The Origin and Consequences of Concentration Dependence in Gel Chromatography

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Concentration dependence of elution volume was determined for Blue Dextran 2000, Dextran 500, Dextran sulphate 500 and bovine serum albumin on columns of Sephadex G-100 equilibrated with sodium phosphate buffer, \( I \) 0.1, pH6.8. From the results for Dextran 500, it was shown that a linear relation exists between elution volume and the corresponding osmotic pressure calculated for the same concentration and incorporating the term containing the second virial coefficient. This relationship was used to predict the concentration dependence of elution volume for bovine serum albumin and myoglobin, proteins that partially penetrate Sephadex G-100. Possible consequences of osmotic effects are considered in relation to various types of column experiments, including differential chromatography.

Concentration dependence of elution volume has been observed in the study of a number of proteins by frontal gel chromatography (Winzor & Scheraga, 1963; Winzor & Nichol, 1965; Gilbert & Kellett, 1971). In addition, its importance has been discussed in relation to the interpretation of the shapes of elution profiles (Winzor & Nichol, 1965) and to the analysis of non-interacting and interacting mixtures (Winzor & Nichol, 1965; Nichol et al., 1967; Gilbert & Kellett, 1971).

In such quantitative studies, the methods employed to allow for the effects of concentration dependence are entirely analogous to those used to correct for frictional effects in sedimentation velocity. However, the effect in chromatography is likely to be osmotic in origin (Edmond et al., 1968; Nichol et al., 1969; Ogston & Wells, 1970). The present work explores this postulate further.

Experimental

Dextran 500 (\( M_w \) 370000, \( M_n \) 186000), Dextran sulphate 500 (sodium salt; 17% S) and Blue Dextran 2000 were obtained from Pharmacia, Uppsala, Sweden. Bovine serum albumin was supplied by Sigma Chemical Co., St. Louis, Mo., U.S.A. Unless specified otherwise, these materials were dissolved directly in sodium phosphate buffer, \( I \) 0.1 (0.025M-Na$_2$HPO$_4$, 0.025M-NaH$_2$PO$_4$, pH6.8), all solutions being prepared by weight.

Two columns of Sephadex G-100, equilibrated with the sodium phosphate buffer, were employed. A flow rate of approx. 12ml/h was used for both larger (2cm x 50cm) and smaller (1.8cm x 20cm) columns, the eluate being divided into fractions of approx. 2ml and 1ml respectively; the volume of each fraction was determined by weight. Frontal gel chromatography was performed at 20°C by the procedure described previously (Winzor & Scheraga, 1963; Winzor & Nichol, 1965), the volume of solution applied being 100ml and 30ml for the larger and smaller columns respectively; these loading volumes ensured the existence of plateau regions of original compositions in the elution profiles. Polysaccharides were monitored polarimetrically in a Perkin–Elmer model 141 polarimeter, and bovine serum albumin was determined by the biuret procedure (Gornall et al., 1949). Low concentrations (<150μg/ml) of carbohydrate were measured by the anthrone method (Roe, 1955).

Results and Discussion

Polysaccharides

Fig. 1 presents results obtained with three different polysaccharides studied on the larger column. Since each of these solutes is sufficiently large to be essentially excluded from the gel phase in the presence of salt (Edmond et al., 1968; Ogston & Wells, 1970; Wells, 1972), the variation in elution volume is a direct measure of changes in the void volume. In turn, such changes must arise as a consequence of shrinkage of gel beads and it is therefore relevant to examine the results in terms of eqn. (1):

\[
\pi \frac{1}{RT} = \frac{c}{M_n} + A_2 c^2 + A_3 c^3 + \cdots
\]  

