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AN EXPERIMENTAL AND THEORETICAL INVESTIGATION OF BOUNDARY SPREADING IN GEL CHROMATOGRAPHY

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SUMMARY

Gel filtration of an initially sharp front of a single, non-interacting solute yields an elution profile which is no longer sharp. The nature of this boundary spreading has been investigated experimentally by frontal gel chromatography of a series of purified proteins through columns of Sephadex G-100. The extent of boundary spreading is independent of flow rate in the range 2.2–8.8 ml/cm²/h, and is linear with respect to mean elution volume. It is concluded that under these conditions adoption of complex, non-equilibrium theories of chromatography is not essential for the description of the boundary spreading, which may be satisfactorily accounted for by a simple length-dependent random-flight model. For the particular system under investigation this modified theoretical treatment, yielding Gaussian elution profiles rather than Gaussian distributions of concentration with length within the column, is preferred to the existing random-flight models of chromatography.

INTRODUCTION

Recent reports of osmotic[1] and Gibbs–Donnan[2] effects in Sephadex chromatography have substantiated the basic tenets of the original concept of gel filtration[3–5], whereby migration of a solute is considered to result from continuous attainment of partition equilibrium between a mobile liquid phase and a phase within the gel medium in which the solute is stationary. However, a more complex model, involving free diffusion in the mobile phase and non-attainment of partition equilibrium, has been used[6] to describe *boundary spreading* in Sephadex chromatography. Our studies of this latter phenomenon were prompted by an interest in predicting the boundary spread for chemically interacting systems; clearly, ability to use the simpler equilibrium concept of gel filtration would greatly facilitate such calculations.

We have therefore experimentally investigated the character of boundary spreading in gel chromatography by running a series of pure, non-interacting proteins through columns of Sephadex G-100. Under the conditions of these experiments omission of terms for the effects of free diffusion and of deviations from partition equilibrium still leads to a satisfactory operational description of the observed spreading. A simple random-flight theory of chromatography, which differs from that proposed by BERAN[7], provides an adequate description of the present data.

EXPERIMENTAL

Frontal gel filtration experiments were performed on a 19.5 × 2.4 cm column of Sephadex G-100 equilibrated with phosphate buffer, pH 6.8, I 0.10 (0.025 M NaH_2PO_4, 0.025 M Na_2HPO_4), the flow rate of the effluent being maintained at 40 ml/h unless otherwise specified. In the main series of experiments the column effluent was collected as 1.3 ml fractions, but in the work on cytochrome c continuous monitoring by means of a Beckman DB recording spectrophotometer was employed to examine more exactly the form of the elution profile. The proteins used were commercial samples, but the thyroglobulin, human γ-globulin and bovine serum albumin preparations were purified by a prior zonal experiment on the same column. Applied concentrations (c_0) of the various proteins were selected to yield an absorbance of approximately 0.4 at 280 nm or, in the case of cytochrome c, at 410 nm because of its greater absorptivity at the latter wavelength. In all experiments protein concentrations were sufficiently low (< 0.6 mg/ml) for effects of concentration dependence in gel filtration[8] to be neglected.

RESULTS AND DISCUSSION

From Fig. 1 it is evident that within experimental accuracy coincidental results were obtained with ovalbumin at flow rates of 10, 20 and 40 ml/h. Thus boundary spreading depends to a negligible extent on the time taken by the front in traversing

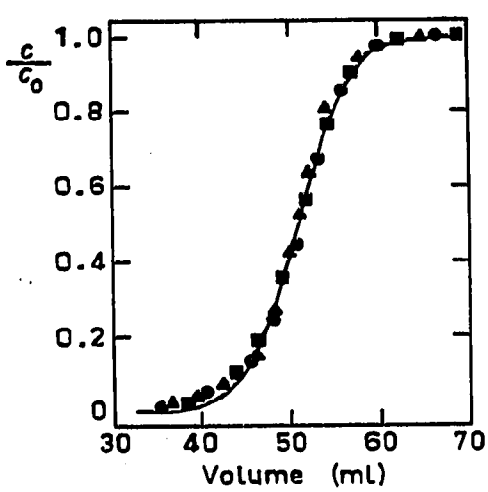


Fig. 1. Effect of column flow rate on the advancing elution profile obtained in frontal gel filtration of ovalbumin on a 19.5 × 2.4 cm column of Sephadex G-100 equilibrated with phosphate buffer, pH 6.8, I 0.1. ■, 40 ml/h; ●, 20 ml/h; ▲, 10 ml/h.

the column. Because of this insensitivity of profiles to flow rate, it is thus not *essential* to employ complex models of chromatography such as those proposed by GIDDINGS AND MALLIK[6] or VINK[9], which consider the effects of diffusion and non-equilibrium. Even though this insensitivity of profiles to flow rate may well reflect the mutual cancellation of non-equilibrium effects, clearly a theory is adequate which predicts elution profiles dependent on distance travelled rather than on time elapsed in the course of migration: random-flight[7,10] and theoretical plate[11] models meet this requirement.

