Osmometry with Single Sephadex Beads

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1. The measurement of osmotic pressure by means of a single bead of Sephadex (Edmond, Farquhar, Dunstone & Ogston, 1968) has been made more precise by immobilizing the bead on a fine needle. The design, calibration and use of the osmometer are described. 2. The method is particularly suitable for measuring high osmotic pressures in solutions of high-molecular-weight solutes, which must not penetrate the Sephadex to a significant extent. 3. With Sephadex G-50 the limit of precision is about 1.5 cmH₂O and the lower limit of molecular weight for a solute of compact molecular form is about 10⁵. 4. The time required for each equilibration is less than 10 min. 5. An impaled bead can be stored in the dry state without affecting its calibration. 6. Measurements on a sample of polyvinyl alcohol, degree of polymerization stated as 1750 ± 50, gave $\bar{M}_n$ 43 000 ± 3000 and A_2 (6.0 ± 0.24) × 10⁻⁴.

Edmond, Farquhar, Dunstone & Ogston (1968) described the use of single beads of Sephadex for measuring the osmotic pressures of solutions of non-penetrating solutes; their method was subsequently used by Edmond & Ogston (1968, 1970). In their method the bead is left free to move within the chamber when one solution is replaced by another and therefore different diameters of the same bead may be measured on different occasions. The variation of different diameters of the same bead (by 0.2-0.3% in Sephadex G-50 and up to 2% in Sephadex G-200) introduces an error into the method considerably greater than that of measuring a given diameter. We have been able to improve the accuracy of the method substantially by impaling a single bead on a fine needle, ensuring that the same diameter is always measured. This makes it possible to measure osmotic pressures accurate to 1-1.5 cmH₂O with a Sephadex G-50 bead (with correspondingly greater accuracy with beads of higher grade). The time required for each equilibration is 10 min or less.

EXPERIMENTAL

Construction of the osmometer

The osmometer (Fig. 1a) was constructed from a flat microscope slide, and pieces of microscope slide (1050-1150 μm thick), haemocytometer coverslip (400-500 μm), and ordinary coverslip (about 150 μm), cemented with Araldite adhesive (Ciba Ltd., Basle, Switzerland). Near one end of the main channel (A) (about 4 mm wide, 1 mm deep and 35 mm long), is a platform (B) about 450 μm high. A cover glass (C) (dotted outline in the Figure) can

be clamped in place by screws in the stainless-steel brackets (D), that are cemented to the top of the slide. A jig (Fig. 1b), about 4 mm wide and 10 mm long, in the form of a rectangular funnel, is made from pieces of ordinary coverslip for the base and top (dotted in the Figure), and pieces of haemocytometer coverslip as spacers, leaving a tube (EE) about 100 μm wide and 450 μm high down the centre. The jig slides freely along the channel A; it serves to locate the needle while it is being cemented in place, and later to hold the bead when it is being impaled.

The needle is made by etching a length of 2-3 mm at the end of a piece of tungsten wire (diameter 130 μm) to a final diameter of 20-40 μm, with a sharp point at the tip, by the method of Hubel (1957). The needle is placed in a spot of adhesive on the platform B, with the point facing along the channel; while the cement is still plastic the needle and jig are manipulated under a dissecting microscope so that the needle passes centrally through the tube EE. When the adhesive has set, the jig is withdrawn.

Use of the osmometer

Dry Sephadex beads are suspended in water for at least 1 h. A small drop of the suspension is transferred to a cavity slide and examined under a dissecting microscope (×20 magnification). When a suitable bead (300-400 μm in diam. and reasonably spherical) has been isolated, it is transferred by pipette to the funnel of the jig. The jig is then placed in the channel and moved carefully along it until the needle pierces and passes through the bead into the tube EE (Fig. 1). When the jig is withdrawn the bead remains transfixed on the needle. Finally, the coverglass is clamped in place, with a little silicone grease under it to prevent creeping of solution from the channel.

