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CALCULATION OF HYDRODYNAMIC PARAMETERS OF RANDOM COIL POLYMERS FROM SIZE EXCLUSION CHROMATOGRAPHY AND COMPARISON WITH PARAMETERS BY CONVENTIONAL METHODS

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SUMMARY

A new method of treating data from size exclusion chromatography, originally evaluated for native proteins, has been extended to random coil polymers using data from dextran and polyethylene glycol as specific examples. Columns are calibrated with globular proteins of known molecular weight. Multiplication by a constant transforms the abscissa to molecular radii. The constants in the equation $r = gM^Z$ are then evaluated directly from the size exclusion chromatographic data and compared with the corresponding constants obtained from viscometry, sedimentation velocity and light scattering, relating Stokes radius, root mean square end-to-end distance and radius of gyration to molecular weight.

INTRODUCTION

An alternative method for treating data from size exclusion chromatography (SEC) has recently[1] been proposed and tested with native proteins. The new treatment is based on an equation which appears to relate the parameters of elution volume to those of size with improved linearity when compared with other equations currently in use, and leads to the precise determination of two constants which correspond closely to the limiting values of molecular sizes or molecular weights for which resolution occurs based on the primary separation process.

While nearly all of the experimental data[1] were obtained with native proteins, the theory was extended to encompass the treatment of polymers having a conformation approximating that of a random coil. This was done in anticipation of SEC experiments we had planned with polysaccharides and other water-soluble polymers. At about the time the manuscript[1] was submitted, a paper appeared[2] from the Central Research Laboratory of Toyo Soda containing high quality data of the type we had proposed to acquire to extend our experimental verification to this class of macromolecules. Dr. Y. Kato very kindly made his original data available to be used for this purpose. Since this is the first time that this approach has been applied to macromolecules with extended conformations, a detailed treatment of the original data is presented.

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RESULTS*

The elution volumes of proteins as well as small molecules for the estimation of V_t are presented as received from Dr. Y. Kato in Table I. In our previous publication[1], we derived two equivalent equations, numbers 3 and 4, and pointed out that preference would depend on whether V_0 or V_t could be determined with greater accuracy. Experience has convinced us that this is true of V_t. Thus, while our earlier[1] treatment was based on eqn. 3, we use eqn. 4 here, which is as follows:

$$\frac{V_e^{1/3} - V_t^{1/3}}{V_0^{1/3} - V_t^{1/3}} = F_{(v)} = \frac{M^{1/3} - A^{1/3}}{C^{1/3} - A^{1/3}} \tag{1}$$

Here, V_e is the elution volume of a protein of molecular weight M, V_0 is the interstitial volume outside the spherical gel beads, and V_t is the total volume available to solvent. Elution times taken from the recorder are treated as described earlier[1]. The constants G and A are equal to the upper and lower limits of the molecular weights of globular proteins that are resolved by the primary size exclusion process.

TABLE I

ELUTION VOLUMES AND MOLECULAR WEIGHTS FOR PROTEINS

Protein	*Molecular weight*	*Elution volume (ml)*		
		G4000 SW	*G3000 SW*	*G2000 SW*
Impurity with large molecular weight (V_0)	–	19.35	18.85	22.37
Thyroglobulin	660,000	30.15	20.03	–
γ-Globulin	156,000	37.97	28.62	23.43
Bovine serum albumin trimer	201,000	–	24.35	–
Bovine serum albumin dimer	134,000	36.55	26.80	–
Bovine serum albumin monomer	67,000	39.27	30.90	25.52
Ovalbumin	43,000	40.87	33.63	27.53
Peroxidase	40.200	41.00	33.50	27.18
β-Lactoglobulin	35,000	41.35	34.65	28.80
Myoglobulin	16,900	43.33	38.18	32.28
Ribonuclease	13,700	44.53	39.53	33.37
Cytochrome *c*	12,400	43.77	39.23	33.47
Glycylglycylglycylglycine	246	46.78	43.50	39.93
Glycine	75	47.35	44.38	41.20

The protein data are plotted according to this equation in Fig. 1. Owing apparently to reversible adsorption, γ-globulin behaves on SEC as a molecule of much smaller molecular weight. This can be clearly seen in Fig. 1 and has also been observed by others[1–3]. Data on γ-globulin were not included in calculating the regression line.

