exponent for g_M of 1.0 for a free-draining polymer and 0.5 for a nondraining polymer. Hence, one can generalize these expressions as having a form shown

in

$$([\eta]_b/[\eta]_l)_M = g_M^e \tag{7}$$

where the exponent can vary from 0.5 to 1.5, depending upon the particular theorical assumptions used in the model development.

For experiments employing SEC/LALLS, comparisons of linear and branched molecules are actually being made at constant molecular size (or elution volume), rather than molecular weight and thus one can conveniently define a branching parameter, g_v, as

$$([\eta]_b/[\eta]_l)_v = (M_l/M_b)_v = g_v \tag{8}$$

The relationship between g_v and g_M can be derived as shown below. The intrinsic viscosity of a linear polymer of the same molecular weight as a branched polymer can be described as

$$([\eta]_l)_{M_b} = KM_b^a \tag{9}$$

where K and a are the Mark–Houwink parameters of the linear polymer in the same solvent. Substituting eq. (8) into eq. (9) gives

$$([\eta]_l)_{M_b} = K(M_l/g_v)^a \tag{10}$$

As M_l^a for a linear polymer is

$$M_l^a = [\eta]_l/K \tag{11}$$

(where K and a are the same as before), this relationship can be substituted for M_l^a in eq. (10), yielding

$$([\eta]_l)_{M_b} = ([\eta]_l/g_v^a)_v \tag{12}$$

By definition

$$g_M^e = ([\eta]_b/[\eta]_l)_M$$

Therefore,

$$= [\eta]_b/([\eta]_l/g_v^a)_v$$

and by rearrangement

$$g_M^e = ([\eta]_b/[\eta]_l)_v g_v^a$$

$$= g_v^{a+1} \tag{13}$$

Finally,

$$g_M = (M_l/M_b)_v^{(a+1)/e} \tag{14}$$

Thus the branching parameter g_M can be calculated by comparing the molecular weight of the eluting material and the linear homolog at each elution volume V.

The single assumption made in the derivation of eq. (14) is that the sample being analyzed is monodisperse. Peak broadening effects of SEC may be negligible.[1] If the analytical method is to be employed in comparisons of mixtures of linear and branched polymer chains in the detector cell at same elution volume, it is necessary that the analysis be performed on polydisperse samples. For one specific case, Hamielec and Ouano[14] have derived the following relationship:

$$([\eta]_m/[\eta]_l)_v = \left(M_l/(\bar{M}_n)_m\right)_v \tag{15}$$

where $[\eta]_m$ is the intrinsic viscosity and $(\bar{M}_n)_m$ the number-average molecular weight of polymer mixture in the detector cell. Therefore, with SEC/LALLS alone, we can only obtain a parameter g_v' defined as

$$g_v' = \left(M_l/(\bar{M}_w)_m\right)_v \tag{16}$$

where $(\bar{M}_w)_m$ is the weight-average molecular weight of polymer mixture in the detector cell. In order to obtain the branching frequency of polymer mixtures, it will be required to derive a relationship between g_v' and g_v. This is a subject of current interest in our laboratory, and our results will be reported in subsequent papers.

EXPERIMENTAL

Materials and Sample Preparation

The polysaccharides used in these experiments are all regarded as α-(1,4)-D-glucan polymers with different degrees of branching through α-(1,6)-D-glucan linkages. Amylose is a linear α-(1,4) polysaccharide. The amylose employed in these experiments was purchased from Hayashibara Biochemical Lab. Inc. (Lot No. 71063012) and is reported by the manufacturer as having a nominal degree of polymerization of 110. Two branched polymers (amylopectin and glycogen) were obtained from Sigma Chemical Co. (St. Louis, MO) and used in the branching parameter experiments. In addition, a high amylose corn starch referred to as amylomaize VII supplied by American Maize-Products Co. (Chicago, IL) was examined via these methods. Two standard polymers were also employed: dextran (from Pharmacia) and sodium poly(styrene sulfonate) (NaPSS, from Pressure Chemical).