The bead is immersed in the chosen solution by placing

Fig. 1. Diagrams (not to scale) of (a) osmometer (plan and sections X-X and Y-Y); N, needle; S, Sephadex bead; (b) jig (plan); (c) evaporation cover. For other lettering and dimensions see the text.

solution from a transfer pipette near one end of the chamber formed by the channel A (Fig. 1) and the cover-glass; the solution (about $40\mu\text{l}$ or less) fills the chamber by capillarity. A glass cover (Fig. 1c) is placed over each end of the channel to prevent evaporation. After a suitable interval for osmotic equilibration the microscope (Zeiss NfpK stand; $\times 6.3$ planachromat objective; $\times 6.3$ projective) is focused on the equator of the bead and a photograph is taken on Ilford Pan F or Kodak Panatomic X 35 mm film. After development of the film with May and Baker Promicrol developer, the diameter of the image of the bead is measured at 90° to the needle with a Gaertner toolmaker's microscope, type M2001, As-P.

The solution is removed from the chamber by placing the end of a wick of folded filter paper at one end. This removes 95–98% of the contents of the chamber so that one solution may be directly replaced by another without a serious change of concentration taking place in the chamber. Preferably, however, the chamber is twice washed with the new solution; this has been our routine.

In most of the present work we used Sephadex G-50 beads. These can be impaled without apparent damage, even near the points of penetration of the needle. Sepha-

dex G-25 beads can be impaled, but show some degree of splitting immediately around the needle. With Sephadex G-200 beads, there is a tendency for the needle to thrust the bead down the neck of the jig rather than to penetrate it; this can be prevented by placing a minute piece of cellulose tissue in the opening of the neck.

Once impaled, a bead can be stored almost indefinitely by keeping the slide in 1:15000 merthiolate in water.

All except preliminary measurements were made at $17 \pm 1^\circ\text{C}$.

Materials

Sephadex. This was neutral Sephadex G (Pharmacia Fine Chemicals, Uppsala, Sweden), of 'coarse' grade where available.

Dextran. Dextran 500 (batch T5406, M_w 420000, M_n 153000) was from Pharmacia. Dextran fractions 52.8, 37.5, 27.2 and 19.7 were those used by Edmond & Ogston (1968).

PEG* 20000. This was obtained from Fluka A.-G.

* Abbreviation: PEG, polyethylene glycol.

Chemische Fabrik, Buchs, Switzerland. This was the same material as that used by Edmond & Ogston (1968), wrongly described by them as being from Carbide and Carbon Chemicals Co., New York, N.Y., U.S.A.

PEG 6000. This was the same material used by Nichol, Ogston & Preston (1967) and by Edmond & Ogston (1968), from Carbide and Carbon Chemicals Co., New York, N.Y., U.S.A.

Polyvinyl alcohol. This was obtained from Tokyo Chemical Industry Co. Ltd., Tokyo, Japan. Its degree of polymerization was stated to be 1750 ± 50 . Its moisture content (4.20%) was measured by drying a sample to constant weight at 105°C in air.

Bovine serum albumin. This was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. lot 108B-8170.

Rabbit immunoglobulin G. This was a gift from Dr E. R. B. Graham.

Solutions. These were made up by weight. Dextran and PEG were dissolved in the cold. The polyvinyl alcohol required heating to 100°C on a water bath for some hours. The water contents of the dextrans were determined by measuring the optical rotation of solutions in a 1 dm cell in a Perkin-Elmer model 141 polarimeter at 436 nm taking $[\alpha]_{436}^{25}$ as 389° . The moisture content of bovine serum albumin was determined by measuring the extinction of a suitable solution at 280 nm in a Beckman DU spectrophotometer, assuming a specific extinction coefficient of 6.41. In calculating final concentrations allowance was made for the water contents of the solutes. Concentrations, w/w, were converted into concentrations w/v by use of the partial specific volumes of the solutes (dextrans 0.60; PEG 0.837; polyvinyl alcohol 0.769). The partial specific volume of polyvinyl alcohol was determined by use of a 27 ml pycnometer.

RESULTS AND DISCUSSION

Immobilization of beads. Sephadex beads contain microscopic heterogeneities which look, in a photomicrograph, like craters on the moon's surface. Each bead has a characteristic crater pattern by which any change of orientation is easily recognized. The unchanging crater pattern of an impaled bead shows that it remains in a fixed orientation even during many changes of solution, so that the same diameter is always measured.

