The Osmotic Behaviour of Sephadex and its Effects on Chromatography

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(Received 19 April 1968)

1. Sephadex in bead form shows reversible changes of inner volume when immersed in solutions of a non-penetrating solute. These changes are in accordance with the theory of Flory (1953) for the swelling of gels. This makes possible the use of single beads for measuring the osmotic pressures of polymer solutions, up to or beyond 1 kg./cm.². 2. Measurements of the inner volume of Sephadex G-200 by equilibrium dilution, at various concentrations of dextran 500, gave values in agreement with those obtained from the dimensions of single beads. 3. Measurements by chromatography of the inner volume of Sephadex G-200 gave values that differed slightly but significantly from those obtained by the other methods. Similar small disagreements were found between the dilution and chromatographic values for bovine serum albumin measured in the presence of various concentrations of dextran 500. The major factor that affects the dependence of the chromatographic elution volume (of dextran 500) on concentration is shown to be the change of the inner volume of the Sephadex, with dynamic factors playing a minor role. 4. Contrary to the findings of Ackers (1964), the equilibrium dilution and chromatographic methods gave closely agreeing values for the distribution coefficient, K_{av} , of bovine serum albumin on Sephadex G-200; this cast doubt on his explanation of the nature of chromatographic separations on Sephadex.

This work began with a chance observation by one of us (J.R.D.) that the zonal separation of a mixture of a tissue polysaccharide and a glycoprotein on Sephadex G-200 was greater at higher than at lower concentration of the mixture. We thought that the polysaccharide partly excluded the glycoprotein from the mobile phase, so that the distribution coefficient, K_{av} , and the elution volume, V_e , of the former were increased. We set up a model experiment to test this hypothesis, investigating the effect of dextran 500 (2×10^{-2} g./ml.) on the zonal separation of a mixture of blue dextran 2000 and BSA* on Sephadex G-200. To our surprise the elution volume of the blue dextran, as well as that of the BSA, was markedly increased. Since the blue dextran should not penetrate the stationary phase to a significant extent, this effect could not be due to exclusion of the blue dextran from the mobile phase by the dextran 500.

At first we thought that this anomaly must be due to a dynamic interaction between the dextran and the Sephadex, such as was suggested by Flodin (1961). However, though this factor probably plays some part, the major cause of the phenomenon

proves to be substantial and reversible changes of the inner volume of the Sephadex. This explanation eluded us at first because a change of 15–20% of the inner volume resulted in only 1–2% change in the volume of the packed column.

In this paper we show that dextran 500 penetrates beads of Sephadex G-200 to not more than a very minor extent, that the dependence of its elution volume in frontal chromatography on Sephadex G-200 can be accounted for mainly by the changes of inner volume and that these changes correspond quantitatively to the geometrical shrinkage of the beads in solutions of dextran 500 up to concentrations of 6×10^{-2} g./ml.

We show also that Sephadex of grades between G-50 and G-200 shows reversible shrinkage in solutions of dextrans of concentrations up to 2×10^{-1} g./ml. and that this shrinkage is consistent with Flory's (1953) treatment of the swelling of gels. On this basis we propose the use of single beads of Sephadex for measuring the osmotic pressures of polymer solutions.

We have also made a preliminary investigation of the effect of dextran 500 on the partition of BSA between Sephadex G-200 and mobile solution: the

* Abbreviation: BSA, bovine serum albumin.

results do not accord with those of Ackers (1964), and cast doubt on his explanation of the nature of chromatographic separations on Sephadex.

EXPERIMENTAL AND RESULTS

Materials

Sephadex. This was in bead form as supplied by Pharmacia (Uppsala, Sweden). It was thoroughly washed with distilled water and dried with ethanol and then in air at 55° before use.

Dextrans. Blue dextran 2000 and dextran 500 (batch T5406, $\bar{M}_w$ 420000, $\bar{M}_n$ 153000) were purchased from Pharmacia. Another dextran fraction (dextran 52-8) with $\bar{M}_w$ 77500 and $\bar{M}_n$ 52800 was a gift from Dr K. Granath.

BSA. This was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. (lot no. 86B-1830 or 17B-0530).

