Chromatogram Broadening of Proteins and Dextrans in Size Exclusion Chromatography

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Synopsis

The mechanisms governing the broadening of experimental chromatograms for proteins and paucidisperse dextrans were studied on TSK-G2000SW and TSK-G3000SW columns. Within the conditions studied, the chromatogram variance for all solutes increased linearly with increasing effluent flow rate. As predicted by current theories of the kinetics of size exclusion chromatography, this flow rate dependence is caused mainly by slow mass transport of the solute within the stationary phase of the column. Restricted diffusion within the stationary phase was dependent upon the ratio of solute molecular size to column pore radius and was similar for both proteins and dextrans. In comparison with results for monodisperse proteins, the broader chromatograms produced by dextrans were due to sample polydispersity and not to differences in solute column spreading. Corrections for column spreading on these columns are small for the determination of integral properties of polymers but may be significant when molecular weight distributions are of interest.

INTRODUCTION

Membrane transport experiments utilizing polydisperse polymers analyzed by size exclusion chromatography (SEC) have proven useful for the characterization of synthetic membranes[1-3] and the glomerular basement membrane.[4-6] In comparison with many polymer applications where evaluation of the number- (M_n) and weight-average (M_w) molecular weight is sufficient, an accurate determination of the entire molecular weight distribution is imperative for these experiments. Experimental chromatograms may not represent the true molecular weight distribution since chromatogram broadening results not only from sample polydispersity but also from undesirable instrumental spreading. This broadening of chromatograms is well known, and the errors incurred by neglecting instrumental spreading when determining average molecular weights have been described.[7] The importance of and methods for assessing systematic errors in determinations of molecular weight distributions by neglecting instrumental spreading have not been studied extensively.

The correction of SEC data for instrumental spreading requires the determination of a spreading factor for monodisperse polymers as a function of retention volume, with subsequent calculations providing accurate molecular weight distributions from experimental chromatograms.[8] Investigations of SEC systems, recently reviewed elsewhere,[9] have shown that column spreading is most important, being dependent mainly upon eddy diffusion in the mobile phase and the slow mass transport of the polymer within the stationary phase. The spreading function is, however, not readily

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obtained since the residual polydispersity of polymer standards is difficult to assess. Experimental methods for spreading function determinations that are theoretically independent of polymer polydispersity such as the reverse flow[10] and recycle[11,12] techniques are complicated and technically difficult to perform. Since column spreading due to mass transport within the stationary phase is a function of flow rate whereas spreading due to eddy diffusion and polymer polydispersity are not, such effects can be studied separately. Separation of the latter two determinants of chromatogram broadening is possible only when monodisperse solutes are also studied.

We have recently described[13] the separation of proteins and dextrans on an aqueous SEC column from Toyo-Soda (TSK-G3000SW). The values of M_n and M_w computed neglecting column spreading for low polydispersity dextran standards were similar to those measured independently, suggesting that polymer polydispersity dominated chromatogram shape. In the present investigation, chromatogram broadening is studied for both low polydispersity dextran samples as well as monodisperse proteins as a function of retention volume and effluent flow rate on both the TSK-G2000SW and TSK-G3000SW columns.

EXPERIMENTAL

Materials

All protein samples used without further purification and dextran sample F264 were obtained from Sigma Chemical Co., St. Louis, Mo. or Pharmacia Fine Chemicals, Piscataway, N. J. Dextran fractions of low polydispersity were generously provided by Dr. K. Ebert, University of Heidelberg, West Germany, and Dr. K. Granath, Pharmacia Fine Chemicals, Uppsala, Sweden. Average molecular weights for the dextrans were provided and are presented in Table I. The column buffer was 0.01*M* sodium phosphate, pH

TABLE I
Molecular Parameters of Dextrans[a]

Fraction	M_w	M_n	I	I_c
F264	(264,000)[b]	—	—	—
M87	87,100	85,400	1.03	1.01
M55	55,600	50,800	1.03	1.01
M44	44,000	39,300	1.03	1.01
M41	41,200	38,000	1.03	1.01
M33	33,100	31,100	1.02	1.01
M25	25,600	24,200	1.04	1.01
M20	19,800	19,400	1.02	1.02
M10	10,000	9,200	1.04	1.03
M8	7,800	7,700	1.04	1.03
M6	5,900	5,400	1.08	1.07
M4	3,500	3,200	1.13	1.11
M3	2,650	2,400	1.13	1.10

[a] Weight-average M_w and number-average M_n molecular weights provided with each sample. I is the polydispersity index determined from the chromatogram variance at a nominal flow setting of 1.0 mL/min on the TSK-G3000SW column. I_c is the polydispersity index corrected for column spreading (see text).