It is a common practice[4] to take V_0 as the elution volume of almost any molecule too large to enter the gel. This is probably justified when working with Sephadex, where the practice originated, but in working with the smaller spheres used

*The chromatographic columns that were used in our experiments are products of Toyo Soda Manufacturing Co., Ltd., Tokyo, Japan. Their properties are further described in refs. 1–3.

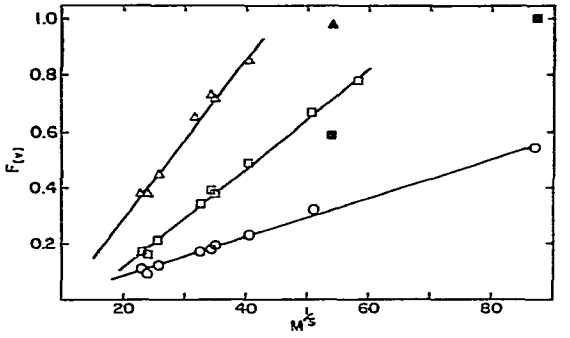

Fig. 1. Data from native proteins (Table I) plotted according to eqn. 1 for G4000 SW (○), G3000 SW (□), and G2000 SW (△). Data presented as solid symbols refer to γ-globulin and a protein used for estimation of V_0. These points were not included in the least-squares analysis.

in this study, size exclusion at the interstices can lead to a small but appreciable error[5]. For G4000 SW, lacking an alternative we used the value 19.35. For G3000 SW, the elution volume of thyroglobulin, 20.03, was used. Unfortunately, thyroglobulin was not run on G2000, but we estimated its elution volume as 23.22 ml from its behavior relative to the high molecular-weight impurity on the G3000 SW column and used that value for V_0.

The length of the regression *here* in Fig. 1 indicates the linear portion of the data. The constants $C^{1/3}$ and $A^{1/3}$ are obtained from the intersection of the regression line with the horizontals located at $F_{(v)} = 1$ and 0, respectively. The values obtained in this way are entered into Table II.

TABLE II

CALIBRATION CONSTANTS OBTAINED FROM FIG. 1

Column	*A*	*C*	r_A (Å)	r_c (Å)
G2000 SW	940	91,000	7.8	36
G3000 SW	2460	340,000	10.7	56
G3000 SW*	3900	330,000	12.5	55
G4000 SW	551	$3.4 \cdot 10^6$	6.5	120

* From Himmel and Squire[1].

For use with macromolecules other than globular proteins, we convert our calibration curve to $F_{(v)}$ *vs.* r. To do this, we merely multiply the constants $A^{1/3}$ and $C^{1/3}$ by the factor $0.794 \cdot 10^{-8}$. This follows from a well known equation from hydrodynamics for native proteins:

$$r = \left(\frac{3\,M\bar{v}}{4\,\pi N}\right)^{1/3} \cdot \left(1 + \frac{w}{\bar{v}\varrho}\right)^{1/3} = 0.794 \cdot 10^{-8}\, M^{1/3} \qquad (2)$$

In this calculation we used the value 0.73 for the partial specific volume, $\bar{v}$, and 0.53 g of water per gram of protein as the hydration, w. These are mean values calculated from an earlier study[6] of the hydrodynamic properties of 21 globular proteins of

known structure. Calibration constants in terms of molecular weights and molecular radii are given in Table II. We point out that these constants also provide an estimate of the limiting values of these parameters for separation by the primary SEC process on the three types of column.

We have now converted eqn. 1 to the form

$$F_{(v)} = ar_p + b$$

where

$$a = \frac{1}{r_c - r_A} \quad \text{and} \quad b = \frac{-r_A}{r_c - r_A}. \tag{3}$$

We assume that all spherical molecules of the same molecular radius will elute at the same elution volume. We also assume that for a given polymer, or biopolymer, the molecular radius is related to the molecular weight by an equation of the form

$$r = gM^Z \tag{4}$$

Entering eqn. 4 into eqn. 3, and taking the logarithm, we have

$$\log (F_{(v)} - b) = \log ag + Z \log M \tag{5}$$

Our objective now is to determine the constants g and Z from SEC data on polymer fractions of known molecular weight. The original data from Kato *et al.*[2] for polyethylene glycol are presented in Table III and plotted according to eqn. 5 in Fig. 2. Values for the constants g and Z are recorded in Table IV.