It appears from Fig. 1 that the elution profile is Gaussian (see below) whereas existing theories of chromatography predict a Gaussian distribution of solute within the column at a given time. These conditions are mutually exclusive.

A Gaussian distribution *within* the column is described by

$$g(x-\bar{x})_t = \frac{1}{\sqrt{(2\pi\sigma^2)}} \exp -(x-\bar{x})^2/2\sigma^2 \qquad (1)$$

The second moment of this distribution, σ^2, is independent of x, though it will in general vary with t (or with $\bar{x}$). Now, if the boundary is sampled at constant x instead of at constant t (as is done in taking eluate from the end of a column), t and therefore $\bar{x}$ vary during the sampling process, with the consequence that σ^2 varies; the resultant distribution is not Gaussian but skew. Conversely, if the elution profile is considered to be "Gaussian", σ^2 must vary with x rather than with t if it is to have a constant value when the boundary is sampled from the end of the column; the distribution within the column is then correspondingly skew.

This relationship is illustrated in Fig. 2. The solid curves show the forms of a distribution for which σ^2 is proportional to ξ, at successive values of the latter quantity; simultaneously, the distribution is migrating in the η direction in such a way that $\bar{\eta}$ is proportional to ξ. The dashed curve shows the skew form (more spread as ξ increases) of the variation of c with ξ at fixed η. If, now, ξ is identified with time and η with distance the solid curves describe successive states, each at a particular time, of a normal (time-dependent) diffusion boundary, while the dashed curve describes the corresponding, skew elution profile. Conversely, if ξ is identified with distance and η with time, the solid curves describe Gaussian elution profiles while the dashed curve describes the correspondingly skew distribution of concentration within the column at a given time.

In order to examine more closely the form of the elution profile, we applied the procedure of CREETH[12], developed for the quantitative evaluation of minor skewness of boundaries, to the data obtained with cytochrome *c*. Fig. 3 summarizes this analysis; the data which span the concentration range of $0.05c_0 < c < 0.95c_0$, are presented in the form of a deviation diagram, the ordinate representing the deviation between Gaussian and observed concentration displacements, while the abscissa is a convenient but complex function (defined by CREETH[12]) related to the difference between the corresponding Gaussian concentration displacements. If the elution profile is Gaussian a value of zero would be obtained for $(\Delta Z - \Delta Z^*)$ irrespective of the concentration levels being compared (curve A). The corresponding plot for the BERAN random-flight model[7], or the GLUECKAUF theoretical plate model[11], is given in curve B, computed with a CDC 3200 computer; both these models predict a Gaussian distribution within the column and therefore a skew elution profile.

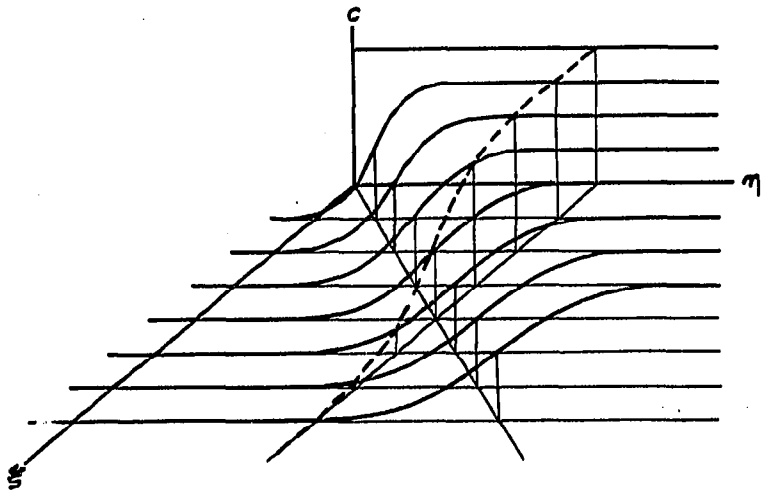


Fig. 2. Schematic representation of the relationship between the concentration distribution within a column at fixed time, and the concentration distribution in an elution profile. Migration in the η direction is proportional to ξ, the spreading being proportional to $\sqrt{\xi}$. If ξ is identified with time, and η with distance the dashed line represents the elution profile from a column of fixed length (η), the solid lines being the Gaussian concentration distributions within a column at different times. Conversely, on interchange of the variables so that ξ now represents distance and η time, the solid lines describe Gaussian elution profiles for columns of different lengths, the dashed line now indicating the non-Gaussian concentration distribution within the column at a fixed time.