[b] An approximate viscosity-average molecular weight.

7.0, containing either 0.15*M* (for dextrans) or 0.05*M* (for proteins) ammonium acetate prepared in filtered glass distilled water.

Chromatography

Chromatography was performed using a Waters Model 6000A solvent delivery system (Waters Associates, Milford, Mass.) with a Waters Model U6K sample injector. The columns used were either a TSK-G2000SW or a TSK-G3000SW (60 cm × 0.75 cm i.d.) supplied by Cole Scientific, Calabasas, Calif. Both columns contain nearly spherical particles with diameters d_p of approximately 10 μm and pore radii of 65 and 120 Å for the TSK-G2000SW and TSK-G3000SW columns, respectively.[14] Column effluents were monitored with a Waters Model R401 differential refractive index detector. Voltage output from the detector was monitored with an Omniscribe strip chart recorder (Houston Instrument, Austin, Tex.) and an Apple II+ microcomputer (Apple Computer, Inc., Cupertino, Calif.) configured with 48K bytes random access memory and a 12-bit resolution analog-to-digital converter option (Interactive Microware, Inc., State College, Pa.).

All samples were prepared at 5 mg/mL and applied to the column in 10–50 μL volumes. Under these conditions solute concentration and volume dependent effects were absent.[13] Proteins and dextrans were monitored by differential refractometry, and all experiments were performed at ambient temperature. The column flow rate was varied by adjusting the nominal flow pump setting between 0.3 and 1.2 mL/min. During each experimental run the average flow rate was measured by collecting effluent from the distal end of the column for a fixed period of time. The average flow rate was calculated by weighing the effluent (assuming a density of 1.0022 g/mL), and the results for two different solvent delivery systems are presented in Table II. Solute retention volumes were computed by multiplying the retention time by the average flow rate.

Detector voltages were digitized by the 12-bit analog-to-digital converter and recorded by the Apple II+ computer. Corrections for a linear base line drift were provided automatically by the data acquisition program or selected by the user after inspection of the chromatogram. More details of the chromatographic experiments and data acquisition have been presented elsewhere.[13]

Data Analysis

Protein and dextran samples were eluted at all flow rates as nearly symmetrical Gaussian curves. Each chromatogram was fitted to the following function that contains three adjustable parameters:

TABLE II
Measured Average Flow Rates for Two Different Waters Model 6000A Solvent Delivery Systems (Values ± SEM in mL/min)

Flow pump setting	System 1	System 2
0.3	0.289 ± 0.001	0.280 ± 0.002
0.6	0.588 ± 0.008	0.583 ± 0.009
1.0	0.983 ± 0.003	0.985 ± 0.007
1.2	1.191 ± 0.027	1.190 ± 0.026

$$g(\nu) = (A/\sqrt{2\pi\mu_2}) \exp[-(\nu - \mu_1)^2/2\mu_2] \tag{1}$$

where ν is the retention volume and the parameters A, μ_1, and μ_2 are the total area under the chromatogram, the peak retention volume, and the variance of the curve from its peak, respectively. Best estimates of these parameters were obtained by using a nonlinear least squares method[15] adapted for the Apple II+ computer. Such an approach has been recently shown[16] to be preferred over the estimation of chromatographic parameters by integral moments. A more general empirical spreading function[17,18] that allows for skewness was also fit to certain chromatograms. The resulting curve fit was not, however, significantly improved by including extra parameters, and no appreciable skewing of chromatograms was seen at any flow rate. Deviations from an ideal Gaussian profile were usually evident as an elevation in the tails of the chromatogram. The peak retention volume and variance obtained in this manner are similar to those obtained by using a simple search for the apex of the profile and by measuring the half-width of the peak.[7] All parameters reported are the average of at least two determinations.

