EXCLUSION IN HYALURONATE GELS

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ABSTRACT Osmotic pressures of solutions of hyaluronate (HA) (mol wt 117,000) and mixtures of HA and bovine serum albumin (BSA) in phosphate-buffered saline, pH 7.2, were measured with a membrane osmometer. The data were fit with a virial expansion in integral powers of total nondiffusible solute concentration. Values of number average molecular weight were calculated for HA and the mixtures from the first virial coefficients. The excluded volume of HA in the single nondiffusible solute solution was calculated from the second virial coefficient extracted from the data on the HA solution. The excluded volume of HA with respect to BSA was estimated from the "osmotic parameters" of HA and BSA by an approach developed in 1976 by Shaw. The resulting excluded volume of HA with respect to BSA was compared with those obtained from a lightly cross-linked HA gel and from solutions of HA (mol wt 1.5×10^6) studied in 1964 by Laurent. The development of this cross-linked HA gel and its subsequent calibration are described.

INTRODUCTION

In a previous paper we reported a method for interpreting osmotic pressure data from solutions of one and two nondiffusible components in terms of excluded volume (1). In the present work we describe our application of this approach to solutions containing hyaluronate (HA) of small molecular size (mol wt 117,000). The osmotic virial coefficients obtained for HA are compared with those given by Cleland and Wang (2). The derived excluded volume for HA with respect to BSA is compared with that measured by Laurent (3) in solutions of HA of larger molecular size (mol wt 1.5×10^6) and with that measured in cross-linked HA gels developed by Laurent et al. (4) and ourselves.

The cross-linked HA gel column is per se a useful system for studying the excluded volume and properties of HA in interactions with other physiologically important molecules, as illustrated by Iverius (5) for plasma lipoproteins. Therefore, we have detailed the method we used for constructing our version of the cross-linked HA column and have compared the resulting gel column with that of Laurent (3).

METHODS

The hyaluronate (HA) was purchased from Biotrics, Inc. (Arlington, Mass.); maximum protein contamination was 0.48%.[1] The HA used in the osmometry had a weight average mol wt (M_w) of 117,000 and limiting viscosity number [η] of 325 ml/g.[1] The HA from which the column gel was crossed-linked had M_w 1.8×10^6 and [η] 2,980 ml/g.[1] Bovine serum albumin (BSA)

[1]David A. Gibbs, Sc. D., Biotrics, Inc.; [η] measured at HA concentration 1% in phosphate-buffered saline, pH 7.2.

TABLE I

VIRIAL COEFFICIENTS AND STANDARD ERRORS FOR HA AND BSA SOLUTIONS

Nondiffusible solutes				ΔC*	Virial coefficients‡			
Weight fraction		Mole fraction						
BSA w_2	HA w_4	BSA x_2	HA x_4		A_1	A_2	A_3	A_4
				g/100 ml				
1	0	1	0	0–2%	2.63 ± 0.03*			
				0–10%	2.44 ± 0.10	0.12 ± 0.02	0	0.001 ± 0.0001
0	1	0	1	0–0.3%	3.79 ± 0.09			
				0–1%	2.52 ± 0.53	4.34 ± 1.65	2.88 ± 1.21	0
0.50	0.50	0.43	0.57	0–0.6%	3.27 ± 0.31			
				0–1%	(1.8–2.6)	(1.6–3.2)		
0.30	0.70	0.25	0.75	0–0.35%	3.12 ± 0.10			
				0–1%	(1.6–2.5)	(2.9–6.8)		

*Concentration range used in the construction of a particular curve.
‡See Eq. 1.

was the same utilized previously (1). The other species applied as samples to the column were fluorescein isothiocyanate dextrans 20, 40, and 70 and proteins ovalbumin, ribonuclease A, and chymotrypsinogen A, all obtained from Pharmacia Fine Chemicals, Inc, (Piscataway, N.J.).

