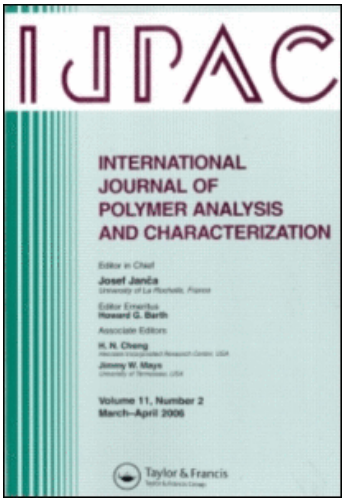

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Assessment of Branching in Polymers by Size Exclusion Chromatography with Light Scattering, Viscosity, and Refractive Index Detection

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Assessment of Branching in Polymers by Size Exclusion Chromatography with Light Scattering, Viscosity, and Refractive Index Detection

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The objective of this work was to assess the methods for determining the degree of long-chain branching in polysaccharides by light scattering and viscosity techniques in combination with size exclusion chromatography (SEC). The samples used were branched dextrans and the reference linear polymers were pullulans. The first method involved determining the ratio of the radii of gyration for branched and linear polymers having the same molecular weight by SEC with multi-angle laser light scattering (MALLS). In the second and third methods, branching was determined by the ratio of the intrinsic viscosities (IV) of branched and linear polymer having the same molecular weight for the SEC slices as well as for the whole polymer, using different values for the structure and solvent factor, ε. The average frequencies obtained for long-chain branching for the whole polymers by light scattering, as well as by viscometry and for the slices by viscometry, agreed well with each other and with those reported from methylation and NMR studies, about 7.5 per 1000 anhydroglucose units. The use of SEC/MALLS/IV was shown to be a useful approach to determine quantitatively the degree of long-chain branching in polysaccharides.

KEY WORDS SEC, Light scattering, viscometry, branching, polysaccharides.

INTRODUCTION

Although numerous studies of long-chain branching in polymers have been published, the use of aqueous size exclusion chromatography (SEC) in combination with multi-angle laser light scattering (MALLS) and viscosity detection is relatively new [1–3]. Kuo et al. [4–5] as well as Styring et al. [6] determined the degree of chain branching in poly(vinyl acetates) using SEC with viscosity and differential refractive index (RI) detectors. Pang and Rudin [7] characterized the degree of long-chain branching in low-density polyethylenes as a function of molecular weight using SEC with low-angle laser light scattering (LALLS), viscosity and RI detectors, and compared their results with those from NMR. Lesec and Millequant [8] studied the degree of branching in model star-branched copolymers of poly(methyl methacrylate) and poly(t-butylacrylate) using SEC with LALLS, viscosity and RI detection. Yu and Rollings [9] applied SEC/LALLS

to determine the molecular weight, molecular weight distribution (MWD), and degree of branching in polysaccharides in 0.5N NaOH aqueous solution. Huber [10] also characterized the branching in hydroxyethyl starches in 0.05M NaCl using SEC/LALLS and discussed qualitatively the type of branching. All of the above methods assumed the applicability of universal calibration.

In our previous paper [11], pullulans and dextrans were characterized by SEC, with MALLS, intrinsic viscosity (IV), and RI detection in terms of their absolute MWD, Mark-Houwink relationships, and radius-molecular weight relationships that described the conformational coefficients as well as the degree of chain branching in dextrans in a qualitative manner. With the above system, it is also possible to determine quantitatively the degree of long-chain branching in polymers by several approaches based on the ratio of the intrinsic viscosities of branched and linear polymers as well as the ratio of radii of gyration. These measurements are not possible with the LALLS system, since LALLS does not provide the angular dependence of scattered light and the radius of gyration. Accordingly, it was the objective of this work to assess the various techniques available in a SEC/MALLS/IV system to determine quantitatively the degree of long-chain branching in dextrans and compare the results obtained with those from NMR and methylation studies.

Because the molecular radii and molecular weights could be determined directly, no assumptions about the validity of universal calibration had to be made. This was important in the current work because the characterization of biopolymers in aqueous media has the additional complexity over organic SEC due to sample support interactions. These include hydrogen bonding, hydrophobic interactions, complex formation, ion exclusion of like charges, as well as ion inclusion from Donnan equilibrium of charged macromolecules. These interactions can modify from ideality the size exclusion behavior of the sample and have been described before [11–14]. Because of these complications, the applicability of universal calibration in aqueous media needs to be verified for each individual case [15].