The peak retention volume of the solute μ_1 can be rendered dimensionless by using the dimensions of the column in a number of ways. Two relevant parameters of interest in this study are given by

$$K = \frac{\mu_1 - V_0}{V_T - V_0} \tag{2}$$

and

$$k = \frac{\mu_1 - V_0}{V_0} = K\frac{V_T - V_0}{V_0} \tag{3}$$

where V_0 and V_T are the column void volume and total solvent penetrable volume of the column, respectively. For each column V_0 was obtained from the peak retention volume of a high molecular weight dextran fraction (F264). The value of V_T was defined as the peak retention volume of β-alanine. The former parameter K is most useful for presenting data with predictions from geometrical models of column separation, whereas the latter k is most relevant when considering the kinetics of column spreading.[19]

Dextran calibration curves for both the TSK-G2000SW and TSK-G3000SW columns were determined at a nominal flow setting of 1.0 mL/min. The molecular weights assigned to the peak retention volume were corrected for residual polydispersity by equating them to $(M_w \times M_n)^{1/2}$.[13] The calibration curves, determined by polynomial least squares regression, were described by

$$\log M = 4.81 - 2.85K + 3.45K^2 - 2.92K^3 \tag{4}$$

for the TSK-G2000SW column and by

$$\log M = 5.06 - 2.97K + 4.26K^2 - 3.83K^3 \tag{5}$$

for the TSK-G3000SW column. The latter equation differs only slightly from that previously described for a different column of the same type.[13] Equations (4) and (5) that show the different separation ranges for the two columns are similar to those reported by others.[20]

RESULTS AND DISCUSSION

Retention Volume

The effect of varying effluent flow rate on the peak retention volume of proteins and dextrans for the TSK-G3000SW column is shown in Figure 1. The retention volume for all solutes increases slightly as effluent flow rate increases. The positive slopes are statistically significant as determined by linear regression and greater for dextrans than for proteins. The increase for those solutes used to estimate V_0 and V_T was such that the dimensionless parameters in eqs. (2) and (3) decreased with increasing flow rate, but the trend is of lesser magnitude. Similar results (not shown) were obtained on the TSK-G2000SW column.

Rokushika and co-workers[21] have studied the influence of flow rate on retention volume of several proteins for flow rates ranging from 0.1 to 3.0 mL/min on the TSK-G3000SW column. They reported no flow rate dependence of retention volume for the proteins studied. Meredith and Nathans[22] also reported a flow rate independence of retention volume for globular proteins on this column. They described, however, a decrease in retention volume with increasing flow rate for the highly asymmetrical protein fibrinogen. The change in peak retention volume with flow rate described here is small, less than 2% for all samples studied, and may have been neglected by others. These results support the contention that the main mechanism governing retention on these columns is size exclusion.

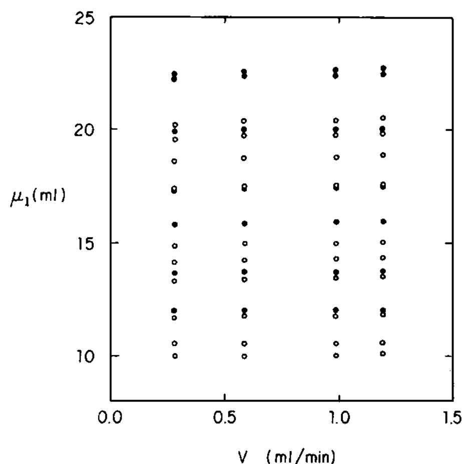

Fig. 1. Peak retention volumes μ_1 obtained on the TSK-G3000SW column as a function of effluent flow rate $\dot{V}$: (●) proteins and other monodisperse solutes; (○) polydisperse dextrans. In increasing retention volume order, the solutes are: F264, M87, M55, β-galactosidase, M33, albumin dimer, M25, M20, albumin, ovalbumin, M10, M6, M4, ribonuclease, M3, sucrose, β-alanine.

The slight flow rate dependence of peak retention volume cannot be explained by chromatogram skewing[23] since such effects were not observed at any flow rate examined. Although experimental conditions were chosen to minimize ionic interactions between solutes and the column,[13] residual adsorption or hydrophobic interactions may still be present. In all subsequent calculations the mean retention volume was used.

