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CONCENTRATION-DEPENDENT MIGRATION IN GEL CHROMATOGRAPHY OF REVERSIBLY POLYMERIZING SOLUTES: THE OSMOTIC BEHAVIOR OF POLYACRYLAMIDE GELS

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The osmotic behavior of gels derived from polyacrylamide has been measured in order to establish the validity of assuming linear concentration dependence of bead shrinkage upon osmotic pressure in quantitative studies of reversibly associating solutes by gel chromatography on Bio-Gel. The concentration dependence of elution volume predicted with due allowance for this osmotic shrinkage yields theoretical curves that provide good descriptions of experimental results obtained in chromatography of α-chymotrypsin on Bio-Gel P-30 equilibrated with acetate-chloride buffer, pH 3.86, I 0.20; and also of bacterial α-amylase on Bio-Gel P-150 equilibrated with 0.10 M NaCl-0.015 M calcium acetate–0.010 M EDTA, pH 7.0. For the former system the osmotic effect has negligible consequences on the quantitative interpretation of the results. With the α-amylase system, however, consideration of the osmotic effect is necessary to obtain even a qualitative indication of the existence of the monomer–dimer equilibrium

1. Introduction

In quantitative studies of a reversibly polymerizing solute ($nA \rightleftharpoons C$) by frontal chromatography [1] the elution volumes of the individual species, V_A and V_C, must be assigned values before the measured weight-average elution volume, $\overline{V}_w$, can be interpreted in terms of the relevant association equilibrium constant. Thereafter, the method is readily applied to solutes such as hemoglobin [2], aryl sulfatase [3], pig liver carboxylesterase [4], and α-chymotrypsin at pH 4 [5,6], for which the major changes in the degree of association occur in the concentration range 0–1 g/liter, and for which the variation of $\bar{V}_w$ with $\bar{c}$, the total solute concentration, may thus be considered to reflect solely the effect of the chemical equilibrium. However, osmotic shrinkage of the gel beads [7,8] introduces slight concentration dependence of elution volume for non-interacting solutes [1,9,10], and hence rigorous quantitative interpretation of $(\bar{V}_w, \bar{c})$ data also requires specification of the dependences of V_A and V_C upon $\bar{c}$.

The first procedures devised to allow for this non-chemical dependence of elution volume upon concentration [11,12] were based on an empirical assumption that V_A and V_C were linear functions of $\bar{c}$. A subsequent, more rigorous approach [13] entailed the use of a theoretical expression for the concentration dependence of elution volume that results from osmotic shrinkage of the gel phase. Its application to results obtained with α-chymotrypsin [6] and α-amylase on Bio-Gel is the aim of the present investigation; but first it has been necessary to establish the extent of osmotic shrinkage that occurs in gels derived from polyacrylamide, since previous quantitative studies of osmotic effects [7,14] have been confined to dextran (Sephadex) gels. Part of the present investigation has appeared in thesis form [15].

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2. Experimental

2.1. Materials

Sephadex G-50 (batches 1662, 4861, 5041 and 6701) was obtained from Pharmacia, Uppsala, Sweden, and Bio-Gel P-30 (batch 135772) and P-150 (batch 135112) from Bio-Rad Laboratories, Richmond, California, U.S.A. Prior to use the gels were washed copiously with distilled water to remove any water-soluble impurities, and then washed with ethanol. Finally they were dried in air at 50°C [7].

Blue dextran and dextran 2000 (batch 6038) were also obtained from Pharmacia. These materials were dissolved directly in 0.2 I phosphate buffer (0.05 M NaH_2PO_4–0.05 M Na_2HPO_4), pH 6.8, and the solutions dialysed exhaustively against more of the same buffer.

Bacterial α-amylase (type IIA, four times crystallized and freeze-dried) was obtained from Sigma Chemical Co., St. Louis, Mo., and used without further purification. The enzyme was dissolved directly in 0.10 M NaCl–0.015 M calcium acetate–0.010 M EDTA, pH 7.0, and dialysed against more of the same medium.