TABLE III

ELUTION VOLUMES AND MOLECULAR WEIGHTS FOR POLYETHYLENE GLYCOLS

Polyethylene glycol	*Molecular weight*	*Elution volume (ml)*		
		G4000 SW	*G3000 SW*	*G2000 SW*
SE-150	1,400,000	19.23 (V_0)	–	–
SE-70	730,000	1923.		–
SE-30	320,000	19.95	18.78 (V_0)	–
SE-15	160,000	22.53	1i.91	–
SE-8	80,000	26.96	19.41	22.07 (V_0)
SE-5	46,000	30.59	20.80	22.22
SE-2	23,000	33.97	23.00	22.83
PEG 6000	7500	39.11	30.65	26.35
PEG 4000	3000	43.06	37.35	32.36
PEG 1540	1500	44.96	40.57	36.29
PEG 1000	1000	45.64	41.86	37.87
PEG 600	600	46.42	43.08	39.61
PEG 400	400	47.02	44.07	40.92
PEG 200	200	47.77	45.12	42.20
Ethylene glycol	62	48.25	45.93	42.59

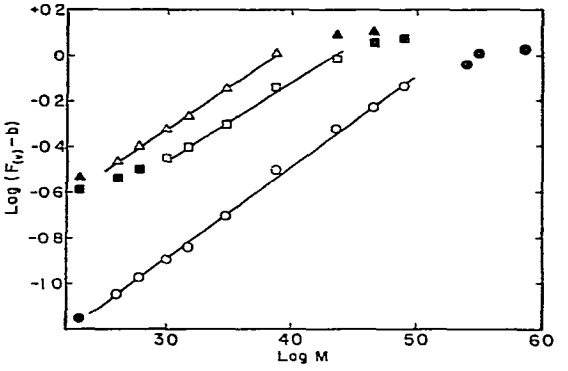

Fig. 2. Data from polyethylene glycol fractions (Table III) plotted according to eqn. 5 for G4000 SW (○), G3000 SW (□), and G2000 SW (△). Data presented as solid symbols lie outside the linear region.

TABLE IV

HYDRODYNAMIC PARAMETERS FOR POLYETHYLENE GLYCOL OBTAINED BY SEC AND HYDRODYNAMIC METHODS

All dimensions are in Å.

From SEC M	0.87 $M^{0.40}$ G4000 SW	1.38 $M^{0.35}$ G3000 SW	1.02 $M^{0.37}$ G2000 SW	Mean
300	8.5	10.1	8.4	9.0
3000	21.4	22.7	19.7	21
30,000	53.8	50.9	46.3	50
From $s_{20,w}$ M	0.144 $M^{0.59}$ Stokes r	0.22 $M^{0.59}$ R_g	0.53 $M^{0.59}$ $\langle r^2 \rangle^{1/2}$	
300	4.2	6.4	15	
3000	16	25	60	
30,000	63	96	226	
From $[\eta]$ M	0.25 $M^{0.5}$ Stokes r	0.37 $M^{0.5}$ R_g	0.91 $M^{0.5}$ $\langle r^2 \rangle^{1/2}$	
300	4.3	6.4	16	
3000	14	20	50	
30,000	43	64	157	

The original data of Kato *et al.*[2] on dextran fractions are not tabulated here in the interests of brevity, but they are presented in Fig. 3, plotted according to eqn. 5. Values for the constants g and Z are recorded in Table V.

It is of interest to compare the hydrodynamic constants obtained by SEC with those obtained by conventional methods. The sedimentation coefficient is related to the frictional coefficient, f, by a well known equation due to Svedberg

$$s^0_{20,w} = \frac{M(1 - \bar{v}p)}{Nf} \tag{6}$$

TABLE V

HYDRODYNAMIC PARAMETERS FOR DEXTRAN OBTAINED BY SEC AND BY CONVENTIONAL METHODS
All dimensions are in ångstroms.

"Radius" from SEC: $r = gM^Z$				
	0.93 $M^{0.36}$	0.69 $M^{0.40}$	1.39 $M^{0.36}$	
M	G4000 SW	G3000 SW	G2000 SW	Mean
3000	16.6	17.0	24.8	19
30,000	38.0	42.6	58.3	46
300,000	87.1	107.0	130.0	108
From light scattering				
	0.44 $M^{0.43}$	0.66 $M^{0.43}$	1.61 $M^{0.43}$	
M	Stokes r	R_g	$\langle r^2 \rangle^{1/2}$	
3000	14	21	50	
30,000	37	56	136	
300,000	100	150	365	
From sedimentation				
	0.160 $M^{0.54}$	0.24 $M^{0.54}$	0.59 $M^{0.54}$	
M	Stokes r	R_g	$\langle r^2 \rangle^{1/2}$	
3000	12	18	45	
30,000	42	63	154	
300,000	145	218	535	
From viscometry				
	0.28 $M^{0.47}$	0.43 $M^{0.47}$	1.05 $M^{0.47}$	
M	Stokes r	R_g	$\langle r^2 \rangle^{1/2}$	
3000	12	19	45	
30,000	36	55	133	
300,000	105	161	394	

