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ESTIMATION OF MOLECULAR WEIGHT DISTRIBUTIONS BY GEL CHROMATOGRAPHY

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SUMMARY

An improved method of boundary analysis is described whereby the molecular weight distributions of polydisperse solutes may be estimated by frontal gel chromatography. Its application to elution profiles obtained with a dextran fraction on Sephadex G-200 demonstrates the necessity of allowing for the effects of eddy diffusion, a phenomenon that has usually been neglected in gel chromatographic analyses of molecular weight distributions.

INTRODUCTION

The size and heterogeneity of macromolecules, both natural and synthetic, are of interest to the biochemist and to the polymer chemist. Following on from the development of the ultracentrifuge, BALDWIN and coworkers[1–3] devised suitable procedures for the analysis of boundary spreading in velocity sedimentation experiments on polymers. In principle, gel chromatography should provide an alternative procedure for examining molecular size distributions, and, indeed, there have been several such applications of the technique (see, *e.g.*, refs. 4–6). However, the reported data are at best approximate, since no account has been taken of the zonal spreading that occurs with a single solute; in this sense the analysis adopted resembles that proposed by SIGNER AND GROSS[7] for systems where spreading during velocity sedimentation is not affected significantly either by diffusion or by dependence of the sedimentation coefficient upon concentration of solute. Investigations with Sephadex indicate that, for this particular gel medium at least, neglect of concentration dependence in gel chromatography may be justified by use of sufficiently low concentrations of solute[8], but that the analog of free diffusion cannot be ignored[9].

The purpose of this communication is to present frontal gel filtration data obtained with a polydisperse dextran fraction on Sephadex G-200; and to thereby illustrate a more rigorous procedure for examining the heterogeneity of polymers by gel filtration under conditions where boundary spreading of single solutes may be described by a simple random-flight model of chromatography[9].

EXPERIMENTAL

Dextran fractions (T-500, T-80, T-40, T-20 and T-10) were obtained from Pharmacia, and were used without further fractionation. Boundary analyses were performed on data obtained with T-40 ($M_n = 22300$, $M_w = 41000$ according to manufacturer's specifications), the other fractions being used either for the determination of the void volumes of the columns (T-500) or for calibration of elution data in terms of molecular weights (T-10 to T-80, inclusive). The various dextrans were dissolved directly in 0.1 *M* NaCl, the solvent used throughout this investigation.

Frontal gel filtration experiments were performed on columns 2.5 cm in diameter of Sephadex G-200 believed to be approximately identical in all respects except for length (and therefore volume). All experiments were done on the same Sephadex column, the variation in bed volume being effected by successive removals of the top layer of the gel bed; the adequacy of this procedure for obtaining approximate equivalence of column packing and of flow uniformity has been established previously[9]. Unless otherwise specified, the flow rate of the column was maintained at 60 ml/h throughout the series of experiments, the eluate being monitored continuously by means of an automated orcinol procedure. The sensitivity of this colorimetric assay was such that a 30 μg/ml solution of polysaccharide could be used for these studies, a concentration sufficiently low for effects of concentration dependence in Sephadex chromatography[8] to be neglected.

METHOD OF BOUNDARY ANALYSIS

Gel filtration of an initially sharp front of a single, non-interacting solute yields an elution profile which is no longer sharp. The following method of boundary analysis, which takes into account this dispersion of elution volume, is an adaptation of the procedures developed for the analysis of velocity sedimentation[3] and moving boundary electrophoretic[10] data. Basically, these procedures for analyzing patterns obtained with freely migrating systems require determination of an experimental quantity $g^*(u)$, the apparent fraction of solute with velocity u after migration for time t; the true value $g(u)$ is then obtained by extrapolation of $g^*(u)$ to infinite time, a plot of $g^*(u)$ *versus* $1/t$ being used for this purpose[11]. Previous studies have established that in Sephadex chromatography elution volume is the analog of velocity in a single-phase migration experiment[12], and column length the corresponding analog of time[9]. In this connection it is noted that elimination of time as a variable in gel chromatography implies the relatively insignificant role of free diffusion in the mobile phase of the gel bed; the insensitivity of elution profiles to flow rate of Sephadex columns (Fig. 1; see also Fig. 1 of ref. 9) makes possible this neglect of non-equilibrium effects.