2.2. Estimation of concentrations

Concentrations of dextran solutions were determined refractometrically at 546 nm on the basis of a specific refractive increment of 0.151 ml/g [7,16–18]. Blue dextran was also estimated spectrophotometrically at 625 nm. Concentrations of α-amylase were based on absorbance measurements at 280 nm and an extinction coefficient ($A^{1\%}_{1\,cm}$) of 22.3 [19].

2.3. Partial specific volumes of Sephadex and Bio-Gel

The partial specific volumes ($\bar{v}_s$) of Sephadex and Bio-Gel were measured by the standard pycnometric procedure in a 50-ml S.G. bottle with reagent grade ethanol as the liquid phase. The value of 0.61 ± 0.01 ml/g for Sephadex G-50 compares favorably with that of 0.611 ml/g for the parent dextran [20]. From measurements with Bio-Gel P-150 and P-30, a value of 0.72 ± 0.01 ml/g was obtained for $\bar{v}_s$ of the cross-linked polyacrylamide gels: a value of 0.7 ml/g was used by Ogston and coworkers [21].

2.4. Measurement of inner volume

Dilution experiments of similar design to those described by Ogston and Silpananta [22] were performed at 20°C in test tubes fitted with Quickfit stoppers. To each tube containing a known weight w_s (400–800 mg) of Sephadex G-50 or the relevant grade of Bio-Gel were added weighed amounts of 0.2 I phosphate buffer, pH 6.8, and the same buffer containing dextran 2000 (70 g/liter), the total volume of the liquid added being approximately 9 ml. After addition of approximately 3 ml (V_a) of the phosphate buffer containing blue dextran (0.5 g/liter) the tubes were reweighed to give a more precise estimate of V_a and then left to equilibrate for 20 h. A sample of the mobile phase (supernatant) from each tube was then obtained by filtration and its absorbance at 625 nm (A_m) measured. Conversion to volumes, of the weights of solutions added, was effected by means of the expression $\rho = \rho_b + (1 - \bar{v}\rho_b)c$ where ρ_b denotes the density of the phosphate buffer (1.008_2 g/ml), c the concentration (g/ml) of dextran (dextran 2000 plus blue dextran) and $\bar{v}$ the partial specific volume of the dextran, which was taken as 0.611 ml/g [20]. The total volume of the slurry (V_t) was then obtained by adding a term ($\bar{v}_s w_s$) for the volume of the gel matrix. Inner volumes (V_i), expressed on a unit weight basis, were obtained from the expression $V_i = [V_t - (V_a A_a / A_m)]/w_s$, where A_a denotes the absorbance at 625 nm of the blue dextran solution added to the gel system.

2.5. Osmotic pressure measurements

A Wescan CSMI recording membrane osmometer was used to measure the osmotic pressures of solutions of dextran 2000 (0–60 g/liter) in 0.2 I phosphate buffer, pH 6.8. The aim of these experiments, conducted at 25°C, was to provide a direct experimental estimate of the osmotic pressures pertinent to the measured inner volumes.

Osmotic pressures appropriate to the inner volume measurements were obtained by substituting the relevant dextran concentration c (g/ml) into the expression $(\Pi/RT) = (c/M_n) + A_2 c^2$, where Π denotes the osmotic pressure at temperature T, M_n the number-average molecular weight, and A_2 the conventional osmotic second virial coefficient. The experimental plot for evaluating the last two parameters is shown in

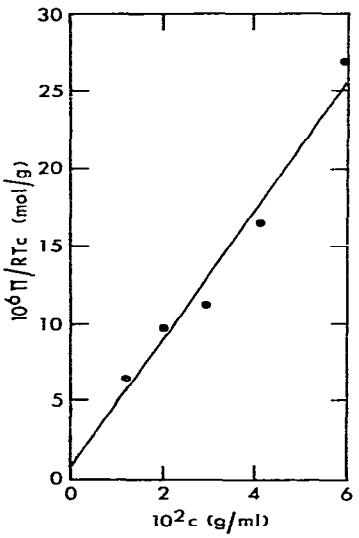

Fig. 1. Dependence of the osmotic pressure of dextran 2000 solutions upon solute concentration. Osmotic pressures of solutions of dextran 2000 dialysed exhaustively against 0.2 1 phosphate buffer, pH 6.8, were measured at 25°C.

