Macromolecules 1989, *22*, 1891–1896

Failure of Universal Calibration for Size Exclusion Chromatography of Rodlike Macromolecules versus Random Coils and Globular Proteins

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Received June 17, 1988; Revised Manuscript Received October 3, 1988

ABSTRACT: "Universal calibration" plots of $[\eta]M$ versus the chromatographic partition coefficient K were constructed for macromolecules corresponding to four conformational types: flexible chains, spheroids, semiflexible rods, and rigid rods. In order to avoid solute-packing interactions, the neutral polysaccharides pullulan and schizophyllan were chosen as representative of the first and last conformations. The stationary phase and the mobile phase were selected so as to minimize electrostatic and hydrophobic effects on the elution of the other two macromolecules: globular proteins and DNA. While $[\eta]M$ (i.e., the viscosity radius, R_η) uniformly determines K for flexible chains and globular proteins, these two species elute later than do rodlike macromolecules of equal R_η. Other dimensional parameters previously suggested, viz., the radius of gyration and the mean projection length, fail even more severely to unify the elution behavior of radially symmetric and asymmetric macromolecules. These findings suggest that the "SEC radius" may not correspond to any dimension obtained from dilute solution behavior.

Introduction

Size exclusion chromatography (SEC) developed initially along two separate paths. For methodological and parochial reasons, the literature related to protein separations on polysaccharide or poly(acrylamide) gels, i.e., "gel filtration", was artificially separated from the field that deals with the characterization of synthetic polymers on rigid or semirigid porous supports, i.e. "gel permeation chromatography". Models for the chromatographic process have tended to diverge as well, although all treatments focus on the chromatographic partition coefficient:

$$K = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_e is the solute retention volume, V_0 the column void volume, and V_t the total permeable volume of the column. K may thus be viewed as the effective fraction of the pore volume to which the solute has complete access or, alternatively, as the equilibrium constant for the transfer of solute between the intrapore volume and the bulk solvent.

In the biochemical literature, K is frequently assumed to yield the frictional coefficient and is therefore thought to be related to diffusion measurements.[1] Consequently, the Stokes radius, R_s, is generally viewed as the fundamental dimensional parameter[2,3] for protein SEC. On the other hand, polymer chemists prefer to use the equivalent hydrodynamic radius,[4] which is proportional to the product of intrinsic viscosity and molecular weight. This last quantity was shown by Benoit et al. to determine chromatographic retention for a variety of statistical chain polymers[5] and has subsequently been called the "universal calibration parameter". As pointed out by Casassa,[6-8] the equivalent hydrodynamic radius (R_η), the root-mean-square radius of gyration (R_G), and the root-mean-square end-to-end distance (r_{rms}) of random coil macromolecules all depend on $n^{1/2}l$ for any statistical chain comprising n units of length l; consequently, all three dimensional parameters will apply equally well to macromolecules with Gaussian segment distributions. A thorough description of these various dimensional parameters vis-à-vis SEC was recently provided by Potschka, with particular focus on nonflexible-chain biopolymers.[9]

In addition to experimentally accessible quantities, such as R_η, R_s, and R_G, other dimensional parameters for SEC have been put forward. Giddings et al.,[10] modeling the stationary phase as an assembly of intersecting planes, used the mean molecular projection or "external diameter" ($\bar{L}$); they pointed out that R_G would consequently fail for molecules with divergent nonradial symmetry. Casassa showed that a completely different model for flexible chains in cylindrical pores[6,7] produces a similar finding of a "universal" parameter given by the mean molecular projection on a line ($\bar{X}$) and also predicted a "significant difference in the $[\eta]M$ calibration between compact spheroidal particles and more extended structures, with no great sensitivity to molecular flexibility in the latter class".[8]

Interest in validiating some of the foregoing hypotheses has prompted a number of recent studies. The technological development of new packings for aqueous SEC has done much to stimulate such investigations. A truly wide range of macromolecular shape factors—i.e., rods, spheres, and random coils—is more readily obtained in aqueous media; however, the aqueous SEC packings formerly available were characterized by poor chromatographic efficiency and/or strong adsorption effects. The former drawback hindered precise determination of peak retention volumes; the latter, more seriously, introduced "nonideal" separation effects, thus invalidating studies intended to elucidate the exclusion process.