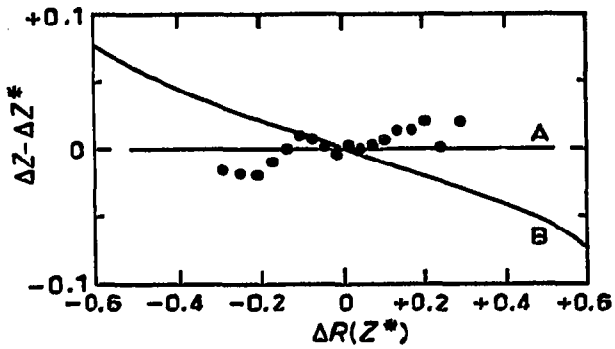


Fig. 3. Analysis of the advancing elution profile of cytochrome *c* for deviation from a Gaussian distribution. The circles denote experimental data, while curves A and B refer to the theoretical deviation plots predicted by the two random-walk models of gel chromatography. Experimental conditions as in Fig. 1 (flow rate of 40 ml/h) except that the eluate was monitored continuously.

Although the results deviate significantly from curve A (Student's *t*-test, $0.01 > P > 0.001$), it is clear that they lie much closer to curve A than to curve B. We therefore conclude that the elution profile is Gaussian. The small deviations could have arisen from a minor systematic error in the experiments, or possibly from the use of a strongly basic protein, which may migrate by virtue of a combination of solid–liquid and liquid–liquid partition; but the observation of a qualitatively similar trend in less precise data obtained with ovalbumin seems to preclude the latter explanation.

A random-flight model

We take up the suggestion of BERAN[7] that dispersion arises simply through granularity of the column. We assume that local partition equilibrium of solute is

instantaneously established and that solute in the gel phase is axially immobile. While the mobile solution moves with a mean axial velocity, elements of its volume (and therefore any solute molecules they contain) take more or less devious routes relative to the mean path; they therefore require varying times to traverse a given axial length of column. λ is a length of column, small in relation to the total length l, but large enough for the migrations of molecules in successive λ's to be statistically independent. The average time required to traverse λ is τ, but the times required by individual molecules are symmetrically dispersed about τ, the root-mean-square dispersion (see OGSTON[13]) being $\alpha\tau$. Then provided that $n = l/\lambda$ is large, the distribution of time t required to traverse the whole column is

$$g(t-\bar{t}) = \frac{1}{\sqrt{\{2\pi n(\alpha\tau)^2\}}} \exp -(t-\bar{t})^2/2n(\alpha\tau)^2 \qquad (2)$$

Since

$$V_e = \dot{V}t$$

where $\dot{V}$ is the rate of volume flow through the column and

$$\bar{V}_e = \dot{V}\bar{t} = \dot{V}n\tau \qquad (3)$$

it follows that

$$g(V_e - \bar{V}_e) = g(t-\bar{t})\frac{dt}{dV_e}$$

$$= \frac{1}{\sqrt{\{2\pi(\alpha^2/n)\bar{V}_e^2\}}} \exp -(V_e - \bar{V}_e)^2/2(\alpha^2/n)\bar{V}_e^2 \qquad (4)$$

Since α and n are both constants for a given column it follows that eqn. 4 is of exactly the same form as eqn. 1 but with elution volumes replacing x; hence Gaussian elution profiles and skew distributions of concentration within the column (see above) are predicted for this model. We note that BERAN[7] has used an essentially similar model, but has arrived at the converse result, namely that the distribution within the column is Gaussian, and, therefore, that the distribution of V_e must be skew. This difference seems to have resulted from his assuming that the elementary axial distances travelled, rather than the elementary times for a given axial distance, are symmetrically distributed about a mean. Although either treatment seems plausible theoretically the results given above favor our version, for this particular chromatographic system.