Osmometry[2]

Osmotic pressures were measured on single-solute solutions of HA (M_w 117,000) in phosphate-buffered saline (0.15 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4 in H_2O at pH 7.2) and binary mixtures of HA and BSA in the same buffer (Table I) with the membrane osmometer[3] (1). Sodium azide (0.02%) was a component of all solutions. Osmotic pressures below 1 cm H_2O were measured by a special transducer built into the osmometer by the manufacturer.

The osmotic pressure data were fitted with a virial expansion in integral powers of concentration:

$$\pi = A_1C + A_2C^2 + 0(C^3) + \cdots \quad (1)$$

where π is the osmotic pressure (in millimeters of mercury), and C is the total nondiffusible solute concentration (grams/100 ml solution). A_1 and A_2 are virial coefficients. At a given temperature, A_1 is determined by number average mol wt of nondiffusible solute, A_2 is a function of two-body nondiffusible solute interactions; interactions involving three nondiffusible solute molecules would contribute to A_3, the third virial coefficient, etc. (6).

The number average mol wt, M_n, in grams per mole, was calculated from Eq. 2:

$$M_n = (0.75 \times 10^{-5})RT/A_1, \quad (2)$$

[2]Equations in this section are taken from ref. 1.
[3]Instrumentation for Physiology and Medicine, Inc., San Diego, Calif.

where R is the gas constant, T is the absolute temperature, and A_1 is the first virial coefficient, defined by Eq. 1. M_n, the number average mol wt of total nondiffusible solute, is given by Eq. 3 for a solution containing two nondiffusible[4] solute components:

$$M_n = x_2M_2 + x_4M_4 = (w_2 + w_4)/(w_2/M_2 + w_4/M_4), \quad (3)$$

where M_J is the number average mol wt of component J, w_j is the weight fraction of nondiffusible solute consisting of component J, and x_J is the weight fraction of nondiffusible solute consisting of component J, and x_J is the corresponding mole fraction. Relative mole fractions were calculated from the number average mol wt and relative weight fractions by Eq. 4:

$$x_J = (w_J/M_J)/(w_2/M_2 + w_4/M_4). \quad (4)$$

The number average mol wt (Eqs. 2 and 3) is the ideal, or low concentration, value.

Excluded volume, u (cm^3/molecule), was calculated from osmotic pressure data from single-solute solutions of HA with Eq. 5:

$$u = (2)(1.34 \times 10^7)M_n^2A_2/N_ART \quad (5)$$

where N_A is Avogadro's number.

The osmotic parameter $D/2$, defined as an average equivalent molecular radius by Eq. 6, was calculated for HA. D is the average equivalent center-to-center separation of the two interacting "spherical" molecules, either of the same species, as in this example, or of two different species, such as HA and BSA (below).

$$D/2 = \tfrac{1}{2}(\tfrac{3}{4}\pi)^{1/3}u^{1/3} \quad (6)$$

$D/2$ was estimated for binary solutions of HA and BSA by averaging the geometric mean of $D/2$ for HA and the radius of gyration of BSA with the arithmetic mean as discussed in Shaw (1). An estimated excluded volume for HA-BSA was then calculated from the estimated $D/2$ for HA-BSA by Eq. 6.

Gel Column Chromatography

HA (M_w 1.8 × 10^6)) was cross-linked according to the general procedure of Laurent et al. (8). A detailed description of our procedure, based on that of Laurent, follows.

Salt was removed, and the HA was precipitated in absolute alcohol, washed with anhydrous ethyl ether, and dried. To every 0.8 g of HA, 7.2 ml of 0.2 M NaOH was added, and the resulting solution was refrigerated overnight. The solution was brought to room temperature; 0.8 ml of 1,2,3,4-diepoxybutane was added, and then 12 mg of sodium borohydride. The mixture was held at 50 ± 2°C for 3 h to produce a gel. The wet gel was added to 250 ml of 0.02% sodium azide, neutralized with acetic acid, allowed to swell at 4°C for 24 h, and chopped in a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) for 30 s at maximum speed. Gel particles in the range 250–500 μm were selected by successive filtration through 500- and 250-μm screens, and a total volume of 150 ml of settled particles was obtained. The particles were resuspended in phosphate-buffered NaCl (pH 7.2 and pI 0.15); and the volume decreased to 50 ml, with an average gel concentration of 1.5%.

