The Thermodynamics of Interaction between Sephadex and Penetrating Solutes

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1. We have measured the partition coefficients of bovine serum albumin with Sephadex grades G-100, G-150 and G-200, and of a dextran ($\bar{M}_n$ 19700) and a polyethylene glycol ($\bar{M}_n$ 8000) with Sephadex G-200. We have also measured the effects of these solutes on the inner volumes of the grades of Sephadex. 2. The results can be described with fair consistency by means of a simple thermodynamic treatment that makes use of the virial coefficients of Sephadex and of the solute, and of a coefficient that expresses their interaction. This coefficient is related to the 'exclusion volume' of Sephadex for the solutes. 3. The Sephadex G-200–polyethylene glycol system shows anomalies of behaviour that are ascribed to the occurrence of 'incompatible' phase separation within the Sephadex beads.

Edmond, Farquhar, Dunstone & Ogston (1968) showed that the inner volume of Sephadex in bead form is decreased progressively by increasing the external osmotic pressure due to a non-penetrating (wholly excluded) solute, and that this process can be satisfactorily represented in terms of the inner osmotic pressure of the Sephadex and the negative entropy of its distension. They showed also that the effect of a solute that partly penetrates the gel is less than that of a wholly excluded solute at the same external osmotic pressure; this would be expected because solute within the gel will contribute to the internal osmotic pressure.

We have made further measurements on the behaviour of Sephadex with some partly penetrating solutes, with the object of finding whether the behaviour of these systems also can be described in simple thermodynamic terms. Three solutes (BSA,† a dextran fraction of mol.wt. 19.7×10^3 , and polyethylene glycol, mol.wt. 8×10^3) were chosen as having roughly the same molecular dimensions but very different thermodynamic characteristics.

The inner volumes of the Sephadex and the partition coefficients of the solutes were measured over a range of concentration of each solute.

EXPERIMENTAL

Materials

Sephadex. Samples of Sephadex G-200, G-150 and G-100 were from the same batches as those used by Edmond *et al.* (1968). Before use, the Sephadex was

thoroughly washed in water, dehydrated with ethanol and dried at 70°C in a vacuum oven. Table 1 summarizes the relevant data on these samples; these include the values of A_2 and A_3 for Sephadex G-150 and the values of P , which were measured but not quoted by Edmond *et al.* (1968).

BSA. This was Sigma lot 108B-8170. As it contained not more than 5% of dimer (tested by disc electrophoresis on polyacrylamide gel) it was used without further purification, dissolved in 0.2M-NaCl.

Dextran 19.7. This was the fractionated sample supplied by Dr K. Granath, described by Edmond & Ogston (1968). The aqueous solution was used.

Polyethylene glycol. This was the PEG 6000 sample used by Nichol, Ogston & Preston (1967) and by Edmond & Ogston (1968). The aqueous solution was used.

Table 2 summarizes the data on these substances.

Methods

Measurement of concentrations. Concentrated solutions were diluted by weight with 0.2M-NaCl (BSA) or with water (dextran and polyethylene glycol) to concentrations suitable for measurement. The concentrations (w/v) of diluted BSA solutions were measured by absorption at 280nm with a Beckman DU spectrophotometer assuming $E_{1\%}^{1\text{cm}}$ 6.41. The concentrations (w/v) of diluted dextran solutions were measured with a Perkin-Elmer model 141 polarimeter at 436nm; the rotation was calibrated with dextran dried to constant weight, giving 3.89×10^2 deg ml g⁻¹. The concentrations (w/v) of diluted polyethylene glycol solutions were measured refractometrically (Cecil & Ogston, 1951), with use of a specific refractive increment 0.137ml/g (Nichol *et al.* 1967). Concentrations were converted between weight/volume and weight/weight, where required, by use of the density of the solvent and the partial specific volume of the solute (Table 2).

