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The Interaction between Polysaccharides and other Macromolecules

4. THE OSMOTIC PRESSURE OF MIXTURES OF SERUM ALBUMIN AND HYALURONIC ACID*

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Ogston & Phelps (1961) found that hyaluronic acid markedly affects the partition of diffusible macromolecules between solutions of the polysaccharide and buffer. The authors explain the phenomenon as a steric exclusion of macromolecular solutes from solutions containing randomly coiled hyaluronic acid chains. From these results one can expect that hyaluronic acid and similar substances would exert a significant influence on the thermodynamic properties of solutions containing other macromolecules. The investigation reported in the present paper shows that solutions containing both hyaluronic acid and serum albumin have osmotic pressures in excess of the sum of osmotic pressures of solutions containing hyaluronic acid and serum albumin separately at the same concentrations.

The influence of the 'effective volume' of a solute of finite concentration on the osmotic pressure of a solution has been treated by a number of authors (see, for example, Scatchard, 1946; Doty & Edsall, 1951; Edsall, 1953; Flory, 1953). Christiansen (1960), using the experimental results of Jensen & Marcker (1958), discussed the phenomenon in hyaluronic acid solutions. All these treatments, however, have been limited to the problem of binary systems containing the solvent and a single species of macromolecules. Ogston (1962) discussed from a theoretical point of view the thermodynamic properties of ternary systems with special reference to buffer solutions containing hyaluronic acid and serum albumin. The study of this problem was approached independently by the present authors. Experimental work was planned and carried out by one of us (T. C. L.) and a theoretical treatment of the thermodynamic properties of systems containing hyaluronic acid and serum albumin was made by the other (Ogston, 1962). The present paper combines the two approaches.

* Part 3: Laurent & Persson (1963).

EXPERIMENTAL

Materials

The human serum albumin was the same as that used by Laurent & Persson (1963). Its moisture content was determined to be 5·1% by drying for 7 hr. over phosphorus pentoxide at 30° in high vacuum and a stock solution of known concentration was then made up by weighing.

Protein-free hyaluronic acid was prepared from umbilical cord by procedures based on Scott's work (Scott, 1960; Laurent, Ryan & Pietruszkiewicz, 1960). Its limiting viscosity number, 2400 ml./g., corresponded to a mol.wt. $1{\cdot}5 \times 10^6$ (Laurent *et al.* 1960). The concentrations of the stock solutions of hyaluronic acid were determined by the carbazole method (Dische, 1947; Laurent *et al.* 1960).

The samples for measurements were prepared by mixing the stock solutions and the solvent in the desired proportions. The solutions were weighed rather than pipetted because of the high viscosity of the hyaluronic acid.

Method

Osmometric measurements were performed in commercial static capillary osmometers described by Hellfritz (1951). The capillaries had bores of 0·6 mm. Each pair of capillaries used in experiments was tested for uniformity by measuring the capillary rise on immersion in toluene at different depths. Visking cellophan casing, size 36/32 (Visking Co., Division of Union Carbide and Carbon Corp., Chicago 38, Ill., U.S.A.), was used as the membrane material. The same roll of casing was used throughout the investigation. The osmometers were assembled as described by Hellfritz (1951) with a silicone lubricant used to seal the joints. To charge the osmometers with the highly viscous hyaluronic acid solutions without the formation of air bubbles, the following technique was adopted. Each solution was centrifuged and then withdrawn from the centrifuge tube with a pipette with a capillary 5 cm. in length and 0·1 cm. in width at its tip, and delivered into the osmometer through the capillary under a pressure of 1–2 kg./cm.^{2}. Toluene was layered over the water surfaces in both the capillaries and the osmometer vessel. The experiments were performed in a constant-temperature bath at 20·0° and readings were taken with a cathetometer. Equilibrium was usually reached after about 48 hr. but occasionally took longer. The osmotic pressure was calculated from the differences in the

liquid levels in the two capillaries and the densities of the solution, the solvent and the toluene. The densities of the solutions were calculated from the concentrations and partial specific volumes of albumin and hyaluronic acid. No correction for asymmetry pressure was made.

All determinations were made in a solvent of 0·5 M-sodium chloride, 6·67 mM-phosphate buffer, pH 7·6, and 25% saturated 5,7-dichloro-8-quinolinol The latter is a bacteriostat and chelating agent.

RESULTS

Terminology. Ogston's (1962) terminology is used. Hyaluronic acid is represented as component 3, serum albumin as component 2 and water as component 1. Concentrations measured in g./ml. are represented by c_3 etc., whereas molal concentrations are m_3 etc. The osmotic pressure of a solution of serum albumin (dynes/cm.^{2}) is written $^2\pi_1$, of hyaluronic acid $^3\pi_1$ and of a mixed solution $^{2,3}\pi_1$. (In all experiments the membrane was permeable only to solvent.) The solvent (water plus salt) is regarded as a single component.