The universality of R_η for linear and branched water-soluble polymers has been clearly established for pullulan, amylopectin, amylose, and poly(ethylene oxide) (PEO) on PW gel (Toyo Soda)[11] and for PEO, pullulan, and dextran on the same packing.[12] More significantly, Frigon et al.[13] compared the retention of globular proteins and dextran fractions on a TSK-G3000SW column (Toyo Soda) in 0.5 M ammonium acetate/0.01 M sodium phosphate, pH 7.0 buffer (the importance of the mobile phase will become evident later in our discussion). An empirical third-order fit to the protein data, presented as R_s versus K, produced a line differing substantially from that calculated for dextrans; i.e., at equivalent R_s, the chromatographic partition coefficient (K) was smaller by some 10–20% for the latter solutes. Data for dextrans and proteins were more nearly congruent as plots of log $[\eta]M$ versus K, from which the authors concluded that R_η, not R_s, was likely to be the appropriate size parameter for dextrans and proteins, earlier observations[14,15] notwithstanding. However, it is important to note that Frigon's protein data exhibit deviations of nearly 8% in K around their least-squares curve, and that 4 of the 10 proteins shown on the universal

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calibration plot exhibit K values 5–10% larger than those of the dextrans (the other coincide). Such effects suggest the presence of nonideal solute-packing interactions. While Frigon et al. attempted to suppress ionic effects by the addition of excess electrolyte, electrostatic interactions between silica-based supports such as TSK-SW and proteins with extreme pI values may be impossible to eliminate.[16]

Price and co-workers also tested universal calibration for spheres and random coils[17] by attempting to compare the retention of polystyrene latex particles with linear polystyrene. Although both solutes could be chromatographed on porous glass, it was necessary to elute the latter with THF, while the mobile phase for the former was dilute aqueous phosphate buffer with sodium nitrate, containing sodium dodecylbenzenesulfonate, at ionic strengths ranging from 0.008 to 0.25 M. Latex retention times were extrapolated to infinite ionic strength in order to suppress electrostatic repulsion of the solute from the highly charged packing. Plots of log $[\eta]M$ versus extrapolated retention times for latex spheres diverged from similar plots (involving normal retention times) for linear polystyrene, but the difference was less than that predicted on the basis of $\bar{X}$. Despite the considerable scatter in the data, Price et al. concluded that R_η, and not $\bar{X}$, determines retention times for coils and spheres.

According to Casassa,[8] a more meaningful test of universal calibration might involve rodlike molecules, as well as spheres and coils. The relevant relationships are given by eq 10, 11, and 14 in ref 8, i.e., for spheres,

$$[\eta]M = (5\pi N_A/12)\bar{X}^3 = 7.88 \times 10^{23}\bar{X}^3 \qquad (2)$$

for random coils

$$[\eta]M = \Phi(\pi^{3/2}/64)\bar{X}^3 = 3.43 \times 10^{23}\bar{X}^3 \qquad (3)$$

and for rods

$$[\eta]M = (2\pi N_A/5)\Omega\bar{X}^3 = 7.57 \times 10^{23}\Omega\bar{X}^3 \qquad (4)$$

Here Ω is a slowly decreasing function of the axial ratio (e.g., $\Omega = 0.27$ for $p = 200$), and all quantities are in cgs units. Thus, if $\bar{X}$ is the fundamental size parameter, R_η is not. A detailed comparison of ellipsoidal or spheroidal macromolecules (globular proteins) with rodlike polymers (e.g., tropomyosin, α-actinin, DNA) on Superose (Pharmacia) and PW (Toyo Soda) columns was carried out by Potschka.[9] Tropomyosin, DNA, α-actinin, and spectrin display values of K 2–16% smaller than those for globular proteins of identical Stokes radii. Congruent plots are, however, obtained if R_η is plotted versus K. From this last finding, it was possible to reject both R_s and $\bar{X}$ as universal parameters.[9]

Considerable discord is apparent from the preceding survey. Theoretical treatments[6–8,10] appear to predict failure of the viscosity radius (R_η) for macromolecules of drastically different shape factors. Experimental results, although sometimes ambiguous, generally lend credence to universal calibration via the hydrodynamic volume parameter, i.e., R_η.

It should be evident that a variety of problems obstruct both theoretical progress and experimental verification. With regard to theoretical models, the uncertain geometry of the pores remains a vexing problem. The best defined pore structure is probably that of porous glass. Waldman-Meyer[18] has recently shown that the simple geometric expression for spherical solutes in cylindrical pores

$$K = (1 - R/r)^2 \qquad (5)$$

fits the data for random coils on CPG porous glass quite well, if one uses the polymer's viscosity radius for R and porosimetry results for the pore radius, r. This result at least suggests that CPG behaves as if it were comprised of cylindrical pores. The pore structure of semirigid gels, however, is more complex and harder to characterize.[19] Even if the geometries of the solutes and the pores were well-defined, the limitations of current understanding of the behavior of macromolecules in small, confined domains could prove inadequate to the theoretical task. With regard to experimental evaluation, the major difficulties are likely to be (1) assuring complete suppression of all nonsteric factors, e.g., electrostatic and hydrophobic solute–substrate interactions, and (2) avoiding solute aggregation.