Chromatogram Variance

The chromatogram variance μ_2 of proteins and low polydispersity dextrans for the TSK-G3000SW column at a nominal flow setting of 1.0 mL/min is shown in Figure 2, where the variance normalized by the inclusive volume of the column ($V_T - V_0$) is plotted as a function of the dimensionless retention volume K. The results for monodisperse solutes (●) demonstrate that column spreading generally increases with decreasing retention volume. A rapid decrease in column spreading is observed, however, for solutes that elute near the column void volume. Chromatogram variance for dextrans (○) is consistently larger than for monodisperse solutes, suggesting that sample polydispersity contributes significantly. Column spreading was greater for all solutes on the TSK-G2000SW (results not shown) than for the TSK-G3000SW column; however, a similar relationship between dextrans and monodisperse proteins was also observed on this column.

Figure 3 illustrates the effect of effluent flow rate on chromatogram variance of various proteins and dextrans for the TSK-G2000SW column. Here, chromatogram variance relative to the peak retention volume μ_2/μ_1^2 is approximately a linear function of effluent flow rate. This ordinate is equivalent to $1/N$, where N is the number of theoretical plates.[7,23] The dependence of chromatogram variance on the TSK-G2000SW and TSK-

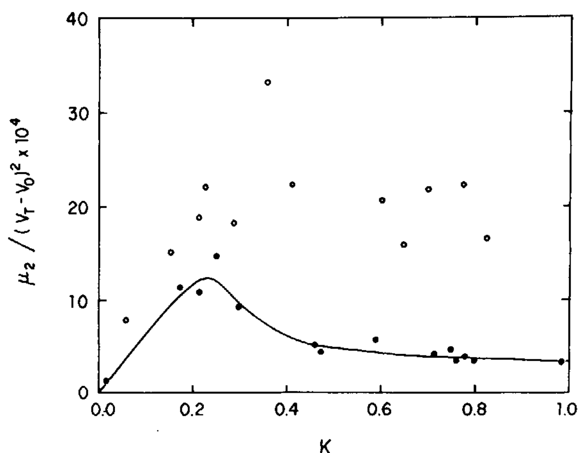

Fig. 2. Normalized chromatogram variance determined at a nominal flow setting of 1.0 mL/min plotted as a function of the dimensionless retention volume K on the TSK-G3000SW column: (○) dextran samples (Table I); (●) proteins, the following in increasing order of retention: thyroglobulin, β-galactosidase, glutamate dehydrogenase, glucose oxidase, albumin dimer, transferrin, albumin, ovalbumin, trypsin inhibitor, chymotrypsinogen, α-lactalbumin, ribonuclease, cytochrome c, and vitamin B-12. The curve is hand-drawn to represent chromatogram variance for monodisperse solutes.

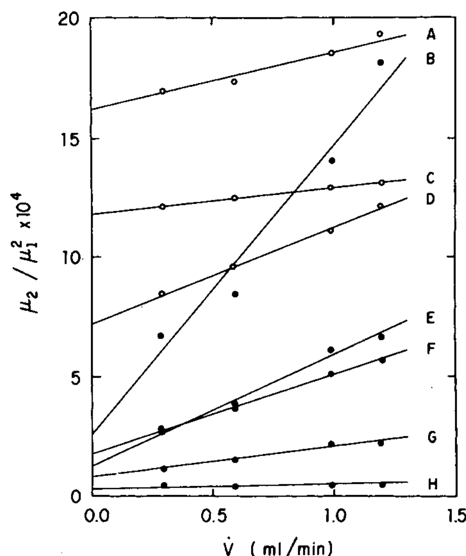

Fig. 3. Chromatogram variance normalized by the peak retention volume μ_2/μ_1^2 plotted as a function of effluent flow rate $\dot{V}$ on the TSK-G2000SW column: (●) proteins; (○) dextrans. The lines are those obtained by linear regression and are labeled as follows: (A) M25; (B) albumin dimer; (C) M3; (D) M41; (E) albumin; (F) ovalbumin; (G) ribonuclease; (H) sucrose.