THEORETICAL CONSIDERATIONS

The equations for determining the degree of long-chain branching are based on the theory that branching decreases the radius of gyration of a polymer chain at constant molecular weight. The branching parameter g is defined from the random flight model by the square of the ratio of the radii of gyration (R) of branched and linear polymers having the same molecular weight [1,16]:

$$g = \frac{\langle R^2 \rangle_b}{\langle R^2 \rangle_L} \quad \text{at constant } MW \tag{1}$$

Furthermore, g can be related to the parameter g' obtained from the hydrodynamic properties, defined as the ratio of intrinsic viscosities of the branched and linear polymer at constant molecular weight and is about equal to g^ϵ [1]:

$$g' = \frac{[\eta]_b}{[\eta]_L} \approx g^\epsilon \quad \text{at constant } MW \tag{2}$$

where ε is the branching structure and solvent factor, and has different values according to various theories [1,17]. The values are as follows:

$$\varepsilon = 3/2 \quad \text{Flory - Fox model [18 - 19]} \tag{3}$$

$$\varepsilon = 1/2 \quad \text{Zimm and Kilb model [20]} \tag{4}$$

$$\varepsilon = 3/4 \quad \text{Roovers [1], Bohdanecky [21], in general} \tag{5}$$

Historically, if the Flory-Fox equation, $[\eta] = \phi R^{3/2}/M$, is valid for branched polymers, then the exponent in Equation (2) is 3/2, and the 3/2 rule is obtained [19,22]. Zimm and Kilb [20] recommended a value of 1/2 based on their bead and string model and their normal coordinate calculation method. They demonstrated that the $g^{1/2}$ rule gave more accurate values for the degree of chain branching for star-shaped polymers under theta conditions. In good solvents, the value of the exponent was about one. Berry et al. [23] demonstrated the applicability of the $g^{3/2}$ rule to comb-type polymers. Later, Berry [24,25] suggested that the value of the exponent might be variable, depending on the polymer and branching type. More recently, Bohdanecky [21] and Roovers [1] recommended the 3/4 rule for general use. Once ε is known, the number of long-chain branches per molecule (B) can be calculated for different branching types from published equations [1,16,20]. For example, for random trifunctional branching in polydisperse samples B is related to the branching parameter g by the following equation [20,22]:

$$g_w = \frac{6}{B}\left[\frac{1}{2}\cdot\left(\frac{2+B}{B}\right)^{1/2}\cdot \ln\left[\frac{(2+B)^{1/2}+B^{1/2}}{(2+B)^{1/2}-B^{1/2}}\right]-1\right] \tag{6}$$

In this work, three experimental methods based on solution properties are assessed and compared with the NMR and methylation techniques. The first method is based on the ratio of the mean-square radii obtained from SEC/MALLS for the whole polymer as described in:

$$g\,(LS,\ \text{whole}) = \frac{\langle R^2\rangle_b}{\langle R^2\rangle_L} \quad \text{at constant } MW \tag{7}$$

In order to determine the degree of long-chain branching by this approach, the radius of gyration and molecular weight for the whole branched sample are needed as well as the radius-molecular weight relation for the corresponding linear polymer, from which the radius of the linear polymer having the same molecular weight and composition as the branched polymer can be calculated. In this work, the weight average radius of gyration was used. The corrections for the differences in the MWD's of the branched and linear samples were small, in the order of a few percent, and were not applied.

The second method is based on the ratio of intrinsic viscosities of branched and linear sample having the same molecular weight as obtained from SEC and viscometry according to:

$$g'\,(IV,\ \text{whole}) = \frac{[\eta]_b}{[\eta]_L} \approx g^{\varepsilon} \quad \text{at constant } MW \tag{8}$$

where ε is equal to 3/4 or 3/2 for the polymers tested. In this case, the intrinsic viscosity and molecular weight of the whole branched polymer is required as well as the intrinsic viscosity-molecular weight relation for the corresponding linear polymer.

The third method is based on the ratio of intrinsic viscosities of the chromatographic slices of the branched and linear samples having the same molecular weight, as obtained from SEC and viscometry:

$$g_i'\,(IV,\ \text{slices}) = \frac{[\eta]_{b,i}}{[\eta]_{L,i}} \approx g_i^{\varepsilon} \quad \text{at constant MW} \tag{9}$$

In this case also, the intrinsic viscosity and molecular weight of the slices of the branched polymer, as well as the viscosity-molecular weight relation of the corresponding linear polymer, are required. The ε values are the same as before. It should be noted that in the first two methods, g was calculated manually, whereas in the third, the g_i were obtained from the Viscotek software.

