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Molecular size analysis of capsular polysaccharide preparations from *Streptococcus pneumoniae*

Bohumil Bednar [a] and John P. Hennessey, Jr. [b,*]

Departments of Biological Chemistry [a] and Analytical Research [b], Merck Research Laboratories, West Point, Pennsylvania (USA)

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ABSTRACT

Purified capsular polysaccharide preparations from *Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were analyzed by high performance size exclusion chromatography (HPSEC) with multi-angle laser light scattering (MALLS), specific viscosity (SV), and refractive index (RI) detection to determine the molecular size and molar mass of each of the pneumococcal (Pn) polysaccharides. The M_w's of the polysaccharides ranged from a low of 606 kg/mol for Pn4 to a high of 1145 kg/mol for Pn9V, and the z-average radii of gyration ranged from 59 nm for Pn14 to 72 nm for Pn18C. Estimations of molar mass of the highly anionic polysaccharides (all but Pn14) by the universal calibration approach were unsuccessful, resulting in a 27–53% overestimate of the M_w's, though application of Mark–Houwink–Sakurada coefficients calculated from the HPSEC–MALLS/SV/RI data resulted in estimates of M_w that were in agreement with the MALLS estimates for all but the Pn4 preparation. These results emphasize the need for direct measurement of both molecular size and intrinsic viscosity distributions for definitive characterization of the molar mass, hydrodynamic volume, rigidity, and drainage of complex biological polymers such as the pneumococcal polysaccharides.

INTRODUCTION

Pioneering work by Heidelberger[1] and co-workers in 1945 demonstrated that capsular polysaccharides from *Streptococcus pneumoniae* were immunogenic and, when used as human vaccines, provided type-specific protection against pneumococcal (Pn) infections. Since that time the capsular polysaccharides from 23 serotypes of *S. pneumoniae* as well as several other bacteria (e.g., *Haemophilus influenzae* type b, *Neisseria meningitidis* groups A, C, W135, and Y, and *Salmonella typhi*) have been purified for use in commercial vaccine preparations[2–4]. Chemical characterization of these and other capsular polysaccharides has been relatively thorough in defining their composition and repeating unit structure. It has been established that the primary structures of the capsular polysaccharides are respon-

* Corresponding author.

sible for their serological specificity, but conformational factors can also play a role in this specificity[5,6]. There is sufficient experimental evidence that polysaccharides, like proteins, can form ordered (helical) structures in which both inter- and intra-chain associations are involved, and that such polysaccharides undergo temperature-induced order–disorder transitions[7–11]. Rees[8] also showed that secondary structure can be responsible for some physical as well as biological properties of polysaccharides. Although laser light scattering studies indicate that the group C meningococcal polysaccharide behaves like a random coil in solution[12], helical structures have been identified in oriented fibers and films of selected capsular polysaccharides[13,14].

Physical parameters that have been documented as important in the immunogenicity of capsular polysaccharides are the molar mass and molar mass distribution[4,15–17]. Kabat and Bezer[15] found that in the molar mass range of 90 000 g/mol and above, dextran remains an excellent immunogen, whereas in the range of 50 000 g/mol and below, it exhibited relatively poor immunogenicity. Howard and co-workers[16] have obtained similar results for the type 3 pneumococcal polysaccharide.

While many other aspects of polysaccharide characterization have been relatively thoroughly explored, physical investigations of capsular polysaccharides (e.g., molecular size and weight distribution, rigidity, drainage, shape, and structure) have been less extensively pursued. Such physical studies have been for the most part limited to low-pressure chromatographic analyses that define either relative elution volumes for the polysaccharide preparations or at best yield molar mass estimates relative to secondary polymer standards (e.g., dextran). Given that the intrinsic viscosities, and therefore the hydrodynamic volumes, of polysaccharides of a given molar mass can vary by more than an order of magnitude, such estimates of molar mass can be very misleading. Additionally, such measurements cannot provide definitive information about other critical hydrodynamic properties of polysaccharides such as size, rigidity, segment density, and drainage of the polymer coil.

In the field of synthetic polymer chemistry methods for measurement of these parameters are well defined and are routinely applied to polymers in solution. Specifically, high performance size-exclusion chromatography (HPSEC) with on-line refractive index (RI), multi-angle laser light scattering (MALLS), and specific viscosity (SV) detection can be used to directly define the distribution of molecular size, molar mass, hydrodynamic volume, and intrinsic viscosity for such preparations[18–23]. These parameters can in turn be used to define changes in the segment density, rigidity, and structure of the polysaccharide molecules in solution as a function of molecular size or mass. From this defined base it is then possible to develop a simplified and rigorous method for the routine characterization of preparations of the polysaccharide in terms of molecular size and molar mass.

In this study HPSEC–MALLS/SV/RI analysis was used to provide basic physical characterizations of seven purified pneumococcal polysaccharides, with a

view to developing a simplified method for routinely monitoring of the molecular mass distribution of these polysaccharide preparations. Such characterizations are invaluable in understanding the hydrodynamic behavior of the polysaccharides in aqueous solutions, and in developing these complex compounds for use in human vaccine formulations.