A bead, once impaled, does not slip along the needle; a shrunken bead has axial lobes adhering to the needle, with an approximately spherical central section (Plate 1). Although the ends of the lobes may creep slightly along the needle, this does not affect the reproducibility of diameter measurements at 90° to the needle.

Time required for osmotic equilibration. An exceptionally large Sephadex G-50 bead ($524\mu\text{m}$ diam. in water) was impaled and equilibrated in the cell in water. The water was removed and quickly replaced by a solution of PEG 20000 (0.109 g/ml; $\pi/RT = 3.816 \times 10^{-5} \text{ mol/ml}$). Photographs were taken at intervals over a period of 20 min. The PEG solution was then removed and replaced by

Fig. 2. Bead diameter of Sephadex G-50, expressed as d/d_0 (d is the diameter in solution, d_0 in water), plotted against time of immersion in PEG 20000 solution (●) and subsequently in water (○).

water (without rinsing the cell) and the re-swelling process followed. The results are shown in Fig. 2. The diameters became constant, within the experimental error, within 7 min. In all subsequent measurements, 10 min or more was allowed for equilibration even with smaller beads. However, since equilibration is essentially a diffusion process, the time of equilibration should vary as the square of the bead diameter: e.g., 4 min should be sufficient for a $300\mu\text{m}$ diameter bead.

Criteria of penetration of the bead by solute. If the solute penetrates the bead to a significant extent, the internal osmotic pressure of the bead is increased, not only by the direct contribution of the solute to the internal osmotic pressure, but also by the contribution due to the thermodynamic interaction between the internal solute and the Sephadex (Ogston & Silpananta, 1970). These effects will lead to underestimation of the osmotic pressure of the external solution. Calculation on the basis of the interaction coefficients measured by Ogston & Silpananta (1970), shows that the partition coefficient of the solute (K_{av}) should be less than 0.01 if the external osmotic pressure is to be measured with reasonable accuracy. Such a low value of K_{av} would be difficult to measure directly. However, three other criteria are available.

(i) Non-penetrating solutes of differing molecular size, whose osmotic characteristics are already known, should give points lying on a single curve of bead diameter against osmotic pressure, whereas a solute that penetrates to a significant extent will give points lying above this curve. Measurements made on the same Sephadex G-50 bead show (Fig. 3) that though measurements with Dextran 500 and PEG 20000 lie on the same curve, those with Dextran 52.8 lie slightly above it and those with Dextrans 37.5 and 19.7 and with bovine serum albumin lie substantially above it. As would be

Fig. 3. Diameters of a single bead of Sephadex G-50, expressed as d/d_0 , plotted against osmotic pressure of the solution (●, PEG 20000; ○, Dextran 500; ▲, Dextran 52.8; △, Dextran 37.5; ■, Dextran 19.7; □, bovine serum albumin in 0.2M-NaCl). Values for Dextran 27.2 fell between those for Dextran 37.5 and Dextran 19.7, but are not shown.

expected, the deviations with the dextrans increase as the molecular size falls. By this criterion, Dextran 52.8, whose effective radius is about 55 Å (Edmond & Ogston, 1968) is near the lower limit of molecular size for measurement with Sephadex G-50 (cf. Edmond *et al.* 1968; Edmond & Ogston, 1968). However, PEG 20000, which has a slightly lower effective radius (Edmond & Ogston, 1968), does not appear to penetrate Sephadex G-50 to a significant extent.

(ii) Since solvent (water) has a higher rate of diffusion in Sephadex than that of a penetrating macromolecular solute, the former will equilibrate more rapidly than the latter. As a result, a Sephadex bead brought from solvent into solution will show a transient shrinkage to a size smaller than the final value; conversely, a bead fully equilibrated in solution will show a transient over-swelling on being brought into solvent. This is illustrated in Fig. 4, which shows three experiments performed as described earlier for PEG 20000; we see that Dextran 37.5 and PEG 6000 penetrate Sephadex G-50 significantly (contrast PEG 20000, Fig. 2), whereas Dextran 52.8 shows only slight penetration. A similar experiment showed that rabbit immunoglobulin G does not penetrate Sephadex G-50.

