Quantitative Interpretation of Concentration-Dependent Migration in Gel Chromatography of Reversibly Polymerizing Solutes

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A theoretical expression is derived for concentration dependence of elution volume in the gel chromatography of a non-interacting solute. Experimental results for bovine serum albumin on Sephadex G-100 are shown to be in good agreement with the predicted gel-chromatographic behaviour. The theoretical treatment of concentration dependence is extended to include a solute undergoing rapid reversible polymerization ($nA \rightleftharpoons C$). Computer simulation of gel-chromatographic data for monomer–dimer systems on Sephadex G-100 is used to illustrate the deficiencies of earlier empirical approaches, and also the potential of the present treatment, of allowing for non-chemical concentration dependence in gel chromatography of polymerizing solutes.

The ability of frontal gel chromatography to provide a weight-average elution volume ($\bar{V}_w$) for a polymerizing solute ($nA \rightleftharpoons C$) has been recognized for over a decade (Winzor & Scheraga, 1963, 1964), but quantitative interpretation of $\bar{V}_w$ in terms of the relevant equilibrium constant requires assignment of elution volumes to the individual oligomers A and C. To this end satisfactory procedures have been devised (Ackers & Thompson, 1965; Winzor *et al.*, 1967) for systems in which chemical equilibrium is effectively the only factor causing the variation of $\bar{V}_w$ with $\bar{c}_2$, the total solute concentration. However, the detection of slight concentration dependence of elution volume for non-interacting proteins (Winzor & Scheraga, 1963; Winzor & Nichol, 1965; Gilbert & Kellett, 1971) has meant that rigorous application of the above treatments is confined to a solute concentration range of 0–2 mg/ml, where the individual elution volumes V_A and V_C may be regarded as effectively constant.

In attempts to allow for non-chemical V – c dependence two procedures (Ackers, 1967; Chiancone *et al.*, 1968) have been devised in which the elution volumes V_A and V_C are both considered to be linear functions of $\bar{c}_2$. Both treatments are empirical and differ in the relative magnitudes assigned to k_A and k_C , the respective coefficients of concentration dependence for A and C.

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The aim of the present investigation has been to develop a more rigorous approach to this problem of allowing for non-chemical V – c dependence, now that its source has been traced to osmotic shrinkage of the beads comprising the gel-chromatography column (Edmond *et al.*, 1968; Nichol *et al.*, 1973). Theoretical relationships are derived which describe the concentration dependence of V_A and V_C in terms of experimentally determinable parameters. Computer simulation of gel-chromatographic data for monomer–dimer systems is used to illustrate the deficiencies of the earlier empirical approaches (Ackers, 1967; Chiancone *et al.*, 1968) and also the potential of a new procedure of allowing for non-chemical concentration dependence in gel chromatography of polymerizing solutes.

Theory

Concentration dependence of non-interacting solutes

Nichol *et al.* (1973) have established that the concentration dependence of elution volume of a non-interacting solute should not be expressed as a linear function of solute concentration. However, although their treatment suffices to establish the non-linearity, eqn. (8) of Nichol *et al.* (1973) does not describe correctly the V – c relationship because linear dependence was assumed for its derivation. We now

derive a more rigorous expression for the quantitative description of this concentration dependence.

From the definition of the partition coefficient K_{av} (Laurent & Killander, 1964) the elution volume V_e of a single non-interacting solute is given by:

$$V_e = V_b - V_s(1 - K_{av}) \quad (1a)$$

where V_b is the bed volume of a column and V_s the volume of stationary phase. Since V_b and K_{av} are both relatively insensitive to solute concentration (Edmond *et al.*, 1968; Ogston & Silpananta, 1970), the major cause of concentration dependence of V_e is the change of V_s that results from osmotic shrinkage of the gel beads (Edmond *et al.*, 1968; Nichol *et al.*, 1973). Indeed over the osmotic pressure range 0–0.5 mosm it is reasonable to consider that V_b and K_{av} are constants. Since V_s is simply the weight of gel matrix (w_s) divided by its concentration (c_s), eqn. (1a) may also be written as:

$$V_e = V_b - \frac{w_s}{c_s}(1 - K_{av}) \quad (1b)$$

Evaluation of concentration dependence of V_e thus requires an expression for c_s in terms of c_2 , the concentration of solute. An expression for this purpose is obtained by combining eqns. (3)–(5) of Edmond *et al.* (1968) with eqn. (6) of Ogston & Silpananta (1970): the result is eqn. (2):