From eqn. 4 the second moment of the distribution of V_e is

$$\sigma_{V_e}^2 = \frac{\alpha^2\bar{V}_e^2}{n} \qquad (5)$$

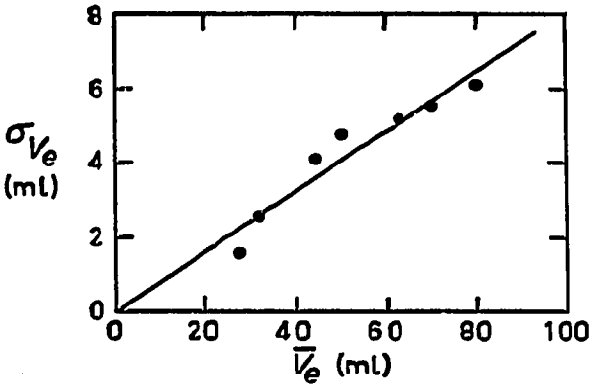
which indicates a linear relationship between σ_{V_e} and $\bar{V}_e$ for different solutes on the same column. A test of this prediction is shown in Fig. 4, which summarizes data for a series of purified proteins, a Gaussian profile being obtained with each solute. It is noted that derivation of an expression analogous to eqn. 5 from the BERAN model (see, *e.g.* ref. 14) requires the untenable assumption that the concentration distribution within the column at a given time and the elution profile are *both* Gaussian.

For the same solute on columns that differ only in length, $\bar{V}_e$ and n are both proportional to l so that

$$\sigma_{V_e}^2 \propto l \tag{6}$$

and the coefficient of variation

$$\frac{\sigma_{V_e}}{\bar{V}_e} \propto l^{-1/2} \tag{7}$$



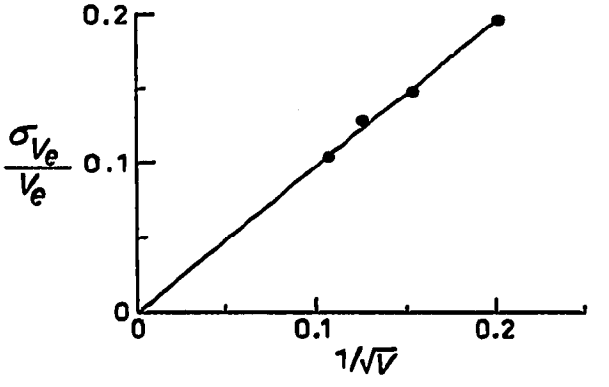


Fig. 4. Dependence of the standard deviation of the elution profile, σ_{V_e}, on the median elution volume, $\bar{V}_e$, for a series of purified proteins, the value of σ_{V_e} being taken as the difference between $\bar{V}_e$ and the elution volume at which $c = 0.84c_0$; the experimental data refer to thyroglobulin, human γ-globulin, bovine serum albumin monomer, ovalbumin, α-chymotrypsinogen, ribonuclease and lysozyme, respectively, reading from left to right.

Fig. 5. Effect of column volume (length) on the spreading of elution profiles obtained with ovalbumin on a column 2.4 cm in diameter of Sephadex G-100, equilibrated with phosphate buffer, pH 6.8, I 0.10, and eluted at a flow rate of 40 ml/h; the eluate was monitored continuously in these experiments.

In order to test experimentally the validity of eqn. 7 a series of frontal experiments with ovalbumin as solute and Sephadex G-100 as gel medium were performed on columns believed to be approximately identical in all respects except for length (and therefore volume). All four experiments were done on the same Sephadex column, the variation in bed volume (length) being effected by successive removals of the top layer of the gel bed; flow rate of the column was maintained at 40 ml/h throughout the series of experiments. The data so obtained exhibited good agreement with the predictions of eqn. 7 (see Fig. 5), and thus provide experimental confirmation of its validity under conditions where approximate equivalence of column packing may be assumed. This assumption might not be justified in the comparison between a small analytical and a large preparative column. Fig. 5 thus confirms the suggestion of GIDDINGS AND MALLIK[6] that the resolution of fronts or zones should be improved by increasing the length of a column, other things being equal.

In summary, we conclude (i) that a random-flight model provides a simple and adequate description of the dispersion process in gel chromatography of proteins on Sephadex G-100, (ii) that it is possible to formulate two versions of the random-flight theory, which predict different forms for the shape of the elution profile, and (iii) that for the particular chromatographic system under investigation the results

support the treatment that predicts Gaussian elution profiles rather than Gaussian distributions within the column.

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