G3000SW columns as a function of flow rate has been studied by others,[14,21] who report a slightly nonlinear dependence of chromatogram variance or plate height on flow rate. A similar flow rate dependence has also been observed with other SEC columns.[23] Such nonlinearity, however, occurs only at high flow rates, and the present experiments are limited to the linear region.

The present results are consistent with existing theories of SEC where chromatogram variance has been shown[9] to be described by the following equation

$$\frac{\mu_2}{\mu_1^2} = \frac{2\lambda d_p}{L} + \frac{k d_p^2}{30(1 + k)^2 V_0 D_s} \dot{V} + H_p \quad (6)$$

where H_p is the contribution of polymer polydispersity to chromatogram variance. Equation (6) results from assuming the linear velocity v is related to the effluent flow rate $\dot{V}$ by

$$v = \dot{V}L/V_0 \quad (7)$$

The values of L, D_s, and λ denote the column length, solute diffusion coefficient in the stationary phase, and a constant characteristic of the column packing, respectively. Equation (6) describes chromatogram variance as a sum of terms describing dispersion processes resulting from eddy diffusion in the mobile phase of the column, mass transport within the stationary phase and the polydispersity of the polymer. This expression shows that chromatogram variance is a linear function of effluent flow rate with an

intercept dependent upon the particle size of the column packing and the polydispersity of the polymer. Such behavior is shown in Figure 3, where for truly monodisperse solutes the intercepts are small compared with the intercepts for polydisperse dextran samples.

The slopes and intercepts obtained by linear regression of μ_2/μ_1^2 on $\dot{V}$ are shown in Table III for both the TSK-G2000SW and TSK-G3000SW columns. For each monodisperse solute the intercept is similar for both columns as predicted by eq. (6). This intercept is not constant but increases with solute molecular weight. Although eq. (6) predicts a value independent of molecular weight, other workers[24,25] have shown that a constant term describing chromatogram broadening due to eddy diffusion in the mobile phase is not consistent with all experimental observations. Working with columns containing nonporous glass particles, those investigators have shown that eddy dispersion is a weak function of flow rate and is dependent on solute molecular weight. In the present study such effects are apparently small and are assumed to affect only the value of the intercept.

Restricted Diffusion

The dependence of chromatogram variance on flow rate is inversely related to the diffusion coefficient of the solute in the stationary phase of the column. From the slopes in Table III and eq. (6) the diffusion coefficient D_s

TABLE III
Slopes and Intercepts (± Standard Error) Obtained by Linear Regression of Chromatogram Variance μ^2/μ_1^2 on Effluent Flow Rate V as Shown in Figure 3

Column	Solute	Slope (min/mL) × 10^4	Intercept × 10^4
TSK-G2000SW	Sucrose	0.10 ± 0.03	0.39 ± 0.03
	Ribonuclease	1.23 ± 0.15	0.86 ± 0.14
	Ovalbumin	3.33 ± 0.10	1.76 ± 0.09
	Albumin	4.64 ± 0.24	1.29 ± 0.22
	Albumin dimer	12.25 ± 2.01	2.62 ± 1.81
	M3	1.10 ± 0.21	11.81 ± 0.19
	M10	0.27 ± 0.26	12.46 ± 0.23
	M25	2.08 ± 0.54	16.63 ± 0.48
	M41	4.07 ± 0.18	7.22 ± 0.16
TSK-G3000SW	Sucrose	0.05 ± 0.03	0.48 ± 0.03
	Ribonuclease	0.88 ± 0.09	0.57 ± 0.08
	Ovalbumin	1.69 ± 0.08	1.07 ± 0.07
	Albumin	1.85 ± 0.09	0.82 ± 0.08
	Albumin dimer	3.94 ± 1.37	2.76 ± 1.23
	β-Galactosidase	12.45 ± 0.65	2.28 ± 0.58
	M3	0.57 ± 0.10	2.27 ± 0.09
	M6	0.50 ± 0.33	5.59 ± 0.30
	M10	0.93 ± 0.18	7.92 ± 0.16
	M20	2.02 ± 0.61	8.40 ± 0.55
	M25	2.42 ± 0.51	20.10 ± 0.46
	M33	2.83 ± 0.40	10.63 ± 0.36
	M55	5.89 ± 0.32	10.15 ± 0.29
	M87	1.35 ± 0.47	6.25 ± 0.42