$$(A_2)_s c_s^2 + (A_3)_s c_s^3 + A^* K_{av} c_s c_2 - [(1 - K_{av}) c_2 / M_2] - (A_2)_2 (1 - K_{av})^2 c_2^2 - P[(\bar{v}_s c_s)^{\frac{1}{3}} - \bar{v}_s c_s / 2] = 0 \quad (2a)$$

$$P = \frac{[(A_2)_s (c_s^0)^2 + (A_3)_s (c_s^0)^3]}{[(\bar{v}_s c_s^0)^{\frac{1}{3}} - \bar{v}_s c_s^0 / 2]} \quad (2b)$$

$(A_2)_s$ and $(A_3)_s$ denote the second and third virial coefficients of the gel matrix and $\bar{v}_s$ its partial specific volume: A^* is an interaction coefficient (Ogston & Silpananta, 1970). M_2 is the molecular weight of solute, the second virial coefficient of which is $(A_2)_2$: terms involving higher virial coefficients may also be added. P is a structure constant that may be evaluated from eqn. (2b) in which c_s^0 denotes the internal gel concentration in the absence of solute. Although it is possible in principle to use eqn. (2) to evaluate c_s for each value of c_2 , the presence of higher and fractional powers of c_s makes such an approach impractical.

We can, however, simplify the problem by noticing that the first, second and sixth terms of eqn. (2a) depend only on c_s and therefore have the same magnitudes for a given value of c_s , whether the solute penetrates the gel or not. It follows that the algebraically summed values of these terms for any specified c_s can be obtained by considering the variation of c_s with the osmotic pressure of a non-penetrat-

ing solute, $(\pi/RT)_e$. Further, since values of $(A_2)_s$, $(A_3)_s$ and c_s^0 are available for the various grades of Sephadex (Edmond *et al.*, 1968) eqns. (2a) and (2b) may be used to determine the dependence of c_s on $(\pi/RT)_e$. Such plots are shown in Fig. 1. Clearly linearity of bead shrinkage with respect to $(\pi/RT)_e$ may be assumed for Sephadex G-75 over the whole osmotic pressure range shown (0–0.4 mosm), the plot being satisfactorily described by:

$$\text{either} \quad c_s^0/c_s = 1 - D(\pi/RT)_e \quad (3a)$$

$$\text{or} \quad c_s/c_s^0 = 1 + D(\pi/RT)_e \quad (3b)$$

For Sephadex G-100 and G-200 the plots are also approximately linear provided that the extent of bead shrinkage is less than about 8%. This proviso could become a limiting factor in the application of eqn. (3) to gel chromatography on Sephadex G-200

Fig. 1. Dependence of Sephadex-bead shrinkage on the osmotic pressure of an excluded solute

The solid lines have been calculated on the basis of eqn. (2) and the following magnitudes of parameters: $(A_2)_s = 2.0 \times 10^{-4}$, $(A_3)_s = 1.8 \times 10^{-3}$ and $c_s^0 = 0.0971$ for Sephadex G-75; $(A_2)_s = 1.3 \times 10^{-4}$, $(A_3)_s = 1.3 \times 10^{-3}$ and $c_s^0 = 0.0764$ for Sephadex G-100; $(A_2)_s = 1.7 \times 10^{-4}$, $(A_3)_s = 7 \times 10^{-3}$ and $c_s^0 = 0.0365$ for Sephadex G-200. The broken lines are included to accentuate the curvilinearity of the plots for Sephadex G-100 and G-200.

because of the lower osmotic pressure at which the assumed linearity of bead shrinkage with $(\pi/RT)_e$ becomes an untenable approximation. It could also be noted in relation to the previous formulation (Nichol *et al.*, 1973) that

$$D = (1/V_s^0) dV_0/d(\pi/RT)_e$$

where $V_s^0 (= w_s/c_s^0)$ is the volume of stationary phase at infinite dilution of solute; thus the experimentally based assumption that $dV_0/d(\pi/RT)_e$ is constant (Nichol *et al.*, 1973) is consistent with eqn. (3).