(1)
which describes the concentration dependence of osmotic pressure $\pi$ at temperature $T$ across a membrane impermeable to the solute in terms of the number-average molecular weight $\bar{M}_n$ and the conventional second and third virial coefficients, $A_2$ and $A_3$, where $c$ (g/ml) is the concentration. In the limit of infinite dilution, $\pi$ tends to zero, and in the absence of osmotically induced bead shrinkage the observed elution volume of all excluded solutes should be identical; this is borne out by the results in Fig. 1, which can be extrapolated to a common ordinate value (48.4–48.5 ml) despite the markedly different variations of excluded volume, $V_0$, with $c$. It is also clear from eqn. (1) that $\pi$ is an approximately linear function of $c$ only in the event that terms involving the virial coefficients are negligible. Although the results for Blue Dextran seemingly exhibit this type of behaviour, the smaller Dextran 500 and its highly charged derivative do not. For Dextran 500 values of $\bar{M}_n$ (186 000) and $A_2$ ($3.4 \times 10^4$ mol·cm$^3$·g$^{-2}$) are available (Edmond et al., 1968), and the curvilinear plot of $(\pi/RT)$ against $c$ may be calculated from eqn. (1); in the range of dextran concentration studied the contribution of the third term, based on the value of $A_3$ obtained by Edmond et al. (1968), was deemed sufficiently small to warrant its neglect. This may be correolated with Fig. 1 to obtain corresponding values of $V_0$ and $\pi/RT$, which are plotted in Fig. 2. From eqn. (6) of Edmond et al. (1968) it is feasible in principle to calculate the dependence of $V_0$ on $\pi/RT$. The dependence so predicted is virtually linear over the range of $\pi/RT$ shown in Fig. 2, but is twice as great as that observed. In this connexion, it is noted that the magnitude of the predicted slope relies heavily on the values used for the internal concentrations and virial coefficients of the Sephadex gel; the values employed were those of Edmond et al. (1968), who point out their uncertainty. The important point emerges, however, that the experimental plot (Fig. 2) is also linear, which strongly supports the contention that the observed concentration dependence of $V_0$ is a direct consequence of osmotically induced bead shrinkage. The plot is analogous to those of relative bead size against $\pi/RT$ presented by Ogston & Wells (1970) in their study of single Sephadex beads, but in the present instance refers to a much smaller concentration range of excluded solute, where the first term.

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**Fig. 1.** Concentration dependence of excluded volume, $V_0$, obtained with Blue Dextran 2000 (△), Dextran 500 (●) and Dextran sulphate 500 (■) on Sephadex G-100

The column (2 cm x 50 cm) was equilibrated with sodium phosphate buffer, $I$ 0.1, pH 6.8.

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**Fig. 2.** Relationship between the excluded volume, $V_0$, of Dextran 500 and the osmotic pressure calculated for the corresponding concentration

For details see the text.
in eqn. (1) is comparable in magnitude with the second. In this concentration range perturbation of individual bead size would be difficult to detect, but is manifested as an accumulative effect in column experiments. Although values of the virial coefficients for Dextran sulphate 500 are unavailable, the existence of two charged residues per glucose residue should enhance the value of $A_2$ by a positive and more-pronounced Donnan contribution. Accordingly, the more-pronounced upward curvature observed in Fig. 1 for Dextran sulphate 500 is not surprising. The apparent linearity of the plot for Dextran Blue is at first sight unexpected, since it is not suggested that the virial coefficients for this derivative are zero. Certainly, the smaller slope at low concentrations, where the first term in eqn. (1) predominates, is expected on the basis of a higher value of $M_c$. In addition, the observation that values of $A_2$ tend to decrease with increasing size of dextrans (Edmond & Ogston, 1968; Edmond et al., 1968) suggests that the second term of eqn. (1) is also of smaller magnitude than that for Dextran 500. Thus, although the second term may become larger than the first for Dextran Blue at lower concentrations than with Dextran 500, the smaller absolute change in $V_e$ may make this relative effect more difficult to detect experimentally.