From methylation and sequential analysis studies of Larm et al. [26], and NMR studies of Wolfrom et al. [27] and Colson et al. [28], the degree of total branching in dextrans is about 5% or 50 branches per 1000 anhydroglucose units (AGU). Since 40% of the side chains are one unit long, and about 45% are two units long, then about 15% of the branches contain more than two units [29]. Assuming that long-chain branches may be considered to consist of more than two units, then there are 7.5 long-chain branches per 1000 AGU in dextrans. If Equations (7–9) are valid and the experiments are accurate, then the degree of long-chain branching obtained by all four methods should agree.

In theory, two other methods are available to determine long-chain branching. One is based on the g_i obtained from SEC slices and light scattering to determine molecular radii for each slice, but branching parameters could not be calculated with the current software. The other technique is called the mass method and is based on the ratio of molecular weights of linear and branched polymers at constant elution volume [3,7,9,30]

$$g_m' = \left(M_1/M_b\right)_v^{a+1} \tag{10}$$

where a is the Mark Houwink exponent. This method is based on the validity of universal calibration for the linear and branched samples. Because in this experiment, the dextrans and pullulans were run at different dates, the universal calibration plots for both samples did not coincide, so that this approach could not be assessed at this time.

EXPERIMENTAL

Materials

Standard pullulans having weight average molecular weights (M_W) of 48.0, 100, 186, 380 and 853 × 10^3 g/mol with a polydispersity (M_W/M_n>) of about 1.1 were obtained from J. M. Science (Buffalo, NY). Standard dextrans having M_W of 97, 165, 325, 750 and 1750 × 10^3 g/mol with a polydispersity of about 1.6 were obtained from American Polymer Standards Corp. (Mentor, OH). The chemical structures of these polymers are given in Figure 1 and show the primarily α-1,4 and α-1,6 linear linkages of pullulans, as well as the α-1,6 linear

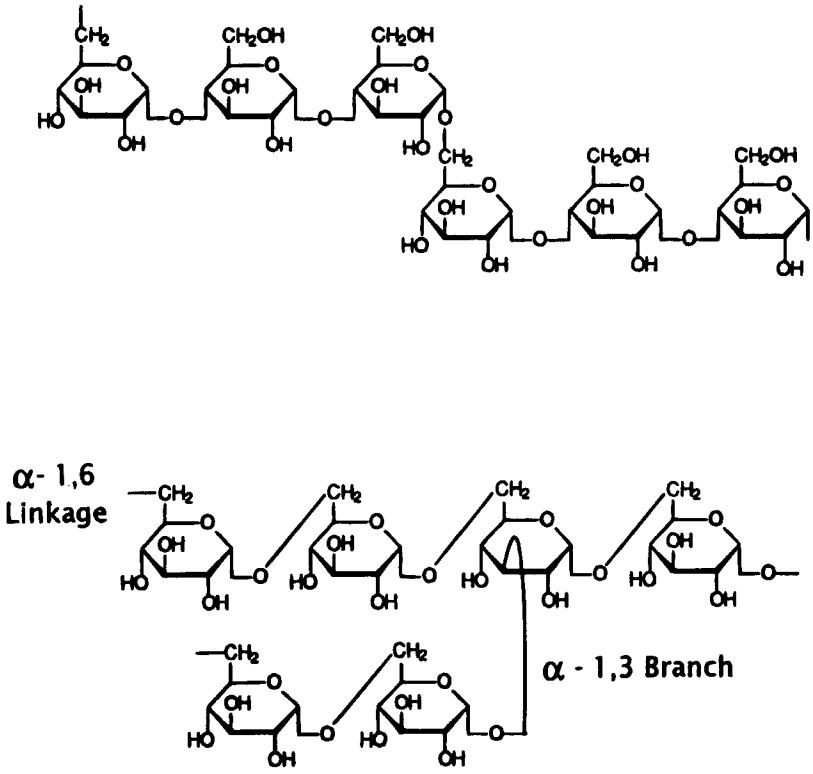

FIGURE 1 Chemical structures of linear pullulan (top) and branched L. mesenteroides B-512 dextran (bottom).

and α-1,3 branches of dextrans. It is assumed that the pullulans are suitable linear reference polymers for the dextrans. The water used to prepare the mobile phase was 18×10^6 Ω-cm purified with the Barnstead Nanopure^R II apparatus. Other materials were reagent grade.