All solutions for injection were prepared in degassed 0.5*N* NaOH aqueous solution, the same solvent used as the SEC eluent. Polysaccharides solutions were prepared for injection by dissolving known mass quantities of material and diluting to volume with the solvent.

Instruments

Our SEC/LALLS system (see Fig. 1) consisted of three Waters Associates' devices which are 6000A solvent delivery pump, U6K sample injector and R401 differential refractive index (DRI) detector, an LDC/Milton Roy KMX-6 LALLS detector and our self-packed TSK Fractogel HW (Toya Soda Chemical Co., Japan) columns.[8] TSK Fractogel are hydrophilic, semirigid spherical

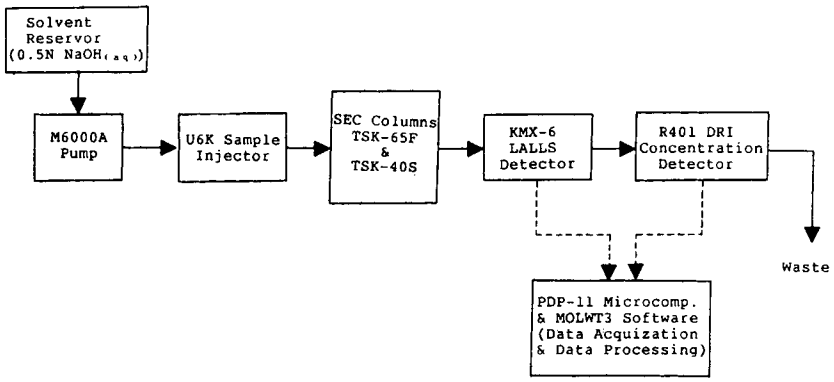

Fig. 1. Block diagram of low-angle laser light scattering detector (LALLS) coupled online with size exclusion chromatograph (SEC).

gels manufactured from vinyl polymers and designed especially for low-pressure, aqueous SEC. They are also chemically stable between pH 1 and 14 and offer good resistance to microbial attack. In order to maintain linearity over the fractionation range, it was determined that a 450 mm TSK-65F column should be coupled to a 225 mm TSK-40S column.[15] In both cases we used 8-mm I.D. stainless steel 304 tubing and Parker column end fittings. Columns were packed using a Micromeritics 705-A stirred slurry column packer.[16] Each column was packed at a flow rate of 0.6 mL/min with an approximately 0.3 volume fraction initial concentration resin slurry of 0.5*N* NaOH aqueous solution. The combined column system (with flow rate 0.08 mL/min) exhibited a plate count of 3900 plates/m relative to a theoretical plate count of 4300 plates/m according to the procedure of Yau et al.[1] The experiments were run at ambient temperature.

The LDC/Milton-Roy KMX-6 LALLS photometer with a flowthrough sample cell was serially connected with the DRI detector, as described by other authors.[17-21] The KMX-6 light source is a 2 mW HeNe laser, which produces polarized radiation with a wavelength of 6328 Å. Scattering intensity data were collected at an appropriate KMX-6 annulus (6–7°). The mobile phase was filtered through an on-line 0.2-μm Fluoropore filter (Millipore Corp.) just before the LALLS cell. Values of specific refractive index increment (dn/dc) and the resultant LALLS optical constant K employed are listed as follows: dextran, dn/dc = 0.142 mL/g,[22] K = 1.46E-7 mol cm^2/g^2; amylose (or amylopectin, glycogen, and starchs), dn/dc = 0.146 mL/g, K = 1.55E-7 mol cm^2/g^2; and NaPSS, dn/dc = 0.140 mL/g, K = 1.42E-7 mol cm^2/g^2. The values of dn/dc for amylose and NaPSS were estimated by comparing the integrated DRI response with a sample whose dn/dc value is known (such as dextran) and using

$$\frac{(\text{total injected mass/integrated DRI response})_k}{(\text{total injected mass/integrated DRI response})_u} = \frac{(dn/dc)_u}{(dn/dc)_k} \quad (17)$$

Where the subscripts u and k denote unknown and known samples, respectively.