[4] Even-numbered subscripts are traditionally reserved for the nondiffusible solutes (7).

The resuspended gel particles were degassed under vacuum and packed into a column (1.6 cm × 7.18 cm). The downward flow rate was measured as 1.3 ml/h. The column was inverted and operated under ascending flow conditions at 1.0 ml/h with a peristaltic pump.

To this column were applied samples of FITC Dextrans 20, 40, and 70 and the proteins ovalbumin, BSA, ribonuclease A, and chymotrypsinogen A. Partition coefficients, K_{av}, for these species were calculated from the elution data from eq. 7 (9).

$$K_{av} = (V_e - V_o)/(V_t - V_o) \tag{7}$$

V_o is the void volume, and V_t the total volume of the column, while V_e is the elution volume of the sample species.

Excluded volumes in the column gels, V_{ex} (in milliliters per gram), were derived from the partition coefficients and gel concentration, C_R (grams per milliliter), by the formula of Laurent (10):

$$V_{ex} = (1 - K_{av})/C_R \tag{8}$$

Eqs. 7 and 8 are both derived from a solution model consisting of two phases: a gel phase of volume, $V_t - V_o$, and a liquid phase of volume, V_o.

(We symbolize excluded volume by u [Eq. 5] when expressed per molecule and by V_{ex} [Eq. 8] when expressed per gram of gel.)

RESULTS

Osmometry

For each mixture of solute components, the osmotic pressure data were fitted with Eq. 1 over two overlapping ranges in total nondiffusible solute concentration: over the low-concentration, linear portion and over an expanded range including the linear portion (Table I). Our curve-fitting procedures are described in Shaw (1). The data are shown in Fig. 1. The difference between the two curve fits was not significant over the lower concentration range because of the scatter in the data (see standard errors in Table I). The scatter in the data probably is due to polydispersity in the HA (below) and the relatively high viscosity of samples at concentrations approaching 1%. Viscous solutions of HA are difficult to apply as samples to the osmometer, as well as to dilute, mix, etc. The data for HA in single-solute solutions were easier to fit than the data from mixtures, for which we were able to estimate only a range of values for A_1 and A_2 over the expanded concentration range (Table I). (We had the opposite experience with chondroitin sulfate: the data from mixtures were smoother [1]).

Values of M_n were calculated by substitution of the low-concentration virial coefficients (3.79, HA; 3.27, BSA/HA 0.50/0.50; 3.12, BSA/HA 0.30/0.70) into Eq. 2. To obtain an average value of M_n (i.e., M_4) for HA, we substituted the value of M_n previously derived for the BSA (67,000, ref. 1) for M_2 into Eq. 3 written for each solute type. The results were: M_4 = 48,000 ± 2%, (w_2 = 0; w_4 = 1); M_4 = 48,500 ± 9%, (w_2 = 0.50; w_4 = 0.50); M_4 = 56,000 ± 3%, (w_2 = 0.30; w_4 = 0.70). The value of M_4 averaged over these three results is 51,000 ± 10%. The ratio M_w/M_n for this lot of HA is, therefore, 2.3, indicating a relatively polydisperse sample.