As would be expected, the osmotic pressures of hyaluronic acid solutions increase non-linearly with concentration (Fig. 1: lower curve). When $^3\pi_1/c_3$ is plotted against c_3 (Fig. 2: lower curve) a straight line is obtained, expressed by:

$$\frac{^3\pi_1}{c_3} = 5{\cdot}9 \times 10^4 + 6{\cdot}3 \times 10^7\, c_3 \qquad (1)$$

This is of the form [Ogston (1962), taking $v_1 = M_1$ for water]:

$$\frac{1000}{RT}\frac{^3\pi_1}{m_3} = 1 + (\tfrac{1}{2}d)\, m_3 \qquad (1a)$$

with the molecular weight of hyaluronic acid, M_3, equal to $4{\cdot}13 \times 10^5$ and d, which represents the second virial coefficient of hyaluronic acid, equal to $8{\cdot}8 \times 10^5$.

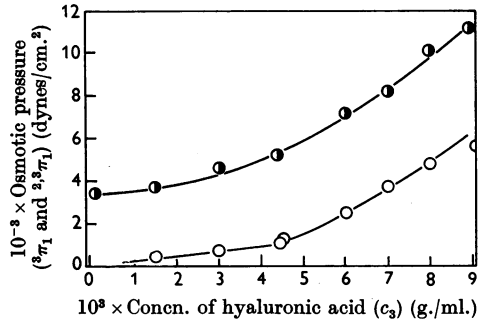

Fig. 1. Osmotic pressure of hyaluronic acid (○) and of mixtures of $9{\cdot}5 \times 10^{-3}$ g. of albumin/ml. and hyaluronic acid (◑) plotted against the hyaluronic acid concentration. Details are given in the text.

The osmotic pressures of solutions of albumin are shown in Fig. 3 (lowest curve). $^2\pi_1/c_2$ plotted against c_2 (Fig. 4: lowest curve) is expressed by:

$$\frac{^2\pi_1}{c_2} = 3{\cdot}43 \times 10^5 \qquad (2)$$

which is equivalent to:

$$\frac{1000}{RT}\frac{^2\pi_1}{m_2} = 1 \qquad (2a)$$

with $M_2 = 7{\cdot}1 \times 10^4$, in fair agreement with published data (see Edsall, 1953).

The osmotic pressures of mixed solutions were measured in three series of experiments. In the first, the albumin concentration was constant at $9{\cdot}5 \times 10^{-3}$ g./ml. and the hyaluronic acid concentration was varied between 1·5 and 9×10^{-3} g./ml. at the same concentrations as were used with hyaluronic acid alone (Fig. 1: upper curve). In the other two series, the hyaluronic acid concentrations

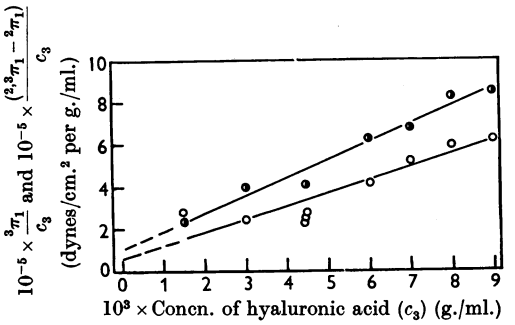

Fig. 2. Reduced osmotic pressure of hyaluronic acid (○), and $(^{2,3}\pi_1 - {}^2\pi_1)/c_3$ for a series of measurements, where the albumin concentration was $9{\cdot}5 \times 10^{-2}$ g./ml. (◑), plotted against the hyaluronic acid concentration. Details are given in the text.

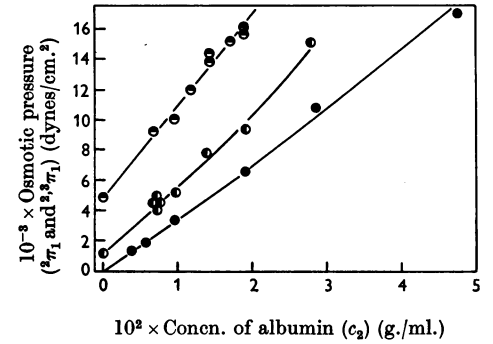

Fig. 3. Osmotic pressure of albumin (●) and of mixtures of albumin and $4{\cdot}4 \times 10^{-3}$ g. of hyaluronic acid/ml. (◐) and 8×10^{-3} g. of hyaluronic acid/ml. (◓) respectively, plotted against the albumin concentration. Details are given in the text.

were kept constant at 4·4 and 8·0 × 10^{-3} g./ml. respectively at the same concentrations of albumin as were used for albumin alone (Fig. 3: upper two curves). The same results are also plotted as $({}^{2,3}\pi_1 - {}^{3}\pi_1)/c_2$ against c_2 in the two upper curves of Fig. 4.