EXPERIMENTAL

Polysaccharide purification and preparation.—Purified pneumococcal capsular polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were obtained from Merck Manufacturing Division (repeating unit structures shown in Fig. 1). These polysaccharides were purified from *S. pneumoniae* cultures using a series of alcohol fractionation steps such as described by Williams and Chase[24]. Samples were dissolved in deionized water at a concentration of 10 mg/mL, mixed by inversion overnight, and then transferred to dialysis bags (MW cutoff 12–14 kDa). The samples were dialyzed at 4°C versus 200 vol of 0.1 M sodium phosphate containing 0.05% (w/v) sodium azide, pH 7.2, overnight, with three changes of the dialysis buffer. After dialysis the samples were filtered through 0.22-μm nylon-66 filters (Rainin), diluted to $\sim$1 mg/mL, and stored at -20°C until used.

For each polysaccharide type analyzed a reference standard solution was prepared in which the starting polysaccharide powder was subjected to thermal gravimetric analysis to determine residual volatiles content, and then the powder was dissolved in deionized water at a concentration of 1.0 mg dry weight per mL. The concentration of polysaccharide in each working sample was determined by integrating the area under the HPSEC–RI profile and comparing that to the area under the RI profile for a known volume of the respective reference standard.

Measurement of dn/dc.—Analysis of the change in refractive index as a function of the change in polysaccharide concentration was done with a Brice–Phoenix interferometric refractometer set at 546 nm and 30°C. Values were extrapolated to 633 nm via a Cauchy dispersion.

HPSEC analysis.—All samples and standards were stored at 4°C and analyzed at a concentration of 0.5 mg/mL. A 50-μL sample was injected in all cases. HPSEC analysis was performed on two HPLC systems utilizing the same set of columns (Waters Ultrahydrogel guard column, two 0.7×30 cm Ultrahydrogel linear columns) and a mobile phase consisting of 0.1 M sodium phosphate with 0.05% (w/v) sodium azide, pH 7.2. The columns were maintained at 30°C. The digitized detector signals from the two HPLC systems were analyzed following synchronization of the retention times of the two RI profiles.

HPSEC–RI/SV analysis was performed using a Spectra–Physics HPLC pump (model 8700), a Waters model 715 UltraWISP autosampler equipped with a cooling unit, a Waters model 410 refractive index detector, and a Viscotek model 100 differential viscometer. The differential viscometer, designed as a fluid analogue of a Wheatstone bridge[25], measures the pressure difference across the

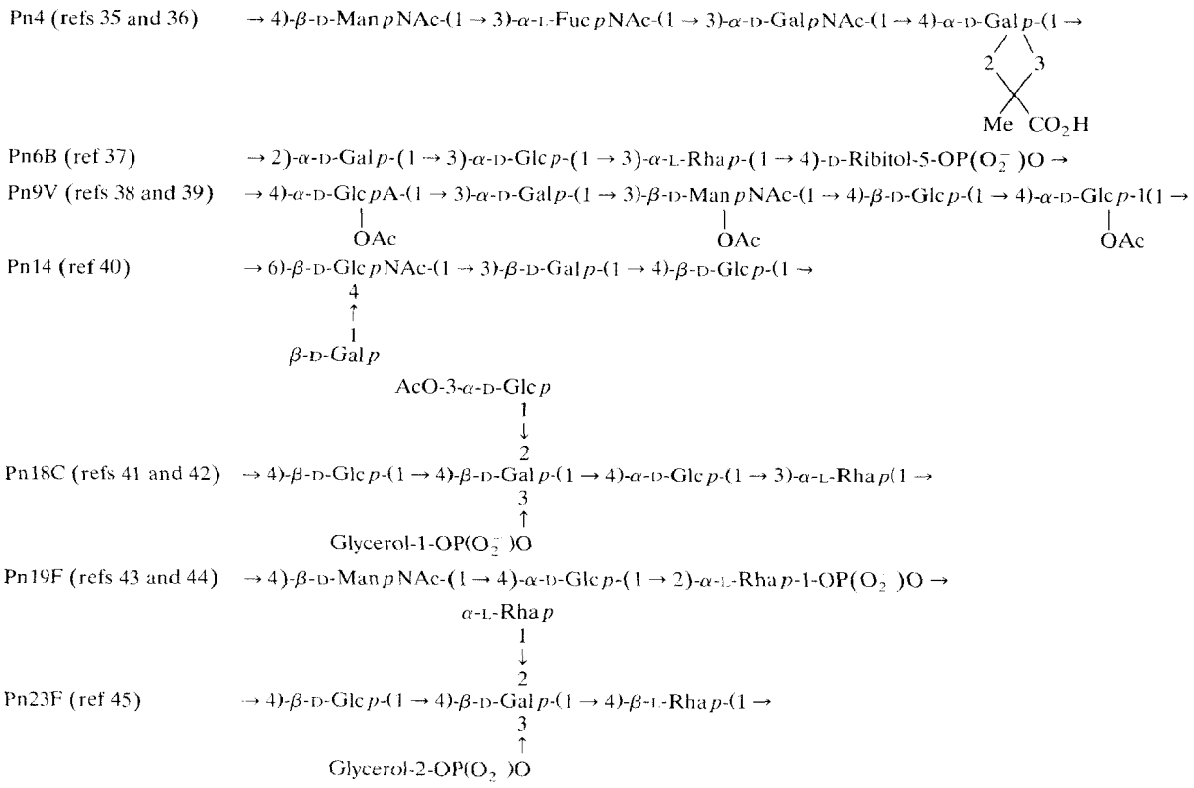

Fig. 1. Repeating unit structures for the pneumococcal polysaccharides.