Eluate volumes in each elution profile were first converted to distribution coefficients K_{av} as defined by eqn. 3 of LAURENT AND KILLANDER[13]; for simplicity of presentation the *av* subscript is omitted hereafter in this manuscript. A partition coefficient increment ΔK was then chosen such that twenty increments spanned the region where solute concentration varied with K in the most diffuse c–K profile. The polydisperse solute was then considered to consist of this number of components, with partition coefficients given by the mean values of K within the individual increments. The apparent fraction of solute i with partition coefficient K_i could then

be calculated from the expression $g^*(K_i) = \Delta c_i/c_0$, where Δc_i denotes the change in solute concentration across the i'th increment, and c_0 the initial or plateau concentration of solute in the experiment. $g(K_i)$ was then estimated from the ordinate intercept (inferred by extrapolation) of a plot of $g^*(K_i)$ *versus* $1/V_0$, the reciprocal of the void volume. Repetition of this procedure for each K_i thus permitted the construction of a $g(K)$–K distribution profile corrected for effects of "diffusion". This profile was then converted to a molecular weight distribution, $g(M)$ *versus* M, by reference to a K–log M_w calibration plot obtained with dextran fractions of known weight–average molecular weights, $\bar{K}$, the weight-average distribution coefficient, being obtained from the first moment of the elution profile[14].

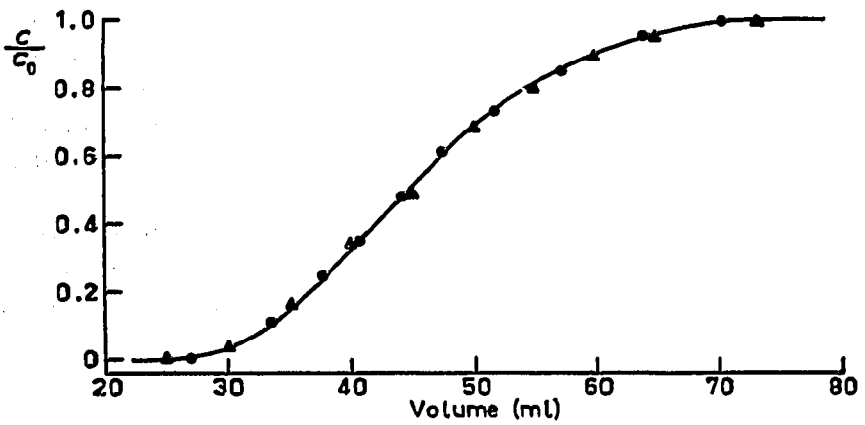

Fig. 1. Effect of column flow rate on the advancing elution profile obtained in frontal gel filtration of Dextran 40 on a 16 × 2.5 cm column of Sephadex G-200 equilibrated with 0.1 M NaCl. ●, 36 ml/h; ▲, 60 ml/h.

APPLICATION OF THE BOUNDARY ANALYSIS

Fig. 2 presents advancing elution profiles obtained with Dextran 40 on Sephadex G-200 columns of different lengths; as expected, the absolute extent of boundary spreading decreases with decreasing column length. However, expression of the

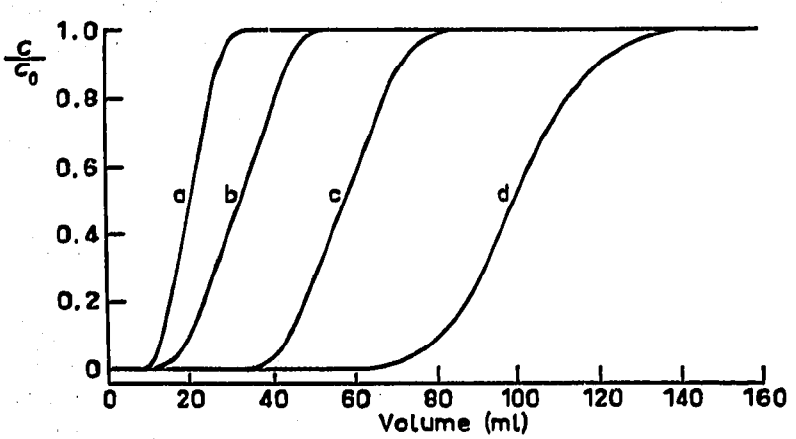

Fig. 2. Advancing elution profiles obtained in gel filtration of Dextran 40 on Sephadex G-200 columns with the same diameter (2.5 cm) but with different lengths: (a) 6.7 cm; (b) 8.7 cm; (c) 17.7 cm; (d) 31.0 cm. Column flow rate, 60 ml/h.