Since we have replaced the first, second and sixth terms in eqn. (2a) by $(\pi/RT)_e$ in eqn. (3), it follows that $(\pi/RT)_e$ for a penetrating solute is given by eqn. (4):

$$(\pi/RT)_e = [(1 - K_{av.})c_2/M_2] + (A_2)_2(1 - K_{av.}^2)c_2^2 - A^*K_{av.}c_sc_2 \quad (4)$$

in which the variable c_s is conveniently eliminated by use of eqn. (3b) to obtain:

$$(\pi/RT)_e = \frac{(1 - K_{av.})c_2 + (A_2)_2M_2(1 - K_{av.}^2)c_2^2 - A^*M_2K_{av.}c_s^0c_2}{M_2(1 + A^*K_{av.}Dc_s^0c_2)} \quad (5)$$

Combination of eqns. (1b), (3a) and (5) then leads to eqn. (6) for the relationship between elution volume and solute concentration:

$$V_e = V_b - V_s^0(1 - K_{av.}) \left\{ 1 - \frac{D[(1 - K_{av.})c_2 + (A_2)_2M_2(1 - K_{av.}^2)c_2^2 - A^*M_2K_{av.}c_s^0c_2]}{M_2(1 + A^*K_{av.}Dc_s^0c_2)} \right\} \quad (6)$$

Alternatively, we may adopt a more conventional method of expressing concentration dependence, namely:

$$V_e = V_e^0[1 + f(c_2)] \quad (7a)$$

where V_e^0 is the limiting elution volume of solute as $c_2 \rightarrow 0$. Conversion of eqn. (6) into this format shows that

$$f(c_2) = \frac{V_s^0 D(1 - K_{av.})c_2}{V_e^0 M_2} \left[\frac{1 - K_{av.} + (A_2)_2 M_2(1 - K_{av.}^2)c_2 - A^* M_2 K_{av.} c_s^0}{1 + A^* K_{av.} D c_s^0 c_2} \right] \quad (7b)$$

experimental value of D that was much smaller than the 3.0×10^5 inferred from Fig. 1. The experimental value was influenced by measurements of void volume beyond the range of pressure for which c_s^0/c_s is linear in $(\pi/RT)_e$. Since the consequent curvilinearity of the $V_0 - (\pi/RT)_e$ plot was obscured by experimental error, the measured value of D was presumably not based on the limiting tangent as $(\pi/RT)_e \rightarrow 0$ and would accordingly be of smaller magnitude.

Concentration dependence of polymerizing solutes

Consider the gel chromatography of a solute comprising monomer and single higher polymer in rapidly established equilibrium ($nA \rightleftharpoons C$) governed by association constant X . Since the concentration scale g/ml is used in the osmotic pressure equations, it is convenient to express X in terms of the same scale, so that it has dimensions $\text{ml}^{n-1} \cdot \text{g}^{1-n}$. The total solute concentration $\bar{c}_2$ is then given by:

$$\bar{c}_2 = c_A + c_C = c_A + Xc_A^n \quad (8a)$$

in which the use of concentrations rather than activities implies self-cancellation of activity coefficient terms ($\gamma_C = \gamma_A^n$). Adams & Fujita (1963) have noted that this self-cancellation may be achieved by expressing thermodynamic non-ideality in the form:

$$\ln \gamma_A = BM_A \bar{c}_2 + \text{higher terms in } \bar{c}_2 \quad (8b)$$

$$\ln \gamma_C = nBM_A \bar{c}_2 + \text{higher terms in } \bar{c}_2 \quad (8c)$$

However, we do not agree with their interpretation of B as a concentration-independent parameter with identical values for A and C : instead we regard it as the mean virial coefficient of the polymerizing solute at any particular solute concentration under consideration.