fig. 1, the linearity of which justifies the neglect of higher terms in the expression for (Π/RT) over the concentration range of this experiment. The straight line is the theoretical relationship for a system with $M_n = 1.2 \times 10^6$ and $A_2 = 4.2 \times 10^{-4}$ mol · cm^3 · g^{-2}. Because of the uncertainties inherent in osmotic pressure measurements on such large molecules, this relationship does not necessarily provide an accurate characterization of the dextran sample in terms of its molecular weight and virial coefficient; but it *does* provide a reasonable, though possibly only empirical, description of the experimental results, and hence a means of evaluating the osmotic pressure, Π, pertinent to any given value of dextran concentration, c, in the concentration range examined.

2.6. Gel chromatography of α-amylase on Bio-Gel P-150

Solutions of bacterial α-amylase (0–2 g/liter) in 0.10 M NaCl–0.015 M calcium acetate–0.010 M EDTA, pH 7.0, were subjected to frontal gel chromatography [1] on a column (2.3 × 26 cm) of Bio-Gel

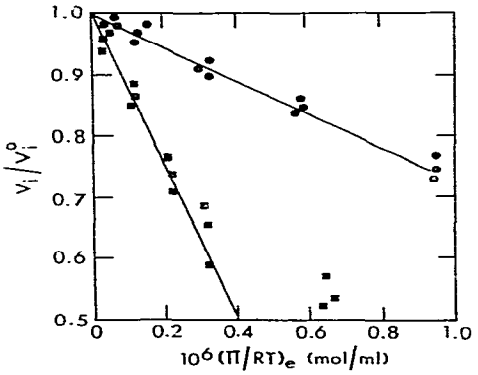

Fig. 2. Effect of osmotic pressure on the inner volume of crosslinked polyacrylamide gels. Inner volumes, V_i, were obtained from dilution experiments with dextran 2000 (0–60 g/liter) as excluded solute, the corresponding values of the external osmotic pressure, Π_e, being derived from fig. 1. Symbols denote experimental points for Bio-Gel P-30 (•) and P-150 (■). The straight lines represent the relationships used to evaluate the parameter D in eq. (2) for the application of eq. (1) to gel chromatographic data on polymerizing enzyme systems.

P-150 equilibrated with the same medium. The column effluent, maintained at a flow-rate of 15 ml/h, was collected in 1-ml fractions, the volumes of which were determined by weight, and analysed spectrophotometrically at 280 nm. The weight-average elution volume, $\bar{V}_w$, was obtained from the centroid [23] of the advancing elution profile.

3. Results and discussion

3.1. Measurements of osmotic shrinkage

Results of experiments on the swelling of Bio-Gel P-30 and P-150 are presented in fig. 2, where the inner volume, V_i, has been expressed relative to its value, V_i^0, in the absence of any external osmotic pressure: inner volumes (V_i^0) of 7.3 ml/g and 10.2 ml/g were obtained for Bio-Gel P-30 and P-150 respectively. Several points should be noted in connection with these results. (i) Dextran 2000 has been used as the source of external osmotic pressure in preference to dextran 500, since Ogston and coworkers [7,14] have commented upon problems resulting from partial penetration of Sephadex and Bio-Gel by dextran mole-

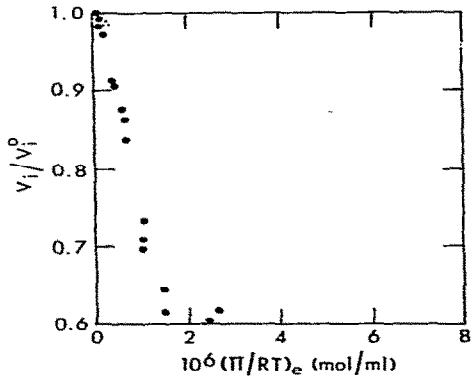

Fig. 3. Variation of the inner volume of Sephadex G-50 with osmotic pressure. Solid symbols (•) denote the present results, obtained as in fig. 2, and open symbols refer to results obtained from diameter measurements on individual beads: ○, data from ref. [7]; △, result reported by Ogston and Wells [14].