Equipment

The liquid chromatograph was a Waters 150C ALC/GPC operated at 30°C equipped with four TSK PW columns from TosoHaas (Montgomeryville, Pa), as shown in Table I. A Wyatt Technology Corp. (Santa Barbara, CA) model Dawn^R F MALLS photometer was used with a Uniphase (San Jose, CA) argon-ion laser model 2011 at 514.5 nm. The viscosity detector was Viscotek model 100 (Porter, TX) and the differential refractometer of the 150C ALC/GPC was used as the RI detector. The Viscotek software employed was Unical^R 4.05 and the Dawn Software Manual Version 1.01 was used.

Procedures

The detectors were connected in series in the following sequence: SEC-MALLS-viscometer-RI-waste. This configuration was used to maximize the RI signal of the very dilute solutions and to ascertain that both the LS and RI detectors observe the same mass

TABLE I

Experimental Conditions for SEC/LS/IV

Columns	TSK 7.5 mm × 30 cm columns
	1. G2500 PW (1×10^{4}*, 100Å)
	2. G3000 PW (1×10^{5}d, 200Å)
	3. G4000 PW (3×10^{5}d, 500Å)
	4. G5000 PW (1×10^{6}d, 1000Å)
Temperature	30°C for SEC and viscometry RT for Dawn-F
Flow Rate	1 mL/min
Sample Concentration	1–4 mg/mL
Injection volume	0.1 mL
dn/dc @ 514.5 nm	0.147 mL/g

*polyethylene oxide exclusion limit in daltons

in solution. Details of the experimental conditions are given in Table I. The mobile phase employed was aqueous 0.1 *M* $NaNO_3$ with 0.025M KH_2PO_4 and was clarified by filtration with 0.22-μm GS type cellulose nitrate filter (Millipore Corp., Bedford, MA.) and the polymer solutions were filtered through 0.45-μm disposable nylon 66 syringe filters (Altec, Deerfield, III.). Light scattering, IV, and concentration chromatograms were obtained and interpreted as described below. The MALLS was calibrated with HPLC grade toluene. The normalization constants and the delay volumes were determined with a 23×10^3 g/mol pullulan having a nominal radius of about 5 nm as a standard. The RI constant, which relates the RI units to sample concentration, was determined by the dn/dc method in which a sample of known refractive index increment and concentration was used to calibrate the RI detector.

Refractive Index Increment

The dn/dc was determined with an Optilab model 903 (Wyatt Technology) at a wavelength of 514.5 nm. The value used for pullulans and dextrans (Table I) was 0.147 mL/g, the same as that obtained in 0.2M $NaNo_3$ [11].

RESULTS

Mark-Houwink equations for pullulans in terms of the experimental and supplier values for the molecular weights and intrinsic viscosities are as follows:

For pullulans:

Experimental (0.1M $NaNO_3$, 0.025M KH_2PO_4, pH 7):

$$[\eta] = 1.70 \times 10^{-4} M^{0.72} \tag{11}$$

Supplier data (0.05M Na_2SO_4):

$$[\eta] = 2.22 \times 10^{-4} M^{0.66} \tag{12}$$

Similarly, the Mark-Houwink equations for dextrans in terms of the experimental and supplier values for the molecular weights and intrinsic viscosities are as follows:

For dextrans:

Experimental : $$[\eta] = 4.76 \times 10^{-3} M^{0.34} \quad (13)$$

Supplier data : $$[\eta] = 1.13 \times 10^{-2} M^{0.28} \quad (14)$$

Figures 2 and 3 give the double logarithmic plots of the RMS radii versus M_W for pullulans and dextrans, respectively, and the equations from the experimental and theoretical plots are as follows:

For pullulans : $$R_{Ex} = 5.04 \times 10^{-2} M^{0.48} \quad (15)$$

$$R_{Th} = 1.67 \times 10^{-2} M^{0.58} \quad (16)$$

For dextrans : $$R_{Ex} = 1.12 \times 10^{-1} M^{0.38} \quad (17)$$

$$R_{Th} = 4.32 \times 10^{-2} M^{0.45} \quad (18)$$

The theoretical values of the radii in the figures were calculated from the Ptitsyn-Eisner equation [11,31,32]:

$$[\eta]M = \Phi \left(1 - 2.63\gamma + 2.86\gamma^2\right)\left(6^{1/2} R_g\right)^3 \quad (19)$$

where $\Phi = 2.86 \times 10^{23}$, $\gamma = (2a - 1)/3$, and a is the Mark-Houwink exponent. The differences between the experimental and theoretical values are caused by errors in the RMS radii which are attributed to the difficulty of removing all the dust particles from aqueous media as well as to a lower limit of about 10 nm. Although the Ptitsyn–Eisner equation only applies to linear polymers, it is interesting that the calculated values do not deviate much from the experimental ones for the branched dextrans.