During a sample run on the SEC/LALLS system the analog data from the DRI and LALLS detector were collected and digitized through an A/D converter CMX10A, using the SEC/LALLS software package MOLWT3 (LDC/Milton-Roy). With the same software package, those collected data can be processed to give the molecular weight (M_v) at each elution volume. The $\overline{M}_n$, $\overline{M}_w$, and $\overline{M}_z$ of samples were calculated based on M_v as described in the theoretical background section.

RESULTS AND DISCUSSION

Figure 2(a) shows an example of SEC/LALLS chromatograms collected from the DRI and LALLS detectors for amylose DP-110. Based on these detector responses, the molecular weight distribution $\overline{M}_w$ and $\overline{M}_n$ of this sample have been calculated and shown in Figure 2(b). Our data indicate that the calculated $\overline{M}_w$ and $\overline{M}_n$ values are 2.2×10^5 and 2.6×10^4, respectively. The manufacturer's reported nominal molecular weight value is 1.8×10^4 (DP = 110), which was obtained by end-group analysis, a measurement of $\overline{M}_n$.

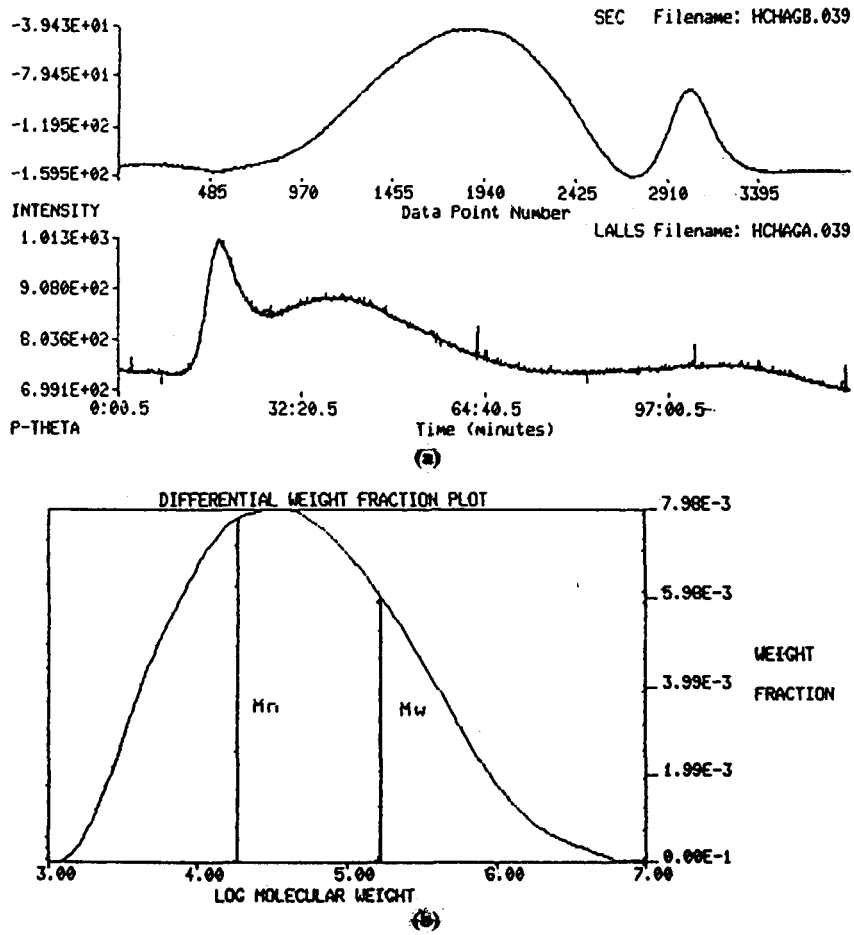

Fig. 2. (a) Typical SEC/LALLS response from DRI and LALLS detectors for amylose and (b) $\overline{M}_w$, $\overline{M}_n$, and molecular weight distribution (MWD).