The elution volumes of A and C are related to their respective partition coefficients K_A and K_C by eqns.

$$V_A = V_b - V_s(1 - K_A) \quad (9a)$$

$$V_C = V_b - V_s(1 - K_C) \quad (9b)$$

$$V_A = V_A^0[1 + f_A(\bar{c}_2)] \quad (9c)$$

$$V_C = V_C^0[1 + f_C(\bar{c}_2)] \quad (9d)$$

(9a) and (9b), and their dependences on total solute concentration by eqns. (9c) and (9d):

Fig. 2 represents a replot of the experimental results for bovine serum albumin that were shown in Fig. 4 of Nichol *et al.* (1973), together with the relationship predicted by eqn. (7): clearly there is good agreement. The previous discrepancy between experiment and theory (Nichol *et al.*, 1973) reflected (i) use of an incorrect expression for $f(c_2)$ because of the assumed constancy of $f(c_2)/c_2$, and (ii) use of an

Fig. 2. Comparison of the predicted and experimental concentration dependence of elution volume for bovine serum albumin on Sephadex G-100

The experimental results are replotted from Fig. 4 of Nichol *et al.* (1973) and refer to frontal experiments on columns (2cm x 50cm; ●) and (1.8cm x 20cm, ■) of Sephadex G-100 equilibrated with sodium phosphate buffer, pH 6.8, I 0.10M. The solid line represents the theoretical relationship predicted by eqn. (7) with the following magnitudes of parameters: $V_e^0/V_e^0 = 1.73$, $K_{av} = 0.15$, $(A_2)_2 = 1.4 \times 10^{-4}$, $A^* = 4.0 \times 10^{-4}$, $M_2 = 69000$ (all taken from Nichol *et al.*, 1973), $c_s^0 = 0.0764$ and $D = 3.0 \times 10^5$ (see Fig. 1).

Eqns. (9a–d) are the direct counterparts of eqns. (1a) and (7a) for a non-interacting solute, and similar treatment leads to the relationships

$$f_A(\bar{c}_2) = \frac{V_s^0 D(1 - K_A)}{V_A^0} (\pi/RT)_e \quad (10a)$$

$$f_C(\bar{c}_2) = \frac{V_s^0 D(1 - K_C)}{V_C^0} (\pi/RT)_e \quad (10b)$$

$$f_A(\bar{c}_2) = \frac{V_s^0(1 - K_A)}{V_A^0(1 - K_C)} f_C(\bar{c}_2) \quad (10c)$$

We thus require an expression for $(\pi/RT)_e$.

Consideration of thermodynamic non-ideality in relation to the osmotic pressure of an equilibrium mixture of A and C is complicated by the necessity to consider not only the virial coefficient for the non-ideality of A arising from the presence of a finite concentration of A, i.e. $(A_2)_{AA}$, and its counterpart $(A_2)_{CC}$, but also a cross-term coefficient $(A_2)_{AC}$ to reflect non-ideality of A due to the presence of C and vice versa. In general, amalgamation of these terms into a non-ideality correction of the form $(\bar{A}_2)_2 \bar{c}_2^2$ is not possible, since the relative contributions of the

three virial terms change with $\bar{c}_2$ owing to variation in the proportions of A and C. The expression for the second virial coefficient of a single non-interacting solute (Tanford, 1961) is a complex function of molecular volume and charge effects. For a polymerizing solute the charge contribution is the same for A and C provided that charge is conserved on polymerization, and hence a virial term of the simpler form is justified for a highly charged solute, particularly at low ionic strength, conditions under which the molecular co-volume terms contribute negligibly to the non-ideality: but under such conditions, the Donnan redistribution of buffer ions complicates the definition of the ionic environment (Nichol *et al.*, 1969). Further, proteins such as β -lactoglobulin (Townend & Timasheff, 1956, 1960; Townend *et al.*, 1960) and haemoglobin (Ackers & Thompson, 1965; Chiancone *et al.*, 1968) reversibly polymerize under conditions of moderate ionic strength and relatively low net charge, under which conditions molecular co-volume contributions may be expected to dominate the magnitudes of the virial coefficients. For example, in buffers of ionic strength 0.1 M the charge terms would contribute less than 10% to the virial coefficients of haemoglobin over the pH range 5.5–9.5. We shall therefore concentrate on a system with A and C as uncharged spherical molecules, in the belief that this extreme situation will often be encountered, or approximated to, in practice.