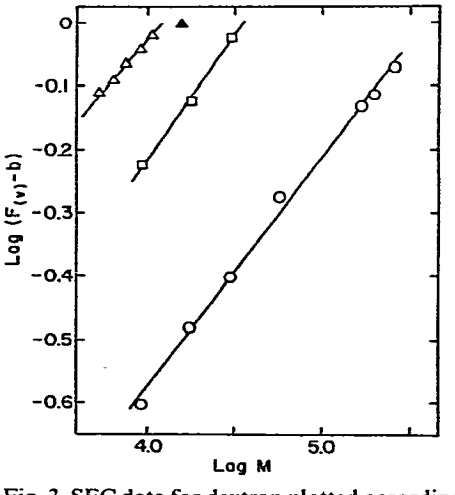

Fig. 3. SEC data for dextran plotted according to eqn. 5. Data reported for G4000 SW (○) and G3000 SW (□), were calculated from the elution volume of fractions with rather narrow molecular-weight distributions, while the G2000 SW (△) data were obtained from the integral elution curve of a sample with a broad molecular-weight distribution. See ref. 2 for details.

The frictional coefficient, f, is related to the Stokes radius by

$$f = 6\,\pi\eta r \tag{7}$$

where η is the viscosity of the solvent. Wan and Adams[7] summarized the original sedimentation velocity data of Granath[8] for dextran by the equation $s^0_{20,w} = 2.24 \cdot 10^{-15}\,M^{0.46}$, and $\bar{v} = 0.593$. Entering these values into the above equations, we have the desired relationship between molecular weight and Stokes radius

$$r_s = 0.160\,M^{0.54}\ \text{Å} \tag{8}$$

These values are entered into Table V.

The frictional coefficient of a non-draining random coil is related[9] to the root mean square (r.m.s.) end-to-end distance by

$$f = 5.1\,\eta_0\,\langle r^2\rangle^{1/2} \tag{9}$$

Substitution into eqn. 6 yields

$$\langle r^2\rangle^{1/2} = 0.59\,M^{0.54}\ \text{Å} \tag{10}$$

Finally, the radius of gyration R_g is related to the r.m.s. end-to-end distance by the equation

$$R_g = \frac{\langle r^2\rangle^{1/2}}{6^{1/2}} \tag{11}$$

Thus $R_g = 0.24\,M^{0.54}$ Å

The following viscosity data on dextran at 20°C are from Granath[8]

$$[\eta] = 0.243\,M^{0.42} \tag{12}$$

A well known[9] theoretical relationship between the intrinsic viscosity and r.m.s. end-to-end distance is

$$[\eta] = \varphi_c\,\langle r^2\rangle^{3/2}/M \tag{13}$$

where φ_c is a universal constant having the value *ca.* $2.1 \cdot 10^{23}$ when $[\eta]$ is in $cm^3\ g^{-1}$ and $\langle r^2\rangle^{1/2}$ is in cm. Setting the theoretical relationship, eqn. 13 is equal to the empirical representation of experimental results, eqn. 12, we have

$$\langle r^2\rangle^{1/2} = 1.05\,M^{0.47}\ \text{Å} \tag{14}$$

Application of eqn. 11 yielded $R_g = 0.43\,M^{0.53}$.

Finally, light-scattering data on dextran by Senti *et al.*[10] could be represented by the equation

$$R_g = 0.66\,M^{0.43}\ \text{Å} \tag{15}$$

Again, eqn. 11 was used to calculate $\langle r^2\rangle^{1/2}$.

From eqns. 7 and 9, we have $r_s = 0.270\,\langle r^2\rangle^{1/2}$. Constants calculated in this way are also in Table V.