While a satisfactory understanding of the migration process in SEC may be a distant goal, it is clearly desirable to reach a conclusion on the universality of the viscosity radius. We have therefore chosen to study the retention of macromolecules of differing shape factors, choosing the solutes, packing, and mobile phase with the goal of avoiding solute–packing interactions. Using low MW probes,[20] we have found Superose to be appreciably less hydrophobic than other packings, and the ionic content of this gel appears to be low. Since neutral polysaccharides display the most ideal chromatographic behavior on SEC supports in general, we employed the linear polysaccharide pullulan as a random coil, and the triple-helical polysaccharide schizophyllan,[21–23] which has been shown to behave in aqueous solution as a stiff rod.[24,25] Globular proteins were used to represent the class of spheroidal particles; a detailed study was conducted to establish conditions which would assure the absence of adsorptive effects for these solutes. Lastly, DNA was selected—despite its intense charge density—as exemplifying the class of flexible rods. While the polydispersity of pullulan fractions and of the proteins could be neglected, it was necessary to take the molecular weight distribution into account for some of the DNA samples and for all of the schizophyllan fractions.

Experimental Section

1. **Materials.** Proteins obtained from Sigma were thyroglobulin (T-9145), apoferritin (A-3660), catalase (C-10), bovine serum albumin (A-8654), ovalbumin (A-8259), myoglobin (M-0630), lysozyme (L-4631), and cytochrome C (C-7150). Unpurified Type III DNA from salmon testes was also from Sigma (D-1626). A 123 base pair (bp) DNA ladder was from Bethesda Research Laboratories (Gaithersburg, MD). A narrow distribution 48 bp DNA sample was kindly synthesized by Prof. A. S. Benight of the University of Illinois, Chicago. Narrow distribution pullulan MW standards were from Shodex (SDK Showa-Denko USA, New York). A 1.0-g sample of broad MWD schizophyllan (hereinafter "schizophyllan A"), MW 420 000, courtesy of Dr. T. Yanaki (Taito Co., Kobe, Japan), was used to prepare samples of lower MW, as explained below. MW fractions of schizophyllan, gifts from Prof. A. Teramoto, Osaka University, Osaka, Japan, were used without further purification. In Table I are shown MW values for the various externally characterized samples of this study. Also shown are viscosity radii calculated from the expression[4]

$$R_\eta = [3\pi/4([\eta]M/0.025N_A)]^{1/3} \qquad (6)$$

where the units of $[\eta]$ and R_η are dL g^{-1} and cm, respectively.

2. **Sonication of Schizophyllan.** Solutions of schizophyllan A, 5 mg mL^{-1} in 0.01 M NaOH + 0.20 M NaCl, were exposed to 30–40 W of 20-kHz sound for a total of 1–19 h, according to the degree of chain scission desired.[24] The samples were sonicated with a Branson Model L sonifier cell disruptor (equipped with a $^1/_4$-in. probe and powered by an Ultrasonics Model W185D unit) while immersed in an ice bath at ca. 5 °C. Bursts of 6-min duration were alternated with pauses to permit the sample to cool. After sonication, each sample was neutralized with HCl and dialyzed against distilled water in Spectrapor 6 - 8000 "molecular weight cutoff" tubing for 36 h, before lyophilization.

Table I
Characteristics of Macromolecular Solutes Used for SEC Calibration

sample	MW	M_w/M_n	R_e, Å
thyroglobulin	669 000		83
apoferritin	467 000		61
catalase	232 000		52
bovine serum albumin	66 000		36
ovalbumin	44 000		28
myoglobin	17 000		19.8
lysozyme	13 900		18
cytochrome C	12 400		16.3
aprotinin	6 750		15
pullulan P-800	853 000	1.14	293
pullulan P-400	380 000	1.12	191
pullulan P-200	186 000	1.13	128
pullulan P-100	100 000	1.10	90
pullulan P-50	48 000	1.09	60
pullulan P-20	23 700	1.07	41
pullulan P-10	12 200	1.06	28.5
pullulan P-5	5 800	1.07	19.4
dextran FDR-7314	114 000	1.18	70
dextran RMI-8630	57 730	1.08	53
dextran 7314	25 600	1.06	36
dextran PD-T5558	2 280	1.15	14

3. Purification and Sonication of DNA. Type III salmon testes DNA was dissolved by vigorous stirring overnight at 4 °C in 10 mM Tris buffer + 1 mM EDTA. The resultant 5–10 mg mL^{-1} solution was emulsified with an equal volume of phenol: $CHCl_3$:isoamyl alcohol (25:24:1 v/v) by shaking for 15 min. (The phenol was saturated with Tris/EDTA buffer first.) After centrifugation at moderate speed for 15 min, the upper aqueous phase was extracted with an equal volume of $CHCl_3$:isoamyl alcohol (24:1) and then adjusted to 0.30 M sodium acetate. The addition of two volumes of 100% ethanol precipitated the DNA, which was then rinsed with pure ethanol and dried at 50 °C under vacuum. MW degradation of DNA was accomplished by sonication of 12.5 mg mL^{-1} DNA in Tris/EDTA buffer at 5 °C. Three-minute sonication periods were alternated with 5-min cooling pauses. Total sonication times ranged from 48 to 548 min. Sonicated samples were dialyzed and lyophilized as described above.