spreading in terms of the reduced variable K, which takes into account the variation in column length (volume), leads to the converse result, *viz.*, that the largest column yields the sharpest profile, the apparent distribution becoming progressively more diffuse with decreasing column volume (Fig. 3). Representative plots of the extrapolations involved in obtaining $g(K_i)$, the true fraction of solute with partition coefficient K_i, from the apparent values of $g^*(K_i)$ are illustrated in Fig. 4, the complete extrapolated distribution being designated by the solid line in Fig. 3. Finally, this profile is converted to a molecular weight distribution by incorporation of the $\bar{K}$–log M_w calibration plot, the relevant abscissa scale for analysis of the heterogeneity in terms of molecular size being indicated at the top of Fig. 3.

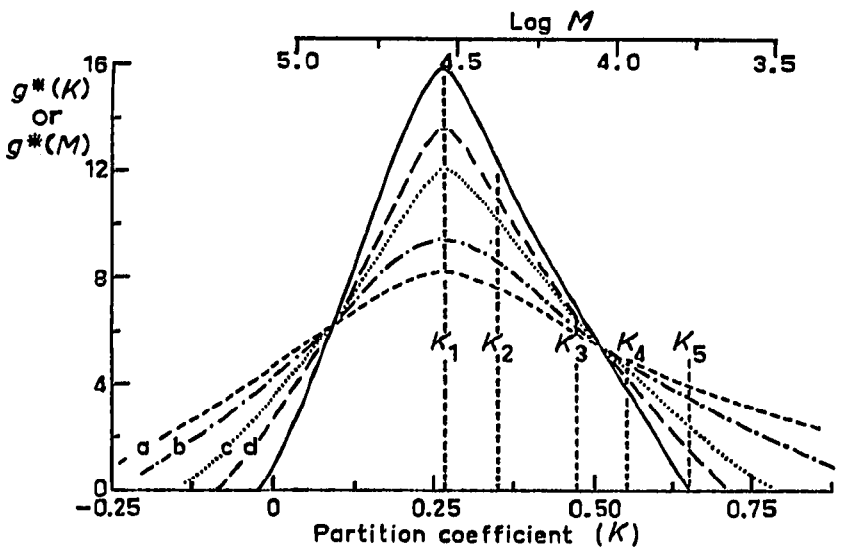

Fig. 3. Boundary analysis of the elution profiles shown in Fig. 2, the solid line referring to the distribution obtained by extrapolation of the experimental data to infinite column length (see text).

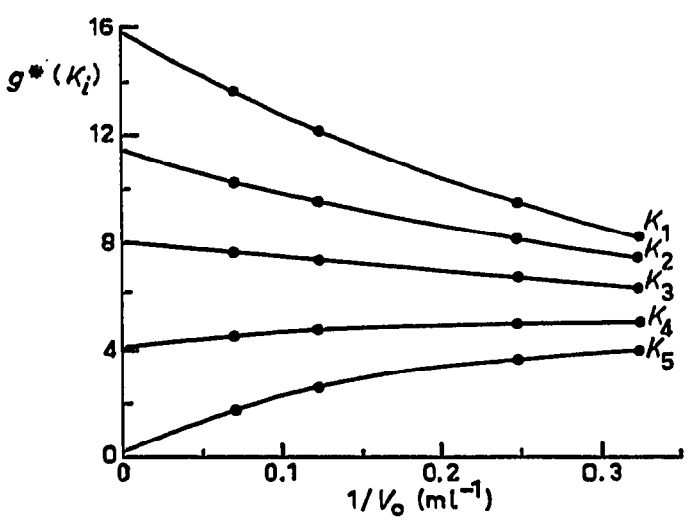

Fig. 4. Representative plots illustrating the extrapolations involved in the estimation of the polydispersity of Dextran 40 by Sephadex chromatography; the experimental data are taken from Fig. 3, in which the relevant values of K are indicated.

DISCUSSION

The data depicted in Fig. 3 demonstrate unequivocally that some account should be taken of the chromatographic analog of diffusion (eddy diffusion[15]) before much reliance can be placed on molecular weight distributions inferred from gel chromatographic profiles; but apparently only MOORE AND HENDRICKSON[16] have made any attempt to do so. In this connection the present method of eliminating the effects of such boundary spreading by extrapolation of data to infinite column length appears to be applicable more readily than their procedure, which involves interpretation of a profile reflecting both polydispersity and eddy-diffusive spread. As in the case of its counterparts in electrophoresis and velocity sedimentation, the present analysis is of limited value for systems in which the "diffusional" spread exceeds that reflecting polydispersity, since low estimates of $g(K)$ in the region of the maximum in the distribution profile are then obtained (see Table I of ref. 3).