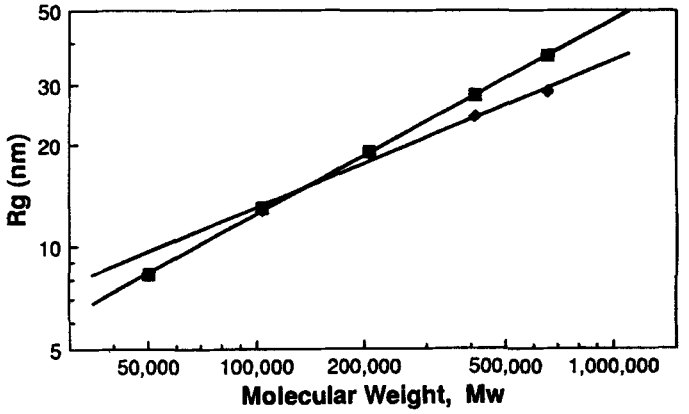

FIGURE 2 Double logarithmic plots of RMS radius versus molecular weight of pullulans based on SEC/light scattering data (diamonds) and as calculated with the Ptitsyn-Eisner equation (Eqn. (16) (squares)).

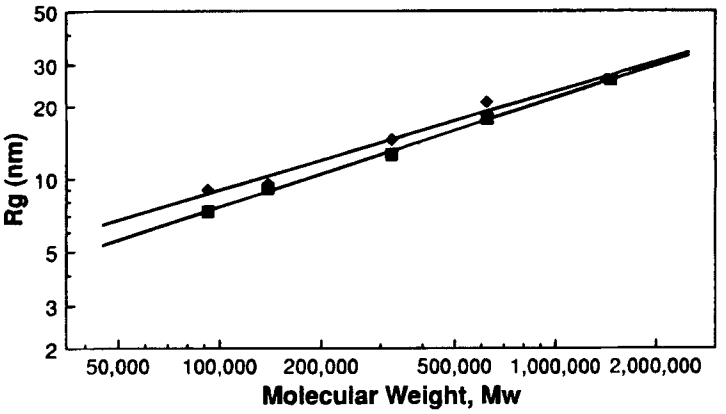

FIGURE 3 Double logarithmic plots of RMS radius versus molecular weight of dextrans based on SEC/light scattering data and as calculated with the Ptitsyn-Eisner equation (Eqn. (16)).

In Figure 4, the Mark-Houwink plots of experimental values for pullulans and dextrans are compared and they demonstrate the lower intrinsic viscosity values obtained for the dextrans relative to the pullulans.

In Figure 5, the experimental RMS radii vs MW data for the pullulans and dextrans are plotted and further demonstrate the lower radii for the dextrans relative to those for the pullulans. The lower intrinsic viscosities and radii for the dextrans is a reflection of the degree of long-chain branching that can be calculated as described above.

In Figure 6, the branching parameter g for five dextrans obtained by the three procedures is plotted as a function of molecular weight: g (LS, whole) was obtained from the ratio of radii of gyration obtained from the light scattering detector for the whole dextran sample relative to that of pullulan; g' (IV, 3/2 rule) was obtained from the viscosity detector for the whole polymer according to the 3/2 rule; g' (IV, 3/4 rule) was obtained from the viscosity detector for the whole polymer according to the 3/4 rule. It is observed that the g values obtained from light scattering and viscometry are in good agreement when the 3/2 rule is applied, but the agreement is poor when the 3/4 rule is applied. The 1/2 rule would yield even poorer agreement.

Figure 7 is a semi-log plot of the g factor obtained from the intrinsic viscosity of the SEC slices versus MW using the 3/2 rule. The value of the branching parameter decreases with molecular weight for each sample as expected.

The branching frequency λ—defined in terms of the number of branches per 1000 anhydroglucose units (AGU), and calculated from Equation (20)—is shown in Figure 8:

$$\lambda = RU \cdot B_w \cdot 1000/MW \tag{20}$$

where RU is the mass of an AGU, which is 162 in this case, B_w is the weight-average number of trifunctional branches per molecule obtained from the g_i of Figure 7 and Equation 6. The fluctuations in g and λ in Figures 7 and 8 are attributed to the unsmoothed fluctuations in the viscosities of the dextran samples, because the Mark-Houwink constants for pullulan were used to calculate branching by the Viscotek software. Although the branching frequency increases with MW as expected, the higher MW fractions exhibit a lower branching frequency, as explained below.