Although this result is in fairly good agreement with our data, two reasons could exist for the discrepancy: (I) the band broadening and detector cell dispersion effects discussed in the theoretical background section would cause a measured value by SEC/LALLS to be greater than its true value and (II) systematic inaccuracies in the chemical end-group measurement are possible. (Hayashibara used a combination of the Somogyi method and the anthrone method for their chemical end-group determinations.) This example therefore demonstrates that SEC/LALLS is an applicable and convenient method for determining linear polysaccharides average molecular weights and molecular weight distributions.

Once the data files have been established for a given sample, the molecular weights M_v at each elution volume V are obtained. These M_v and V data points can be used to calculate a linear relationship between $\log(M_v)$ and V (see Fig. 4) as done by traditional analysis:

$$\log(M_v) = p + qV \tag{18}$$

Here p and q represent the y-axis intercept and slope of the $\log(M_v)$ vs. V plot. With the relationship $[\eta] = K \cdot M^a$ and knowledge of the Mark–Houwink coefficients K and a, an universal calibration curve may be constructed

$$\log[\eta]M_v = p' + q'V \tag{19}$$

where

$$p' = (1 + a)p + \log K \tag{20}$$

$$q' = (1 + a)q \tag{21}$$

The values of the Mark–Houwink coefficients employed for calculations are listed in Table I. Figure 3 shows the resultant curve modeled from eq. (19) with $p' = 14.65$ and $q' = -1.037$ for amylose (solid line) in 0.5N NaOH aqueous solution. Similar data for dextran and NaPSS are shown, respectively, by open circles and open squares in Figure 3 using the same SEC/LALLS system. As can be seen, there is good agreement between these three polymers. These results establish the universality of the SEC/LALLS method. The method is therefore applicable for broadly distributed polymer samples having widely varying solution characteristics. With this relationship

TABLE I
Mark–Houwink Coefficients of Polymers in 0.5N NaOH Aqueous Solution

Polymers	Temp (°C)	K (dL/g)	a	Reference
Amylose	20	3.65E-5	0.85	24
Dextran	20	1.32E-3	0.48	25
NaPSS	20	1.98E-5	0.83	25
Glycogen	22	1.80E-6	0.70	Present studies
Amylopectin	22	2.30E-5	0.68	Present studies

