The Exclusion of Protein by Hyaluronic Acid

MEASUREMENT BY LIGHT SCATTERING

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SUMMARY

Light scattering measurements have been made on solutions of hyaluronic acid in the presence and absence of bovine serum albumin. The results are interpreted as showing an exclusion of bovine serum albumin from molecules of hyaluronic acid, the magnitude of which agrees with most other measurements of exclusion. The theory of the effect of heterogeneity of exclusion is given. The apparent heterogeneity that is observed is explained on statistical grounds. The results suggest that the main cause of exclusion is steric. No significant effect of bovine serum albumin on the configuration of the hyaluronic acid was observed.

Ogston and Phelps (1) showed by equilibrium dialysis that various solutes, including proteins, are partly excluded from solutions of hyaluronic acid; that is, the equilibrium concentration of diffusible solute in a hyaluronic acid solution is less than that in a diffusate in equilibrium with it. Ogston has discussed the steric (2) and the thermodynamic aspects (3) of this phenomenon; further thermodynamic discussion is given by Preston, Ogston, and Davies (4). Laurent (5) has confirmed the phenomenon both by equilibrium dialysis and by gel filtration, although the results that he obtained differed quantitatively from those of Ogston and Phelps (1). The predictions of Ogston (3) were tested by Laurent and Ogston (6), who reported measurements of osmotic pressures of mixed solutions of hyaluronic acid and bovine serum albumin; as Laurent (5) points out, these were in quantitative agreement with his own partition measurements. Preston et al. (4) also report osmotic pressure measurements on mixed solutions that are in substantial agreement with those of Laurent and Ogston (6). Similar effects occur in systems when the nondiffusible solute is a branched or linear polymer other than hyaluronic acid (5–7).

As we have pointed out (8) the measurement of light scattering allows this phenomenon to be observed at a molecular level and we now report measurements on hyaluronic acid in the presence of bovine serum albumin.

THEORY

The extrapolation of light scattering data on hyaluronic acid in buffer solution, to zero concentration and zero angle, by the method of Zimm (9) allows its weight average molecular weight to be measured, since

\[ \lim_{c_1 \to 0} \frac{k(dn/dc)_1}{\Delta R(0)_1} = \frac{1}{M_1} \]

where \( k \) is a constant, \( (dn/dc)_1 \) is the specific refractive increment of hyaluronic acid in a buffer measured against a diffusate, \( \Delta R(0)_1 \) is the extrapolated excess scattering, and \( M_1 \) is the weight average molecular weight. Similar extrapolation of the excess scattering due to hyaluronic acid in a solution of BSA of constant concentration, with the assumption that \( M_2 \) is unchanged, then gives

\[ \lim_{c_1 \to 0} \frac{k(dn/dc)_2}{\Delta R(0)_2} = \frac{1}{M_2} \]

The value of \( (dn/dc)_2 \) can be obtained from this. The subscript 12 refers to solution of BSA, 2, in buffer, 1. If BSA is excluded from the domains occupied by the hyaluronic acid molecules to a degree expressed by \( \varepsilon \) ml per g of hyaluronic acid, then (8)

\[ (dn/dc)_1 = (dn/dc)_2 - \varepsilon c_2 (dn/dc)_1 \]

where \( c_2 \) is the concentration of BSA and \( (dn/dc)_2 \) is its specific refractive increment in buffer solution. Thus \( \varepsilon \) can be determined.

This treatment does not allow for possible heterogeneity, two aspects of which should be considered: first, heterogeneity of the hyaluronic acid with respect to \( (dn/dc)_2 \), and, secondly, with respect to \( \varepsilon \). First, considering the effect of variation of \( (dn/dc)_2 \), Equation 1 is written

\[ \Delta R(0)_1 = \sum (dn/dc)_1 M_1 c_1 = \frac{k \sum (dn/dc)_1^2 M_1 c_1}{\sum M_1 c_1} \]

This shows that to determine the weight average molecular weight of a solute heterogeneous with respect to \( (dn/dc) \), we should use the mean square z-average value of \( (dn/dc) \). However, refractometric measurements actually give the weight
average value of (dn/dc3)1,w. The apparent value of the molecular weight which is obtained is therefore not M1,q, but

\[ M_1,q \approx \frac{(dn/dc3)_{f,s}^2}{(dn/dc3)_{t,s}^2} \]  