capillaries between sample plus solvent and solvent alone. This difference in pressure (ΔP) is converted into a measure of specific viscosity (η_{sp}) using the equation $\eta_{sp} = 4\Delta P/(P_i - 2\Delta P)$, where P_i is the inlet pressure to the capillary bridge and is held constant. The intrinsic viscosity $[\eta]$ is calculated from the η_{sp} value and the refractive index detector's measure of sample concentration (c) using the equation $[\eta] = \eta_{sp}/c$, where c is assumed, for all practical purposes, to be close to zero. Chromatographic data were acquired and analyzed using Viscotek's Unical V3.12 software.

HPSEC–RI/MALLS analysis was performed using a Waters model 715 Ultra-WISP autosampler, a Waters model 510 HPLC pump, a Waters model 410 refractive index detector, and a Dawn-F MALLS photometer (Wyatt Technology Corp.). The Dawn-F photometer measures the light scattered from a flowing sample at 15 angular positions in the range of 15–150°. A chromatographic peak is typically divided into 500–1000 slices. For each slice, a weight average molar mass, M_w, is calculated together with a z-average radius of gyration, R_g. The initial slope of the reciprocal scattering function K^*c/R_θ, as a function of $\sin^2(\Theta/2)$, yields $R_g^2 = (3\lambda_0)/(16\pi^2 n_0^2)k_1$, where k_1 is the slope as $\Theta \to 0$. The intercept of this extrapolated line with the K^*c/R_Θ axis is $K_0 = 1/M + 2A_2c$, where R_Θ is the excess Rayleigh ratio, K^* is a physical constant proportional to the square of the refractive index increment, c is the polymer concentration, and A_2 is the second virial coefficient[26]. Values of A_2 for several slices of the sample measured were estimated using a Zimm plot incorporated in ASTRA™ software (typical values were in the range from 5 to 10×10^{-4} cm^3/g). Chromatographic data were acquired and analyzed using Wyatt Technology's ASTRA™ and EASI™ software.

Narrow molecular weight range polyethylene oxide (PEO), dextran (American Polymer Standards), and pullulan (Polymer Laboratories) standards were used to characterize the performance of the HPSEC columns and as "control" samples for these analyses.

RESULTS AND DISCUSSION

Column calibration and characterization.—Fig. 2a shows a "universal calibration" plot for the PEO, dextran, and pullulan standards run on the Ultrahydrogel linear columns in the sodium phosphate mobile phase, based on vendor-supplied data on molecular masses. The experimental data were best fitted with a third-order polynomial equation. Data for the two smallest PEO standards do not conform to the universal calibration line, in agreement with the previous findings of Lesec and Volet[27]. In contrast, Nagy[28] found no such discrepancy for PEO or polyethylene glycol standards run on Toyo Soda TSK-PW columns in an aqueous mobile phase.

A universal calibration curve was also constructed using intrinsic viscosity data from the HPSEC–RI/SV analysis and molecular mass data from the HPSEC–RI/MALLS analysis of pullulan standards (Fig. 2b). These data are well fitted

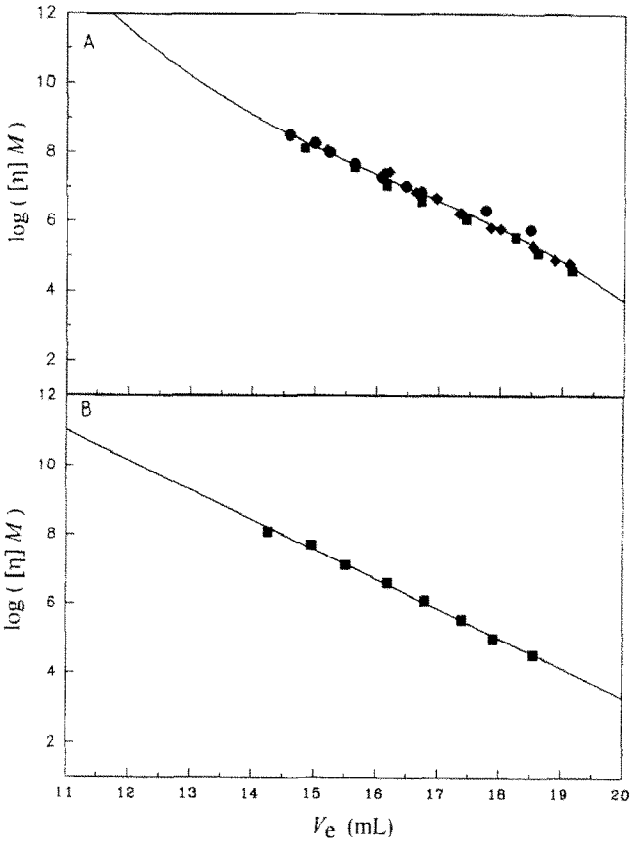

Fig. 2. Universal calibration plots for HPSEC analysis using Waters Ultrahydrogel linear columns and 0.1 M sodium phosphate, pH 7.2 as the mobile phase. A, Universal calibration plot derived from HPSEC–RI/SV analysis of pullulan (■), dextran (♦), and polyethylene oxide (●) molecular mass standards using vendor-supplied values of molecular mass. B, Universal calibration plot derived from analysis of pullulan polysaccharides using M_w values determined by HPSEC–RI/MALLS analysis.