Measurement of inner volumes. This was done by

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† Abbreviation: BSA, bovine serum albumin.

measuring the diameters of single beads of Sephadex, as described by Edmond *et al.* (1968), giving ample time (up to 1 h) for the solute to equilibrate between the solution and the bead. The inner volumes/g were calculated from the values in water (Table 1) and the ratio of each bead diameter to that in water. Diameters in 0.2M-NaCl were indistinguishable from those in water.

Measurement of partition coefficients. Amounts of Sephadex (0.06–0.5 g), such as to leave about 1 ml of clear supernatant at the end of equilibration, were weighed into 5 ml stoppered cylinders. Weighed amounts of stock solution of solute and of solvent (0.2M-NaCl or water), if required, were added. The contents of the cylinders were equilibrated with repeated mixing for 2 or more days at 23–25°C. After they had stood overnight, samples of supernatant solutions were withdrawn and diluted by weight, for determination of their final concentrations. The relevant value of the inner volume/g of Sephadex was read from a plot of inner volume versus external solute concentration (Fig. 1) to give the total inner volume in each tube. Thence the total outer volume, the total solute in the outer volume and (by difference from the total solute added) the total solute in the inner volume were calculated. Finally, the partition coefficients were expressed as K_{av} . (Laurent & Killander, 1964), the ratio of weight of solute/total inner volume to weight of solute/total outer volume. In each set of experiments the concentration of the stock solute solution was measured (after weight-dilution) by the same method as that used for the supernatants. All measurements were made to a precision of 1 part in 500 or better.

Table 1. *Characteristics of grades of Sephadex*

Values are quoted from Edmond & Ogston (1968 and unpublished work).

Sephadex grade	Inner volume (ml/g in water)	Virial coefficients		Structure constant ($10^6 P$)
		$10^4(A_2)_s$	$10^3(A_3)_s$	
G-100	13.1	1.3	1.3	4.0
G-150	20.1	2.9	3.9	4.0
G-200	27.4	1.7	7	2.12

THEORY

Partition. For partition equilibrium of solute (2) between the inner (') and outer (") phases we have:

$$\mu'_2 = \mu''_2 + V_2 \Delta\pi \quad (1)$$

where both chemical potentials are referred to the same standard state (1 atm), V_2 is the molar volume of solute and $\Delta\pi$ is the difference of pressure between the phases. From the osmotic pressure relationship (see below):

$$\Delta\pi = RTPf \quad (2)$$

It follows that:

$$\ln(m'_2 \gamma'_2) = \ln(m''_2 \gamma''_2) + V_2 Pf \quad (3)$$

P is a constant that depends on the internal structure of the Sephadex and f is a function of the partial specific volume, $\bar{v}_s$, and internal concentration, C_s g of Sephadex/ml, defined by:

$$f = (\bar{v}C_s)^{1/3} - \bar{v}C_s/2$$

Fig. 1. Variation of the inner volumes (ml/g) of grades of Sephadex with the concentration of solute in the external solution. ■, Sephadex G-200-BSA; ▲, Sephadex G-200-dextran 19.7; ●, Sephadex G-200-polyethylene glycol 6000; □, Sephadex G-150-BSA; △, Sephadex G-100-BSA.

Table 2. *Characteristics of solutes*

Solute	$10^{-3} \bar{M}_n$	Virial coefficients		Partial specific volume	Effective molecular radius (Å)†
		$10^4(A_2)_2$	$10^3(A_3)_2$		
BSA	73*	0.85††	0	0.75	35
Dextran 19.7	19.7‡	5.85‡	1.92‡	0.60‡	37
Polyethylene glycol 6000	8.0§	25	16.7	0.837	30

* Davies (1966).

† Scatchard, Batchelder & Brown (1946).

‡ Edmond & Ogston (1968).

§ Alexandrowicz (1959).

|| Nichol *et al.* (1967).