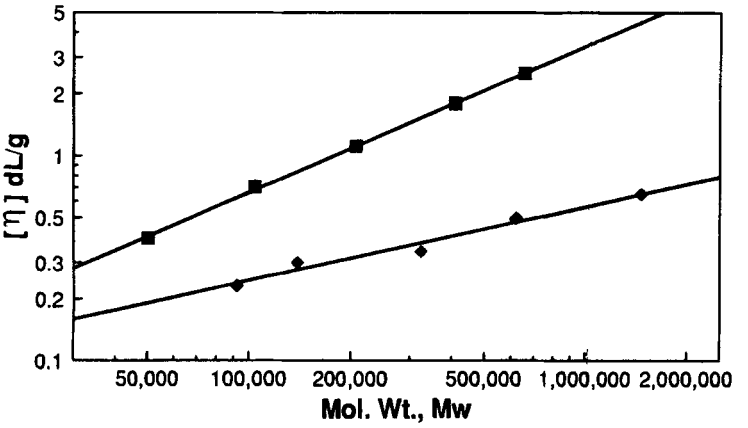

FIGURE 4 Comparison of experimental Mark-Houwink plots for dextran (diamonds) and pullulan (squares) samples.

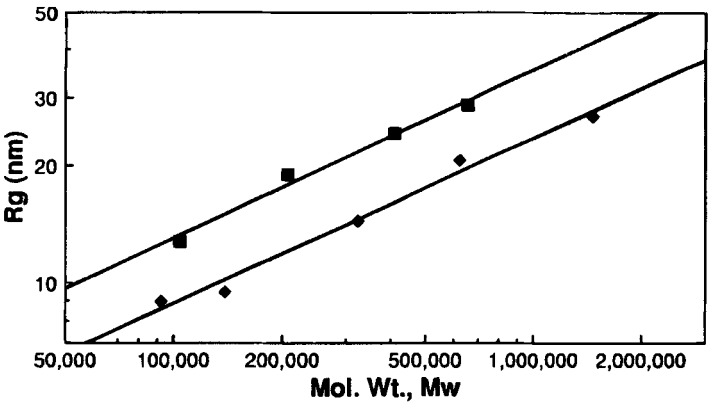

FIGURE 5 Comparison of double logarithmic plots of RMS radius vs molecular weight for dextran (diamonds) and pullulan (squares) samples.

In Figure 9, the branching frequencies obtained for the various dextran whole samples by light scattering and viscometry, as well as for slices are compared to those obtained from NMR and methylation studies. The latter is depicted by the solid line at 7.5 long-chain branches per 1000 AGU. It is observed that the values obtained by light scattering, viscometry, and chemical methods are within the same range.

DISCUSSION

For pullulans, the coefficients k and a in Equation (11) are in agreement with those reported by Tsujisaka and Mitsuhashi [33] in water (2.36×10^{-4} and 0.66) as well as by Kawahara et al. [34] in water (2.36×10^{-4} and 0.66), and by Bahary et al. [11] in 0.2M $NaNO_3$ (4.22×10^{-4} and 0.64). The higher intrinsic viscosities in sodium nitrate and the

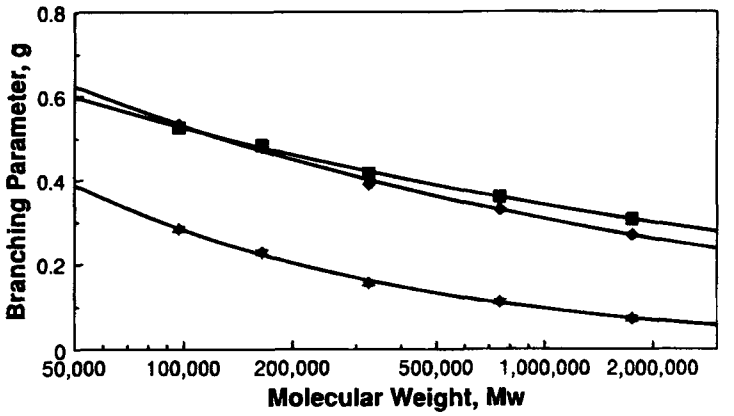

FIGURE 6 Semi-logarithmic plots of the branching parameter g versus molecular weight for whole dextran samples obtained from light scattering and viscosity detectors after SEC: squares, g (LS, whole); diamonds, g (IV, 3/2 rule); stars, g (IV, 3/4 rule)).