cules comprising the low end of the chain length distribution present in dextran 500. (ii) The present results provide a direct measure of any changes in inner volume V_i, whereas measurements of changes in bead diameter [7,14] reflect changes in $V_i^{1/3}$. However, the consequently greater sensitivity of the present type of measurements is offset by their greater inherent imprecision. The scatter of results is thus larger than in corresponding plots based on diameter measurements on individual gel beads [7]. (iii) In conformity with the results obtained for Sephadex [7], the extent of gel shrinkage is smaller for the more highly crosslinked polyacrylamide sample (P-30) studied.

The extents of shrinkage observed for these two grades of Bio-Gel are much greater than those reported [7] for comparable grades of Sephadex. However, although this observation seemingly implies that polyacrylamide gels are more susceptible to osmotic shrinkage than their dextran counterparts, a parallel study of Sephadex G-50 yielded results that differed markedly from those reported previously [7]. The magnitude of the discrepancy is emphasized in fig. 3, which compares the present results for G-50 (•) with data (○) contained in fig. 3 of ref. [7]; a single observation (△) by Ogston and Wells [14] is also included. This difference between results seems too large to reflect only the experimental inaccuracy inherent in the present method of study. Accordingly it must be attributed to non-identity of the osmotic characteristics of the Sephadex G-50 samples studied. In this connection it is noted that the present batch of Sephadex G-50 (batch 5041) is characterized by an inner volume (V_i^0) that is 50% larger than that of the sample used in the earlier studies [7,14]. The greater susceptibility of the present batch of Sephadex G-50 to osmotic shrinkage is thus consistent with it forming a more distended gel.

Measurements of V_i^0 for four batches of Sephadex G-50 yielded values of 7.4 ml/g batch (4861), 7.5 ml/g (batch 6701), 7.8 ml/g (batch 5041), and 8.4 ml/g (batch 1662), which emphasizes the extent of variation that exists between the swelling properties of different preparations of the same grade of Sephadex. Such differences are not confined to G-50, a factor evident from the reports of 13.1 ml/g [7] and 11.1 ml/g [24] for the inner volume of Sephadex G-100. Quantitative measurements of the osmotic properties of gels should thus be regarded as characteristics of particular batches rather than of particular grades of Sephadex or Bio-Gel.

3.2. Prediction of concentration-dependence in chromatography on Bio-Gel

As mentioned in the Introduction, the aim of this study is to obtain a quantitative theoretical description of the concentration-dependence of elution volume in chromatography of two reversibly dimerizing solutes, α-chymotrypsin and α-amylase, on Bio-Gel. To this end expressions have been derived [13] for predicting effects of concentration-dependent migration in gel chromatography of a solute undergoing reversible polymerization ($nA \rightleftharpoons C$); they permit the calculation of values of the species elution volumes, V_A and V_C, corresponding to the total solute concentrations $\bar{c}$, whereupon the weight-average elution volume $\bar{V}_w$ may be determined. The relevant equations are,

$$V_A = V_A^0(1 + f_A(\bar{c})), \quad V_C = V_C^0(1 + f_C(\bar{c})), \qquad (1a)$$

$$f_C(\bar{c}) = \frac{V_s^0 D(1 - K_C)}{V_C^0 n M_A (1 + A_A^* K_A D c_s^0 c_A + A_C^* K_C D c_s^0 c_C}$$

$$\times \{nc_A(1 - K_A - A_A^* M_A K_A c_s^0)$$

$$+ c_C(1 - K_C - nA_C^* M_A K_C c_s^0)$$

$$+ v_e[4n(1 - K_A^2)c_A^2 + 4(1 - K_C^2)c_C^2$$

$$+ (1 + n^{1/3})^3(1 - K_A K_C)c_A c_C]\}. \quad (1b)$$

$$f_A(\bar{c}) = f_C(\bar{c})V_C^0(1 - K_A)/V_A^0(1 - K_C). \quad (1c)$$

In these expressions V_A^0 and V_C^0 denote the elution volumes of monomer and polymer, respectively, in the absence of any external osmotic pressure. K_A and K_C are the partition coefficients [25] corresponding to elution volumes V_A^0 and V_C^0 from a column with stationary phase volume V_S^0, its volume under conditions (zero osmotic pressure) where the internal gel concentration is c_s^0. M_A denotes the molecular weight of monomer, and c_A, c_C the concentrations (g/ml) of monomer and polymer, respectively, which are governed by an association equilibrium constant X' (liter^{n-1} · g^{1-n}). Four parameters, namely D, v_e, A_A^*, and A_C^*, remain to be defined and considered.