The results of the first series of experiments are also shown in the upper curve of Fig. 2, where $({}^{2,3}\pi_1 - {}^{2}\pi_1)/c_3$ (at corresponding values of c_2) is plotted against c_3; this plot in comparison with the lower curve of Fig. 2 can be regarded loosely as showing the changed osmotic behaviour of hyaluronic acid resulting from the presence of 9·5 × 10^{-3} g. of albumin/ml.

The intercept values from Fig. 4 are plotted in Fig. 5 against c_3, together with some other values at finite concentration of albumin, in the form of $({}^{2,3}\pi_1 - {}^{3}\pi_1)/c_2$ (at corresponding values of c_3); this plot, in comparison with the upper curve of Fig. 2, can be regarded loosely as indicating the changed osmotic behaviour of albumin resulting from the presence of hyaluronic acid.

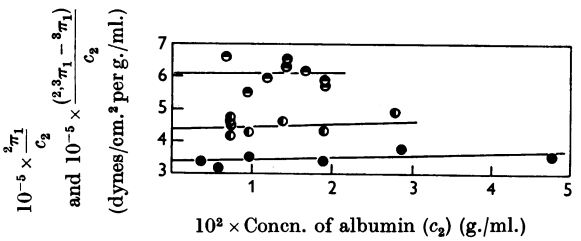

Fig. 4. Reduced osmotic pressure of albumin (●), and $({}^{2,3}\pi_1 - {}^{3}\pi_1)/c_2$ for two series of measurements, where the hyaluronic acid concentrations were 4·4 × 10^{-3} g./ml. (◐) and 8·0 × 10^{-3} g./ml. (◓) respectively, plotted against the albumin concentration. Details are given in the text.

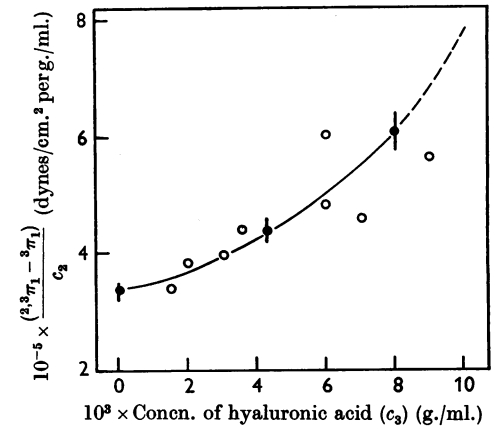

Fig. 5. Changed osmotic behaviour of albumin resulting from the presence of hyaluronic acid. ●, Intercept values from Fig. 4; ○, measurements made at an albumin concentration of 9·5 × 10^{-3} g./ml. Details are given in the text.

DISCUSSION

General

A constant exchange and partition of water and low-molecular-weight solutes occurs between the extracellular space of connective tissue and the vascular system. It has been assumed that the process is regulated by the hydrostatic and colloid osmotic pressures in these compartments (Evans, 1941). To elucidate this mechanism it is necessary to ascertain the magnitude of the role of the main macromolecular components of the ground substance of connective tissue, i.e. polysaccharides and proteins, in maintaining and controlling osmotic pressures. The osmotic behaviour of mixtures of serum albumin and hyaluronic acid has been investigated as a first approach. Albumin was selected for study as it is the component which contributes most of the colloid osmotic pressure of serum and has been identified in the extracellular space of several connective tissues. Experiments were performed in a medium of high ionic strength to minimize charge interaction between the solute molecules and also to decrease the contribution of the Donnan effect to the osmotic pressure. In this way an interaction due to steric exclusion would give the main effect.

The accuracy of the osmometric determinations is indicated by the measurements made on pure serum albumin; a molecular weight in close agreement with other published reports was obtained from the experimental data. A greater amount of scatter was observed in the results from experiments with hyaluronic acid solutions; this is probably a reflexion of the difficulties of working with an extremely viscous material.