It is emphasized that omission of mathematical expressions from the present communication indicates their redundancy, and not the lack of a sound theoretical basis for the method of obtaining the $g(K)$–K distribution. We drew attention to the fact that in Sephadex chromatography of a single solute diffusion in the mobile phase of the column could be neglected, the extent of boundary spreading being explained satisfactorily in terms of a simple random-walk treatment of chromatography[9]. With this particular model the analogy between elution profiles and concentration–distance distributions in freely migrating systems is completely rigorous, and, consequently, adaptation of the equations derived for the latter[1–3,10,11] to describe Sephadex elution profiles merely requires the substitution of elution volumes for velocities[12] and of column lengths for times[9]. The conversion from a $g(K)$–K profile to a molecular weight distribution is, of course, empirical, reliant solely upon an experimentally determined relationship between K and M.

The present method of boundary analysis may therefore be applied with a fair degree of confidence to profiles obtained with Sephadex as the chromatographic medium, or indeed with any other gel system yielding elution profiles which are insensitive to column flow rate. Time dependence of elution profiles[17] could result from significant contributions of diffusion in the mobile phase and/or non-equilibrium effects[15], both of which would render more difficult the theoretical treatment inasmuch as terms in t as well as l would have to be incorporated. However, in the event of significant diffusional contributions such spreading is proportional to $\sqrt{t}$, which is in turn dependent on $\sqrt{l}$ provided column flow rate is maintained constant. Since the time-independent dispersion is also proportional to $\sqrt{l}$, it follows that effects of free diffusion would be eliminated by the extrapolation procedure adopted in the above boundary analysis. The other postulated source of time-dependent elution profiles represents a much more complex situation[15], for which the present method of analysis may not be valid. Thus we do not anticipate that the present method of estimating molecular weight distributions will apply necessarily to all gel data, but rather that this study will prompt closer scrutiny of the conditions prevailing in the various gel chromatographic systems and development of the relevant methods of boundary analysis.

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REFERENCES

1 J. W. WILLIAMS, R. L. BALDWIN, W. M. SAUNDERS AND P. G. SQUIRE, *J. Am. Chem. Soc.*, 74 (1952) 1542.
2 R. L. BALDWIN, *J. Am. Chem. Soc.*, 76 (1954) 402.
3 R. L. BALDWIN, *J. Phys. Chem.*, 58 (1954) 1081.
4 D. J. HARMON, *J. Polymer Sci.*, Part C (1965) 243.
5 K. A. GRANATH AND B. E. KVIST, *J. Chromatog.*, 28 (1967) 69.
6 A. WASTESON, *Biochim. Biophys. Acta*, 177 (1969) 152.
7 R. SIGNER AND H. GROSS, *Helv. Chim. Acta*, 17 (1934) 726.
8 D. J. WINZOR AND L. W. NICHOL, *Biochim. Biophys. Acta*, 104 (1965) 1.
9 G. E. HIBBERD, A. G. OGSTON AND D. J. WINZOR, *J. Chromatog.*, 48 (1970) 393.
10 R. L. BALDWIN, P. M. LAUGHTON AND R. A. ALBERTY, *J. Phys. Chem.*, 55 (1951) 111.
11 L. J. GOSTING, *J. Am. Chem. Soc.*, 74 (1952) 1548.
12 L. W. NICHOL, A. G. OGSTON AND D. J. WINZOR, *J. Phys. Chem.*, 71 (1967) 726.
13 T. C. LAURENT AND J. KILLANDER, *J. Chromatog.*, 14 (1964) 317.
14 D. J. WINZOR, in S. J. LEACH (Editor), *Physical Principles and Techniques in Protein Chemistry*, Part A, Academic Press, New York, 1969, p. 451.
15 J. C. GIDDINGS AND K. L. MALLIK, *Anal. Chem.*, 38 (1966) 997.
16 J. C. MOORE AND J. G. HENDRICKSON, *J. Polymer Sci.*, Part C, (1965) 233.
17 S. A. BARKER, B. W. HATT, J. B. MARSTERS AND P. J. SOMERS, *Carbohyd. Res.*, 9 (1969) 373.