can be obtained. It is noted that the accuracy of the slopes for proteins is greater than for dextrans since the change in chromatogram variance with flow rate for dextrans is relatively small. Figure 4 shows the results of this computation, where the logarithm of the ratio of the stationary phase diffusion coefficient D_s to the solution diffusion coefficient D_m is plotted as a function of the ratio of Stokes radius R_s to pore radius R_p for both proteins (●) and dextrans (○) on both columns. The value of the solution diffusion coefficients for proteins were derived from literature values and for dextrans from the following relationship[13]:

$$D_m = 7.04 \times 10^{-5} (M_w)^{-0.47} \tag{8}$$

where D_m has the units of cm²/s. The Stokes radii were determined from the solution diffusion coefficients by the following equation

$$R_s = kT/6\pi\eta D_m \tag{9}$$

where k is the Boltzmann constant, T is the absolute temperature, and η is the solution viscosity. The physical data for the proteins used here were summarized previously.[13] The diffusion coefficients in the stationary phase are reduced from their values in solution with greater reductions occurring as the ratio of Stokes to pore radii increases. Similar reductions were obtained for both proteins and dextrans.

The reduced rate of diffusion in porous media due to its tortuosity and the constriction of the pores is termed restricted diffusion. Previous studies with SEC in organic solvents using low polydispersity polystyrene standards have shown that restricted diffusion within the stationary phase is present,

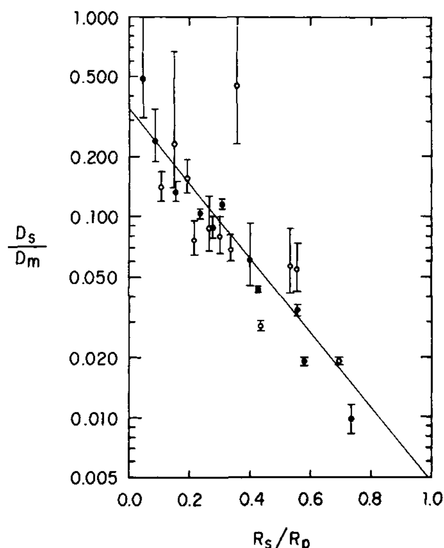

Fig. 4. The logarithm of D_s/D_m for all proteins (●) and dextrans (○) obtained on both columns plotted as function of the ratio of Stokes to pore radii R_s/R_p. Error bars denote standard errors obtained by linear regression of μ_2/μ_1^2 on $\dot{V}$ as shown in Figure 3 and described in Table III. The solid line was obtained by linear regression.

but there is no agreement over the molecular weight dependence of this phenomena. Van Kreveld and van den Hoed[23] evaluated the diffusive term for mass transport within porous silica particles and found D_s/D_m to decrease from 0.31 to 0.12 as the molecular weight of the polystyrene standards increased. Knox and McLennan[19] and Dawkins and Yeadon[26] using microparticulate SEC systems found a similar but more pronounced dependence of D_s/D_m on molecular weight. The values obtained by these workers ranged between 0.167–0.059 and 0.144–0.082. Klein and Grüneberg[27] have also found restricted diffusion to be present on columns of both VITX–glass and TSK–polystyrene. This phenomenon was, however, independent of partition coefficient K for $0.35 \leqslant K \leqslant 1$, but increased restricted diffusion was apparent for values of $K < 0.35$ on the polystyrene column. Lastly, Chiantore and Guaita[28] have studied chromatogram broadening on LiChrospher silica gels and found restricted diffusion of polystyrene standards to be independent of molecular weight on each column. However, the values of D_s/D_m were dependent on the mean pore size of the gels for different columns.

Many previous investigations were limited to a study of only two or three different polymers on each column. The scatter in the present data suggests that conclusions based upon so few solutes should be regarded as tenuous. Much of the scatter in the values obtained for D_s/D_m may result from neglecting the flow rate dependence of eddy diffusion. In their extensive study of column dispersion, Knox and McLennan[19] have shown that after correction for the flow rate dependence of mobile phase eddy diffusion their values of D_s/D_m were more self-consistent. Additional experiments at very low flow rates may resolve this issue.