Flory (1953) has shown that the three virial coefficients $(A_2)_{AA}$, $(A_2)_{CC}$ and $(A_2)_{AC}$ may be regarded as expressing the co-volumes that are mutually excluded by neighbouring molecules. By adopting the simplified approach (Edmond & Ogston, 1968) that the spheres are impenetrable, we may then write the external osmotic pressure as:

$$\begin{aligned} (\pi/RT)_{\text{ext.}} &= \frac{c_A}{M_A} + \frac{c_C}{nM_A} + (A_2)_{AA}c_A^2 + (A_2)_{CC}c_C^2 + (A_2)_{AC}c_Ac_C \\ &\quad (11) \end{aligned}$$

M_A denotes the monomeric molecular weight, and the three virial coefficients are related to the molecular volumes of A and C by eqns. (12a–12c).

$$(A_2)_{AA} = \frac{2\pi N}{3M_A^2} (2r_A)^3 = \frac{4v_e}{M_A} \quad (12a)$$

$$(A_2)_{CC} = \frac{2\pi N}{3n^2M_A^2} (2r_C)^3 = \frac{4v_e}{nM_A} \quad (12b)$$

$$(A_2)_{AC} = \frac{4\pi N}{3nM_A^2} (r_A + r_C)^3 = (1 + n^3)^3 \frac{v_e}{nM_A} \quad (12c)$$

N is Avogadro's number, r_A and r_C represent the effective radii of A and C respectively, and v_e denotes the corresponding effective specific volume, assumed

identical for A and C. In the event that charge effects are also important each virial coefficient is increased by addition of the appropriate charge term to the co-volume contribution (Tanford, 1961).

The solute contribution to the internal osmotic pressure therefore requires description by three virial coefficients and two-gel interaction (A^*) terms. By analogy with eqn. (5) for a non-interacting solute we may write the following relationship for $(\pi/RT)_c$:

$$(\pi/RT)_c = [(1 - K_A)c_A/M_A] + [(1 - K_C)c_C/nM_A] + (A_2)_{AA}(1 - K_A^2)c_A^2 + (A_2)_{CC}(1 - K_C^2)c_C^2 + (A_2)_{AC}(1 - K_A K_C)c_A c_C - A_A^* K_A c_A c_C - A_C^* K_C c_C c_A \quad (13)$$

In eqn. (13) A_A^* and A_C^* are gel-interaction coefficients (Ogston & Silpananta, 1970) for A and C respectively, which may be evaluated from the relationships:

$$A_A^* M_A = \frac{(r_s + r_A)^2}{r_s^2} \bar{v}_s \quad (14a)$$

$$n A_C^* M_A = \frac{(r_s + r_C)^2}{r_s^2} \bar{v}_s \quad (14b)$$

where r_s , the radius of a matrix fibre, may be taken as 0.6 nm (6 Å) for Sephadex (Siegel & Monty, 1966; Ogston *et al.*, 1973). Combination of eqns. (3b), (10b), (12) and (13) yields the following expression for $f_C(\bar{c}_2)$:

$$f_C(\bar{c}_2) = \frac{V_s^0 D(1 - K_C)}{V_C^0 n M_A (1 + A_A^* K_A D c_s^0 c_A + A_C^* K_C D c_s^0 c_C)} \left\{ n c_A (1 - K_A - A_A^* M_A K_A c_s^0) + c_C (1 - K_C - n A_C^* M_A K_C c_s^0) + v_e [4n(1 - K_A^2)c_A^2 + 4(1 - K_C^2)c_C^2 + (1 + n^2)^3(1 - K_A K_C)c_A c_C] \right\} \quad (15)$$

Explicit expressions for $f_C(\bar{c}_2)$ and hence $f_A(\bar{c}_2)$ via eqn. (10c) permit the description of V_A , V_C and the weight-average elution volume $\bar{V}_w$ as a function of $\bar{c}_2$ (via eqns. 9c, 9d and 16 respectively):

$$\bar{v}_w = \frac{(c_A V_A + c_C V_C)}{\bar{c}_2} \quad (16)$$

Discussion

The first point to emerge from this theoretical treatment of non-chemical concentration dependence in the gel chromatography of reversibly polymerizing solutes is the existence of a simple relationship between the two concentration-dependence functions $f_A(\bar{c}_2)$ and $f_C(\bar{c}_2)$ (eqn. 10c). Both of the empirical procedures prove to be correct qualitatively but imprecise quantitatively in this respect, since Ackers (1967) assumed that $f_A(\bar{c}_2) = (V_C^0/V_A^0)f_C(\bar{c}_2)$, and Chiancone *et al.* (1968) that $f_A(\bar{c}_2) = f_C(\bar{c}_2)$. However, a further theoretical deficiency of the earlier approaches is the assumption that $f_A(\bar{c}_2) = k_A \bar{c}_2$ and

$f_C(\bar{c}_2) = k_C \bar{c}_2$, whereas the coefficients k_A and k_C are not constants but rather are functions of monomer and polymer concentrations and thus vary with $\bar{c}_2$ (eqn. 15). The possible extent of these variations with $\bar{c}_2$ is examined by considering the theoretical gel-chromatographic behaviour of a solute undergoing reversible dimerization.