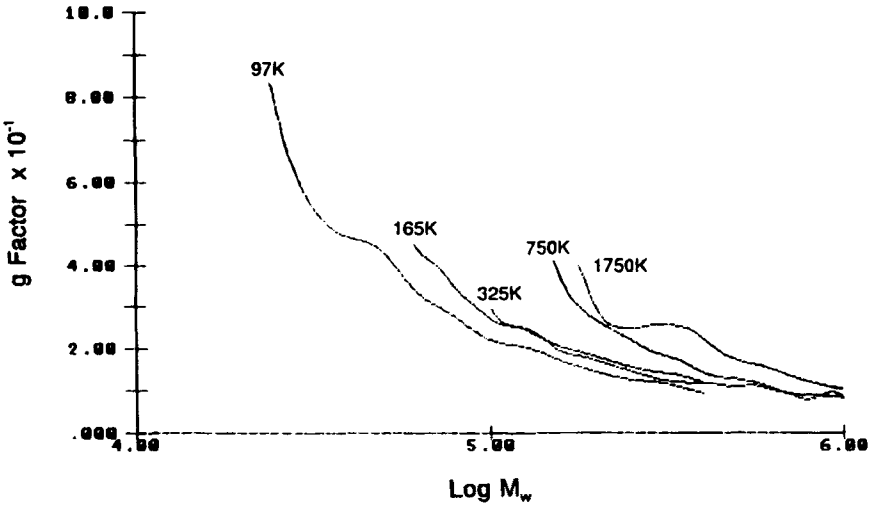

FIGURE 7 Semi-logarithmic plots of the branching parameter g_i (IV, slice) versus molecular weight for slices of five dextran samples obtained from viscosity and Viscotek software after SEC.

phosphate buffer, as well as the higher exponent in the phosphate buffer suggest that these are better solvents for pullulan than water.

For linear fractions of dextrans in water, Senti et al. [35] report values of 97.8×10^{-3} and 0.50 and for branched dextrans the value of the exponent was 0.2. Cerney et al. [36] report values of 10.3×10^{-3} and 0.25 for branched dextrans in methanol/water. The results of this work are in agreement with those reported for dextrans and the exponent corresponds to that for a highly branched coil, about 0.0–0.5 [37].

The conformational coefficients, 0.48 and 0.38, obtained from the double logarithmic plots of RMS radii vs M_w for pullulans and dextrans, respectively, are in agreement with

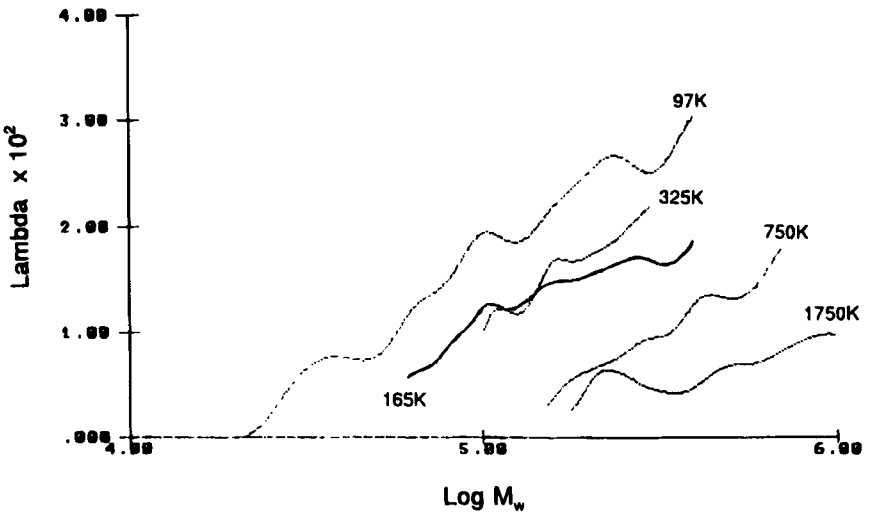

FIGURE 8 Semi-logarithmic plots of branching frequency λ versus molecular weight for slices of five dextran samples. The λ values were calculated from values of g_i (IV, slice) using Equation (6) for B_w and Equation (17) relating λ to B_w.