Analytical methods

Solutions. Solutions of dextrans were made up by weight, with heating to 100° as required. Conversion of the apparent w/w concentrations into true w/v concentrations was performed on the basis of the dry weights of the solids (measured by drying over P_2O_5 for 1 hr. at 110° and 1–2 mm. Hg pressure) and the partial specific volumes (see below). Subsidiary determinations of the concentration of dextran were done refractometrically (Cecil & Ogston, 1951) with the use of a measured specific refractive increment of 0.153 ml./g., polarimetrically in a 10 cm. cell at 436 m μ or 536 m μ in a Perkin-Elmer model 141 polarimeter, or by the phenol-H₂SO₄ method of Dubois, Gilles, Hamilton, Rebers & Smith (1956).

Blue dextran. This was estimated spectrophotometrically at 625 m μ or at 265 m μ .

BSA. Concentrations were determined spectrophotometrically at 280 m μ by using the value 6.6 for the specific extinction coefficient, $E_{1\text{cm}}^{1\%}$, of BSA (Kronman & Foster, 1957).

Partial specific volumes. The values for the dextrans were measured pycnometrically: all had identical values of 0.60 for $\bar{v}$. The value of $\bar{v}$ for Sephadex was taken to be the same as that of the parent dextran (0.60).

Direct measurement of inner volume of Sephadex G-200 beads

This was done by weighing a suitable quantity of washed beads ($118 \pm 2 \mu\text{g.}$) on to a cleaned and weighed cavity microscope slide. A little water was then added to the beads: after they had swelled fully and air bubbles had been removed, the cavity was completely filled with water and closed with a coverslip; the latter was sealed with immersion oil. After a further 12 hr. a systematic grid of photomicrographs was made, including all the beads; this was done with a Zeiss NfpK microscope, $\times 2.5$ objective and $\times 6.3$ projective, on 35 mm. film. The diameter of each bead (totalling 749 in number) was then measured on a Gaertner toolmakers' microscope, type M2001, As-P. The scale magnification was determined by photographing a stage micrometer scale. From these measurements the inner volume of beads/dry weight (in water) was calculated to be 28 ml./g. (Table 2).

Fig. 1. Diagram (plan and section X-X, not to scale) of the chamber used for measuring single beads of Sephadex. For lettering and dimensions see the text.

Measurement of volume changes of single Sephadex beads in solutions of dextrans

Method. The chamber used (Fig. 1) was developed through a series of preliminary trials. It was constructed from a flat microscope slide and slips of haemocytometer coverslip, 350–550 μ in thickness, cemented with Araldite adhesive (Ciba Ltd., Basle, Switzerland). A channel (A), 3–4 mm. wide and about 500 μ deep, connects two open wells (B and C). A glass barrier (D) is placed across one end of channel A, leaving a horizontal slit about 4 mm. long $\times$ 1 mm. wide $\times$ 100 μ deep. Irrigating solution can flow readily from well B to well C through channel A, but beads of suitable size are unable to pass the barrier D. A small drop of a dilute suspension of beads in water is initially placed in well B, and quickly inspected microscopically to see whether it contains a suitable bead; this should be spherical, undamaged, 300–400 μ in diameter and easily distinguished (by its size) from any other beads present. If a suitable bead is not present, a new sample is taken; if a suitable bead is present, it is washed into the channel A with water and (if necessary) by tilting the slide. It is then inspected more carefully ($\times 6.3$ objective, $\times 10$ ocular) for sphericity. At any time, beads can be removed from the channel by washing in reverse, from well C to well B.

In earlier experiments the beads were measured with a $\times 6.3$ objective and a micrometer eyepiece, with effective overall magnification $\times 10$. In later experiments, photographs were made ($\times 6.3$ objective, $\times 6.3$ projective) on to 35 mm. film, and measured on the Gaertner comparator. The precision of measurement of bead diameter was better than 0.1 μ . Two diameters, at 90°, were measured and the mean taken. Different diameters for a 'good' bead of Sephadex G-50 were the same to 1 part in 300–400; beads of Sephadex of larger grade showed non-sphericity up to 1–2%.

The irrigating solution was changed by first removing the previous solution from well C until none was left in channel A and well B; the new solution was successively added to well B and withdrawn from well C until the solution in channel A had been replaced at least 20 times. The slide was then covered to prevent evaporation and left for 10 min. for the bead to equilibrate with solution. If necessary, the

Fig. 2. Variation of the diameter of Sephadex beads with the concentration of dextran 500. Sephadex grades used: A, G-50; B, G-75; C, G-100; D, G-150; E, G-200. d_0 is the bead diameter in water and d the diameter in dextran solution.

bead was brought clear of the barrier by a small reverse flow of solution (well C to well B) before it was photographed.