with a first-order polynomial. The differences between these two universal calibration plots for the Ultrahydrogel linear columns is attributable to the differences between the molecular masses of the pullulan standards defined by the vendor and those measured by our analyses for narrow fractions at the peak maximum.

Analysis of pneumococcal polysaccharides.—Preliminary HPSEC analyses of the pneumococcal polysaccharides were performed at a variety of sample polysaccharide concentrations to insure that viscosity-related artifacts did not impact our results and that sample recovery was complete. None of the polysaccharides examined showed altered elution volumes or peak shapes on varying the sample concentrations from 0.2 to 1 mg/mL. Variation of the ionic strength of the mobile phase (from 0.05 to 0.5 M sodium phosphate) or the flow rate (0.2 to 1.5 mL/min) also did not significantly change the elution volumes or elution profiles of the samples. Additionally, plots of peak area versus mass injected onto the column

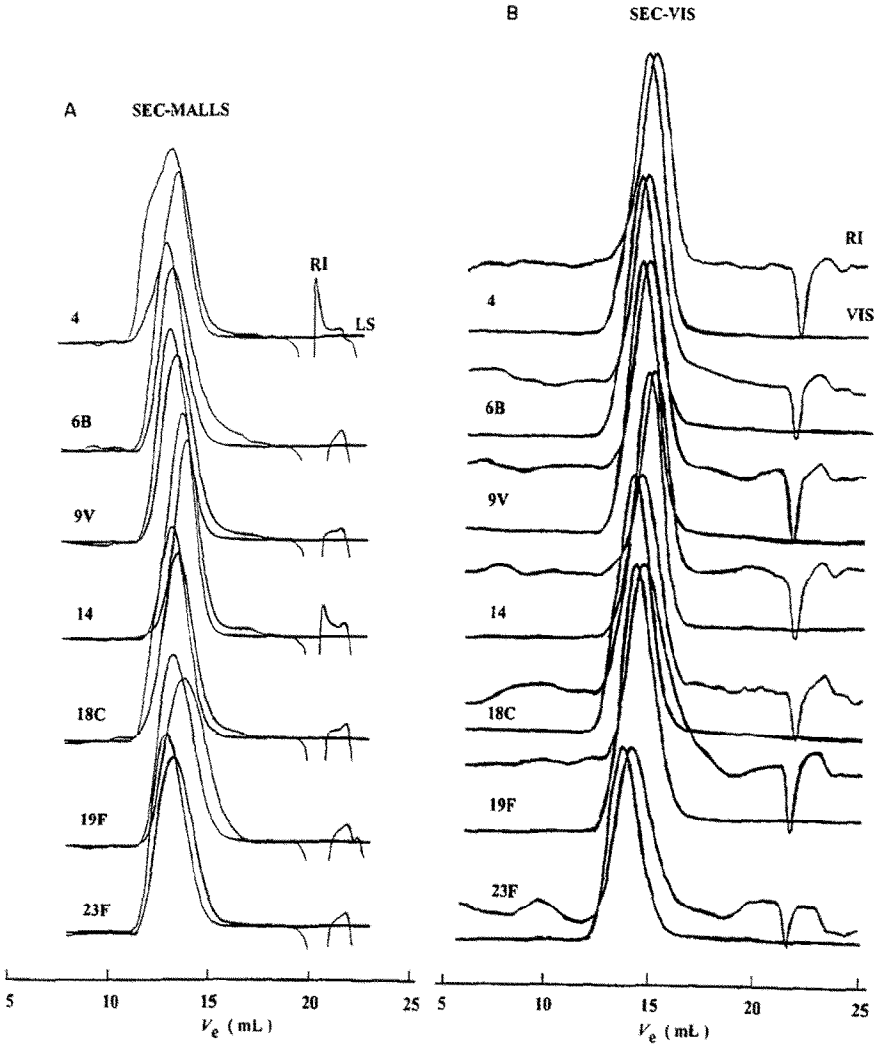

Fig. 3. HPSEC elution profiles for the pneumococcal polysaccharides on the Waters Ultrahydrogel linear columns with the 0.1 M sodium phosphate, pH 7.2 as the mobile phase. For each analysis the peak in the RI profile is at a slightly greater elution volume as expected. A, HPSEC–RI/MALLS (at 90°) profiles. B, HPSEC–RI/SV profiles.

were linear with comparable slopes for all polysaccharides (data not shown), indicating complete recovery of the samples in these HPLC systems.