(iii) For the same reason, when a bead is taken through a series of solutions of a penetrating solute of increasing concentration, being left in each only long enough for equilibration of the solvent, its diameter in each solution will be less than when it is taken through them in reverse order. Use of too

Fig. 4. Bead diameter of Sephadex G-50, expressed as d/d_0 , plotted against time of immersion in solution (●) and subsequently in water (○). (a) Dextran 37.5, 0.094 g/ml; (b) Dextran 52.8, 0.091 g/ml; (c) PEG 6000, 0.048 g/ml.

short equilibration times with a non-penetrating solute will have the opposite effect.

Coincidence of values obtained in the two directions (with sufficient time to equilibrate solvent) is therefore evidence that penetration is not significant. We therefore followed the routine of making each set of measurements from solvent through to the most concentrated solution, and then back in the reverse order. This criterion (because of the partial equilibration of solute that takes place) is less sensitive than the other criteria.

Osmotic pressures of solutions. These were calculated by use of the equation:

$$\frac{\pi}{RT} = \frac{c}{\bar{M}_n} + A_2 c^2 + A_3 c^3 \quad (1a)$$

$$= \frac{c}{\bar{M}_n} + \frac{\alpha_1^*}{2} c^2 + \frac{2\alpha_2^*}{3} c^3 \quad (1b)$$

where $\bar{M}_n$ is the number-average molecular weight, c is the concentration (g/ml) of solution, A_2 and A_3 are the conventional virial coefficients and α_1^* and α_2^* are the non-ideality coefficients defined by Nichol *et al.* (1967). Values of α_1^* and α_2^* for the dextrans and PEGs were those determined, by

EXPLANATION OF PLATE I

Photomicrograph of an impaled Sephadex G-50 bead, equilibrated in (a) water, and (b) PEG 20000 (0.109 g/ml); notice the characteristic 'crater pattern'.

Table 1. *Summary of constants for solutes used*

Material	$10^{-3} \bar{M}_w$ (g mol ⁻¹)	$10^{-3} \bar{M}_n$ (g mol ⁻¹)	$10^3 \alpha_1^*$ (mol cm ³ g ⁻²)	$10^3 \alpha_2^*$ (mol cm ⁶ g ⁻³)
Dextran 500	420 **	153 **	0.682§	5.03§
Dextran 52.8	77.5¶	52.8¶	1.02 §	2.99§
Dextran 37.5	50.5¶	37.5¶	1.11 ‡	2.71‡
Dextran 27.2	36.5¶	27.2¶	1.09 ‡	2.89‡
Dextran 19.7	26.5¶	19.7¶	1.17 ‡	2.88‡
PEG 20000		19.4‡	5.18 ‡	20.2 ‡
PEG 6000		8.0*	5.0	20
Bovine serum albumin in 0.2M-NaCl		73.2†	1.0 ‡	

* Alexandrowicz (1959).

† Davies (1966).

‡ Edmond & Ogston (1968).

§ Edmond *et al.* (1968).|| Nichol *et al.* (1967).

¶ K. Granath (personal communication).

** Values supplied by Pharmacia.

Fig. 5. Calibration curve for a single Sephadex G-50 bead obtained with solutions of PEG 20000. Bead diameter in PEG solution is expressed as d/d_0 versus osmotic pressure of PEG solution.

sedimentation equilibrium, by Nichol *et al.* (1967), Edmond *et al.* (1968) and Edmond & Ogston (1968). The values are summarized in Table 1.

Calibration. Since individual beads of a given grade differ significantly from each other it is necessary to calibrate each bead, over the range of osmotic pressure required, with solutions of a solute known not to penetrate and whose osmotic properties are known. We know that PEG 20000 does not penetrate Sephadex G-50 (Fig. 2); this conclusion is supported by the coincidence of the calibration curves obtained with Dextran 500 and with PEG 20000 (Fig. 3). Because solutions of PEG 20000 are less viscous for a given osmotic pressure, we used these for routine calibration of Sephadex G-50 beads. The ratio of the bead diameter to that in solvent (d/d_0) is plotted against π/RT : Fig. 5 is a typical example.