Results. In preliminary experiments it was found that 10 min. is amply sufficient for equilibration with a new solution. Dr B. N. Preston (personal communication) told us that in making similar observations on beads of Bio-Gel (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) he had noticed that on bringing a bead into a more concentrated solution of dextran 500 an initial rapid shrinkage was followed later by some degree of swelling to a final equilibrium size, and that on bringing a bead from a dextran solution back into water a transient swelling beyond its initial size in water occurred. This behaviour indicated some penetration of the bead by dextran, occurring slowly in relation to its exchange of water. We looked carefully for this effect with Sephadex G-200 beads; the effect, though present, was very small; it was undetectable with Sephadex G-50.

Different beads of the same grade showed slightly different degrees of volume change, though this appeared not to be correlated with the size of the bead. The degree of volume change varied markedly with the grade of Sephadex, the lower grades changing volume less than the higher grades under the same conditions. Representative curves are shown in Fig. 2.

Osmotic pressure and bead volume

A comparison of the effects of solutions of different dextrans on the sizes of individual beads showed that it is the osmotic pressure of the solution (see below) that determines the bead volume provided that the solute does not penetrate the Sephadex to a significant extent. Thus (Fig. 3) dextran 500 and dextran 52.8 give points for Sephadex G-50 lying on a common curve when bead size is plotted against osmotic pressure. However, with Sephadex G-100 (Fig. 4) the values for dextran 52.8 lie above the curve obtained with dextran 500, showing substantial penetration by the former.

Fig. 3. Variation of the bead size of Sephadex G-50 with osmotic pressure. The bead size is expressed as the reciprocal ratio of the inner concentration of Sephadex, c_g (g./ml.), to its value c_g^0 in water: ●, external solute dextran 500; ○, external solute dextran 52.8.

Fig. 4. Comparison of the effect of penetrating and non-penetrating solutes on the variation of bead size of Sephadex G-100 with osmotic pressure. The bead size is expressed as the reciprocal ratio of the inner concentration of Sephadex, c_g (g./ml.), to its value c_g^0 in water: ●, external solute dextran 500; ○, external solute dextran 52.8.

Evidence discussed below suggests that dextran 500 does not significantly penetrate even Sephadex G-200.

Measurement of the osmotic pressures of dextran. This was done by measurements of the non-ideality coefficients, α_2^* and α_3^* , by sedimentation equilibrium as described by Nichol, Ogston & Preston (1967), with the use of their

Table 1. Values of 'non-ideality' coefficients determined by sedimentation equilibrium: details of experiments and other data

	Dextran 500	Dextran 52.8
$10^{-3}\bar{M}_w$	420	77.5
$10^{-3}\bar{M}_n$	153	52.8
Column height (cm.)	0.43	0.28
Cell thickness (cm.)	0.15	0.15
$10^{-3} \times$ Initial speed (rev./min.) (duration, hr.)	59.78 (3)	59.78 (3)
$10^{-3} \times$ Final speed (rev./min.) (duration, hr.)	35.60 (9)	39.46 (6)
$10^3 \times$ Initial concn. (g./ml.)	48.0	29.5
$10^3 \times$ Final concn. at meniscus (g./ml.)	0	0
$10^3 \times$ Final concn. at bottom (g./ml.)	181	132
α_1^*	6.82×10^{-4} *	1.02×10^{-3} *
α_2^*	5.03×10^{-3} *	2.99×10^{-3} *

* Estimated by eqn. (9) of Nichol *et al.* (1967).

eqn. (9) (Table 1). From these the osmotic pressures were calculated by:

$$\frac{\pi}{RT} = \frac{c}{\bar{M}_n} + \frac{\alpha_1^*}{2}c^2 + \frac{2\alpha_2^*}{3}c^3 \equiv \frac{c}{\bar{M}_n} + A_2c^2 + A_3c^3 \quad (1)$$

where c is the concentration (g./ml.) and A_2 and A_3 are the conventional second and third virial coefficients.

Chromatography

Methods. Chromatographic experiments were carried out with a 43.5 cm. $\times$ 2 cm. column of Sephadex G-200. The column was equilibrated with buffer solution (10m-moles of KH_2PO_4 , 10m-moles of K_2HPO_4 and 60m-moles of KCl /l. of solution, pH 6.76 and I 0.1) or with appropriate solutions of dextran made up in this buffer. Solutions for chromatography were always prepared by dissolving materials directly in this buffer. The flow rate was adjusted to near 4 ml./cm.²/hr. and fractions of constant volume (in the range 1.40–2.00 ml.) were collected with an automatic fraction collector (Paton Industries Ltd., Beaumont, S. Austral., Australia). The size of the fractions was checked by weighing tubes selected at random; the fraction weights were constant to within ± 0.02 g.