These findings also establish that, in the HPSEC analyses, separation mechanisms other than size exclusion (e.g., ion exclusion, ion repulsion, ion exchange, hydrophobic interactions, and hydrogen bonding), originating from specific solute–matrix interaction[19], do not contribute significantly.

Figs. 3a and 3b show the RI, MALLS (at 90°), and SV detector profiles for all polysaccharides studied. There are no indications of unexpectedly broad distribu-

tions in the chromatograms, which again suggests that size-exclusion is the predominant mechanism for the chromatographic resolution of the pneumococcal polysaccharides in this HPSEC system. The profiles of Pn4 in Fig. 3 have a shoulder at the leading edge, which is especially pronounced in the MALLS trace. Given that the response of the MALLS detector is predominantly dependent on molar mass and can thus reflect very low concentrations of very high molar mass molecules[20–22], these data suggest that the Pn4 preparation contained a low concentration of molecules with very high molar mass. Both the RI and the SV detectors, which are less molar mass-dependent than the MALLS detector, show profiles for Pn4 having a much less pronounced shoulder.

Molecular size and molar mass distributions.—Molecular size and molar mass distributions were determined using the integrated data from the two independent HPSEC systems, one including MALLS and RI detectors and the other including SV and RI detectors.

HPSEC–RI / MALLS.—The data from the RI and MALLS (responses for all angles; for details, see Experimental) detectors, shown in Fig. 3a (MALLS only at 90°), were used to calculate mean square radius of gyration and molar mass distributions for each polysaccharide preparation. From these distributions, values for the z-average radius of gyration (R_g), the number (M_n) and weight (M_w) average molar masses, and polydispersity (M_w/M_n) were derived (see Table I). Such calculations required the determination of specific refractive index increments, dn/dc, also shown in Table I for the pneumococcal and pullulan polysaccharides measured. The values are in good agreement with dn/dc values for similar polysaccharides published elsewhere[12]. The molar mass distributions of the Pn9V, Pn18C and Pn14 polysaccharide preparations are narrow, as reflected by the patterns shown in Fig. 4 and by the low values of M_w/M_n (< 1.5) in Table I. In contrast the molar mass distributions of 6B and 19F polysaccharides are quite broad ($M_w/M_n = \sim 2$), with the Pn6B preparation showing a small population of

TABLE I

Molecular size and molar mass parameters for pneumococcal and pullulan polysaccharides. The relative standard error for all values is $\leqslant 5\%$

Polysaccharide	dn/dc (cm^3/g)	M_n (kg/mol)	M_w (kg/mol)	M_w/M_n	R_g (nm)	$[\eta]$ (cm^3/g)
Pn4	0.130	357	606	1.69	70	464
Pn6B	0.131	434	926	2.13	61	272
Pn9V	0.132	821	1145	1.40	66	215
Pn14	0.139	753	1100	1.46	59	181
Pn18C	0.130	564	770	1.37	72	560
Pn19F	0.136	359	700	1.95	63	314
Pn23F	0.136	733	1106	1.51	68	421
Pul-880	0.137		1010		42	114
Pul-440	0.137		444		25	91

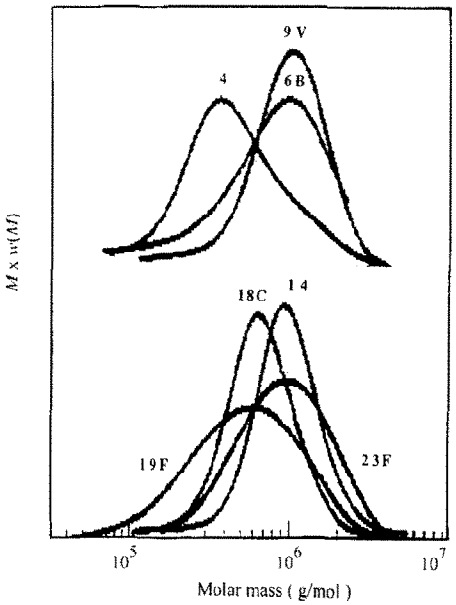

Fig. 4. Molar mass distribution profiles for the pneumococcal polysaccharides as determined by HPSEC–RI/MALLS.

larger molecules and Pn19F showing a wider distribution of molar masses. This wider distribution of Pn19F may be due to its inherently greater susceptibility to hydrolytic cleavage (personal observations), although the relative width of the molar mass distribution profiles may be a function of both the purification process and the biosynthesis of the respective polysaccharides.

HPSEC–RI/MALLS analysis of all polysaccharide preparations provided a measure of their radii of gyration, listed in Table I. The highest values of R_g were found for the Pn18C, Pn4, and Pn23F preparations, whereas Pn14, the only polysaccharide examined that did not carry a negative charge in its repeating unit, had the lowest R_g value. The values of R_g for the Pn6B, Pn9V, and Pn19F polysaccharides are only slightly higher than the R_g for Pn14. The variation in R_g values probably reflects differences in the primary, and possibly the secondary and tertiary structures of the different polysaccharides analyzed, as well as differences in the expansion of the polymer coils in solution.