4. Viscosity. Intrinsic viscosities of schizophyllan and DNA fractions were measured with a Schott-Gerate AVS 300 Ubbelohde dilution viscometer at 25.0 °C ± 0.1 °C. All solutions were filtered twice (0.20 μm) or until no particles could be detected in the beam of a 20-mW HeNe laser. Solvent efflux times ranged from 99 s for water to 105 s for 0.01 M NaOH + 0.20 M NaCl. The precision of the efflux times was ±0.03 s.

Solutions of DNA and schizophyllan were prepared and analyzed in a similar fashion. Each polymer sample was dried (50–60 °C, 2 h), cooled in a dessicator, and weighed. The procedure was repeated until the weight was constant to within 0.2 mg. The dry polymer was then diluted to 11.00 mL with the desired filtered solvent. Schizophyllan solutions were sonicated (30 W, 6 min) at this point to break up any aggregates that persisted through solution. (Quasi-elastic light scattering measurements performed on these solutions proved that the samples were stable with respect to aggregation for at least 4 h beyond this point.) The starting solutions were diluted in the viscometer, and the resulting value of the reduced specific viscosity was extrapolated to give $[\eta]$. To confirm accuracy of dilution and initial concentration, a 10.00-mL aliquot of the final solution was dried to constant weight, and after correction for solvent, the initial concentration was calculated. Agreement between the two concentration determinations was better than 3%.

5. Size Exclusion Chromatography. A prepacked Superose 6 column, 1.00-cm i.d. × 30 cm (Pharmacia USA, Piscataway, NJ) with a MW range of 5000–500 000 (proteins) was found to have a plate count of 4100 at a typical flow rate of 0.35 mL min^{-1}, as determined by injection of 5% D_2O. Eluant was delivered with a piston pump (NSI-33R, Milton Roy, Riviera Beach, FL) through a Rheodyne 200-μL loop injector (Cotati, CA). Protein and DNA samples were detected with a UV detector (Model 153, Altex, Berkeley, CA) equipped with a 254-nm-wavelength kit (Model 150-13). All other samples were detected with a differential refractometer (R-401, Waters, Framingham, MA). A Rheodyne 0.20-μm precolumn filter was used to reduce particulates. Flow rates were determined by weighings of timed collections of column eluant and were reproducible to ±0.1%. All injections were made in triplicate, and the average of the three peak retention volumes was used.

The sonication of schizophyllan was monitored by SEC, using a mobile phase consisting of 0.10 M NaOH + 0.20 M NaCl.[26] Retention data for universal calibration plots were obtained in pH 5.5 phosphate buffer with a total ionic strength of 0.38 M (9:1 sodium chloride:sodium phosphate). (Selection of the mobile phase is discussed below.) Solvents were degassed and filtered before use. Schizophyllan solutions (3–4 mg mL^{-1}) were sonicated for 6 min at 30 W, centrifuged (5 min at moderate speed), and filtered (0.20-μm disposable nylon syringe tips) immediately prior to injection. Protein samples (2–5 mg mL^{-1}) were injected after filtration through 0.45-μm filters. The concentrations injected for pullulan and for DNA were 4–6 mg mL^{-1} and 0.5–1.0 mg mL^{-1}, respectively.

The column void volume, determined with a 3 mg mL^{-1} injection of blue dextran, was 7.84 mL. The total column volume was 22.42 mL, determined from the retention peak of D_2O.

Results

1. Mobile-Phase Selection. In previous studies, the deviation of data for several proteins from a smooth plot gave evidence of adsorption effects.[9,13] The addition of excess electrolyte may suppress electrostatic adsorption but can also induce hydrophobic protein–gel interactions; thus, K for lysozyme at pH 7 on a variety of derivatized silica packings displays a minimum with respect to ionic strength, I, in the vicinity of $I \cong 0.3$–1. These minimum values for K range from 0.7 to 1.7,[27] so the results clearly indicate that there is no ionic strength at which both electrostatic and hydrophobic (or "salting out") effects can be neglected for proteins on these packings. However, a non-silica-based support such as Superose with a lower surface density of ionizable groups might produce more nearly ideal chromatography for proteins, particularly with the proper selection of pH and ionic strength. Since the proteins used here differ widely with respect to isoelectric point and hydrophobic character, the optimization of the mobile phase was not straightforward. More specifically, an increase in ionic strength will suppress ionic interactions but may increase partitioning; decreasing the pH should reduce the concentration of negative groups on the packing but increase the positive charge on the proteins. An empirical approach was taken with the goal of having elution data for all proteins fall on a single curve, which, according to eq 5, would result in a linear fit to a plot of $K^{1/2}$ versus R_η. While it is not evident that any conditions would fully remove all nonsteric effects, fortuitous coincidence with eq 5 of the data for a variety of proteins would seem unlikely.