Elution volumes were transformed, when necessary, into partition coefficients, K_{av} , defined by the relationship (Laurent & Killander, 1964):

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \quad (2)$$

where V_e is the elution volume of the species in question, V_t is the total volume of the gel bed calculated from the dimensions of the column and V_0 is the void volume of the gel bed. K_{av} allows a system to be described independently of individual experimental conditions. For convenience, the elution volumes are plotted (Figs. 6 and 8) as $V_t - V_0$ for the dextrans and $V_t - V_e$ for BSA, which express the volumes of gel not available to the solute.

Zonal experiments were carried out as described by Andrews (1965); the void and elution volumes were estimated from elution diagrams by extrapolating both sides of the solute peak to an apex (Andrews, 1965); blue dextran 2000 was used for the determination of void volume.

Fig. 5. Effect of concentration of dextran 500 on the zonal separation on Sephadex G-200 of blue dextran 2000 and BSA. —, Experiment in the absence of dextran 500; ----, experiment in the presence of dextran 500 (2×10^{-2} g./ml.). Experimental details are given in the text.

Frontal experiments were performed according to the procedures described by Winzor & Scheraga (1963, 1964) and by Nichol & Winzor (1964); in these experiments sufficient solution was applied to the column to ensure that the resulting elution profile contained a plateau region in which the concentration was equal to the initial concentration. The volume of solution usually applied was 180–190 ml.; median elution volumes, V_e , were calculated. In experiments with dextran 500 alone the trailing ('descending') side of the boundary was used for the calculation of the elution volume; with mixtures, the same boundary was used for dextran 500, but the advancing ('ascending') front was used for the calculation of the elution volume of the other solute (BSA). The values so obtained refer to the condition in the common plateau region. The values of the elution volume for dextran 500 were used for the void volume of the column; this seems justified because the elution volumes for dextran 500 and blue dextran 2000 are not materially different (Figs. 6 and 8).

Zonal experiments. The result of a zonal separation of a mixture of blue dextran 2000 and BSA on Sephadex G-200 in the absence and presence of dextran 500 at concentration 2×10^{-2} g./ml. is shown in Fig. 5. The displacements to higher values of the elution volumes of both the blue dextran and BSA by the presence of dextran 500 are clearly shown; the peak elution volumes suggest an apparent improved separation in the presence of the dextran 500, but this may be deceptive because of the increased boundary spreading. K_{av} values were calculated from the peak elution volumes and are recorded in Table 3. The effect of concentration of dextran 500 on the elution volume of the blue dextran 2000 is illustrated in Fig. 6. In these experiments the column was equilibrated with dextran 500 of the required concentration, a zone of blue dextran 2000 solution (2×10^{-3} g./ml.) in dextran 500 was applied and the blue dextran 2000 was eluted with the same dextran 500 solution. The result shows an approximately linear dependence of the inner volume on dextran 500 concentration. This dependence is compared below (Fig. 6) with that expected from the measurements of individual beads.

The quantitative interpretation of zonal experiments where mixtures are used or the solutes are polydisperse is complicated because of Johnston-Ogston effects (Johnston

& Ogston, 1946; Winzor & Nichol, 1965). For this reason our remaining experiments were all frontal (plateau) experiments in which the interpretation of the results is straightforward.

Frontal (plateau) experiments. Fig. 7 illustrates the effect of concentration on the form of the dextran 500 boundaries. It shows the spreading of the advancing boundaries relative to the trailing boundaries, as well as the displacement of the median elution volumes to higher values, with increasing concentration of dextran 500.

The effect of concentration on the inner volume, $V_t - V_0$, of the column with respect to dextran 500 is shown in Fig. 8. The results agree closely with those obtained in the zonal experiments. The result of one experiment with blue dextran 2000 is included in Fig. 8 to show the close agreement between the elution volumes for dextran 500 and blue dextran 2000. A comparison between this dependence on concentration and that expected from the measurements on individual beads is also included in Fig. 8.

Inner volume of Sephadex G-200. The weight of Sephadex in the column was obtained after washing exhaustively with distilled water, dehydrating with ethanol and drying in air at 55° . By use of the value 94 ml. for $V_t - V_0$, obtained by extrapolation of the data in Figs. 6 and 8 to zero concentration, and the dry weight (3.51 g.), the inner volume was 27 ml./g. of dry Sephadex G-200.