An immediate consequence of the correlation between osmotic pressure, non-ideality and the dependence of $V_e$ on c becomes apparent in the use of differential chromatography (Baghurst et al., 1971) to effect a transfer of a solute from one solvent environment to another of different ionic strength. In the previous study (Baghurst et al., 1971), such an experiment was employed to examine the effect of ionic strength on the extent of association of a polymerizing protein system by comparing the weight-average elution volumes in the two environments. To explore the possible effects of osmotic shrinkage on experiments of this design, Dextran sulphate 500 was selected, since the results in Fig. 1 suggest that osmotic shrinkage would be particularly sensitive to a change in ionic strength (via the Donnan contribution to non-ideality). Dextran sulphate in sodium phosphate buffer, $I$ 0.2, pH 6.8, was applied to the larger column pre-equilibrated with sodium phosphate buffer, $I$ 0.005, of the same pH value. The results are shown in Fig. 3 for two concentrations of the polysaccharide. In the control experiment (Fig. 3a), where a low concentration was employed to minimize the osmotic pressure in both environments, the elution volumes measured on either side of the ionic-strength gradient differed only by the loading volume, $V_L$, and in accordance with conservation of mass a single solute plateau was observed. At the higher concentration (Fig. 3b), bizarre effects were observed in that the plateau of original concentration was attained only after emergence of the phosphate gradient. The rectangle describes an area equal to the total amount of solute applied, and indeed identity of the hatched areas establishes mass conservation in the experiment. Thus, despite the complexity of the profile, the same elution volume in both environments would have been obtained by use of median bisectors of the entire advancing and trailing fronts. This implies that the existence of osmotic effects does not negate the basic comparison of weight-average elution volumes in differential-chromatography experiments, although the advantage of comparing plateau values ahead of and behind the ionic-strength gradient by use of the Johnston–Ogston equation (Johnston & Ogston, 1946; Baghurst et al., 1971) is lost. The lack of a plateau ahead of the ionic-strength gradient in Fig. 3(b) is due to the initial shrinkage of the gel beads in response to the relatively high osmotic pressure consequent on movement of the dextran sulphate into the medium of low ionic strength: the increase in external volume is observed as a decreased solute concentration, which continues until the ionic strength begins to increase, whereupon the process is reversed, resulting in an ‘overshoot’ of the applied concentration.

Proteins

In general, gel-chromatographic studies on proteins are conducted with gel media into which the solute partially penetrates. Even though eqn. (1) is not directly applicable to these cases in its present form, it nevertheless appears that osmotic effects continue to operate, since concentration dependence of $V_e$ has been observed for a number of non-interacting proteins with $K_w$. (Laurent & Killander, 1964) greater than zero (Winzor & Scheraga, 1963; Winzor & Nichol, 1965; Gilbert & Kellett, 1971). A further example is provided by results for bovine serum albumin ($K_w$, 0.15) shown in Fig. 4. The ordinate scale has been normalized by division by $V^*_e$ (the elution volume pertaining to zero solute concentration) to accommodate results from the different columns on the same graph; the larger error bars on experimental points referring to the smaller column reflect the fact that elution volumes were determined with the same precision ($\pm$0.1 ml) in both cases. That this concentration dependence of $V_e$ has resulted from gel shrinkage is evident from Figs. 5(a) and 5(b), which present the elution profiles obtained in frontal gel chromatography of a mixture of Dextran 500 (150 $\mu$g/ml) and bovine serum albumin (7.56mg/ml). In the advancing profile, pure polysaccharide ($K_w$, 0) migrates ahead of the protein, and by virtue of its low concentration provides a measure of $V^*_e$ (the void volume in the absence of bead shrinkage). On the trailing side, however, the Dextran 500 migrates in the presence of protein and thus its elution volume reflects the void volume $V_e$ consequent on osmotic effects; Fig. 5(a) clearly shows a difference between $V^*_e$
Fig. 3. Differential gel chromatography of Dextran sulphate 500 (●) dissolved in sodium phosphate buffer, \( I \) 0.2, pH 6.8, and applied to a column of Sephadex G-100 pre-equilibrated with sodium phosphate buffer, \( I \) 0.005, pH 6.8

The column dimensions were 2 cm × 50 cm. The loading concentrations were 0.001 g/ml and 0.005 g/ml in (a) and (b) respectively. --- indicates the gradients of ionic strength. \( V_e \) and \( V_l \) denote elution and loading volumes respectively. For details of the rectangle (-----) and hatched areas see the text.
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Fig. 4. Concentration dependence of the elution volume for bovine serum albumin on Sephadex G-100 equilibrated with sodium phosphate buffer, \( \text{pH} 6.8 \).