The quantitative criterion for solvent selection was the regression coefficient for the linear fit of $K^{1/2}$ versus R_η. The procedure for maximization of the regression coefficient consisted of a variable size simplex method of Nelder and Mead, reviewed in ref 28. The plot of $K^{1/2}$ versus R_η is shown in Figure 1, from which it appears that protein separations are governed by solute size alone at pH 5.5 and $I = 0.38$ M.

2. Preparation and Characterization of Schizophyllan. Schizophyllan fractions were characterized by a combination of SEC and viscometry. Primary data are shown in Table II. We note that the Mark–Houwink parameters obtained from ref 25 are also consistent with $[\eta]$, M data in ref 24, and that the M–H plot is linear over a range of MW considerably larger than that dealt with here.

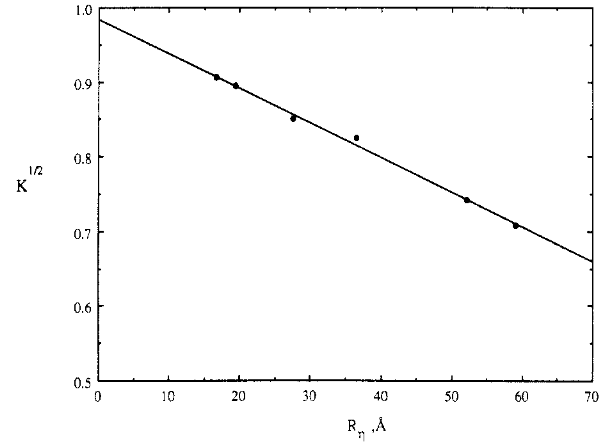

Figure 1. $K^{1/2}$ versus R_η for globular proteins in pH 5.5, $I = 0.38$ M phosphate buffer.

Table II
Primary Characterization of Schizophyllan Fraction

sample	sonication time, min	intrinsic viscosity, dL g^{-1} H_2O	0.01 M NaOH	phosphate buffer	10^{-4} × MW[a]	V_e,[b] mL
III[c]	30	2.17	1.76	1.74	26.8	9.94
238		1.85	1.40	1.38	23.4	10.18
150			0.635	0.640	14.5	11.52
I[c]	320	0.513	0.414	0.444	11.2	12.12
II[c]	1140	0.163	0.127	0.126	5.49	14.13

[a] Obtained from the relationship $[\eta] = 1.82 \times 10^{-7} M^{1.66}$ (ref 25), in conjunction with $[\eta]$ measured in 0.01 M NaOH. [b] Elution volume in 0.01 M NaOH + 0.20 M NaCl. [c] Characterized fraction obtained from Prof. A. Teramoto.

The initial SEC characterization of schizophyllan fractions was carried out in 0.01 M NaOH, since it had been reported that the addition of NaOH, up to 0.02 M, prevents aggregation without disturbing the triple helical structure.[25] However, the resultant high pH could dissociate hydroxyl groups on both the Superose gel and the macromolecular solute, leading to early elution of the latter through electrostatic repulsive effects, or to distortion of the gel matrix. These possibilities were examined as follows. First, the elution volumes of a series of poly(oxyethylene) (PEO) MW standards were found to be identical in 0.01 M NaOH and in 0.01 M NaOH + 0.20 M NaCl. This observation suggests that the gel dimensions are not influenced by charges induced on the packing, a conclusion that is further supported by the finding that the total column volume (V_t) and the interstitial volume (V_0) in these two solvents are the same (within 0.5%) as the corresponding quantities in pH 5.5, $I = 0.38$ M phosphate buffer. On the other hand, plots of log $[\eta]M$ versus V_e for PEO standards and for poly(sodium styrenesulfonate) in 0.01 M NaOH diverged strongly from each other. The ca. 8% earlier elution of the polyanion relative to the nonionic polymer reveals the effect of repulsive interactions between the polyelectrolyte and the ionized gel. If the gel hydroxyl groups dissociate in 0.01 M NaOH, pullulan should behave similarly, and indeed, the elution volumes of pullulan standards were observed to be 10% smaller in this solvent than in neutral phosphate buffer. The addition of 0.2 M NaCl to the basic eluant totally suppressed this effect, and the calibration curve of log $[\eta]M$ for pullulan in 0.01 M NaOH + 0.20 M NaCl was identical with that obtained in pH 5.5, $I = 0.38$ M buffer.