Inherent in eq. (1b) is the assumption that the dependence of c_s^0/c_s (or V_i/V_i^0) upon external osmotic pressure can be expressed by a linear relationship of the form,

$$(c_s^0/c_s) = 1 - D(\Pi/RT)_c. \quad (2)$$

Reference to fig. 2 shows that there is indeed a range of $(\Pi/RT)_c$ over which the observed osmotic shrinkage of each of the polyacrylamide gels can be described in these terms. There is thus no great difficulty entailed in evaluating D as the slope of the limiting tangent (as $\Pi/RT \to 0$) to the appropriate plot in fig. 2. Furthermore, the range of $(\Pi/RT)_c$ for which eq. (2) describes the shrinkage is such that this assumption is likely to be a reasonable approximation for many experimental systems.

The next parameter to be considered is v_e, the effective specific volume (ml/g) of the hydrated solute [13]. In the following applications of the theory to gel chromatography of α-chymotrypsin and α-amylase it is assumed that a reliable estimate of v_e may be obtained from the Stokes radius r_X of either monomer or polymer and the relationship,

$$v_e = 4\pi N r_X^3/3M_X, \quad (3)$$

in which N is Avogadro's number and M_X the molecular weight of the appropriate species.

The quantities A_A^* and A_C^* are gel interaction coefficients [22] for A and C, respectively, and require evaluation from the expressions,

$$A_A^* = [(r_s + r_A)/r_s]^2(\bar{v}_s/M_A),$$

$$A_C^* = [(r_s + r_C)/r_s]^2(\bar{v}_s/nM_A). \quad (4)$$

$\bar{v}_s$ is the partial specific volume of the gel matrix (0.72 ml/g for Bio-Gel), and r_A, r_C denote the effective radii (again assumed to be the Stokes radii) of monomer and polymer, respectively. The relationship $r_C = n^{1/3}r_A$ has been considered to provide a sufficiently precise estimate of the second Stokes radius. r_s, the radius of a matrix fiber, has been taken as 0.6 nm [21, 26].

3.3. Gel chromatography of α-chymotrypsin on Bio-Gel P-30

In a recent study of α-chymotrypsin [6] the dependence of $\bar{V}_w$ upon $\bar{c}$ observed in gel chromatography on Bio-Gel P-30 was presented as confirmatory evidence of a value of 3.5 liter/g for the dimerization constant X' of the enzyme in 0.2 I acetate-chloride buffer, pH 3.86. Certainly, the agreement between experimental points and the predicted concentration-dependence of elution volume was very good in the low enzyme concentration range, as is evident from fig. 4, which re-presents the results and the theoretical description (– – –) reported in fig. 3 of ref. [6]. Furthermore, the comment was made that the disparity between experiment and theory at the highest enzyme concentration reflected the failure of the theoretical plot to take into account the effect of osmotic shrinkage of the gel phase. This assertion can now be substantiated.

Combination of the reported values of 16.4 ml and 12.0 ml for V_A^0 and V_C^0, respectively, with the appropriate stationary phase volume V_S^0 of 36.5 ml yields partition coefficients [25] of 0.11 for K_A and zero for K_C. The Stokes radius of monomer, r_A, is calculated to be 2.4 nm from the molecular weight of 25,000 and a sedimentation coefficient ($s_{20,w}$) of 2.4 S [27]. Substitution of this radius into eq. (3) and (4) yields v_e = 1.4 ml/g and $A_A^* = 7.2 \times 10^{-4}$, the magnitude of A_C^* being irrelevant since all terms containing this parameter disappear because $K_C = 0$. The solid line in fig. 4 presents the theoretical concentration dependence