(Edmond *et al.* 1968); m_2 is the molal concentration of solute and γ_2 its activity coefficient. Expressing the activity coefficients by simplified virial expansions (Ogston, 1962; Edmond & Ogston, 1968), eqn. (3) becomes:

$$\ln m_2' + dm_2' + am_2' = \ln m_2'' + dm_2'' + V_2 Pf \quad (4)$$

where d is the (molal) second virial coefficient of the solute and a is the (molal) interaction coefficient of solute with Sephadex. Actually, the 'molality' of Sephadex (m_s') is of itself meaningless, since its molecular weight is effectively infinite, but the product am_2' is meaningful. We may now convert eqn. (4) into weight/volume terms (without much error, as noted by Edmond & Ogston, 1968) by writing:

$$a = 10^{-3} A^* M_2 M_s$$

where A^* is the interaction coefficient expressed on a weight/volume scale, and:

$$m_s = 10^3 C_s' / M_s$$

where C_s' is the internal concentration (g/ml) of the Sephadex; by replacing the dm_2 terms by corresponding second and third virial terms on a weight/volume scale (C_2), eqn. (4) becomes, with some rearrangement:

$$\ln \left(\frac{C_2'}{C_2''} \right) = \ln K_{av} = M_2 \left(2(A_2)_2(C_2'' - C_2') + \frac{3(A_3)_2}{2} [(C_2'')^2 - (C_2')^2] + \bar{v}_2 Pf - A^* C_s' C_2 \right) \quad (5)$$

where $\bar{v}_2$ is the partial specific volume of solute. Eqn. (5) allows values of A^* to be obtained.

Osmotic equilibrium. This is described by eqns. (3) and (5) of Edmond *et al.* (1968), but to their expression for the internal osmotic pressure we now have to add terms that express the contribution of the solute to the internal osmotic pressure. Accordingly:

$$\left(\frac{\pi}{RT} \right)_{\text{int.}} = (A_2)_s (C_s')^2 + (A_3)_s (C_s')^3 + \frac{C_2'}{M_2} + (A_2)_2 (C_2')^2 + (A_3)_2 (C_2')^3 + A^* C_s' C_2 \quad (6)$$

where the third, fourth and fifth terms on the right-hand side express the 'self' contribution of the internal solute, and the last term expresses the interaction contribution. Combination of eqn. (6) with eqns. (3) and (5) of Edmond *et al.* (1968) again allows A^* to be estimated.

Since each of eqns. (5) and (6) is a thermodynamic statement about an aspect of the same equilibrium system, both must hold good provided that they are adequate with respect to the number of terms included, the values of the coefficients and the approximations made. This can be tested by the

constancy of estimates of A^* obtained by use of each equation, their agreement with each other and with other available estimates.

RESULTS AND DISCUSSION

Fig. 1 shows the variations of the inner volume of the grades of Sephadex with external concentration of solute. As would be expected, the variations are generally less than with non-penetrating solutes (Edmond *et al.* 1968). The effect of the dextran on Sephadex G-200 is greater than that of BSA because of its higher osmotic activity, and the effect diminishes as the Sephadex is more highly cross-linked. The rapid variation of inner volume of Sephadex G-200 with concentration of polyethylene glycol partly reflects the latter's very high osmotic activity, but other factors (see below) have to be considered.

Fig. 2 shows the variation of K_{av} with external concentration of solute. All systems except the Sephadex G-200-polyethylene glycol system show increases of K_{av} with external concentration. At first sight this appears anomalous: since the inner volumes decrease with increasing external concentration of solute, one might expect the Sephadex to become less penetrable by solute and therefore K_{av} to decrease. However, inspection of eqn. (5) shows that this view is too simple. The second (and third) terms on the right-hand side of the equation are positive and the values of $C_2'' - C_2'$ and $(C_2'')^2 - (C_2')^2$ increase with increase of C_2' . If this positive variation is greater than that of the last term (which depends mainly on the magnitude of A^*), then K_{av} will vary positively.

Table 3 shows the values (range and mean) for A^* calculated from the partition relationship (eqn. 5) and from the osmotic relationship (eqn. 6). Those calculated from eqn. (5) are likely to be the more reliable, because this relationship involves the osmotic

Fig. 2. Variation of K_{av} (see the text) with concentration of solute in the external solution. ■, Sephadex G-200-BSA; ▲, Sephadex G-200-dextran 19.7; ●, Sephadex G-200-polyethylene glycol 6000; □, Sephadex G-150-BSA; △, Sephadex G-100-BSA.