Table III
Stability with Respect to Aggregation of Schizophyllan 238 in Various Solvents

time, h	R_s,[a] nm H_2O	0.01 M NaOH + 0.20 M NaOH	pH 5.5, $I = 0.38$ M
1.0	28	27	27
2.0	28	27	27
3.0	28	27	26
4.0	28	27	26
5.0	28	27	27
5.0	28	27	27
6.0	28	28	27

[a] Mean apparent Stokes diameter from cumulants mode analysis of QELS autocorrelation curves obtained on 12.5 mg mL^{-1} solutions.

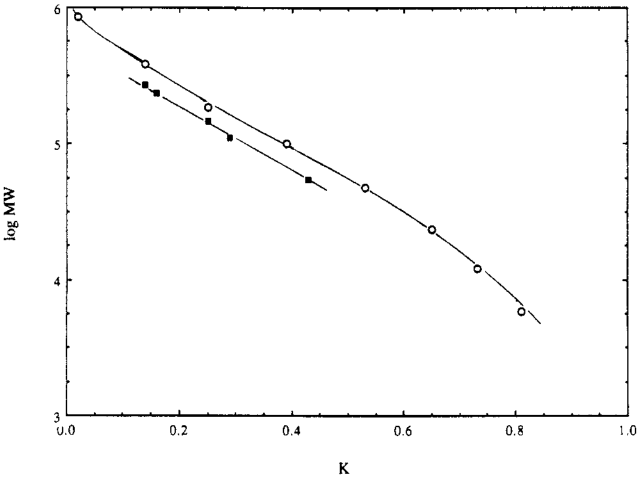

Figure 2. First approximation "primary calibration curve" for schizophyllan fractions (■) (see text for explanation) in pH 5.5, $I = 0.38$ M phosphate buffer. Calibration curve for pullulan (○) shown for comparison.

The initial incorporation of 0.01 M NaOH in the mobile phase was intended to avoid aggregation of schizophyllan.[26] We studied the aggregation of freshly sonicated schizophyllan in several solvents by quasi-elastic light scattering with the results shown in Table III. The QELS results fail to disclose any evidence of aggregation of freshly sonicated schizophyllan over the time frame used for SEC and viscometry measurements. Additional evidence for the absence of aggregation in the buffer selected for universal calibration studies is the finding that SEC chromatograms obtained in pH 5.5, I 0.38 M phosphate and in 0.01 M NaOH are superimposable.

In order to obtain universal calibration plots for broad distribution fractions, M, $[\eta]$, and V_e must all correspond to the same moment of the distribution. Since the intrinsic viscosity already corresponds to the viscosity-average of the distribution, this requirement is best met by using M_v and calculating the corresponding elution volume, V_v.[29] Both of these variables are readily obtained from the chromatogram if the correct calibration curve is available. Thus, the problem reduces to obtaining the "primary" calibration curve from broad MWD fractions for which the viscosities are known, and the following procedure was used. Chromatograms of schizophyllan fractions and monodisperse pullulan standards were obtained in phosphate buffer. By use of the pullulan calibration curve and the value for the Mark–Houwink exponent a of 1.66,[25] viscosity-average molecular weights were calculated from the chromatogram of each fraction, along with the corresponding viscosity-average elution volume, V_v. The true values of M_v, obtained by applying the expression $[\eta] = 1.82 \times 10^{-7} M^{1.66}$ to the viscosities measured in phosphate buffer, were then plotted versus V_v, to yield a "first approximation" primary calibration curve. In principle,

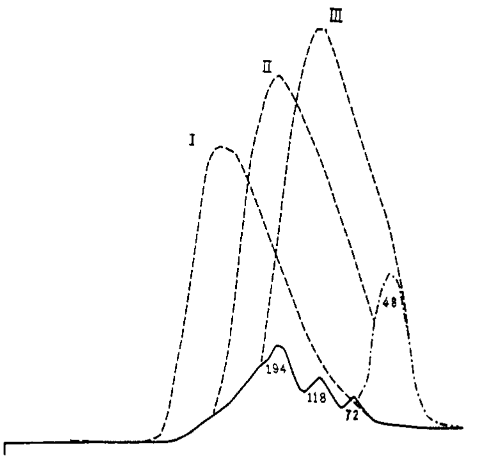

Figure 3. Chromatograms of sonicated fractions DNA I, DNA II, and DNA III; BRL restriction fragments DNA (solid line); and synthesized 48 bp DNA.