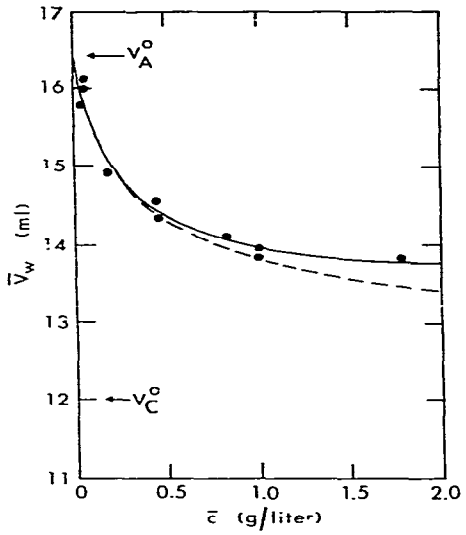

Fig. 4. Effect of osmotic shrinkage on the quantitative prediction of the gel chromatographic behavior of α-chymotrypsin on Bio-Gel P-30. The experimental points, taken from fig. 3 of ref. [6], refer to gel chromatography of α-chymotrypsin on a column (0.9 × 51 cm) of Bio-Gel P-30 equilibrated with 0.2 I acetate-chloride buffer, pH 3.86. The solid line is the theoretical description of the concentration dependence of elution volume with account taken of osmotic shrinkage of the gel (eq. (1)), whereas the broken line is the dependence predicted [6] by neglecting this phenomenon.

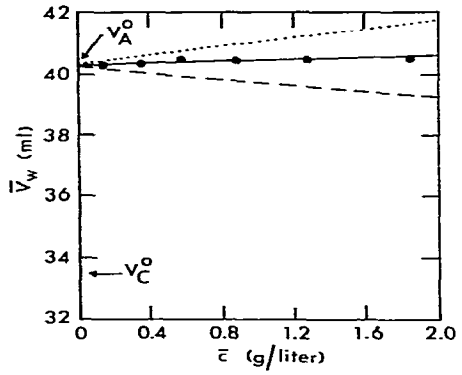

Fig. 5. Quantitative prediction of the gel chromatographic behavior of α-amylase on Bio-Gel P-150. Experimental points denote weight-average elution volumes obtained in frontal gel chromatography of zinc-free α-amylase on a column (2.3 × 26 cm) of Bio-Gel P-150 equilibrated with 0.10 M NaCl–0.015 M calcium acetate–0.010 M EDTA, pH 7.0, and the three curves represent attempts to provide a theoretical description of the results. – – –, a monomer-dimer system with V_A and V_C independent of concentration $\bar{c}$; ——, a monomer–dimer system with account taken (eq. (1)) of osmotic effects; . . . . ., a non-associating monomeric species with account taken of osmotic effects.

of $\bar{V}_w$ predicted on the basis of eq. (1) with the above parameters and values of 0.72 ml/g for $\bar{v}_s$, 0.137 g/ml for c_s^0 (= $1/V_i^0$), and 2.7×10^5 for D (fig. 2). The extent of agreement between theory and experiment is certainly improved by considering the osmotic effect; indeed, the agreement should be regarded as excellent.

3.4. Gel chromatography of α-amylase on Bio-Gel P-150

The preceding section on α-chymotrypsin has served to illustrate that osmotic shrinkage does affect the dependence of weight-average elution volume upon concentration for a reversibly polymerizing solute. For that particular system failure to consider the effect did not affect unduly the conclusion drawn from the study, because sufficient results were obtained in a concentration range where osmotic shrinkage could justifiably be neglected. Attention is now turned to a gel chromatographic study of zinc-free bacterial α-amylase on Bio-Gel P-150, which fails to detect concentration-dependence of $\bar{V}_w$ despite the fact that the enzyme is known to be a monomer-dimer system with $X' = 0.1$ liter/g [19] under the conditions used.