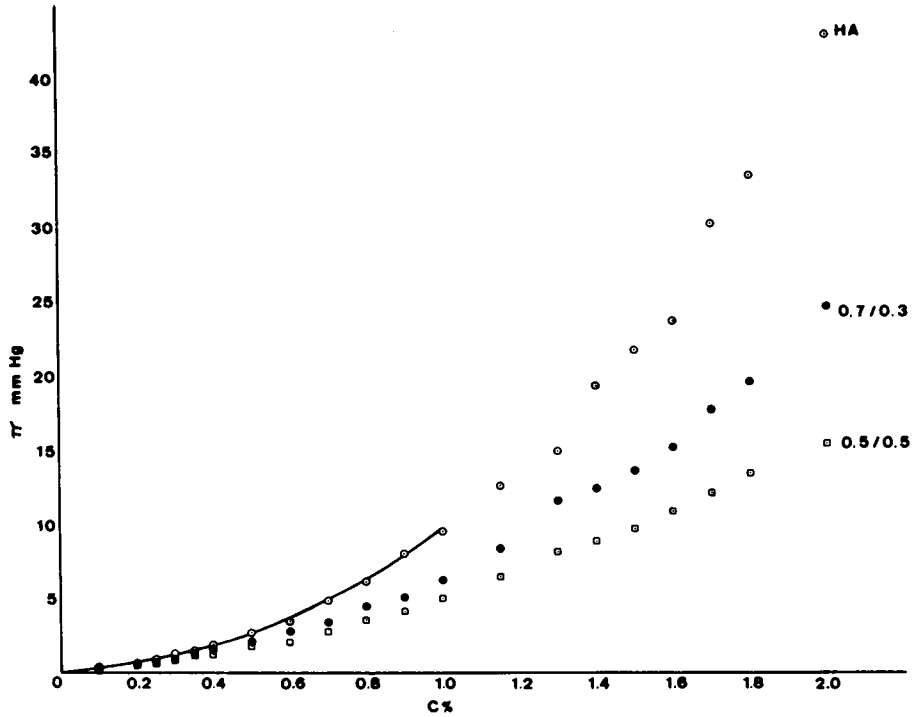

FIGURE 1 Osmotic pressure plotted against total nondiffusible solute concentration for solutions containing BSA and HA. The relative weight fractions are as follows: ⊙, HA 1.0, BSA 0; • HA 0.7, BSA 0.3; ⊡, HA 0.5, BSA 0.5. Each point is the mean of at least three determinations, involving at least two membranes. The line drawn through HA data points lying between 0 and 1% is the computer-generated curve corresponding to the virial coefficients, $A_1 = 2.52$, $A_2 = 4.34$, and $A_3 = 2.88$ (Table I).

From the values of A_2 obtained from the curve fit over the range $0 \leq C \leq 1\%$ and M_n, the excluded volume and effective radius of HA were calculated (Eqs. 5 and 6). The osmotic parameter $D/2$ for HA-BSA calculated from the average of the geometric mean and arithmetic mean of the effective radius of HA (85.0 Å) and the radius of gyration (30.1 Å) of BSA was 54.1 Å. The result is given in Table II, together with the predicted value of excluded volume of HA with respect to BSA, calculated from Eq. 6. The excluded volume of HA with respect to BSA was then converted to milliliters per gram by means of Avogadro's number and the value obtained for M_n of HA; the calculated value is 62.1 ml/g (Table II).

Chromatography

The calibration curves (11) for our hyaluronate column (1.5% HA) are shown in Fig. 2 together with curves for a G-200 column[5] (dextran 5%), Sepharose 6B[5]

[5]L. Beadling, Ph.D., Pharmacia Fine Chemicals.

TABLE II

EXCLUDED VOLUME AND OSMOTIC PARAMETER ($D/2$) CALCULATED FOR BSA-HA

Effective radius		$D/2$	Excluded volume	
Å	Å	Å	$(Å)^3$/molecule	*ml/g*
BSA	HA			
30.1*	85.0‡	54.2	5.3×10^6	62.1

*Radius of gyration (15).

‡Calculated from Eq. 6 and osmotic pressure data.

(6% agarose), and the HA column (1.45%) of Laurent (3). As can be seen, we have chromatographed both FITC dextrans and globular proteins on HA and have thereby obtained two different calibration curves. It can be seen from the figure that the HA curve for FITC dextrans, unlike that for globular proteins, is bounded by the curves of the rather open dextran and agarose gels.