It is to be expected that deviations of the sort observed here will affect osmotic pressures in physiological systems; for example, Ropes, Bennett & Bauer (1939) found osmotic pressures of synovial fluids greater than could be accounted for by the contributions of hyaluronic acid and serum proteins. Caution is required in making predictions about osmotic balances *in vivo* since these will depend on the permeability of the membrane across which the pressure is considered and, in general, on a steady-state rather than equilibrium partition of materials.

Thermodynamic interpretation

The points in Fig. 5 corresponding to the extrapolated values of Fig. 4 (zero concentration of albumin) are fitted by the expression:

$$\frac{{}^{2,3}\pi_1 - {}^{3}\pi_1}{c_2} = 3{\cdot}43 \times 10^5 + 8{\cdot}35 \times 10^6\, c_3 + 3{\cdot}17 \times 10^9\, c_3^2 \qquad (3)$$

and the corresponding molal form is:

$$\frac{1000}{RT}\frac{^{2,3}\pi_1 - {}^{3}\pi_1}{m_2} = 1 + a_1 m_3 + a_2 m_3^2 \qquad (3a)$$

where a_1 and a_2 are constants having values $1{\cdot}01 \times 10^4$ and $1{\cdot}57 \times 10^9$, taking M_2 to be $7{\cdot}1 \times 10^4$ (eqn. $2a$) and M_3 to be $4{\cdot}13 \times 10^5$ (eqn. $1a$). There does not appear to be any systematic departure of the other points from this curve. Now, since:

$$\frac{1000}{RT}\,(^{2,3}\pi_1 - {}^{3}\pi_1) = \left(m_2 + m_3 - \frac{1000}{M_1}\ln \gamma_1^1\right) - \left(m_3 - \frac{1000}{M_1}\ln \gamma_1\right)$$

(where γ_1^1 and γ_1 are the activity coefficients of water in solution of 2 and 3 and in solution of 3 respectively), the forms of eqn. ($1a$), eqn. ($2a$) and eqn. ($3a$) require for the general expression of γ_1:

$$-\frac{1000}{M_1}\ln \gamma_1 = (\tfrac{1}{2}d)\, m_3^2 + a_1 m_2 m_3 + a_2 m_2 m_3^2 \qquad (4)$$

and it follows necessarily for thermodynamic consistency that (Ogston, 1962):

$$\ln \gamma_2 = a_1 m_3 + (\tfrac{1}{2}a_2)\, m_3^2 \qquad (5)$$

$$\ln \gamma_3 = d m_3 + a_1 m_2 + a_2 m_2 m_3 \qquad (6)$$

Finally, the upper curve in Fig. 2 is represented by:

$$\frac{^{2,3}\pi_1 - {}^{2}\pi_1}{c_3} = 1{\cdot}08 \times 10^5 + 8{\cdot}4 \times 10^7\, c_3 \qquad (7)$$

whereas eqn. (4) predicts:

$$\frac{1000}{RT}\frac{^{2,3}\pi_1 - {}^{2}\pi_1}{m_3} = (1 + a_1 m_2) + m_3(\tfrac{1}{2}d + a_2 m_2) \qquad (7a)$$

It is seen that eqn. ($7a$) correctly predicts that the upper curve in Fig. 2 should be linear in c_3, and that its slope and intercept should differ from those of the lower curve, which is expressed by eqn. (1) and eqn. ($1a$). From eqn. (7) and eqn. ($7a$), by using the previously determined value of d, independent estimates are obtained of $a_1 = 0{\cdot}62 \times 10^4$ and $a_2 = 1{\cdot}1 \times 10^9$.

The osmotic results are therefore self-consistent in form and reasonably so with regard to the independent estimates of the parameters. The value of M_3 obtained from the osmotic data is a good deal lower than that of $1{\cdot}5 \times 10^6$ estimated viscometrically. The osmotic estimate is not very accurate, being based on the extrapolation of the lower curve in Fig. 2; one might expect a lower value from an osmotic than from a viscometric estimate, since the former measures a number-average molecular weight. In any case, the value taken for M_3 does not affect the consistency of the data, a change in M_3 merely leading to changes of the numerical values of a_1, a_2 and d.

From the presence of a term in m_3^2 in eqn. (5) which results from the curvature of Fig. 5 and from the difference of slopes in Fig. 2, one would predict that in partition experiments of the type done by Ogston & Phelps (1961) the plots of ln K against c_3 should be curved instead of approximately linear as they found. Also, the value of d ($8{\cdot}9 \times 10^5$) obtained from the osmotic results is much lower than would be expected from the Flory–Huggins treatment (Flory, 1953), namely 3×10^8. We have at present no explanation for these discrepancies; it can be stated, however, that, though the simplification of treating the system as ternary instead of quarternary could explain the low value of d, application of a quaternary treatment would not account for the curvature of Fig. 5.