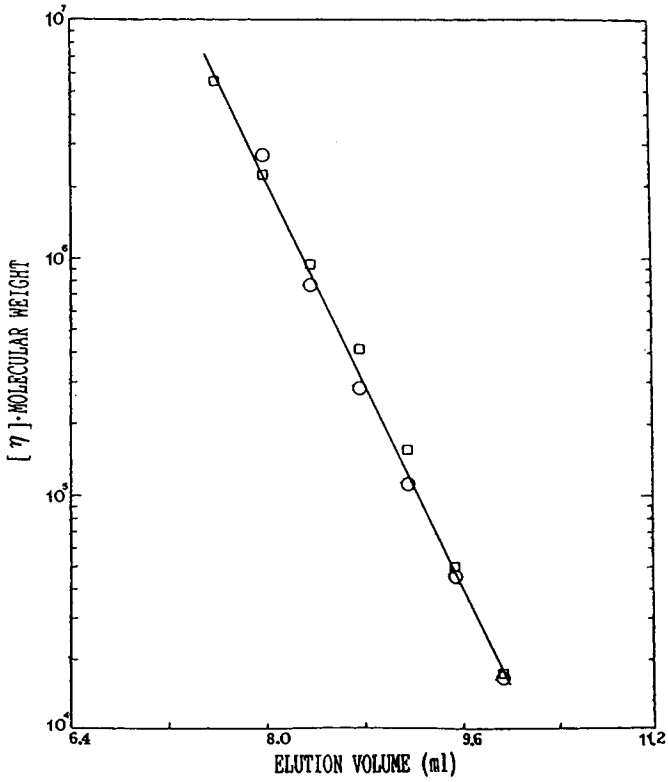

Fig. 3. Universal calibration curve of SEC/LALLS system; the solid line has been derived from amylose calibration curve: (○) dextran; (□) NaPSS.

established, unknown polymers soluble in strong aqueous alkali solvents can also be characterized using eqs. (20) and (21). The values of p' and q' can be obtained from the universal calibration curve, and using the SEC/LALLS analysis, values of p and q can be directly obtained. With knowledge of these values, the Mark–Houwink coefficients of an unknown polymer are obtained with simultaneous solution of eqs. (20) and (21). Hence it is possible to apply SEC/LALLS for determination of Mark–Houwink coefficients indirectly. Cael and coworkers[23] have used this method for cellulose characterization. In Table I, the listed K and a values for glycogen and amylopectin have been calculated by this method from our data and knowledge of the universal calibration curve of Figure 3.

SEC/LALLS is also applicable for the study of branched polysaccharides. The $\log(M_v)$ vs. V data for the four polysaccharides (amylose, amylopectin, glycogen, and amylomaize VII) were analyzed by SEC/LALLS and are shown in Figure 4. Independent chemical analyses indicate that these polymers are primarily α-(1,4)- bonded glucans along the main chain. Amylopectin structure possesses about 4–5% of α-(1,6)- branch points,[26] while the corresponding value for the glycogen structure is approximately 10%.[27] The amylomaize VII material is a corn starch having about 70% amylose and 30% amylopectin, and can therefore be considered a mixed polymer sample. Figure 4 indicates that linear relationships between $\log(M_v)$ and V exist for amylose, amylopectin,