Other grades of Sephadex. Sephadex G-25 shows

too little shrinkage to be used for measuring osmotic pressures in the range studied here. It should be possible to use higher grades of Sephadex with solutes of higher molecular weight, since the lower osmotic pressure for a given weight-concentration of solute would be offset by the relatively greater change of diameter of the bead with osmotic pressure (Edmond *et al.* 1968). This is illustrated in Table 2. Assuming that the absolute error of measuring bead diameter is independent of the grade of Sephadex, the absolute error of measuring osmotic pressure is inversely proportional to the change in bead diameter produced by a given osmotic pressure; this is obtained from the data of Edmond *et al.* (1968). The lower limits of protein molecular weight for $K_{av} \approx 0$ are taken from this work and from Andrews (1964, 1965). The last column of the table shows that the concentration of solute at which the osmotic pressure equals the standard error of its measurement is approximately independent of molecular weight.

Dextran 500 is not suitable for precise calibration of higher grade beads, as its molecular weight distribution includes a significant fraction of smaller molecules. We have found (from time of swelling experiments similar to those shown in Fig. 4) that there is very slight but significant penetration of Sephadex G-100, Sephadex G-150 and Sephadex G-200 beads by Dextran 500; this is consistent with the chromatographic and other results of Edmond *et al.* (1968) on Sephadex G-200.

Osmotic pressures, molecular weight and second virial coefficient of polyvinyl alcohol. Polyvinyl alcohol was chosen as a suitable test solute. It passed the tests for non-penetration, it has not too high a molecular weight and it was expected to have a fairly high second virial coefficient. Twenty-two

Table 2. *Approximate sensitivities of different Sephadex grades*

Grade	d/d_0 in 2% Dextran 500*	$\Delta(d/d_0)$	Absolute error† (cmH ₂ O)	$10^6 \times$ Absolute error† (mol ml ⁻¹)	Protein mol.wt. limit (g mol ⁻¹)	$10^3 \times$ Limit concn.¶ (g ml ⁻¹)
G-50	0.995	0.005	1.3	0.052	$10^5 \ddagger$	5.2
G-75	0.988	0.012	0.54	0.022	$1.1 \times 10^5 \S$	2.3
G-100	0.980	0.020	0.32	0.013	$\sim 4 \times 10^5 \parallel$	5.2
G-150	0.970	0.030	0.22	0.009		
G-200	0.950	0.050	0.13	0.005	$10^6 \parallel$	5

* $10^6 \pi/RT = 0.293$.

† Value for Sephadex G-50 is from this work; values for other grades calculated from the Sephadex G-50 value and the data in column 3.

‡ This work.

§ Andrews (1964).

|| Andrews (1965).

¶ Osmotic pressure = error of its measurement.

Fig. 6. Osmotic pressure of polyvinyl alcohol solutions versus concentration (c , g/ml). The line is that from the statistical fitting (see the text). For experimental details see the text.

measurements were made on ten solutions of concentrations between 0.5 and 6.8×10^{-2} g/ml; values of π/RT were read from a calibration curve obtained with solutions of PEG 20 000, and are shown plotted in Fig. 6. Least-squares fitting to eqn. (1a), omitting the c^3 term, gave:

$$\frac{1}{\bar{M}_n} = (2.32 \pm \text{s.e. } 0.17) \times 10^{-5};$$

$$\bar{M}_n = 43000 \pm 3000; \quad A_2 = (6.0 \pm \text{s.e. } 0.24) \times 10^{-4};$$

standard error of a single determination of osmotic pressure (from the residual variance) 4.6×10^{-8} mol/ml or a standard error of 1.15×10^3 dyn/cm² in π .