Table 3. *Estimates of interaction coefficients A^* and ϵ (see the text) between grades of Sephadex and solutes*

Sephadex	Solute	$10^4 A^*$ (range and mean)		ϵ (limiting exclusion volume of solute by Sephadex) (ml/g)†
		From eqn. (5)	From eqn. (6)	
G-200	BSA	3.3–3.9 (3.6)	3.7–4.1 (4.0)	26
G-150	BSA	3.1–3.7 (3.4)	3.4–4.2 (3.8)	30
G-100	BSA	3.7–4.8 (4.2)	11–15 (13.6)	30
G-200	Dextran 19.7	13–16 (15)	16–18 (17)	30
G-200	Polyethylene glycol	34–39*	41–43*	30

* Values at approx. 20 and 40mg/g external concentration of solute (see the text).

† Calculated from the A^* values in column 3 (see the text.)

properties of the Sephadex only by way of the $\bar{v}_2Pf$ term, which is numerically small. The terms containing $(A_2)_s$ and $(A_3)_s$ in eqn. (6) are, however, numerically large, so that the values of A^* obtained from eqn. (6) are sensitive to errors in the values of $(A_2)_s$ and $(A_3)_s$. In spite of this, the agreements between the two estimates are fairly good, except for the Sephadex G-100–BSA system, where the $(A_2)_s$ and $(A_3)_s$ terms are particularly large. It is noticeable also that the values of A^* for interactions between BSA and the three grades of Sephadex are much the same, in spite of the varying degree of cross-linking.

The interactions can be presented in another way. Since 10^3a is the molar co-volume of interacting species (Edmond & Ogston, 1968) it follows that A^*M_2 is the limiting value ϵ (ml/g) that the exclusion volume for the solute by Sephadex would have at zero (internal) concentration of Sephadex. These values are given in the last column of Table 3. The BSA values agree well with the zero-concentration exclusion value (25ml/g) calculated from the data of Laurent (1963) for the exclusion of BSA by dextran by measurement of solubility. Further, the values for the different solutes are all similar, as would be expected from their similar effective molecular radii.

The Sephadex G-200–polyethylene glycol system has features of special interest. The values for A^* quoted in Table 3 are for external concentrations of solute only up to 40mg/g. Above this concentration, although the values of A^* obtained from eqn. (5) remain of about the same value, those obtained from the osmotic relationship become strongly, and increasingly, negative as the external concentration increases. At the same time, the interiors of the beads, under microscopic examination, lose their optical homogeneity and become granular. This change is particularly obvious under phase-contrast microscopy (Plate 1); it is not seen with either BSA or dextran as solute. These deviations of A^* values are far too great to be ascribed to errors in the virial coefficients of either the polyethylene glycol (which are rather well established) or of Sephadex G-200. The probable explanation is that, as with solutions

of dextran and polyethylene glycol (Albertsson, 1960; Edmond & Ogston, 1968), so with cross-linked dextran (Sephadex) and polyethylene glycol, incompatible phase separation occurs when critical composition limits are surpassed. In fact, at an external concentration of polyethylene glycol of 40mg/g, its internal concentration is about 15mg/g, and the internal Sephadex concentration is 80mg/g, and this pair of values would lie close to the phase-separation curves found by Albertsson (1960) for dextrans and polyethylene glycol 6000. The absence of a sharp break, corresponding to internal phase separation, in either the internal volume or the K_{av} curve (Figs. 1 and 2), is probably due to internal heterogeneity of cross-linking within the Sephadex. The rise of K_{av} values at the highest concentrations of solute (Fig. 2) are certainly real but, because of the complication introduced by phase separation, we are unable to explain these.