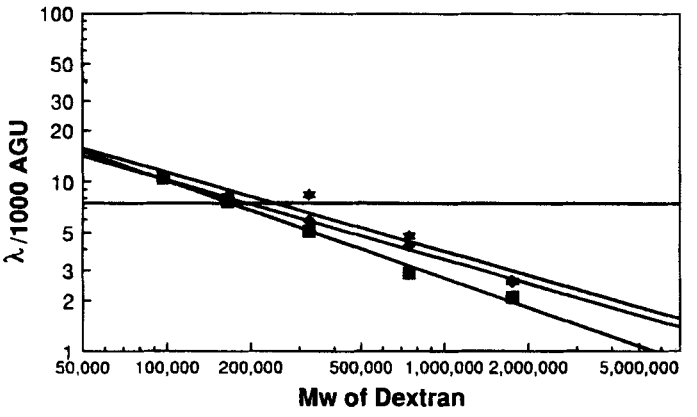

FIGURE 9 Comparison of the average branching frequencies obtained for five dextran samples using the three methods with the value obtained for native dextran by chemical and NMR methods: squares, LS, whole; diamonds, IV, 3/2 rule, whole; star, IV, 3/2 rule, slices; solid line-expected LCB frequency of 7.5 branches per 1000 AGU.

those reported by Jackson et al. [38] in 0.15M sodium nitrate, 0.45 and 0.36. The value obtained for pullulan is close to the range expected for linear polymer coils, 0.5–0.6. and the one for dextran corresponds to the value expected for highly branched coil, 0.33–0.5, as expected. These comparisons confirm the accuracy of the experimental data and impart credence to the branching determinations and the assumptions made as described below.

In calculating the degree of long-chain branching in dextrans using pullulan as a reference linear polymer, three main assumptions were made. The first assumption refers to the use of Equation (6), which relates g to the number of trifunctional branch points in a poly-

disperse sample. Because the branch points in dextran B-512 are trifunctional and the samples are polydisperse according to the SEC chromatograms and to Figure 7, this assumption is justified.

The second assumption is that long-chain branches correspond to greater than two AGU groups. From NMR and SEC studies with polyethylene, a long-chain branch may be considered to consist of a minimum of 6–12 carbon atoms [7,39]. Because the length of each AGU is about 5–6 Å, two AGU's are expected to correspond to about 6–12 carbon atoms. Therefore, the average frequency of long-chain branching in dextrans is 7.5 branches of over two units per 1000 AGU's as given in Figure 9 [29].

The third assumption is that solvent effects are small as the equation relating the ratio of the radii of gyration to g is defined for theta conditions. In the SEC experiments generally good solvents are required. Fortunately, using a good rather than a theta solvent has little effect on quantitative branching determinations [1,3].

One apparent anomaly is the increase in branching frequency λ with molecular weight within each fraction as shown in Figure 8, but a decrease in λ with molecular weight for the higher molecular weight samples (Figures 8 and 9). This may be explained in terms of the procedure used to prepare the samples. Upon fractional precipitation, the branched molecules are more soluble than the linear ones for a given molecular weight, and therefore remain in solution. For this reason, the lower molecular weight fractions have a higher level of branching and are more polydisperse with respect to branching and molecular weight than the higher molecular weight samples (Figure 8).

Based on these assumptions, the branching frequencies obtained for the dextran samples of this work (Figure 9) ranged from 2–10 and bracket the 7.5 obtained from NMR, methylation and sequential degradation, which is encouraging. A value of ε of 3/2 for dextran is reasonable as the branching is extensive and dendritic rather than star shaped. Further, using a good instead of a theta solvent would favor the higher value of ε [1,3].

Because the dextran samples were prepared by fractionation, and all the fractionation data are not available, it is not possible to calculate the average branching frequency for native dextran from this work. However, these results confirm the accuracy of the data, verify the validity of the assumptions, and enhance confidence in polymer solution theory with respect to branching in polysaccharides.

CONCLUSION

Reliable M_W, intrinsic viscosities, and RMS radii for dextrans and pullulans were obtained. The degree of long-chain branching in dextrans from SEC/MALLS for whole polymers was in good agreement with values reported by NMR and methylation. The degree of long-chain branching obtained from SEC/viscometry for whole polymers or from the SEC/viscometry slice method agreed with expected values when the value of the branching structure ε used was 3/2. The use of SEC/MALLS/IV is a useful approach to determine quantitatively the degree of long chain branching in polysaccharides.

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