- and ■ denote experimental points obtained with the 2 cm \( \times \) 50 cm and 1.8 cm \( \times \) 20 cm columns respectively, and the lines are relations predicted theoretically (see the text). The bars represent the experimental error.

(16.4 ml) and \( V_0 \) (17.2 ml). No abnormal concentration change resulting from gel shrinkage (see Fig. 3) was observed in the region of the advancing profile between \( V_0^* \) and \( V_e \) for bovine serum albumin, presumably because of diffusional spreading and consequent partial overlap of the Dextran 500 and albumin boundaries. In contrast, Fig. 5(b) shows that the elution volumes of bovine serum albumin obtained from advancing and trailing profiles are identical (22.2 ml), a result consistent with the fact that bovine serum albumin is virtually the sole contributor to the osmotic pressure of the system.

A third experiment was performed in which the higher concentration of bovine serum albumin (7.56 mg/ml) was used, but the Dextran 500 was replaced with glucose (150 \( \mu \)g/ml). Fig. 5(b) describes the advancing and trailing profiles for protein, and those for glucose are shown in Fig. 5(c). An elution volume of 48.7 ml is obtained from the advancing profile where glucose migrates in the presence of protein, the corresponding estimate from the trailing profile, where glucose migrates alone, being 48.5 ml. These elution volumes are identical within experimental error. This result may be explained simply on...
the basis of the definition of $K_{av}$, (Laurent & Killander, 1964):

$$V_e = V_0 + K_{av}(V_0 - V_0)$$

(2)

where $V_b$ is the bed volume of the column. In the present context, $V_0$ is the void volume of the column under the osmotic conditions operative in the measurement of the elution volume of glucose, and thus $V_0$ must be regarded as a variable, as is evident in Fig. 5(a); it is the value extrapolated to infinite dilution, $V_0^0$, which is the constant characterizing the void volume of a given column. Differentiation of eqn. (2) yields $dV_e/dV_0 = (1 - K_{av})$ on the reasonable assumption that $K_{av}$ and $V_b$ are constants (Edmond et al., 1968). Since $K_{av}$ for glucose is 0.95, it follows for a variation in $V_0$ of 0.8 ml (Fig. S(a)) that $V_e$ should vary by only 0.04 ml, which is indeed undetectable.

This finding bears directly on the use of gel chromatography to investigate the binding of ligands of low molecular weight to macromolecular acceptors (see, e.g., Cooper & Wood, 1968). In these studies a gel is selected to exclude unbound acceptor and all complexes, when osmotic effects may be expected to operate. The experimenter is required to examine the trailing profile where the plateau of original composition is separated from one of pure ligand by dents in all forms of accepot, the median biector of the reaction boundary being associated with a void volume, $V_0$. Determination of $V_0^0$ in a separate experiment therefore gives the required $\Delta V_0$ to permit estimation of $\Delta V_e = \Delta V_0(1 - K_{av})$. Since $V_e$ in the region containing ligand alone is readily measurable, it follows that $V_e$ in the original-mixture plateau may now also be estimated.

**Concentration dependence of partially penetrating solutes**

Since the preceding results indicate that the dependence reported in Fig. 4 is the result of osmotic effects, it is of interest to enquire whether a modified form of eqn. (1) suffices to describe the observed phenomenon. One way of envisaging the effect of partial penetration is to consider that the net osmotic pressure causing bead shrinkage is equal to the difference between the external osmotic pressure defined by eqn. (1) and the internal osmotic pressure defined by eqn. (6) of Ogston & Silpananta (1970), with the first two terms omitted. This formulation solely in terms of solute concentration is consistent with that on which Fig. 2 is based. On noting that the inner concentration is given by the product $K_{av}c$, the differential of the net osmotic pressure with respect to $c$ becomes:

$$\frac{d(\pi/RT)}{dc} = \frac{(1 - K_{av})}{M_n} + 2A_2(1 - K_{av})^2 - A^* K_{av} c_d - A^* K_{av} c \frac{dc}{dc}$$