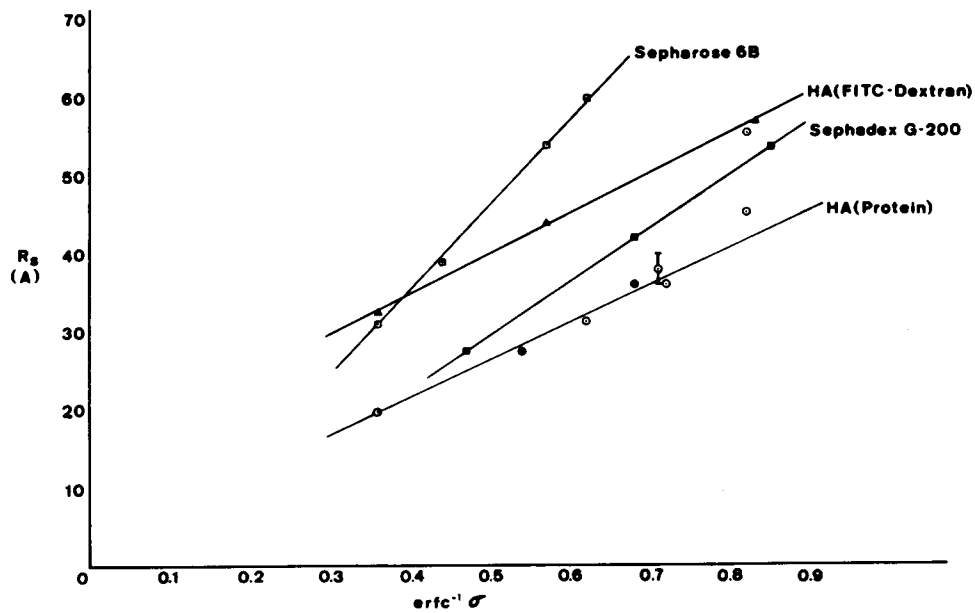

FIGURE 2 The Ackers calibration plot (11) for our HA gel column (1.5%): ▲ FITC dextrans, ●globular proteins. We have also included some points taken from Laurent's calibration of his HA gel column with globular proteins (○) (3). Also shown, for purposes of comparison, are FITC-dextran calibrations of a 6% Agarose (⊡) and a 5% dextran column (■). The buffer for all columns was 0.15 M NaCl, pH 7.2, with the exception of that of the HA column of Laurent, which was 0.1 M NaCl, pH 8.5. R_S is the Stokes radius (in Ångstroms). σ is a type of partition coefficient and closely approximates K_{av} (Eq. 7) for these gels. σ was calculated from the elution data by the definition given by Ackers (11): $\sigma = K_{av}(1 + \overline{V}/W_r)$ where $\overline{V}$ is the partial specific volume and W_r is the water regain of the gel. The error bar represents the maximum uncertainty in Stokes radius for proteins chromatographed by Laurent.

DISCUSSION

Osmometry

The success of the method utilized to obtain number average mol wt and excluded volumes from osmotic pressure data depends on the smoothness of the data. Factors encountered by us in this study and previously (1) that affect the smoothness of osmotic pressure data taken with a membrane osmometer include the molecular weight and the degree of polydispersity of the nondiffusible species, and the sample viscosity.

We have limited ourselves to relatively small nondiffusible species (HA, chondroitin sulfate [CHS], dextran T110 [D110], and BSA) with mol wt $\lesssim$ 120,000. Data from solutions of high mol wt ($\simeq 10^6$) are more difficult to interpret, since the standard error may be comparable to the magnitude of the first virial coefficient.