The weight average molar mass for the pneumococcal polysaccharides examined by HPSEC–RI/MALLS ranges from ~ 600 000 to ~ 1 200 000. Pn4 has the lowest values of M_n and M_w of all polysaccharides examined in this study (see Table I), though the M_z value is comparable with the M_z values of other polysaccharides (data not shown), thus reflecting the pronounced leading edge on the molar mass distribution profile (see Fig. 4).

HPSEC–RI / SV.—Integration of the chromatograms obtained by HPSEC–RI/SV analysis (see Fig. 3b) provided values of intrinsic viscosity, $[\eta]$, for all the

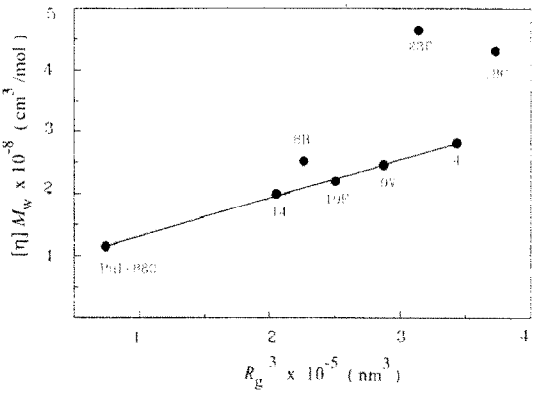

Fig. 5. The relationship between z-average radius of gyration (R_g) and hydrodynamic volume ($[\eta]M_w$) for the pneumococcal polysaccharides.

polysaccharide preparations (see Table I). These values in general correlate with the R_g values obtained by HPSEC–RI/MALLS analysis in that high $[\eta]$ typically corresponds to high R_g, though Pn9V shows a significant discrepancy from this trend.

In an attempt to compare the "hydrodynamic" behavior of polysaccharide macromolecules[29], we have plotted $[\eta]M_w$ vs. R_g^3 (see Fig. 5). The values for the linear, or largely linear pneumococcal polysaccharides Pn14, Pn19, Pn9V, and Pn4, and Pul-880 fit well on a straight line, suggesting that these polymer molecules display similar hydrodynamic behavior. Pn6B, also a linear polysaccharide, deviates slightly from this linear relationship. However, the pneumococcal polysaccharides with ramified repeating units, Pn23F and Pn18C, show significantly different behavior, with a greater ($[\eta]M_w$) to R_g^3 ratio than seen for the other polysaccharides, indicative of much stronger intramolecular hydrodynamic interactions.

Calculation of Mark–Houwink–Sakurada coefficients.—Fig. 6 shows log–log plots of $[\eta]$ versus M_w for the pneumococcal and pullulan polysaccharide preparations. These plots were obtained by using the molar mass profiles calculated from the HPSEC–RI/MALLS analyses and the $[\eta]$ profiles calculated from the HPSEC–RI/SV analyses, and synchronizing the two data sets based on overlaying of the respective RI profiles. In each case the data are confined to a range where the signal-to-noise ratio is greater than 10 for both analyses, but in each case the range spans at least an order of magnitude on the molar mass axis, which is enough to provide a reliable linear least-squares regression analysis, and thus reliable estimates of the coefficients K and a of the Mark–Houwink–Sakurada (MHS) equation (see Table II).

It is important to stress that the MHS coefficients were calculated from data covering a specific range of molar masses and that they are not necessarily valid outside this range. Some polyelectrolytes or worm-like polymers have been found to exhibit curvature or even abrupt changes in the slope of MHS plots[30–32] over a broad range of molar masses. This has resulted in some papers reporting different

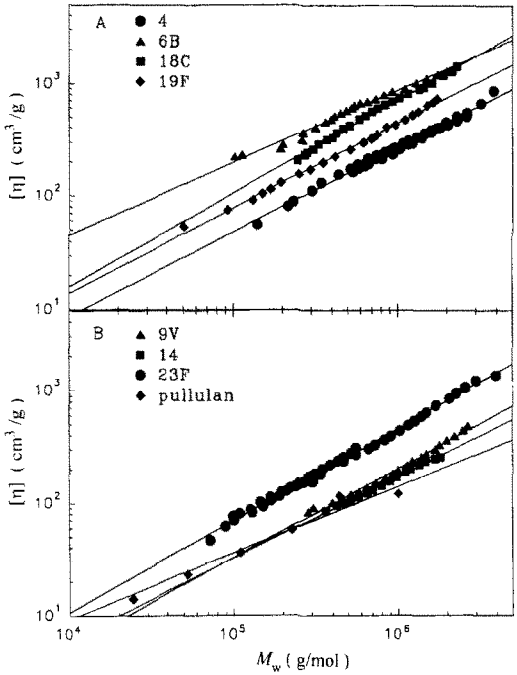

Fig. 6. Mark–Houwink–Sakurada plots for the pneumococcal and pullulan polysaccharides.

MHS coefficients for a given polymer over different molar mass ranges[32]. We have as yet no definitive experimental evidence that this is the case for the pneumococcal polysaccharides examined here, but we do detect slight curvature in the MHS plot for the Pn18C preparation (see Fig. 6a). Additional experiments with polysaccharide preparations having lower molar masses will be necessary to support or eliminate such possibilities.