Table IV
Characteristics of DNA Samples

sample	sonication time, min	$10^{-4}M_v$[a]	$[\eta]$,[b] dL g^{-1}
I	48	31.6	1.90
194		12.9	0.583
II	168	11.0	0.474
118		7.82	0.302
III	558	5.80	0.205
72		4.77	0.158
48		3.20	0.092

[a] Values for fractions I, II, and III were obtained from $[\eta]$ in conjunction with the relationship $[\eta] = 1.05 \times 10^{-7}M^{1.32}$;[30] others provided by BRL Inc. [b] Values for fractions I, II, and III measured in phosphate buffer; others calculated from $[\eta]$–M relationship noted above.

this process could be repeated until the resulting calibration curves converged.[29] In the present case, when the first approximation calibration curve, shown in Figure 2, was applied to chromatograms of the schizophyllan fractions, values of M_v were obtained which agreed with the intrinsic viscosity molecular weights to within 3%, so no further iteration was required.

3. Characterization of DNA Samples. Chromatograms of sonicated DNA samples ("I", "II", and "III") are shown superimposed on chromatograms of the BRL "ladder", and the synthesized DNA, in Figure 3. The sonicated samples are evidently very broad, and a process somewhat similar to that described above for schizophyllan was required prior to the construction of universal calibration plots. A "primary" calibration curve, constructed by plotting log M versus V_e (peak) for the synthesized and the constriction DNA fragments, was used to calculate the viscosity-average molecular weights of the sonicated fractions from their chromatograms, employing a Mark–Houwink exponent $a = 1.32$.[30] When a plot of these values of M_v versus V_v for the sonicated fractions was superimposed on the plot of log M versus V_e for other samples, a smooth curve resulted. It was therefore concluded that the values of V_v obtained for DNA I, II, and III using this procedure were accurate. Characteristics of the various DNA samples are summarized in Table IV. It is important to note that the Mark–Houwink plot for DNA exhibits curvature for $M_v > 10^5$, and ref 30 reports M–H parameters for different MW ranges. The constants employed by us were obtained from the $[\eta]$–M plot in ref 30 in the appropriate MW range.

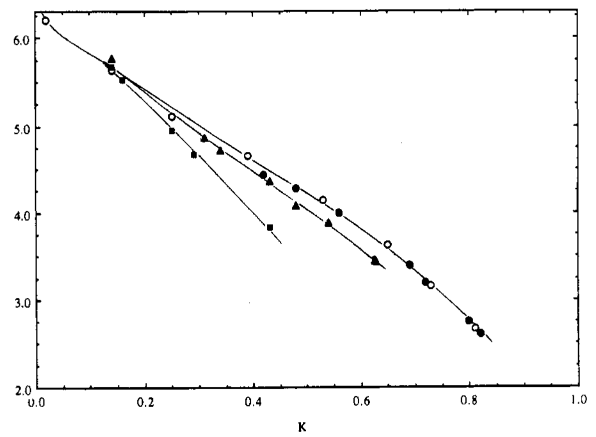

Figure 4. "Universal calibration" plots for pullulan (○), globular proteins (●), DNA (▲), and schizophyllan (■). K for DNA and schizophyllan corresponds to component with viscosity-average molecular weight (see text for explanation).

4. Universal Calibration. Figure 4 shows combined universal calibration curves for proteins, pullulan standards, schizophyllan fractions, and DNA samples, all in pH 5.5, $I = 0.38$ M phosphate buffer. As noted above, the values of M employed for all fractions prepared by sonication (i.e., all schizophyllan fractions, and half of the DNA samples) are viscosity-average molecular weights, M_v; for these samples, K was obtained from the elution volume corresponding to M_v, determined by the procedures described above.

Discussion

Our results for proteins and pullulans are in agreement with Frigon's findings,[13] in that globular proteins and random coil macromolecules form congruent plots of R_η versus K. Our conclusion regarding rodlike macromolecules, however, is at variance with that of Potschka, who found such plots for DNA and rodlike proteins to coincide with data for spheroidal proteins.[9] We note that our R_η values for DNA diverge from the combined random coil and sphere data only in the low MW region. This result can be easily understood in terms of the persistence length of DNA, estimated as ≅ 450 Å.[31] Below ≅ 50 base pairs, DNA behaves as a fairly stiff rod, as evidenced by its Mark–Houwink exponent, $a \cong 1.3$.[30] In the limit of high MW, DNA behaves as a flexible coil with $a \cong 0.7$.[30] We therefore suggest that the discrepancy between our result and Potschka's could arise from the different MW ranges employed, i.e., 3.2×10^4–3.2×10^5 (48–470 bp) in the present study, versus 1.3×10^5–1.5×10^6 (200–2200 bp) in ref 9. This argument is also consistent with our data for schizophyllan. This triple-helical polysaccharide has a persistence length of approximately 1200 Å;[23] diminution in the Mark–Houwink exponent from the rigid rod value of 1.7 is observed only at MW > 10^6, in which range $a \cong 1.1$.[24] Thus, schizophyllan behaves as a rigid rod over the entire MW range investigated here, while DNA exhibits a gradual transition from rodlike to flexible chain behavior with increasing MW. The results in Figure 4 therefore reveal that R_η is not the dimensional parameter that governs the elution of both rods and spherically symmetric macromolecules. Since the equivalent Stokes radius of rods is smaller than the equivalent viscosity radius, the use of R_s in place of R_η will generate yet greater divergence among the three lines of this plot. Hence, R_s does not uniformly determine the retention of spheres and coils.