The hydrodynamic properties of polethylene glycol in aqueous solution have been reviewed by Bailey and Koleske[11]. They include $s_{30,w} = 1.26 \cdot 10^{-15} M_w^{0.41}$ and $\bar{v} = 0.8392$ for the range $10^4 < M_w < 10^7$, and $[\eta] = 0.156 M^{0.5}$ at 25°C for the M_w range 200–8000. These data were treated as described above for dextran and entered into Table IV. For purposes of comparison, we have calculated the SEC radius as well as the Stokes radius, radius of gyration and r.m.s. end-to-end distance for values of molecular weight near the mean and the extremes of the range of separation. These values are also included in Tables IV and V.

DISCUSSION AND CONCLUSIONS

It is interesting to compare the calibration constants for G3000 SW from data obtained in two different laboratories. As seen in Table II, the constants C are virtually identical while the A values are in fair agreement. This suggests that in using similar columns one might use the mean values after checking the results with a couple of standard proteins.

We note that with the exception of γ-globulin, which we discussed earlier, the plots of $F_{(v)}$ *vs.* $M^{1/3}$ and log $(F_{(v)} - b)$ *vs.* log M were linear in the range $A < M < C$. These results confirm our earlier findings, and extend them to water-soluble flexible polymers.

Before turning to a comparison of the hydrodynamic parameters g and Z, let us consider how they were introduced in this method of analysis. We merely assumed that there existed a mathematical relationship of the form $r = gM^Z$ that related a parameter of size, which was not precisely defined, to molecular weight. The constants g and Z are expected to be characteristic of a particular polymer and, especially in the case of polyelectrolytes, of the ionic strength and other properties of the solvent. We[1] and others[12] have expressed uncertainty as to which radius is the appropriate parameter of size. The Stokes radius has most frequently been utilized for this purpose, but Nozaki *et al.*[12] have clearly pointed out that the Stokes radius of fibrous proteins as measured by SEC is in poor agreement with values obtained from sedimentation velocities. To our knowledge, the appropriate parameter of size for polysaccharides and similar water-soluble polymers that determines the elution volume in the primary size exclusion process[13] remains to be defined.

In Table V we note that the mean value of the SEC "radius" for dextran obtained from the three columns agrees rather well with the Stokes radius as determined either from light scattering, sedimentation velocity or viscometry, especially near the middle of the molecular weight range. Poorer agreement near the extremities of the range arises from the fact that the SEC data have a lower exponent than data from the other methods. For polyethylene glycol, Table IV, the mean SEC radius at mid-range is in better agreement with R_g than the Stokes radius and the discrepancy in slopes is even more severe.

With reference to the discrepancies between the results obtained by this method of analysis of SEC data and data from conventional methods, we should point out that for both polymers, this agreement is about as good as the agreement between the various values of R_g, Stokes radius or $\langle r^2 \rangle^{1/2}$ as calculated from different hydrodynamic methods. It is also as good as the agreement between values of $\langle r^2 \rangle^{1/2}$ for polymer chains by four different theories of polymer statistics, as pointed out by

Bailey and Koleske (ref. 11, Table 23.4). We should also point out that the equations we have used are based on linear polymers and dextran, which is known[13] to be at least somewhat branched. For polyethylene glycol, on the other hand, the low molecular weight fractions are so small that the equations we have used may not apply. In particular, eqn. 11 is valid only for high degrees of polymerization. The SEC experiments were done at transient laboratory temperatures, probably *ca.* 23°C. Most of the hydrodynamic data were obtained at 25°C except for the sedimentation velocity data for polyethylene glycol, which were at 30°C. Finally, all conventional hydrodynamic data were extrapolated to infinite dilution while the SEC experiments were done at a sample concentration suitable to the detector and an "effective concentration" that resists definition. All things considered, agreement is probably about as good as could be expected.

In our earlier model for SEC[14], we made use of the concept that the excluded volume of a macromolecule is determined by the distance between the center of a macromolecule and an impermeable barrier at nearest approach. For compact globular proteins this distance is closely approximated by the Stokes radius. For long rods, arguments could be advanced in favor of $L/2$, the Stokes radius of gyration, or the radius of the rod. The data of Nozaki *et al.*[12] approach the fourth of these alternatives. For flexible random coil polymers it seems likely that during the size exclusion process, some deformation may occur within the pores of the beads. If the extent of deformation were to increase with molecular size, this could account for the lower value of the exponent of molecular weight for data from SEC. In considering the data in Tables IV and V we noted that this discrepancy was more serious for polyethylene glycol than for dextran. This concept of deformability is supported by the rather high shear dependence of the viscosity of polyethylene glycol[11]. Dextran, on the other hand, shows little shear dependence in viscometry[13].

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