Partition of BSA. To examine the effect of the concentration of dextran 500 on the partition of BSA, plateau experiments were performed with mixtures of BSA at constant concentration (approx. 2 mg./ml.) and dextran 500. Fig. 8 shows the variation of $V_t - V_e$ with dextran 500 concentration. From the results given in Fig. 8 values for

Fig. 6. Effect of concentration of dextran 500 on the elution volumes of blue dextran 2000 in zonal experiments on Sephadex G-200. ----, Expected variation of $V_t - V_0$ due to bead shrinkage, calculated from the data given in Fig. 2, with the value 93.5 ml. for $V_t - V_0$ at zero concentration of dextran 500; ●, experimental values. Experimental details are given in the text.

Fig. 8. Effect of concentration of dextran 500, in frontal experiments, on the inner volume of Sephadex G-200 for dextran 500 and BSA. ●, Values of $V_t - V_0$ for dextran 500; ○, value of $V_t - V_0$ for blue dextran 2000; △, values of $V_t - V_e$ for BSA. ----, Expected variation in $V_t - V_0$ for dextran 500 due to bead shrinkage, calculated from the data given in Fig. 2, with the value 94.5 ml. for $V_t - V_0$ at zero concentration of dextran 500. Experimental details are given in the text.

Fig. 7. Effect of concentration of dextran 500 on the form of dextran 500 boundaries in frontal experiments on Sephadex G-200. —, Concentration of dextran 500 of 10^{-2} g./ml.; ----, concentration of 2×10^{-2} g./ml. Experimental details are given in the text.

Fig. 9. Effect of concentration of dextran 500, in equilibrium-dilution experiments, on the inner volume of Sephadex G-200 for dextran 500 and BSA. ●, Values for dextran 500 obtained by use of the chemical method of analysis (the error bars represent the standard errors of the estimates of the means); ○, values for dextran 500 obtained by use of the more precise polarimetric method of analysis; △, values for BSA. ----, Expected variation in inner volume for dextran 500 due to bead shrinkage, calculated from the data given in Fig. 2, with the value 27 ml./g. for the inner volume at zero concentration of dextran 500. Because this line coincided with the line calculated for the experimental values, the ordinates were increased by 1 ml./g. Experimental details are given in the text.

Table 2. Inner volumes of different grades of Sephadex

Except where indicated, inner volumes were obtained by measuring the inner volume of the Sephadex in a single dilution experiment and then calculating the inner volume at zero concentration of dextran 500 from the data given in Fig. 2.

Grade, of Sephadex	Inner volume (ml./g.)
G-50	5.1
G-75	10.3
G-100	13.1
G-150	20.1
G-200	27.4
G-200	26.7*, 27.0†, 26.5‡, 28§

* Value obtained by extrapolation from Fig. 6.

† Value obtained by extrapolation from Fig. 8.

‡ Value obtained by extrapolation from Fig. 9.

§ Value obtained by measurement of beads.

K_{av} . at dextran 500 concentrations of 0, 10^{-2} and 2×10^{-2} g./ml. were calculated. These values are given in Table 3; the K_{av} . values increase slightly with increasing concentration.

Dilution experiments

Methods. The procedure was similar to that of Ackers (1964). In its final form, weighed samples of Sephadex were added to measured volumes of water or buffer, the amounts being chosen so as to leave approx. 1 ml. of supernatant solution for analysis in the final stage of the experiment. After equilibration for several days, with occasional shaking, a measured amount of dextran 500 solution or BSA solution (or both) was added. The mixtures were allowed to equilibrate for a further 24 hr., with occasional shaking. They were then centrifuged for 5 min. at 500 rev./min., and allowed to stand for a further 3 hr., after which supernatant solution was taken for analysis. The centrifuging did not appreciably affect the distribution of solute. The volume not available to solute per g. of Sephadex was calculated from the final total volume of the system and the dilution of the added solute. These volumes per g. of Sephadex were independent of the weight of Sephadex taken and the final solution volume. It was found that greater precision was obtained when dextran 500 was estimated polarimetrically or refractometrically than when it was estimated chemically.

Inner volume of Sephadex G-200. The effect of concentration of dextran 500 in the external solution on the inner volume of Sephadex G-200 is shown in Fig. 9. For most of these measurements the dextran was estimated by the less precise chemical method: the mean values $\pm$ s.e.m. of many determinations are given. The results show a decrease in the dextran 500 inner volume with increasing concentration similar to that observed in the chromatographic experiments, giving an extrapolated value of 27 ml./g. at zero concentration.