For purposes of illustration we have calculated the theoretical gel-chromatographic behaviour of haemoglobin, a solute that is known to exist in a state of reversible dimer-tetramer equilibrium at its isoelectric point (Ackers & Thompson, 1965; Chiancone *et al.*, 1968). However, to explore further the possible consequences of non-chemical concentration dependence we have considered values of the association constant X other than the reported magnitude of 20000 ml/g (Chiancone *et al.*, 1968), selecting the additional values 2000 and 200 ml/g. A 50 ml column of Sephadex G-100 was considered and partition coefficients assigned by means of a K_{av} versus $\log M$ calibration plot constructed from Table 1 of Laurent & Killander (1964). On the basis of a molecular weight of 64500 for tetrameric haemoglobin (species C) estimates of 0.36 and 0.18 were obtained for K_A and K_C respectively. These partition coefficients taken in conjunction with a void volume (V^0) of 15.0 ml lead to values of 27.6 ml for V_A^0 and 21.3 ml for V_C^0 . The Sephadex G-100 was considered to be the batch characterized by Edmond *et al.* (1968),

and accordingly values of 0.0764 and 3.0×10^5 were used for c_s^0 and D (Fig. 1) respectively. The method by which the remaining three inter-related parameters v_e , A_A^* and A_C^* were assigned magnitudes requires elaboration, since an experimenter must resort to similar procedures.

In eqn. (12) v_e has been defined as the effective specific volume corresponding to the effective radii r_A and r_C of the two oligomers, and hence use of the partial specific volume as an estimate of v_e would imply that monomer and polymer were both strictly spherical and that neither was hydrated. However, at least two procedures are available for providing values of v_e , r_A and r_C that are more in keeping with their operative functions of defining thermodynamic non-ideality. First, provided that the solute may also be studied under conditions where either A or C is essentially the sole form, measurement of the second virial coefficient under those conditions yields estimates of v_e and the appropriate effective radius via eqn. (12a) or (12b). This approach is illus-

Fig. 3. Evaluation of the second virial coefficient of tetrameric haemoglobin

The experimental points have been calculated from the results reported in Table 1 of Adair (1928), which refers to the osmotic pressure of ovine haemoglobin in phosphate buffer, pH 7.8, 1.0.29M. The solid line denotes our estimate of the limiting tangent as $\bar{c}_2 \rightarrow 0$.

trated in Fig. 3, which presents the osmotic-pressure results of Adair (1928) for haemoglobin at neutral pH. Although haemoglobin dissociates under such conditions, the equilibrium is displaced essentially completely in the direction of tetramer over the concentration range (7–350mg/ml) used by Adair (1928). The slope of the limiting tangent as $\pi/\bar{c}_2 \rightarrow 0$ yields values of 4.8 for the product $(A_2)_{CC}M_C$, 1.20 for v_e and 3.13 nm for r_C . Geometrical considerations ($r_C = n^{1/3}r_A$) then give 2.48 nm for the effective radius of dimeric haemoglobin (r_A). The magnitudes of A_A and A_C^* are then calculated to be 4.90×10^{-4} and 3.60×10^{-4} respectively from eqn. (14). Alternatively, we could adopt the commonly made assumption that the equivalent spherical Stokes radius of a molecule provides a reliable estimate of the equivalent co-volume radius. For haemoglobin, the Stokes radius is 3.13 nm (Table 1 of Laurent & Killander, 1964), a value agreeing exactly with the co-volume estimate quoted above. We should note, however, that the two values are based on different physical models and properties, and may not always be identical.