The apparent value of (dn/dc3)3,s that will be obtained in the presence of BSA is therefore (from Equations 2 and 5)

\[ (dn/dc3)_{f,s}^2 = \lim_{c_2 \to 0} \frac{\Delta R_0(\alpha)_{w}}{k_0 M_2} \]  

\[ = \frac{M_1,q}{M_2} (dn/dc3)_{t,s}^2 \]  

The mean square z-average of (dn/dc3)3,s is obtained from Equation 3.

\[ (dn/dc3)_{t,s}^2 = (dn/dc3)_{t,s}^2 + c_2(dn/dc3)_{f,s}^2 c_2^2 \]  

\[ - 2c_2(dn/dc3)_{f,s} c_2 \epsilon_2^2 \]  

When combined with Equations 5 and 6, this gives

\[ (dn/dc3)_{t,s}^2 = (dn/dc3)_{f,s}^2 + \frac{c_2(dn/dc3)_{f,s}^2 c_2^2}{(dn/dc3)_{t,s}^2} \]  

\[ - \{c_2(dn/dc3)_{f,s}^2 c_2^2 - 2c_2(dn/dc3)_{f,s} \epsilon_2 \epsilon_2^2 \} \]  

If the system is homogeneous with respect to (dn/dc3), this reduces to

\[ (dn/dc3)_{f,s}^2 = (dn/dc3)_{t,s}^2 + c_2^2(dn/dc3)_{f,s}^2 c_2^2 - 2c_2(dn/dc3)_{f,s} \epsilon_2 \epsilon_2^2 \]  

Comparison of Equation 3 in its squared form with Equations 8 and 9 shows that in all cases the variation of (dn/dc3)_{t,s}^2 with c2 is parabolic, (dn/dc3)_{f,s}^2 first decreasing to a minimum and then increasing again; but this minimum is zero only when the system is homogeneous in both respects. Otherwise the minimum value is positive. This fact can be used to estimate the degree of heterogeneity.

**EXPERIMENTS AND RESULTS**

**Materials**—The hyaluronic acid was prepared by ultrafiltration of ox synovial fluid (4); the material contained about 20% protein, which was not removed by further filtration. Bovine serum albumin was purchased from Sigma, Batch A253-69. The buffer used was 0.12 M NaHCO3 in equilibrium with air; this controlled the pH within the range 6.8 to 7.0 (4). All other materials were of analytical quality and glass-redistilled water was used throughout. Solutions of BSA in hyaluronic acid solution and in the buffer were made up by weight for each light scattering experiment, a pair of such solutions of the same BSA concentration was prepared. Each pair was thoroughly dialyzed against the same buffer solution. A check on any change of concentration during dialysis was obtained from the change of weight of the dialysis sac and its contents. The concentration of the hyaluronic acid was determined refractometrically (4). The concentration of the BSA was determined spectrophotometrically.

**Light Scattering** The light scattering measurements were carried out in a Sofica photometer between angles of 30° and 150° with unpolarized light of wave length 436 mp. The stock solutions (mixtures of hyaluronic acid and BSA) and the solvent (solution of BSA) were clarified by centrifugation for 30 min at 29,000 rpm (Spinco model L centrifuge, No. 30 head). Dilutions were made by weight in the photometer cell in a dust-free cabinet. The results were plotted by the method of Zimm (9).

This normally gives the mean molecular weight, as indicated by Equation 1. A similar extrapolation of the excess scattering due to hyaluronic acid in a solution of BSA at constant concentration will lead to a value of (dn/dc3)_{f,s}^{12,s,app}, by use of Equation 2.

Two samples of hyaluronic acid were used in this study, they were of the same composition but were of slightly different average molecular weight. The results of the light scattering measurements are shown in Table I.