Inner volumes of Sephadex G-50, G-75, G-100 and G-150. The inner volumes of these grades of Sephadex were determined at a concentration of dextran 500 in the external solution near 2×10^{-2} g./ml. The inner volumes at zero concentration were then calculated from these values and the data given in Fig. 2. The results are recorded in Table 2; values for Sephadex G-200 are also included.

Partition of BSA. The variation of $V_t - V_e$ for BSA with the concentration of dextran 500 in the external solution is shown in Fig. 9. A decrease in $V_t - V_e$ with increasing concentration occurs; this parallels the results of the chromatographic experiments. From the results obtained (Fig. 9), values for K_{av} . at dextran concentrations of 0, 10^{-2} , 2×10^{-2} and 5×10^{-2} g./ml. were calculated and are recorded in Table 3; the K_{av} . values show a slight decrease

Table 3. Comparison of the K_{av} . values for the partition of BSA on Sephadex G-200 at different concentrations of dextran 500

The values were calculated from the data given in Figs. 5, 8 and 9.

$10^2 \times$ Concn. of dextran 500 (g./ml.)	...	0	1	2	5
Column experiment (Fig. 5)		0.44	—	0.58	—
Column experiment (Fig. 8)		0.43	0.47	0.51	—
Dilution experiment (Fig. 9)		0.48	0.47	0.45	0.35

with increasing concentration, but agree reasonably with those calculated from the results of the chromatographic experiments.

DISCUSSION

Osmotic behaviour of Sephadex

The reversible volume changes of Sephadex beads, determined by the osmotic pressures of external solutions of non-penetrating solutes, can be treated on the basis of the gel-swelling theory of Flory (1953). This regards equilibrium swelling as resulting from a balance between the internal osmotic pressure of the gel and the negative entropy of distension. Since, at equilibrium, the chemical potential of water is the same in the gel as in the external solution, Flory's eqn. XIII-38, with the use of his eqns. XII-27 and XII-31, is equivalent to:

$$\left(\frac{\pi}{RT}\right)_{\text{external}} = \left(\frac{\pi}{RT}\right)_{\text{internal}} - P \left[(\bar{v}_s c_s)^{\frac{1}{2}} - \frac{\bar{v}_s c_s}{2} \right] \quad (3)$$

where $\bar{v}_s$ is the partial specific volume of the Sephadex and c_s is the inner concentration (g./ml.), which is the reciprocal of the inner volume of the gel (ml./g.). P is a constant that depends on the internal structure. Since the molecular weight of the Sephadex is in effect infinite, the internal osmotic pressure may be represented, subject to the limitations noted by Nichol *et al.* (1967), by:

$$\left(\frac{\pi}{RT}\right)_{\text{internal}} = (A_2)_s c_s^2 + (A_3)_s c_s^3 \quad (4)$$

where $(A_2)_s$ and $(A_3)_s$ are the conventional second and third virial coefficients of the Sephadex.

The value of P cannot be reliably estimated *a priori*, but may be eliminated by use of the value of c_s in water (c_s^0), when $(\pi/RT)_{\text{external}}$ (eqn. 3) is zero. Writing:

$$\begin{aligned} f &= (\bar{v}_s c_s)^{\frac{1}{2}} - \bar{v}_s c_s / 2 \\ f_0 &= (\bar{v}_s c_s^0)^{\frac{1}{2}} - \bar{v}_s c_s^0 / 2 \end{aligned} \quad (5)$$

eqn. (3) can be rearranged to:

$$\begin{aligned} \left(\frac{\pi}{RT}\right)_{\text{external}} &= (A_2)_s \left(\frac{c_s^2}{(c_s^0)^2} - \frac{f}{f_0} \right) (c_s^0)^2 \\ &+ (A_3)_s \left(\frac{(c_s)^3}{(c_s^0)^3} - \frac{f}{f_0} \right) (c_s^0)^3 \end{aligned} \quad (6)$$

and treated by least-squares as a bivariate regression to estimate $(A_2)_s$ and $(A_3)_s$. The results (Table 4) show, as might be expected, that these quantities are not very different from those of parent (non-cross-linked) material, represented by dextran 500 ($10^4 A_2 = 3.4$; $10^3 A_3 = 3.3$). The values of $(A_2)_s$