Simulated gel-chromatography results are summarized in Figs. 4 and 5. Fig. 4 shows the dependence

Fig. 4. Effect of total solute concentration on the non-chemical-concentration-dependence coefficient k of monomeric (A) and dimeric (C) species that coexist in rapidly established equilibrium

Values of $k_A = f_A(\bar{c}_2)/\bar{c}_2$ and $k_C = f_C(\bar{c}_2)/\bar{c}_2$ were calculated for the various values of $\bar{c}_2$ on the basis of eqns. (8a), (10) and (15) with the following magnitudes of parameters: $V_s^0 = 35.0$ ml, $V_A^0 = 27.6$ ml, $V_C^0 = 21.3$ ml, $K_A = 0.36$, $K_C = 0.18$, $M_A = 32,250$, $c_s^0 = 0.0764$, $D = 3.0 \times 10^5$, $A_A^* = 4.90 \times 10^{-4}$, $A_C^* = 3.60 \times 10^{-4}$, $v_e = 1.2$. ●, $X = 20000$ ml/g; ■, $X = 2000$ ml/g; ▲, $X = 200$ ml/g; ----, $X = 0$; ····, $X = \infty$. For further details see the text.

of the two frequently used concentration-dependence parameters $k_A = f_A(\bar{c}_2)/\bar{c}_2$ and $k_C = f_C(\bar{c}_2)/\bar{c}_2$ on total solute concentration $\bar{c}_2$, and Fig. 5 the corresponding variation in $\bar{V}_w$, the weight-average elution volume. Three points require comment in relation to Figs. 4 and 5. First, consideration of the coefficients k_A and k_C as constants is clearly undesirable even as an approximation. Secondly, their magnitudes may differ markedly from those of non-interacting solutes of comparable sizes ($X = 0$ and $X = \infty$ for A and C respectively). Indeed, these hypothetical concentration-dependence coefficients of pure A and pure C are the respective limits of k_A as $\bar{c}_2 \rightarrow 0$ and k_C as

Fig. 5. Dependence of the weight-average elution volume for monomer-dimer systems on total solute concentration

Points in Fig. 5 have the same significance as those in Fig. 4, and were calculated from the same expressions plus eqn. (16). The broken lines denote the dependence of V_A (—) and V_C (---) on total solute concentration.

$\bar{c}_2 \rightarrow \infty$. Thirdly, comparison of Figs. 4 and 5 shows that the largest variation in k_A and k_C occurs under conditions where $\bar{V}_w$ exhibits the greatest concentration dependence: but much of the variation occurs at low concentrations where the values of V_A and V_C are relatively insensitive to the magnitudes of the respective concentration-dependence coefficients.

In relation to the possible analysis of $(\bar{V}_w, \bar{c}_2)$ data in terms of the equilibrium constant, we note that Chiancone *et al.* (1968) have used non-linear curve-fitting of their haemoglobin results to solve for the parameters V_A^0, V_C^0, X and k_A (assumed constant and identical with k_C). However, the availability of explicit expressions for $f_A(\bar{c}_2)$ and $f_C(\bar{c}_2)$ (eqns. 10c and 15) simplifies the analysis by decreasing from four to three the number of parameters to be evaluated. Moreover, the fact that no assumptions need then be made about the constancy of, and interrelation between, k_A and k_C means that such curve-fitting should provide more reliable estimates of the required parameters (V_A^0, V_C^0 and X ; or K_A, K_C and X).

Table 1 summarizes the results of a non-linear least-squares regression analysis of the $(\bar{V}_w, \bar{c}_2)$ data shown in Fig. 5. The algorithm of Marquardt (1963)

Table 1. Results of non-linear-regression analysis of the simulated gel-chromatographic data shown in Fig. 5

Numbers in parentheses denote the allowed variation ($\pm$) in the magnitudes of the derived estimates (see the text). X value is divided by the appropriate power of ten. The quantity k is equivalent to $-g$ in the formulation of Chiancone *et al.* (1968).