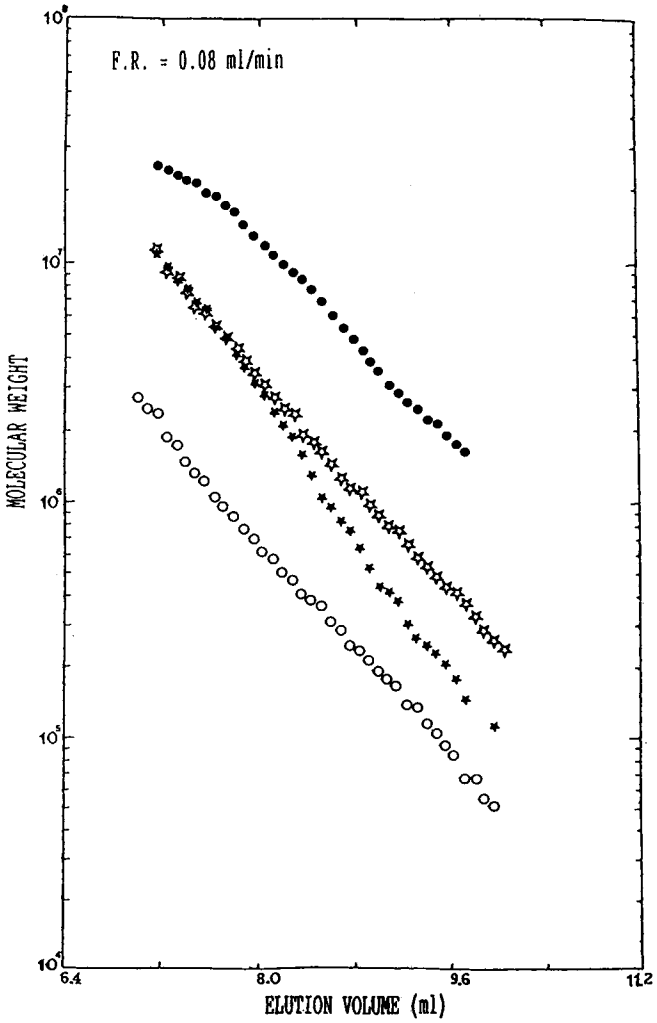

Fig. 4. Plot of $\log(M_v)$ vs. elution volume (V) for four polysaccharides as indicated: (●) glycogen; (☆) amylopectin; (★) amylomaize VII; (○) amylose.

and glycogen, while a more complex relationship of these parameters exists for amylomaize VII. Moreover, Figure 4 indicates that the lines of $\log(M_v)$ vs. V for the three polysaccharides are widely separated and nearly parallel with intercepts increasing with increasing degree of polymer branching. Hence, polymer branching frequency greatly affects the direct molecular weight calibration curve, consistent with our theoretical expectations. Branched molecules, at a common molecular weight, are more compact than the corresponding linear molecule and therefore elute later. As the degree of branching increases (amylose 0% branched; amylopectin 4–5% branched; glycogen 10% branched) at a common elution volume (i.e., hydrodynamic volume) molecular weight increases, as evidenced by LALLS detection of increased number of scattering centers. Therefore, the trends shown in Figure 4 for the polymers examined are totally consistent with the theoretical background presented

above. Amylomaize VII starch shows a noticeable downward curvature of $\log(M_v)$ at low molecular weight range shown in Figure 4. In the high molecular weight range, amylomaize VII has similar molecular characteristics as amylopectin whereas this material is more like amylose at the lower molecular weight range. Such nonhomogeneous characteristics of native starch material have not been established previously.

Nonhomogeneous branching as a function of amylomaize VII starch molecular weight can be more readily seen by transcribing the data into branching parameter values. Such information is presented in Figure 5. Figure 5(a) shows g_v^{-1} (where $g_v = (M_l/M_b)_v$, molecular weight ratio of linear vs. branched polymer) dependence on elution volume for glycogen, amylopectin, and amylomaize VII. In the range examined, glycogen displays g_v^{-1} values indicative of a molecule that is approximately 15–20 times heavier than an equivalent hydrodynamic volume amylose. Correspondingly, amylopectin is 4–5 times heavier than an equilvalent hydrodynamic volume amylose. Values of g_M for these same polysaccharides vs. molecular weight are shown in Figure 5(b). These values have been determined using eq. (14) with $e = 1.0$. These values represent, ideally, the ratios of branched and linear polymer mean-square radii of gyrations. Amylopectin exhibits a mean-square radius of gyration approximately 0.05 the value of an equivalent molecular weight amylose and glycogen a corresponding ratio less than 0.01 that of amylose. Again, amylomaize VII starch displays a strong nonhomogeneous branching characteristic with variation in its molecular weight. The g_M values show a monotonic increasing dependency with decreasing molecular weight. The greatest change occurring between 5 and 10×10^5 daltons. Amylopectin characteristics exist at molecular weight in excess of 10^6 daltons. Branching decreases with decreasing molecular weight. This observation is consistent with experimental evidence[28] that the linear components (amylose) of most starches have a lower molecular weight than their branched components (amylopectin). The molecular weight of amylose in starch is usually less than 10^6. Our data are consistent with this. Interestingly, even for this high amylose containing starch, amylopectin fractions are still confined to the high molecular weight region, and our data in the range of 10^5–2×10^7 do not show any fractions that are purely linear as shown by g_M values.