Table 4. *Estimates of the internal second and third virial coefficients of Sephadexes*

Sephadex	External solute	Virial coefficients	
		$10^4(A_2)_s$	$10^3(A_3)_s$
G-50	Dextran 500 or 52.8	2.9	0.2
G-75	Dextran 500	2.0	1.8
G-100	Dextran 500	1.3	1.3
G-200	Dextran 500	1.7	7

show no significant trend, though those of $(A_3)_s$ appear to decrease with increase of cross-linking. It should be pointed out that, though the values of the ratios c_s/c_s^0 used in eqn. (6) were derived from direct measurements on individual beads, the values of c_s^0 were necessarily average values obtained from measurements of the inner volumes of relatively large samples. These values may not apply exactly to individual beads, and this will introduce error into the estimates of $(A_2)_s$ and $(A_3)_s$.

Penetration of Sephadex by dextrans. The foregoing discussion assumes that the dextran does not significantly penetrate the Sephadex, and therefore exerts its full osmotic pressure. The evidence for this is as follows. (i) The geometrical volume/g. of Sephadex G-200 (28 ml.) is only slightly greater than the values measured by dilution of dextran 500 (27 ml.) and by chromatography (27 ml.). (ii) The elution volume at low or zero concentration of dextran 500 on a column of Sephadex G-200 differs only slightly from that of blue dextran 2000, which has a substantially greater molecular weight (Figs. 6 and 8). (iii) On bringing a Sephadex G-200 bead from water to dextran 500 solution or dextran to water there are only very small transient changes of volume such as would result from penetration of the dextran.

The conclusion applies *a fortiori* to the lower grades of Sephadex. Any discrepancy between the relationship between the Sephadex volume and the osmotic pressures of polymer solutions of differing molecular size is an indication that the smaller molecules are penetrating (compare Figs. 3 and 4).

Use of Sephadex beads as osmometers

The use of a polymer gel for the measurement of osmotic pressure was proposed by Boyer (1945). A spherical bead of Sephadex is particularly suitable for this purpose. It can be calibrated as an osmometer by use of solutions of known osmotic pressure, and then used to determine the osmotic pressures of unknown solutions, provided that neither solute penetrates the Sephadex. This condition is most directly tested by comparing the geometrical

volume/g. of the Sephadex with its apparent inner volume for the solutes in question. To minimize the risk of penetration it is advisable to use the lowest possible grade of Sephadex. In our experience Sephadex G-50 is the lowest grade for which changes of diameter are large enough to be measured with reasonable accuracy.

The method is obviously limited to solutes of fairly high molecular weight. It is not likely to prove as precise as conventional methods and would not be suitable for the determination of molecular weight. Its special advantage lies in its extreme simplicity, the requirement of only small volumes of solution and its usefulness for measuring high osmotic pressures of concentrated solutions. The most concentrated solution of dextran that we have used has an osmotic pressure of nearly 1 kg./cm.².

Chromatography of dextran 500 on Sephadex G-200

In the usually accepted model of chromatography on Sephadex (see, e.g., Laurent & Killander, 1964) the elution volume of a solute depends only on the volumes of stationary (gel) phase and of mobile phase, and the instantaneous partition of solutes between them. All solute in the gel phase is assumed to have zero velocity, and solvent and all solutes in the mobile phase to move at the same velocity. The elution volume of the solute that does not penetrate the gel phase at all (K_{av} , 0) should therefore be directly proportional to the mobile volume (or volume fraction) of the column. If this fraction is itself a function of the concentration of solute, then any lamina of constant concentration of solute moves down the column with a velocity characteristic of that concentration and emerges at a corresponding volume. Since with dextran the osmotic shrinkage of the Sephadex causes the mobile volume to increase with concentration of dextran, the elution volumes will increase with concentration. This will lead to a spreading of the advancing ('ascending') front; a corresponding sharpening of the trailing ('descending') front is expected. Within the range of dextran concentrations that we have been able to study (limited by the viscosity of the solutions), these predictions are strikingly confirmed.

These observations have practical consequences in relation to the use of Sephadex columns, particularly of higher grade and at other than very low concentrations of solute. The median elution volumes can be expected to vary with concentration of a solute (or solutes); also, non-enantiographic spreading of fronts in frontal analysis and asymmetry of zones in zonal analysis are predicted, even with monodisperse solutes. These effects will occur even when the solute partially penetrates the gel phase since, unless it does so completely (K_{av} , 1), it

will contribute to the osmotic relationship between gel and mobile solution.