We note that Polson & Katz (1969) have attempted an analysis somewhat similar to ours. However, instead of taking equality of the chemical potential of solute in the inner and outer phases as the criterion of equilibrium, they have taken equality of 'the osmotic pressure of protein' as the criterion. The latter quantity has in general no exactly definable meaning, though it would be legitimate to speak of 'the contribution' of protein to osmotic pressure. Their treatment is valid only for the case (which they appear to assume) where the gel has no osmotic properties, i.e. where it is a completely rigid structure; their equations are therefore not compatible with eqns. (3) and (4) of Edmond *et al.* (1968).

Properly, they should have used an equivalent of our eqn. (5). Then, putting their B [our $(A_2)_2$ and $(A_3)_2$] and the $\bar{v}_2Pf$ term all equal to zero and noting that their G equals our A^*C_s , we obtain:

$$G = -\frac{\ln K_D}{M_2} \quad (7)$$

to which their eqn. (5) is a rather poor approximation. The values of G calculated from the results of Polson & Katz (1969) by eqn. (7) show a systematic variation

EXPLANATION OF PLATE I

Phase-contrast photomicrographs of a Sephadex G-200 bead in: (a) Dextran 500 (200mg/ml); (b) Dextran 19.7 (100mg/ml); (c) polyethylene glycol 6000 (100mg/ml). The scale line is 10^{-2} cm.

with molecular weight of solute, confirming their surmise that the constancy of their estimates of G is fortuitous.

CONCLUSIONS

The simple thermodynamic treatment here used appears adequate to describe the interactions between Sephadex and penetrating macromolecular solutes. This is likely to be true *a fortiori* at lower solute concentrations, such as those normally used in chromatographic separations, where osmotic changes of the inner volume are unimportant. The description requires the use of no steric model of the structure either of the Sephadex or of the solute molecule, though interpretation of the thermodynamic quantities in terms of such models is legitimate and interesting (see, e.g. Laurent & Killander, 1964; Laurent, 1964, 1967; Giddings, Kucera, Russell & Myers, 1968). The similarity of values of the interaction coefficients of BSA with the grades of Sephadex and with dextrans is parallel to the similarity of internal osmotic coefficients of Sephadex grades and of dextrans found by Edmond *et al.* (1968).

The anomalous behaviour of the Sephadex G-200-polyethylene glycol systems draws attention to the

possible occurrence of phase separations, whether of the 'incompatible' or 'complex coacervation' types, which could complicate chromatographic behaviour.

REFERENCES

- Albertsson, P. (1960). *Partition of Cell Particles and Macromolecules*. Uppsala, Sweden: Almquist and Wiksell.
- Alexandrowicz, A. (1959). *J. Polym. Sci.* **40**, 107.
- Cecil, R. & Ogston, A. G. (1951). *J. scient. Instrum.* **28**, 253.
- Davies, M. (1966). *Makromolek. Chem.* **90**, 108.
- Edmond, E. & Ogston, A. G. (1968). *Biochem. J.* **109**, 569.
- Edmond, E., Farquhar, S., Dunstone, J. R. & Ogston, A. G. (1968). *Biochem. J.* **108**, 755.
- Giddings, J. C., Kucera, E., Russell, C. P. & Myers, M. N. (1968). *J. phys. Chem., Ithaca*, **72**, 4397.
- Laurent, T. C. (1963). *Biochem. J.* **89**, 253.
- Laurent, T. C. (1964). *Biochem. J.* **93**, 106.
- Laurent, T. C. (1967). *Biochim. biophys. Acta*, **136**, 199.
- Laurent, T. C. & Killander, J. (1964). *J. Chromat.* **14**, 317.
- Nichol, L. W., Ogston, A. G. & Preston, B. N. (1967). *Biochem. J.* **102**, 407.
- Ogston, A. G. (1962). *Archs Biochem. Biophys. Suppl.* no. 1, p. 39.
- Polson, A. & Katz, W. (1969). *Biochem. J.* **112**, 387.
- Scatchard, G., Batchelder, A. C. & Brown, A. (1946). *J. Am. chem. Soc.* **68**, 2320.