With the aid of the MHS coefficients calculated above, the HPSEC–RI/SV data can be used to directly calculate the molar mass distribution of the polysac-

TABLE II

Parameters of the Mark–Houwink–Sakurada equation, $[\eta] = KM^a$, for pneumococcal and pullulan polysaccharides in phosphate buffer, pH 7.2 at 30°C

Polysaccharide	K $(cm^3/g) \times 10^3$	a
Pn4	116	0.65
Pn6B	5.64	0.78
Pn9V	3.61	0.79
Pn14	7.69	0.72
Pn18C	7.49	0.84
Pn19F	13.1	0.76
Pn23F	5.81	0.82
Pullulan	39.7	0.59

TABLE III

Comparison of the calculated molar masses of the polysaccharide preparations

Poly-saccharide	HPSEC–RI/SV, MHS coeff.			HPSEC–RI/MALLS	HPSEC–RI/SV, Universal calibration
	M_n (kg/mol)	M_w (kg/mol)	M_w/M_n	M_w (kg/mol)	M_w (kg/mol)
Pn4	259	373	1.44	606	630
Pn6B	780	947	1.21	926	1290
Pn9V	843	1070	1.27	1145	1410
Pn14	1037	1227	1.18	1100	1390
Pn18C	539	697	1.29	770	1040
Pn19F	413	629	1.52	700	940
Pn23F	672	872	1.30	1106	1740

charide sample preparations independent of calibration standards. The results of such calculations are shown in Table III. Assuming the accuracy of the MHS coefficients, these calculated molar mass distributions should theoretically be equivalent to those calculated from the MALLS data. Given that neither of the HPSEC systems was characterized for axial dispersion, values of M_n can be expected to be biased by the individual system parameters (both chromatographic and calculational) and should, therefore, be subject only to relative rather than absolute comparisons. However, M_w values for the various sample preparations should be, and as shown in Table III are, directly comparable. With the exception of Pn4, whose M_w value as derived from MHS coefficients was only 62% of that determined by HPSEC–RI/MALLS, MHS-derived M_w's are 0.79 to 1.12 times the HPSEC–RI/MALLS values, showing fairly good agreement between the two methods. The discrepancy in the Pn4 sample can be attributed to the higher degree of sensitivity with which the MALLS detector picks up the low concentration of high molar mass molecules relative to the SV detector. Table III also shows a comparison of the M_w's for the polysaccharides as calculated from the HPSEC–RI/SV data using the universal calibration method. Values obtained by this method are in reasonable agreement with the HPSEC–RI/MALLS data only for Pn4. The other M_w values from the universal calibration analysis range from 1.23 to 1.57 times higher than those obtained by HPSEC–RI/MALLS, in general a much poorer fit than that resulting from the use of the MHS coefficients to derive M_w's from the HPSEC–RI/SV data.

The apparent failure of the universal calibration analysis of the pneumococcal polysaccharides examined here can in large part be attributed to the lack of suitable high molar mass calibration standards. Though many of the pneumococcal elute very close to the largest pullulan (M_w, 850000) and PEO (M_w, 937500) standards, for a proper third-order fit of the $\log([\eta]M)$ versus elution volume function for the Ultrahydrogel linear columns in the range of interest, at least one calibration standard of $M_w > 1500000$ would be required. It is also possible that

TABLE IV

Physical parameters for characterization of the pneumococcal polysaccharide preparations

Polysaccharide	R_g (nm)	R_g^η [a] (nm)	$R_g/M_w^{1/2}$ (nm)	$R_g^\eta/M_w^{1/2}$ (nm)	D_{Rg} (kDa/nm^3)
Pn18C	72	57	0.082	0.064	0.49
Pn4	70	44	0.090	0.057	0.42
Pn23F	68	58	0.065	0.055	0.84
Pn9V	66	46	0.062	0.043	0.95
Pn19F	63	44	0.075	0.052	0.67
Pn6B	61	46	0.063	0.048	0.97
Pn14	59	41	0.039	0.039	1.28
Pullulan [b]			0.038 ± 0.002	0.037 ± 0.001	

[a] Calculated as the z-average values from calculated $R_{[\eta]}$ and R_g^η versus elution volume profiles for each sample. [b] Values shown represent the average for all pullulan standards examined.

the influence of mechanisms other than size exclusion in the chromatographic separation, i.e., repulsive interactions between the polysaccharide and the column matrix or matrix diffuse double layers, resulting in early elution for very high molar mass polysaccharides, could bias the universal calibration estimates of molar mass.