However, the apparent variation of the inner volume of Sephadex G-200, whether measured from the frontal elution of dextran 500 (Fig. 8) or from zonal elution of blue dextran 2000 (Fig. 6), is significantly greater than the variation of inner volume obtained indirectly by dilution of dextran 500, or directly by measurement of the diameters of individual beads. This discrepancy means that the elution volume becomes increasingly greater than that predicted as the concentration of dextran increases. It can arise only from the difference of conditions between dynamic (elution) and static experiments. Its direction is such as would result from a filtration effect, i.e. a frictional retardation of the macromolecular solute relative to the movement of solvent within the mobile phase.

Chromatography and partition of BSA in the presence of dextran

Preliminary measurements were made for the reason given in the introduction to this paper. They were extended when we came to realize the effect of dextran on bead volume. Much more work is needed, but we have included these results because they indicate some of the complexities that may arise, and suggest ways in which they can be handled.

First, we must consider the question whether the generally accepted view of gel chromatography is correct. Ackers (1964) has questioned the assumption that solute in the gel phase is stationary relative to the direction of flow, proposing a model akin to a real 'gel filtration'. He based his view on disagreements between values of K_{av} , measured by partition and by elution volume, particularly with Sephadex G-200 and including BSA. In a number of careful measurements, by both techniques, we have obtained agreeing values close to his chromatographic value; we have been unable to confirm the much higher value that he obtained by static partition. We therefore see no need to regard the conventional view as unsatisfactory in this respect.

The effect of running BSA on a Sephadex G-200 column in the presence of dextran is to increase its elution volume as the dextran concentration is increased. A part of this effect is due to the effect of dextran on the bead size and thence on the fraction of mobile volume of the column. When allowance is made for this, values of K_{av} can be calculated; values of K_{av} were also obtained by simultaneous equilibrium measurements of the dilution volume for dextran 500 and for BSA. The results show a remarkably small effect of dextran on the partition of BSA.

This relatively small effect of dextran on K_{av} can be qualitatively explained by the gel shrinkage

that the dextran brings about. The effect of this is to concentrate the Sephadex, decrease its average pore size (or effective grade), and so to lessen the degree of penetration of BSA. Thus the two effects of the dextran, namely the exclusion of BSA from the mobile solution and its increased exclusion from the Sephadex, cancel out approximately.

Such changes as there are appear to be in opposite directions, as measured by the two methods (Figs. 8 and 9 and Table 3). The deviation must (as with the case of dextran, discussed above) arise from the difference of conditions between the dynamic and static experiments. Its direction is opposite to that which would result from Ackers's (1964) suggestion, that BSA present in the gel phase has a non-zero mobility. It could, again, be explained by a dynamic retardation of the BSA in the mobile phase, secondary to that of dextran and caused by frictional interaction between them.

We are grateful to Dr K. Granath for the gift of a sample of fractionated dextran and to Dr B. N. Preston for advice; E.E. is the holder of a Research Scholarship at the Australian National University.

REFERENCES

- Ackers, G. K. (1964). *Biochemistry*, **3**, 723.
Andrews, P. (1965). *Biochem. J.* **96**, 595.
Boyer, R. F. (1945). *J. chem. Phys.* **13**, 363.
Cecil, R. & Ogston, A. G. (1951). *J. sci. Instrum.* **28**, 253.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Flodin, P. (1961). *J. Chromat.* **5**, 103.
Flory, P. J. (1953). *Principles of Polymer Chemistry*, p. 576. Ithaca, N.Y.: Cornell University Press.
Johnston, J. P. & Ogston, A. G. (1946). *Trans. Faraday Soc.* **42**, 789.
Kronman, M. J. & Foster, J. F. (1957). *Arch. Biochem. Biophys.* **72**, 205.
Laurent, T. C. & Killander, J. (1964). *J. Chromat.* **14**, 317.
Nichol, L. W., Ogston, A. G. & Preston, B. N. (1967). *Biochem. J.* **102**, 407.
Nichol, L. W. & Winzor, D. J. (1964). *J. phys. Chem.* **68**, 2455.
Winzor, D. J. & Nichol, L. W. (1965). *Biochim. biophys. Acta*, **104**, 1.
Winzor, D. J. & Scheraga, H. A. (1963). *Biochemistry*, **2**, 1263.
Winzor, D. J. & Scheraga, H. A. (1964). *J. phys. Chem.* **68**, 338.