(3)

boundary across which all forms of the acceptor disappear. Thus, on the column, ligand migrates in regions subject to different osmotic effects. However, provided that $K_{av}$ approaches unity, $dV_e/dV_0 \approx 0$, and thus ligand migrates with essentially the same velocity in the two regions. In these cases, the concentration of ligand in the trailing plateau may be equated with the equilibrium concentration of unbound ligand in the original mixture (Nichol & Winzor, 1964). On the other hand, for smaller values of $K_{av}$, a gradient in the concentration of ligand must arise since different velocities of ligand will pertain in the two plateau regions, and the two concentrations may only be related by application of the Johnston-Ogston equation (Johnston & Ogston, 1946), which requires estimation of the two velocities. The gradient in the concentration of ligand will form part of a reaction boundary also involving concentration gra-

where $c_d$ denotes the internal concentration of Sephadex (g/ml) and $A^*$ is the interaction coefficient (Ogston & Silpananta, 1970). Three further points in relation to eqn. (3) require comment. First, terms involving the third and higher virial coefficients have been omitted (as in Fig. 2). Secondly, $K_{av}$ has been assumed constant, an assumption amply justified in the concentration range under study by the results shown in Fig. 2 of Ogston & Silpananta (1970). Thirdly, numerical calculations have shown the final term of eqn. (3) to be of negligible magnitude; this term will accordingly be omitted from further steps. From eqn. (2) it follows that:

$$\frac{d(V_e/V_0^0)}{dc} = \frac{d(V_0/V_0^0)}{d(\pi/RT)} (1 - K_{av})$$

(4)

Combining eqns. (3) and (4), we obtain:

$$\frac{d(V_e/V_0^0)}{dc} = \frac{d(V_0/V_0^0)}{d(\pi/RT)} \left[ \frac{(1 - K_{av})}{M_n} + 2A_2(1 - K_{av})^2 - A^* K_{av} c_d - A^* K_{av} c \right] (1 - K_{av})$$

(5)
where the value of \( \frac{d(V_0/V_0')}{d(\pi/RT)} \) may be obtained from Fig. 2, which also refers to the net osmotic pressure as defined. An alternative expression for \( \frac{d(V_0/V_0')}{dc} \) may be obtained on the basis of the empirical relation:

\[
V_c = V_0(1 + kc)
\]

(6)

where \( k \) is the concentration-dependence coefficient. Combination of eqn. (2), written in terms of \( V_0^* \) and \( V_0^0 \), and eqn. (6) yields:

\[
\frac{d(V_0/V_0^0)}{dc} = k \left[ 1 + \frac{V_b}{V_0^0} - 1 \right] = k \frac{V_0^0}{V_0^0} (1 - K_{sv})
\]

(7)

Thus, from eqns. (5) and (7):

\[
k = \frac{\frac{d(V_0/V_0^0)}{d(\pi/RT)}}{\frac{d(V_0/V_0^0)/d(\pi/RT)}} = \frac{(1 - K_{sv})}{\frac{M_s}{2} + 2A_2(1 - K_{sv})c - A^* K_{sv} c_I} \frac{V_0^0}{V_0^0} (1 - K_{sv})
\]

(8)

It is seen that the parameter \( k \) is a function of \( c \).