**DISCUSSION**

**Excluded Volume**—The marked initial fall and later rise of (dn/dc3)_{f,s}^{12,s,app} with concentration of BSA shown in Table I and Fig. 1 means that there is an exclusion of BSA from the domains occupied by the molecules of hyaluronic acid. The fact that the minimum value is not zero means that the system is heterogeneous with respect to (dn/dc3) or ε or both. However, the degree of heterogeneity is such as to make it unlikely that it could arise to a significant extent from variation of (dn/dc3); if this were to be responsible for the whole effect, (dn/dc3) would have to vary at least in the range 0.042 to 0.258. We have therefore assumed that it arises entirely from variation of ε. A least squares fitting of Equation 9 to the experimental results (forcing the equation through the point c2 = 0, (dn/dc3)_{f,s}^{12,s,app} = 0.0225) gives the values of ε2, c2, and the coefficient variation of ε(σ) (Table II); these values were used to calculate the full curve drawn in Fig. 1. Table II also gives the previously determined values of ε. With two exceptions, the experimental values are in reasonable agreement with each other, and with the value expected from the simple steric model. The original determination of Ogston and Phelps (1) appears to be much too high; at the present time, we cannot explain this result, which was obtained with material similar to that used by ourselves and Preston et al. (4). The very low value of Blumberg and Ogston (10) was tentatively explained by Ogston and Phelps (1) on the basis of the different relationships between the solute under the dynamic conditions of velocity sedimentation and under conditions of equilibrium. This explanation is supported by the work of Laurent and Pietruszkiewicz (11), who showed a strong dy-
namic interaction between hyaluronic acid and other macromolecular solutes in velocity sedimentation.

Preston et al. (4) pointed out that a substantial degree of exclusion of BSA by hyaluronic acid might be expected to arise from the polyanionic characters of both molecules at pH 7, in addition to purely steric exclusion. However, Ogston and Phelps (1) found no apparent effect of changing the ionic strength or pH, or of exclusion at a molecular level also suggests that charge effects are of minor importance.

Variation of Excluded Volume—It is surprising at first to find so great an apparent heterogeneity among molecules of hyaluronic acid with respect to the excluded volume per g. This can be explained, however, on the basis of the statistical distribution of protein molecules in relation to hyaluronic acid molecules, without recourse to the assumption that the latter differ intrinsically among themselves with regard to exclusion. The volume of the domain of a hyaluronic acid molecule was estimated by Preston et al. (4) to be about 1.4 X 10^{-13} ml; a concentration of BSA of 5 X 10^{-3} g per ml (taking its molecular weight as 6.8 X 10^8) corresponds to 4.3 X 10^{10} molecules of BSA per ml, and to 6 X 10^{10} molecules of BSA within each molecular domain of hyaluronic acid. This number, however, will show a statistical fluctuation of Poissonian type, independent of the statistical distribution of hyaluronic acid molecules relative to each other (which gives rise to the excess light scattering). The coefficient of variation of such a distribution is 1/\sqrt{n}, which is (6 X 10^9)^{-1/2}.

This fluctuation of the concentration of BSA molecules within the hyaluronic acid molecules can be viewed as an apparent fluctuation of the excluded volume. A molecule volume of 1.4 X 10^{-13} ml per molecule of hyaluronic acid corresponds to a volume 6 X 10^{10} ml per g (taking the molecular weight of hyaluronic acid as 1.4 X 10^9). The coefficient of variation of the excluded volume would then be 6 X 10^{10}/\sqrt{n} = 0.79. Although this value should vary somewhat with the protein concentration, it is close enough to the experimental value to explain the effect convincingly.

Variation of Radius of Gyration—The validity of the argument of Ogston and Preston (8) with regard to the measurement of the excluded volume by light scattering applies equally to the estimation of the hydrated volume for BSA at zero concentration of hyaluronic acid, at zero concentration of hyaluronic acid, with concentration of bovine serum albumin.

The full line was calculated from Equation 9 with values for k or 99 ml per g and for k^2 = 121 ml per g (Table II).

![Figure 1](http://www.jbc.org) Variation of the square of the apparent specific refractive increment of hyaluronic acid, at zero concentration of hyaluronic acid, with concentration of bovine serum albumin. •, Sample 1; A, Sample 2. The full line was calculated from Equation 9 with values for k or 99 ml per g and for k^2 = 121 ml per g (Table II).

**FIG. 1.** Variation of the square of the apparent specific refractive increment of hyaluronic acid, at zero concentration of hyaluronic acid, with concentration of bovine serum albumin. •, Sample 1; A, Sample 2. The full line was calculated from Equation 9 with values for k or 99 ml per g and for k^2 = 121 ml per g (Table II).

**REFERENCES**

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