Input	Derived parameters			
	By present procedure		By procedure of Chiancone <i>et al.</i> (1968)	
	X (ml/g)	K_A	K_C	k (ml/g)
2×10^4	Error in $\bar{V}_w$ (ml)			
	± 0.02			
	± 0.05			
2×10^3	X (ml/g)	K_A	K_C	k (ml/g)
	2.07 (0.30)	0.361 (0.006)	0.180 (0.001)	3.11 (0.50)
	1.92 (0.63)	0.358 (0.015)	0.180 (0.003)	2.62 (0.93)
2×10^2	X (ml/g)	K_A	K_C	k (ml/g)
	1.88 (1.30)	0.358 (0.030)	0.180 (0.006)	3.63 (2.75)
	1.98 (0.17)	0.360 (0.002)	0.180 (0.002)	2.99 (0.77)
2×10^1	X (ml/g)	K_A	K_C	k (ml/g)
	1.88 (0.35)	0.359 (0.004)	0.178 (0.005)	2.22 (1.38)
	2.11 (0.86)	0.361 (0.009)	0.181 (0.011)	3.00 (4.11)*
2×10^0	X (ml/g)	K_A	K_C	k (ml/g)
	1.92 (0.26)	0.360 (0.001)	0.177 (0.010)	2.40 (2.40)*
	1.65 (0.44)	0.359 (0.002)	0.164 (0.023)	0.77 (1.11)*
2×10^{-1}	X (ml/g)	K_A	K_C	k (ml/g)
	2.15 (1.40)	0.360 (0.005)	0.185 (0.047)	-0.09 (3.11)*

* Analysis that has failed to establish the existence of non-chemical concentration dependence.

as modified by Reich *et al.* (1972) was used in conjunction with a UNIVAC 1108 digital computer. Experimental error was simulated by addition to the $\bar{V}_w$ values of a vector of numbers selected randomly from a normalized population with zero mean and the desired standard deviation. Table 1 reports both the value of each parameter that is obtained by regression analysis and also the maximum extent of variation in its magnitude that is permissible within a 95% confidence limit for the sum of squares of residuals, calculated with the assumption that the minimum is approximately quadratic (Box, 1960; Reich *et al.*, 1972): this uncertainty in each estimated value is recorded in parentheses. The data were analysed on the basis of eqns. (8), (9), (10), (15) and (16), and also on the basis of eqns. (8), (9) and (16) plus the assumption that $k_A = k_C = k$ was an additional constant to be evaluated from the analysis: the k values reported in Table 1 are equivalent to the quantity, $-g$, in the formulation of Chiancone *et al.* (1968), who express concentration dependence as $V_e = V_e^0(1 - gc_2)$.

Several points in relation to Table 1 should be noted. First, either procedure is satisfactory in the sense that the values of X , K_A and K_C used to simulate the data are encompassed within the ranges of allowed magnitudes of the derived estimates. The superiority of the present procedure is thus reflected in the smaller uncertainty associated with a derived estimate rather than in its absolute magnitude. These conclusions also apply when the data are analysed on the basis of the Ackers (1967) assumption that $k_A = (V_e^0/V_A^0)k_C$. Secondly, although the procedure of Chiancone *et al.* (1968) yields a finite value for the concentration-dependence parameter k , in many cases (asterisked) the allowed variation in its magnitude is such that the analysis fails to establish the existence of non-chemical concentration dependence. Further, the final entry in Table 1 shows that a mean value of k may be obtained which represents a physically unreal solution ($k < 0$). A potential danger that stems from the use of the Chiancone *et al.* (1968) analysis is thus the possible misinterpretation of such a result as non-compliance of the experimental system with the simple monomer-single-higher-polymer model. Thirdly, from the experimental viewpoint it is of interest to compare the precision of $\bar{V}_w$ with the consequent accuracy of the derived X . Thus measurement of elution volume to the nearest 0.1 ml (a 0.5% error) has led to an equilibrium constant with an uncertainty that is as much as 70% of its estimated value, even with use of the present analysis. Finally, with sufficiently small values of X the input information would have become unretrievable except for the unrealistic situation devoid of experimental error. However, this should not be construed as an indication of the

fallibility of the analysis procedure but as evidence of the need for an experimenter to obtain results in which the magnitude of the variation in $\bar{V}_w$ is orders of magnitude greater than the random experimental error inherent in each determination of elution volume.

In summary, the present treatment of non-chemical concentration dependence of elution volume in the gel chromatography of a polymerizing solute has yielded explicit expressions for the description of the weight-average elution volume as a function of total solute concentration. Analysis of simulated data for monomer-dimer systems has shown the feasibility of using these expressions for evaluation of equilibrium constants from experimental results provided that the magnitude of the overall change in $\bar{V}_w$ is large compared with the random error associated with each measurement of elution volume.

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