In relation to bovine serum albumin studied under the conditions reported in Fig. 4, the values of the parameters relevant to eqn. (8) are as follows: \( K_{sv} = 0.15; V_0^0/V_0^* = 0.77; M_s = 69,000; A_2 = 1.4 \times 10^{-4} \) (Scatchard et al., 1946); \( d(V_0/V_0^0)/d(\pi/RT) = 3.5 \times 10^5 \) (Fig. 2); \( c_I = 0.076 \) (Table 1 of Ogston & Silpananta, 1970). Substitution of these quantities into eqn. (8) yields \( k = 2.8 + 63c - 2600A^* \) (with \( c \) expressed as g/ml). Use of this relation together with eqn. (6) permitted the construction of the solid and broken lines in Fig. 4. The former is based on a value of zero for \( A^* \) (i.e. a system with no solute–gel matrix interaction), and the broken line refers to the value \( 4 \times 10^{-4} \) for \( A^* \) reported by Ogston & Silpananta (1970). Whereas the solid line describes adequately the experimental results, small but finite values of the interaction parameter \( 0 < A^* < 4 \times 10^{-4} \) would also suffice. From Table 3 of Ogston & Silpananta (1970), values of \( A^* \), determined with single Sephadex beads and hence high solute concentrations, exhibited considerable variability. Regardless of the exact magnitude of \( A^* \), it is evident that the observed concentration dependence of elution volume is satisfactorily described by eqns. (6) and (8). However, the curvature introduced by the term containing \( A_2 \) is sufficiently small to have escaped detection experimentally. Indeed, the experimental points in Fig. 4 could well be described by a linear relation within experimental error, as has been done with other protein systems (Winzor & Nichol, 1965; Gilbert & Kellett, 1971).

It might be considered that the agreement between the theoretical curve and experimental points for bovine serum albumin does not provide a critical test of eqn. (8) in that the degree of penetration of the protein is relatively small. For myoglobin studied on Sephadex G-100, \( K_{sv} \), equals 0.54 (Laurent & Killander, 1964), and Gilbert & Kellett (1971) have reported the concentration dependence of the elution volume obtained under isoelectric conditions, where there is no contribution of the Donnan effect to \( A_2 \). Thus it seems reasonable to consider only the first term in eqn. (8), employing \( V_0^0/V_0^* = 0.41 \) (estimated), \( M_s = 17,800 \) and \( d(V_0/V_0^0)/d(\pi/RT) \) as before. This leads to an estimate of 1.7, the value reported by Gilbert & Kellett (1971). It is recognized that several of the experimental parameters used in these calculations are subject to some uncertainty, but it does appear that the assumptions leading to eqn. (8) are reasonably fulfilled and that the osmotic shrinkage term, \( d(V_0/V_0^0)/d(\pi/RT) \), is a function of the gel (Sephadex G-100 in this instance).

**General Discussion**

All the results in the present work support the findings made on the osmotic shrinkage of Sephadex beads (Edmond et al., 1968; Ogston & Silpananta, 1970; Ogston & Wells, 1970) and extend the concept by suggesting that the effect is amplified in column studies to the extent that concentration dependence of the elution volume may be observed even in a concentration range where non-ideality terms do not dominate in the expression for osmotic pressure. Moreover, it appears that even for a solute that penetrates the gel phase, this concentration dependence may be estimated solely on the basis of the parameters defining the degree of penetration and of the osmotic pressure of a solution of the solute.

In regard to the consequences of osmotic effects, it is necessary to consider experiments of different design. Experiments involving the determination of elution volumes for either molecular-weight estimations (Andrews, 1964) or evaluation of void volumes, require the quantities \( V_0^0 \) and \( V_0^* \), which may be obtained only by use of extremely dilute solutions or by extrapolation of results to infinite dilution. In the study of a single self-interacting solute by frontal analysis, procedures have been devised to allow for linear dependence of the elution volume of each species on total concentration (Ackers, 1967; Chiancone et al., 1968). Viewed in the light of the present findings, this seems to be a reasonable approach provided that the concentration range covered is such
that the terms containing virial coefficients contribute negligibly to the osmotic pressure. Similar reservations apply to the corrections discussed in the study of interactions between dissimilar molecules (Nichol et al., 1967; Gilbert & Kellett, 1971). On the other hand, the effects of concentration dependence in studies of ligand binding will probably present little if any difficulty, since the requirement that the macromolecule be confined to the mobile phase allows selection of a tightly cross-linked gel with minimal osmotic response (Edmond et al., 1968).

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References