Restricted diffusion of macromolecules within porous glass particles and across membranes of well-defined pore structure has also been studied by others. Colton and co-workers[29] have studied both equilibrium partitioning and restricted diffusion of nearly monodisperse polystyrenes within porous glass cubes. At low polymer concentrations, equilibrium partitioning was a function of polymer molecular weight, but restricted diffusion within the pores was independent of polymer molecular weight. The independence of restricted diffusion was observed only with polystyrene standards since restricted diffusion was dependent on the molecular weight of the compact proteins studied. Cannell and Rondelez[30] have observed restricted diffusion of nearly monodisperse polystyrene through porous membranes with a well-defined pore size. They reported an increase in restricted diffusion with increasing polymer molecular weight, but their results were dependent upon polymer concentration. A similar concentration dependence has been observed by Satterfield and co-workers with respect to equilibrium partitioning of polystyrene in porous glass cubes.[31] Restricted diffusion of dextran and ficoll in aqueous solvents through porous membranes has also been studied recently by Deen and co-workers.[2] They have observed that restricted diffusion is a function of the molecular weight of the polymer but is more severe for the more highly branched ficoll than for dextran, suggesting that molecular flexibility may be an important factor.

The present results suggest that restricted diffusion within the stationary phase is a function of the ratio of Stokes to pore radius. For all solutes studied, the best straight line from linear regression analysis is also shown in Figure 4 and is given by

$$\log D_s/D_m = -0.46 - 1.86\, R_s/R_p \tag{10}$$

This empirical exponential relationship has been observed by others[32–34] using different solutes and other porous materials. The magnitude of the slope observed relating the dependence on solute Stokes radius is also similar to the value of 2.0 obtained in one previous study.[34] When a similar correlation was performed based not on Stokes radius but rather the solute radius determined from the hydrodynamic volume of dextran,[13] a similar correlation was obtained. We do not believe that the present data warrant such a fine distinction between radii. These results then indicate that restricted diffusion is similar for proteins and dextrans of the same molecular size. Moreover, they suggest that column spreading for proteins may be used as models for column spreading of other polymers that have the same retention volume.

Column Spreading Errors

The effects of column spreading on molecular weight distribution determinations can be demonstrated most easily by considering a polymer sample with a Gaussian distribution. We denote the chromatogram in the absence of column spreading by $W(\nu)$ defined by

$$W(\nu) = 1/\sqrt{2\pi\mu_p}\, \exp[-(\nu - \mu_1)^2/2\mu_p] \tag{11}$$

where μ_1 and μ_p are the peak retention volume and the chromatogram variance caused by polymer polydispersity, respectively. If column spreading is independent of retention volume, then the observed chromatogram $F(\nu)$ is given by[35]

$$F(\nu) = 1/\sqrt{2\pi\mu_c}\, \exp[-(\nu - \mu_1)^2/2\mu_c] \tag{12}$$

where μ_c is the observed chromatogram variance caused by both polymer polydispersity and column spreading μ_s and is given by

$$\mu_c = \mu_p + \mu_s \tag{13}$$

The percent error E_p incurred by neglecting column spreading can be calculated by combining eqs. (11) and (12) and is given by

$$\begin{aligned} E_p &= 100\,\frac{W(\nu) - F(\nu)}{F(\nu)} \\ &= 100[1/\sqrt{1-\epsilon}\, \exp[-(\nu - \mu_1)^2\epsilon/2\mu_c(1-\epsilon)] - 1] \end{aligned} \tag{14}$$

where ϵ the fractional broadening due to column spreading is defined as

$$\epsilon = \mu_s/\mu_c \tag{15}$$

The relationship described in eq. (14) is shown in Figure 5, where E_p is plotted as a function of ϵ for various values of $(\nu - \mu_1)/\sqrt{\mu_c}$. The values selected for $(\nu - \mu_1)/\sqrt{\mu_c}$ correspond to the retention volumes where the