The results obtained in gel chromatography of α-amylase on a column of Bio-Gel P-150 equlibrated with 0.10 M NaCl–0.015 M calcium acetate–0.010 M EDTA, pH 7.0, are presented in fig. 5. For this column $V_s^0 = 82$ ml, $V_A^0 = 40.3$ ml (fig. 5), and $V_C^0 = 33.5$ ml, a value obtained by chromatography of the enzyme (0.1 g/liter) on the same column in the presence of zinc ion. Values of 0.17 and 0.09 are thus calculated for K_A and K_C, respectively, the void volume of the column having been determined as 26.2 ml. Combination of a sedimentation coefficient ($s_{20,w}$) of 6.7 S [19] with the dimeric molecular weight of 96 000 yields $r_C = 3.5$ nm, whereupon $r_A = 2.8$ nm, $v_e = 1.1$ ml/g (eq. (3)), $A_A^* = 4.7 \times 10^{-4}$ and $A_C^* = 3.5 \times 10^{-4}$ (eq. (4)). The remaining parameters required for appli-

cation of eq. (1) are $c_s^0 = 0.098$ g/ml and $D = 1.2 \times 10^6$ (fig. 2).

The concentration dependence of $\bar{V}_w$ predicted on the basis of eq. (1) is shown as the solid line in fig. 5, which also shows the curve predicted without consideration of the osmotic effect (– – –). With this system the difference between the two theoretical plots may also be regarded as slight; but this slight difference can obviously have a pronounced influence upon the interpretation that is placed on the experimental findings. For example, it could be argued on the basis of the less rigorous theoretical curve (– – –) that the experimental results refute the existence of the monomer-dimer equilibrium, since the discrepancy between theory and experiment exceeds that attributable to experimental error. For the theoretical curve that takes due account of osmotic shrinkage the maximum variation in $\bar{V}_w$ is only 0.4 ml (cf 1.3 ml), a change that can obviously be masked by an experimental error of 0.2 ml in the estimates of $\bar{V}_w$. Furthermore, the experimental results are described better by this theoretical plot than that predicted (eq. (1)) for a non-associating monomer (. . . .). In this case, therefore, consideration of the osmotic phenomenon has been required to obtain even a qualitative indication of the existence of the monomer-dimer equilibrium.

4. Concluding remarks

The present investigation of the osmotic behavior of crosslinked polyacrylamide gels has demonstrated that gel chromatographic results obtained with Bio-Gel for reversibly associating solutes are also amenable to quantitative interpretation by the Baghurst et al. [13] procedure inasmuch as the assumed linear dependence of gel shrinkage upon osmotic pressure (eq. (2)) is likely to be a valid approximation for this gel medium. The study also serves to emphasize the possible existence of pronounced variation in the osmotic characteristics of different batches of the same grade of Sephadex or Bio-Gel. This variability has been detected specifically in Sephadex, but there is no cause to consider that it is confined to dextran gels. Indeed, Batlle [28] has commented on differences between the bed volumes of columns prepared from the same weights of different batches of the same grade of Bio-Gel. In applications of the Baghurst et al. [13] analysis to gel chromatographic data on associating solutes it is thus important to obtain the osmotic characteristics of the appropriate batch of the chromatographic medium.

Although it is superior to the previous approaches [11,12] to the problem of allowing for non-chemical dependence of elution volume upon concentration of a reversibly associating solute, the present procedure [13] is certainly not devoid of criticism. In particular, no account is taken of possible variation in the partition coefficients K_A and K_C governing the distribution of solute between the mobile and stationary phases, whereas some variation is to be expected on the basis of (i) changes in porosity due to changes in the volume of the gel phase, and (ii) considerations of thermodynamic non-ideality [29]. Although it is possible to incorporate the efect of thermodynamic non-ideality [29] into the theoretical treatment [13], the prediction of effects arising from changes in gel porosity poses a more difficult problem unless the extent of gel shrinkage is sufficiently small to warrant their neglect. Such a situation should pertain to the two systems considered above, since the effective osmotic pressure would have led to a maximum change of 2% in c_s (or V_i). For studies of concentrated solutions, where pronounced osmotic shrinkage of any gel phase is likely to occur, a change of chromatographic medium to porous glass beads [29,30] is recommended so that complications arising from changes in the void and stationary phase volumes may be avoided.

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