The branching parameter values presented require further explanation. As discussed in the theoretical background section, in order to calculate g_M for any polymer sample analyzed by SEC/LALLS, the data must be converted from a common elution volume basis to a common molecular weight basis. If peak broadening is negligible, the detected samples can be considered monodispersed and therefore homopolymer characterization can be accomplished. Since g_v is identical to g_v' [see eq. (16)] with this assumption, g_M is a simple function of g_v and the Mark–Houwink coefficients, g_M can be determined. For mixed polymer samples such as amylomaize VII, this procedure cannot be strictly applied. Because the sample being analyzed in the detector cells could be a mixture of linear and branched polymers, the sample is not monodisperse. SEC/LALLS provides only weight-average molecular weight values of mixtures whereas number-average molecular weight values of mixtures are required for comparison [see eq. (15)].[14] Moreover, the relationship of the mixture's intrinsic viscosity and molecular parameters are unknown. Conse-

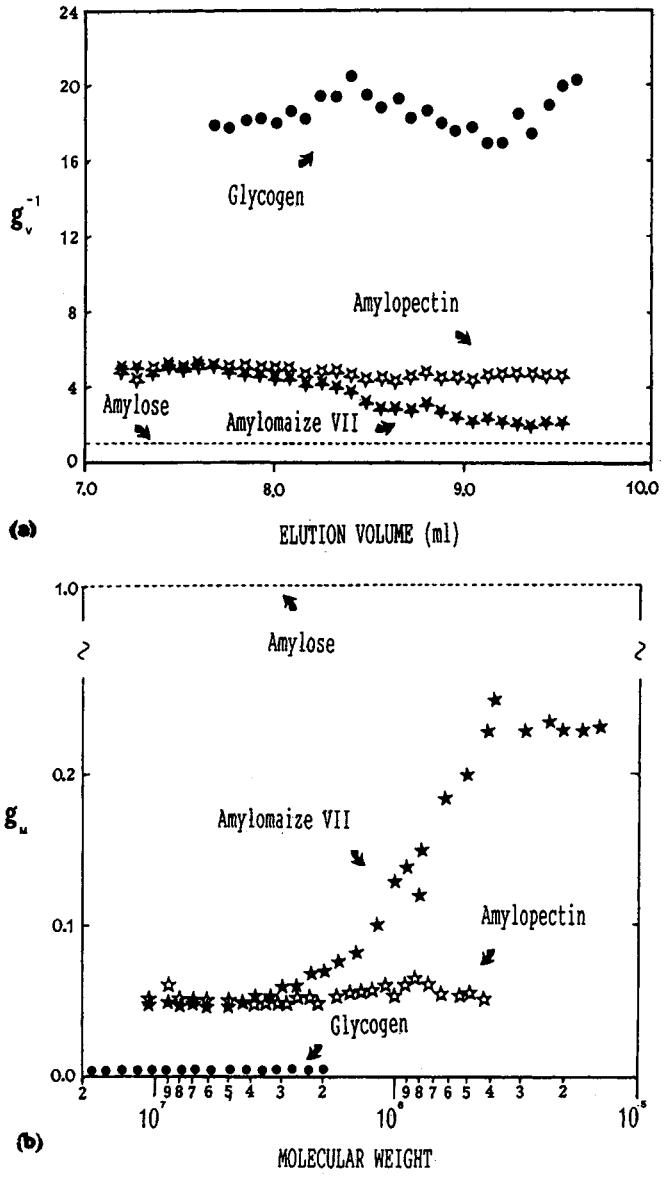

Fig. 5. Branching distribution of polysaccharides (see text): (a) the plot of g_v^{-1} vs. elution volume and (b) the plot of g_M vs. molecular weight for three branched polysaccharides.

quently, mixed polymer sample characterization in terms of the branching parameters, g_v and g_M, cannot be obtained directly at this time. But as indicated in Figure 5, we can qualitatively represent nonhomogeneous branching of amylomaize VII starch with molecular weight. Rigorous quantitation of mixed polymers in terms of the more fundamental parameters g_v and g_M will be the subject of a later paper.

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