Physical properties of the pneumococcal polysaccharides in aqueous solution.— Additional information about the physical properties of these polysaccharides can be derived from the data presented above. Several of these parameters are shown in Table IV. The viscosity radius of gyration, R_g^η, as calculated using the Flory–Fox[33] and Ptitsyn–Eizner[34] relationships (eq. *1*),

$$R_g^\eta = (1/6^{1/2})\left[[\eta]M/\Phi_0(1 - 2.63e + 2.86e^2)\right]^{1/3} \quad (1)$$

where Φ_0 is the Flory universal constant ($= 2.86 \times 10^{21}$) and $e = (2a - 1)/3$, with a being the exponent of the Mark–Houwink–Sakurada equation, provides additional insight as to the molecular size and conformation of these polysaccharide preparations. R_g^η indicates conformational aspects of the polymer coil, as characterized by the MHS coefficient a (see Table II), which reflects the flexibility of the polymer in the nondraining limit (i.e., as the structure comprised of the polymer and incorporated solvent). As such, R_g^η could be a misleading parameter if the polysaccharide structure were either worm-like, rigid-rod-like, or partially drained (i.e., rapid exchange of integral solvent molecules with the aqueous phase). As shown in Fig. 7, R_g^η correlates well with hydrodynamic volume, with the exception of Pn4. This discrepancy is again a reflection of the difference in the M_w predicted by intrinsic viscosity measurements versus that by light scattering (see discussion above), and is resolved if the M_w calculated from the MHS coefficients is used in the calculation of $\log([\eta]M_w)$. Normalization of the values of R_g and R_g^η to molecular mass (as $R/M_w^{1/2}$; see Table IV) shows that the two methods of molecular size determination used here provide equivalent values for each of the neutral polysaccharides examined (pullulan and Pn14 polysaccharide), indicating that the theoretical expectations are met for the nonionic polymers. The discrepan-

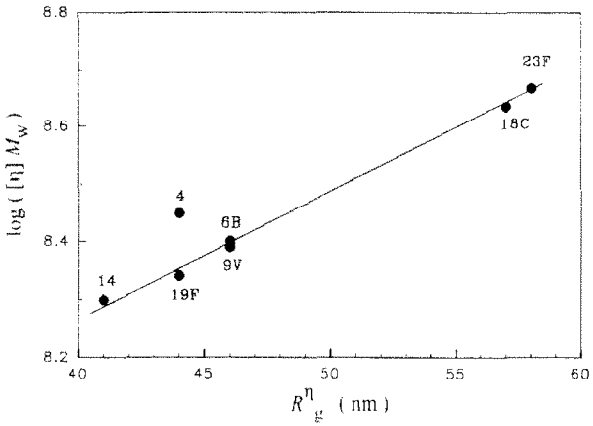

Fig. 7. The relationship between viscosity radius of gyration (R_g^{η}) and hydrodynamic volume ($[\eta]M$) for the pneumococcal polysaccharides.

cies in the absolute and normalized values for the charged polysaccharides (Pn4, Pn6B, Pn9V, Pn18C, Pn19F, and Pn23F) are clear indications that the assumptions made in calculating radius of gyration from the HPSEC–RI/SV data are insufficient to account for the structural interactions inherent to the charged polymers.

Though the parameter $R_g/M_w^{1/2}$ is not corrected for expansion coefficient and sample polydispersity, this molar mass-normalized value is reflective of the relative molecular size of pneumococcal and pullulan polysaccharides and the relative expansion of their polymer chains in solution. Given that the MHS coefficient a (Table II) indicates that, under the conditions of the present study, all of these polysaccharides behave in solution as expanded polymer coils, the discrepancies between the values of $R_g/M_w^{1/2}$ and $R_g^{\eta}/M_w^{1/2}$ for the anionic polysaccharides suggest that these molecules are more highly expanded, generally more rigid in structure, and subject to greater drainage of solvent through the polymer coils than are the uncharged polysaccharides. Moreover, the data for Pn14 and pullulan, both of which are uncharged, show that increased polymer chain rigidity can affect the size of macromolecules in solution at least to the same extent as the charges on the polymer chain.

This phenomenon is more vividly shown in the calculations of relative polymer segment density, shown in Table IV. A polymer segment density function can be derived from the above data by using R_g to calculate molecular volume and dividing this value by M_w. From these calculations it is apparent that Pn4 and Pn18C have the lowest polymer segment density, ~ 40% of the value for Pn14, which is the densest of the pneumococcal polysaccharides examined. Even though Pn14 has a relatively high density, it has only 50% of the density of a pullulan sample of comparable molecular mass, suggesting that while lack of a charge in the repeating unit may result in a more dense and compact structure, other factors (i.e., rigidity of the polymer coil) appear to prevent the Pn14 polysaccharide from achieving the highest density. These segment density estimates again suggest that,

relative to pullulan, the pneumococcal polysaccharides are more rigid and incorporate a larger amount of solvent into their hydrodynamic structure. Other experiments employing different ionic strength solutions could be used to further isolate and explore the contributions of polyelectrolyte effects to polymer chain rigidity.

In conclusion, from these analyses it is apparent that neither HPSEC–RI/MALLS or HPSEC–RI/SV alone is sufficient to allow definitive characterization of the molecular size and molar mass of the pneumococcal polysaccharides in solution. Use of HPSEC–RI/MALLS alone would prevent direct determination of intrinsic viscosity, which would lead to erroneous assumptions about the hydrodynamic volume, rigidity, and drainage of the polysaccharides (especially Pn9V and Pn14). And use of HPSEC–RI/SV alone would result in incorrect calculation of the molar masses of the pneumococcal polysaccharides by the universal calibration approach, due both to the large size of the polysaccharides (relative to commercially available calibration standards) and the polyanionic nature of the molecules. The use of both techniques provides the most comprehensive characterization of the molecular size and molar mass of polymer solutions with a minimum of assumptions.

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