We may also evaluate the mean external length ($\bar{X}$) in the context of the data of Figure 3. To do so, we use Figure 4 to obtain $J \equiv [\eta]M$ at $K = 0.4$ for random coils (pullulan), spheroids (proteins), and rods (schizophyllan), and thence we obtain the various possible ratios J_{rc}/J_s, J_{rc}/J_r,

Table V
Comparison of Experimental and Predicted[8] Values of $[\eta]M$ (at K = 0.40)

ratio ($J = [\eta]M$)	calcd[a]	exptl
$J_{\text{sphere}}/J_{\text{rigid rod}}$	2.60	2.87
$J_{\text{sphere}}/J_{\text{random coil}}$	2.30	1.00
$J_{\text{sphere}}/J_{\text{flex rod}}$	2.00	1.26
$J_{\text{coil}}/J_{\text{rigid rod}}$	1.13	2.87
$J_{\text{coil}}/J_{\text{flex rod}}$	0.87	1.26
$J_{\text{rigid rod}}/J_{\text{flex rod}}$	0.77	0.44

[a] Based on eq 2–4 in conjunction with the assumption that $\bar{X}$ determines K.

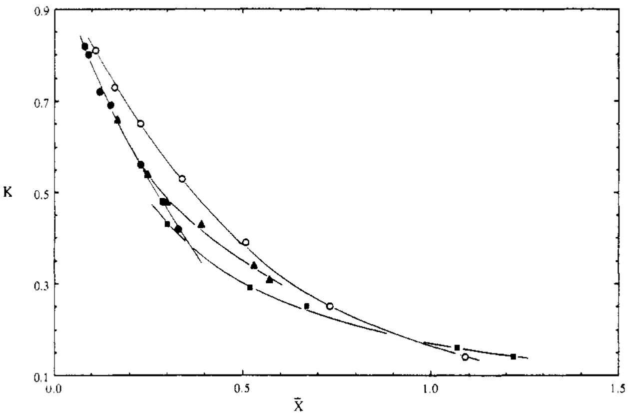

Figure 5. K versus $\bar{X}$; symbols as in Figure 4.

etc., where the subscripts refer to the shape factor. If $\bar{X}$ is the governing parameter, as suggested by Giddings[10] and Casassa,[8] then we may set $\bar{X}_{\text{rc}} = \bar{X}_{\text{s}} = \bar{X}_{\text{r}}$ at any particular value of K, and then use eq 2–4 to solve for $J_{\text{rc}}/J_{\text{s}}$, etc. In Table V the experimental ratios J/J' are compared with the values calculated by using eq 2–4 in conjunction with the assumption that $\bar{X}$ is independent of shape factor at constant K. The discrepancy between the experimental and calculated values is obvious and shows that the mean external length does not uniformly determine the retention volume. One may also calculate $\bar{X}$ for each solute from the measured values of $[\eta]M$ and eq 2–4. When elution data are plotted as K versus $\bar{X}$, in Figure 5, one clearly observes that none of the four data sets are congruent.

Conclusions

There presumably exists some dimensional parameter, R_{SEC}, such that all macromolecules with the same R_{SEC} display identical elution volumes on any chosen column. It has been shown that R_{SEC} does not correspond to any of the SEC size parameters previously suggested, i.e., the hydrodynamic radius, the Stokes radius, or the mean projected length, possibly because the behavior of macromolecules in confined regions is not readily comparable to their properties in dilute solution. An empirical definition of R_{SEC} may arise from forced fit of data such as those in Figure 4 to modified forms of geometric expressions, e.g., eq 5. This phenomenological approach is currently being evaluated.

Acknowledgment. We thank Prof. A. S. Benight for providing the 48 bp DNA sample, Prof. V. Bloomfield for bringing ref 9 to our attention, Prof. D. A. Brant for advice on schizophyllan, Prof. E. F. Cassasa for his illuminating remarks, Prof. H. Edenberg for assistance with DNA purification and characterization, Prof. M. Potschka for helpful conversations, and Profs. T. Yanaki and A. Teramoto for their kind gifts of schizophyllan samples. Support from the Johnson's Wax Fund is gratefully acknowledged.

Registry No. Catalase, 9001-05-2; lysozyme, 9001-63-2; cytochrome C, 9007-43-6; aprotinin, 9087-70-1; pullulan, 9057-02-7; dextran, 9004-54-0; schizophyllan, 9050-67-3.

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