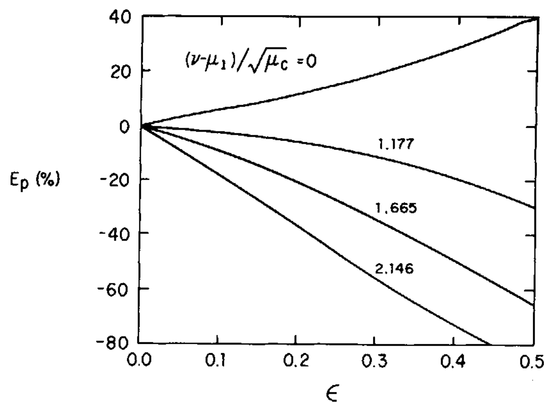

Fig. 5. The percent error E_p incurred by neglecting column spreading plotted as a function of ϵ as defined in eq. (14). The different values of $(\nu - \mu_1)/\sqrt{\mu_c}$ correspond to different heights of the chromatogram that are 1.0, 0.5, 0.25, and 0.1 times its peak value.

chromatogram heights are 1.0, 0.5, 0.25, and 0.1 times the peak value. For each point on the chromatogram the magnitude of the percent error increases with increasing values of ϵ. It should be noted that the percent error can be extremely large for the chromatogram tails. The percent error relative to the peak of the chromatogram or $100[W(\nu) - F(\nu)]/F(\mu_1)$ can also be computed from eqs. (11) and (12). It can be shown that the maximum value of this function is obtained at the peak of the chromatogram. The curve for $(\nu - \mu_1)/\sqrt{\mu_c} = 0$ in Figure 5 can, therefore, be helpful in assessing an upper bound to this error function.

By using proteins as models of column spreading it can be seen in Figure 2 that for the dextran samples examined in the present study approximately 10–75% corrections to the chromatogram variance are required to accurately determine the polymer polydispersity. This statement is in apparent contradiction with our previous work,[13] where M_n and M_w agreed well with those measured independently. This apparent discrepancy, however, is resolved by examining the polydispersity index I given by

$$I = M_w/M_n = \exp[5.3\mu_2 S^2/(V_T - V_0)^2] \tag{16}$$

where S is the slope of the calibration curves described in eq. (4) or (5) and the factor 5.3 results from the change to natural logarithms. For the low polydispersity dextrans studied, the exponent in eq. (16) is small such that the polydispersity index may be approximated as

$$I \simeq 1 + 5.3\mu_2 S^2/(V_T - V_0)^2 \tag{17}$$

For samples with small chromatogram variance, a fairly large correction to μ_2 will result in a relatively minor correction to the polydispersity index and also the average molecular weights. Table I shows the values of the polydispersity index computed by eq. (16) neglecting column spreading on the TSK-G3000SW column at a nominal flow setting of 1.0 mL/min. Also shown are the values corrected for column spreading by subtracting the chromatogram variance of a hypothetical monodisperse solute with the

same retention volume as determined from the curve in Figure 2. The values of I reported previously[13] are larger than described here and are more accurate since they were determined by integration of the chromatograms and are free from a Gaussian distribution assumption. They are described here only to show that corrections for column spreading that decrease chromatogram variance by over 50% have little effect on the polydispersity index. Moreover, since column spreading errors are greatest for polymers of low polydispersity,[7] corrections to average molecular weight determinations in all situations for these columns will be small. Confirmation of these calculations is difficult since this degree of precision is equal to or greater than independent measurements (such as light scattering or osmotic pressure) as suggested recently by others.[36]

Molecular weight distribution determinations may, however, need substantial corrections for column spreading. The errors incurred by neglecting column spreading depend also on the polydispersity of the polymer studied (see Fig. 5). For the low polydisperse dextrans studied here relatively large errors are present if column spreading is not taken into account. Although the methods needed to correct for column spreading lead to difficult numerical problems,[37] a number of techniques are available for this purpose that are sufficient under certain conditions.[8] It is suggested that the magnitude of column spreading errors be assessed when representations of polymer molecular weight distributions are fundamental to an investigation.

CONCLUSIONS

Column spreading on the TSK-G2000SW and TSK-G3000SW columns, including restricted diffusion within the stationary phase of the column, is similar for proteins and dextrans of the same molecular size. The differences in chromatogram width between proteins and paucidisperse dextrans are primarily caused by sample polydispersity. Determination of integral properties of polymers, such as average molecular weights, are only slightly affected when column spreading is neglected. Depending upon the polydispersity of the polymer, however, corrections for column spreading may be substantial when a precise determination of molecular weight distributions are of interest.

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