Exclusion of albumin by hyaluronic acid

The deviations of the osmotic properties of solutions of hyaluronic acid and albumin from ideality may be regarded as resulting from the 'exclusion' of albumin from part of the solution occupied by the hyaluronic acid. If K is the resultant partition coefficient of albumin between buffer solution and solution of hyaluronic acid, and ϵ is the fraction of volume excluded, then:

$$K = \gamma_2 = \frac{1}{1-\epsilon}$$

From eqn. (5) it is seen that ϵ is not a linear function of the concentration of hyaluronic acid, but, at zero concentration of the latter,

$$\epsilon/m_3 \to a_1 \quad \text{or} \quad \epsilon/c_3 \to 1000 a_1/M_3$$

as $m_3 \to 0$, so that ϵ/c_3 has a value of about 25 ml./g. at zero concentration of hyaluronic acid. This exclusion is much lower than the corresponding value derived from the partition data of Ogston & Phelps (1961), and would correspond to much lower partition coefficients.

SUMMARY

1. An investigation of the osmotic behaviour of mixtures of hyaluronic acid and serum albumin has shown that their osmotic pressures are significantly greater than the sum of the osmotic pressures of solutions containing hyaluronic acid and serum albumin separately at the same concentrations.

2. A thermodynamic analysis of the results shows that they are self-consistent in form and reasonably so with regard to independent estimates of certain parameters.

3. The results can be interpreted in terms of an 'exclusion' of albumin from part of the solution occupied by hyaluronic acid. The excluded volume was calculated to be approximately 25 ml. of hyaluronic acid/g. at low polysaccharide concentrations.

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The Interaction between Polysaccharides and other Macromolecules

5. THE SOLUBILITY OF PROTEINS IN THE PRESENCE OF DEXTRAN

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Experimental studies on aqueous mixtures of hyaluronic acid and proteins have indicated that a solute with a large effective volume, such as hyaluronic acid, exerts a profound influence on the relationship between other macromolecular solutes and the solvent (Ogston & Phelps, 1961; Laurent & Ogston, 1963). Ogston & Phelps have interpreted the observed results as a steric exclusion of foreign particles from the hyaluronic acid network, and have shown that this effect is more pronounced the larger the particle. If such a mechanism is operative it would mean that hyaluronic acid affects the activity of other substances and thereby should alter their solubility. To test this hypothesis, the solubilities of different proteins have been measured in the presence of a polysaccharide. Dextran was selected rather than hyaluronic acid to avoid any charge interaction between the macromolecules.

EXPERIMENTAL

Materials

Dextran. Preparations of dextran, a polyglucose, were obtained from AB Pharmacia, Uppsala, Sweden. The two commercially available preparations, dextran-150 and dextran-500, have limiting-viscosity numbers of 36 and 48 ml./g., and weight-average molecular weights of 153000 and 450000 respectively. Through the courtesy of Dr K. Granath, three other preparations with higher molecular weights were obtained, namely dextran I, dextran II and dextran III. These had limiting-viscosity numbers of 94, 117 and 142 ml./g., and molecular weights of $6{\cdot}9 \times 10^6$, 13×10^6 and 25×10^6–50×10^6 respectively. The manufacturer kindly determined the degree of branching and found that approx. 94% of the glucosidic bonds in the various preparations were (1→6) linkages.

Ficoll. Ficoll, which is a synthetic polysucrose with a very compact structure, was also obtained from Dr Granath. It had a molecular weight of approx. 10^6 and a limiting-viscosity number of 14 ml./g.

D-*Glucose and* L-*tyrosine.* These were of analytical grade.

Proteins. The same preparations of human serum albumin, human γ-globulin, human fibrinogen and bovine α-crystallin described by Laurent, Björk, Pietruszkiewicz & Persson (1963) and Laurent & Persson (1963) were employed. Their molecular weights are 69000, 160000, 341000 (see, for example, Phelps & Putnam, 1960) and 830000 (I. Björk, unpublished work) respectively.

Methods

Determination of the solubility of proteins. Stock solutions of proteins (3×10^{-2}–10×10^{-2} g./ml.) were made up in water, 0·1 M-phosphate buffer, pH 7·6, or 0·1 M-acetate buffer, pH 4·7.

Stock solutions (100 ml.) of the dextrans, ficoll and glucose (7×10^{-2}–12×10^{-2} g./ml.) were prepared by dissolving these compounds in a small volume of water; with dextran, this was done at 100°. Then the ammonium sulphate and in most experiments either phosphate buffer, pH 7·6, or acetate buffer, pH 4·7, were added, and the