Precision of measurement. Repeated measurements of a single photograph showed that the standard error of a single measurement of diameter is $4.7 \mu\text{m}$. Since the magnification from object to negative is $\times 40$, this corresponds to a standard

error of $0.12 \mu\text{m}$ in the diameter of the bead or, for a bead of $400 \mu\text{m}$ diameter, a coefficient of variation of 3.0×10^{-4} . The standard error of a value of d/d_0 based on a single measurement of each diameter is therefore 4.2×10^{-4} . From the average gradient of the calibration curve (d/d_0 against $10^6 \pi/RT$), this would correspond to a standard error of 1.4×10^3 dyn/cm² in the estimation of π , or about 1.4 cmH₂O.

Eleven pairs of measurements obtained during forward and reverse passage through a series of solutions of PEG 20 000 up to 0.102 g/ml ($10^6 \pi/RT = 33.9$ mol/ml) gave an estimated standard error of a single diameter measurement of $9 \mu\text{m}$ and ten pairs of measurements on solutions of polyvinyl alcohol up to 0.068 g/ml gave an estimate of $7 \mu\text{m}$; these correspond to standard errors in π of 1.3 and 1.6×10^3 dyn/cm² respectively. The residual variance (see above) estimated by fitting the polyvinyl alcohol values to eqn. (1a) (which is statistically a more efficient estimate of random error) is in good agreement with these estimates.

We made photomicrographs of a particularly small Sephadex G-75 bead at a magnification of $\times 100$; the standard error for a single diameter measurement on the negative was $10 \mu\text{m}$, or the same error of $0.1 \mu\text{m}$ in the bead diameter as at lower magnification. This suggests that the precision is limited by the resolving power of the microscope.

Effect of drying the bead. The accidental drying-out of one osmometer prompted us to test the effect of drying on the osmotic behaviour of the bead. Diameters of a Sephadex G-50 bead were measured in water and in seven different solutions of PEG 20 000 before and after drying the bead over phosphorus pentoxide for one month. On bringing the dried bead back into water it swelled to its original diameter within 15 min. Comparison of the eight

pairs of diameters gave an estimated standard error of measurement of a single diameter of $12\mu\text{m}$. This shows that the calibration of a bead is little if at all affected by drying.

General discussion. Immobilization of the bead has decreased the error of measuring an osmotic pressure to a value not much greater than that which arises from the measurement of a given diameter, which, in turn, is near the resolving power of the microscope. The method is still 10–100 times less accurate than the best conventional techniques of osmometry, but it is accurate enough to be useful for many purposes; it requires no expensive equipment other than a good photographic microscope and comparator; determinations can be made very easily and quickly; and the volume of solution required ($40\mu\text{l}$ in most of our experiments) can easily be decreased to $10\mu\text{l}$, so that only the order of 1 mg of solute is needed. The method is particularly useful for measuring osmotic pressures higher than those accessible to conventional techniques. We have measured osmotic pressures up to about one atmosphere; the upper limit seems to be determined only by the viscosity of the solution.

Because of the severe criterion of non-penetration ($K_{av} \geq 0.01$) required for osmotic measurements, the lower limit of molecular weight of solute which will not significantly penetrate a given grade of Sephadex (the 'exclusion limit' molecular weight) will be greater than the analogous value determined from chromatography. For Sephadex G-50, the exclusion limit molecular weight obtained from osmotic measurements is about 60 000 for dextrans. A protein of the same effective size will have a molecular weight of about 10^5 ; we have shown that rabbit immunoglobulin G, which has a molecular weight of 145 000 (Noelken, Nelson, Buckley & Tanford, 1965), is excluded from Sephadex G-50.

For linear polymers with a randomly coiled configuration in solution, such as PEG and polyvinyl alcohol, the exclusion limit molecular weight is lower than that for dextrans.

In most of our experiments we have used neutral solutes, with no supporting salt. Although it may be expected that salts will interact thermodynamically with Sephadex and will therefore affect the osmotic balance, we have found evidence (A. G. Ogston & J. D. Wells, unpublished work) that this effect is negligible for the purposes of osmotic pressure measurement. Osmotic pressures of polyelectrolyte solutions will of course (as in other methods of colloid osmometry) be subject to the Gibbs–Donnan equilibrium of permeant ions (Edmond & Ogston, 1970).

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