The values of the ratio M_w/M_n for HA, CHS, and D110 previously studied were 2.3, 1.2, and 1.4, respectively. It was possible to interpret the initial slopes of the osmotic pressure data in terms of number average mol wt for each of the following combinations of species: HA, HA and BSA, BSA, D110, D110 and BSA, and CHS and BSA.[6] By combining information from single and binary nondiffusible solute data, we have been able to obtain number average mol wt of BSA, CHS, D110, and HA to within 11%. However, the polydispersity and viscosity of solutions containing HA prevented a direct determination of the excluded volume of HA with respect to BSA, unlike the previous situation with CHS and D110, where viscosity was not a problem and polydispersity less of a problem.

Although the first virial coefficients obtained from fitting the low concentration, linear data from solutions of mixtures of HA and BSA had slightly more scatter than those from single-solute solutions of HA and BSA, they were sufficiently good to allow calculation of the number average molecular weight of HA to within 10% by application of Eqs. 2 and 3 to the coefficients from each solution type and averaging of the results. We have also included the virial coefficients obtained from fitting the mixture data over the range $0 \leq C \leq 1\%$ in Table I, although they were not used in calculations, to illustrate the experimental difficulty.

The value of second osmotic virial coefficient, A_2, derived from single-solute solutions of HA over the expanded concentration range was 4.34 $\pm$ 38% mm Hg/(g/100 ml)^2 (Table I), or $2.34 \times 10^{-3} \pm 38\%$ dyn-cm/g in the units used by Cleland and Wang (2). The ratio $(A_2 M_w)/[\eta]$ takes on the value 0.84 [(dyn-cm) (dalton)]/ml; values in the range 0.9–1.1 were obtained by Cleland and Wang for HA fractions in 0.5 M NaCl. This ratio approaches asymptotically from below the range 1.5–2.0 at large coil expansion (12). It would be anticipated, therefore, that our HA sample also would have an approximate flexible coil configuration at the lower ionic strength of 0.2 M NaCl and also that our HA osmotic pressure data are consistent with that of Cleland and Wang.

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[6]Note that CHS was left off the list as a single solute since there was enough scatter with this substance that it was necessary to introduce an ultracentrifuge determination of M_n (1).

One would intuitively expect that excluded volume should increase with molecular size until effective concentration is high enough that overlap between molecular chains takes place, as discussed by Tanford (13); i.e., overlap reduces excluded volume. Laurent (3) measured the excluded volume of HA with respect to BSA in solutions of HA (M_w 1.5 × 10^6) as approximately 40 ml/g.[7] The excluded volume for BSA in the 1.5% HA gel filtration matrix is 45 ml/g (Fig. 2 and Eq. 8). Both of these results are significantly less than the value of V_{ex} (62.1 ml/g) that we obtain for solutions of HA, M_w 117,000, in the concentration range 0–1%. Thus our result indicates that overlapping between HA molecules may be contributing to excluded volume in 1% solutions of M_w 500,000 and in the gel matrix.

Chromatography

We conclude that the two HA calibration curves for FITC dextrans and globular proteins (Fig. 2) reflect the difference in partition in the gel between the globular and flexible coil configuration, and that the difference in detail between Laurent's cross-linking procedure and ours was not significant with respect to partition of globular proteins. These conclusions are based on the observations that the calibration points for the proteins (*a*) fall on the same straight line for two buffers of differing pH that exceed the isoelectric points of the proteins, but (*b*) fall on a different straight line from that for the uncharged FITC-dextrans. This result is also anticipated from diffusion data indicating that linear and coiled molecules more readily penetrate HA networks than do globular proteins (8).

It can be concluded from the results illustrated in Fig. 2 that the fractionation properties of HA gels such as ours and that of Laurent (3) are not affected by pH changes in the range 7–8 in 0.1–0.15 M NaCl buffers. This is not unexpected, since the charge on the HA is probably shielded, and there is relatively little change in size of the HA coil in this pH range, as shown by the viscosity measurements of Pigman et al. (14)

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[7]Calculated by us from Eq. 8 and his data at 1%.

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