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Short communication

Determination of Stokes radii and molecular masses of sodium hyaluronates by Sephacryl gel chromatography

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Abstract

Stokes radii (R) of sodium hyaluronate fractions were estimated from their available volumes (K) by gel chromatography on a column of Sephacryl S-1000 instead of the Sepharose series previously examined. The relationships between R and K already established for the Sephadex and Sepharose series were also applicable to Sephacryl S-500 and S-1000 gels. R values were determined with the use of bovine serum albumin as a standard of known size and the average radius (5.8 nm) of fibres constructing Sephacryl gels. The molecular masses (M) of the glycosaminoglycan fractions, calculated from their intrinsic viscosities according to Mark–Houwink equations, were in the range $3.9 \cdot 10^4$–$8.8 \cdot 10^5$. Their Stokes radii were in the range 5.9–19.6 nm. The plots of R and $M^{1/2}$ for these fractions gave $R = 0.030\ M^{1/2}$ and $R = 0.016\ M^{1/2} + 4.3$ with M below and above 10^5, respectively. Although an upper limit of M for hyaluronic acid on Sepharose 2B appeared to be about $4 \cdot 10^5$, the use of Sephacryl S-1000 seems to extend this limit to about $1.5 \cdot 10^6$ under the experimental conditions

1. Introduction

A method for determining the molecular mass of sodium hyaluronate by use of its Stokes radius (R), which was calculated from its available volume (K) estimated by Sepharose gel chromatography, was given in a previous paper [1]. R of a substance B [R(B)], for instance, was calculated according to the following equation:

$$\log K(\mathrm{B})/\log K(\mathrm{A}) = \{[R(\mathrm{B}) + r]/[R(\mathrm{A}) + r]\}^2 \quad (1)$$

where K(A) and K(B) are available volumes of substances A and B, respectively; A is a substance with known Stokes radius such as bovine serum albumin. The average fibre radius (r) constructing gel networks was 2.5 nm for the Sepharose series [1].

Molecular masses (M) of hyaluronate samples were calculated from the relationships between M and R, and an upper limit of M for the polymer measurable by gel chromatography seemed to be about $4 \cdot 10^5$ on Sepharose 2B. As hyaluronic acid with $M > 4 \cdot 10^5$ is generally found in soft tissues such as vitreous body of the eye [2] and skin [3], and Sephacryl S-1000 (Pharmacia) is said to have a larger fractination range than that of Sepharose 2B, we tried to investigate whether the method described above is applicable to Sephacryl gels. These gels consist of the fibres produced by cross-linking dextran

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chains allylated perhaps at the position C-2 of appropriate residues with N,N'-methylenebisacrylamide and it is assumed that their networks form stiff and hydrophilic gels. The validity of Eq. 1 was verified at least for Sephacryl S-500 and S-1000 gels by the observation that the radii of the fibres constructing both gels (5.8 nm) agreed with each other; other Sephacryl types (S-100, S-200, S-300 and S-400) were not examined, because the chief interest in this work was the molecular sizes of hyaluronate samples with $M > 4 \cdot 10^5$.

In this paper, we describe a method for determining the Stokes radii of hyaluronates by Sephacryl gel chromatography.

2. Experimental

2.1. Materials

In order to prepare hyaluronic acid fractions with molecular masses $>4 \cdot 10^5$, a sample of sodium hyaluronate (rooster comb) was further fractionated by gel filtration of Sephacryl S-1000. Part (22 mg) of the hyaluronate sample, which was included in the above gel, was dissolved in 22 ml of 0.2 *M* sodium chloride and 2-ml aliquots were chromatographed on a column (54.5 × 2 cm I.D.) of Sephacryl S-1000 equilibrated with the above salt solution. The eluate was divided into five fractions (I–V) in eleven identical runs. Each pooled fraction was lyophilized after dialysis against distilled water. Although each elution pattern of these fractions was still broad on the same column, further fractionation was not done.

Six hyaluronate fractions (B-1, B-3 and E-1 to 4), already characterized in a previous study [1], were also used in this experiment to determine the fibre radius of Sephacryl gels.

The viscosities of six hyaluronate samples (a–f) with different molecular masses above $M = 1.5 \cdot 10^5$ were used to examine the reliability of the Mark–Houwink equations for hyaluronic acid reported by several investigators [4–7].

2.2. Gel chromatography

Gel filtration for the determination of available volumes (K) of hyaluronate samples and bovine serum albumin (BAS) was performed on a column (54.5 × 2 cm I.D.) of Sephacryl S-500 or S-1000 (Parmacia) at room temperature, using 0.2 *M* sodium chloride as the eluent. BSA (Sigma) was used as a standard having a known Stokes radius of 3.5 nm [8]. BSA (1 mg) or hyaluronate fractions (1–1.5 mg) were applied in 1 ml of 0.2 *M* sodium chloride. Column eluents were collected in 2-ml fractions.

Blue dextran 2000 (Pharmacia) of concentration 10 mg/ml was used to determine the void volume of a given column (54.5 × 2 cm I.D.), which was not detected as a peak, but as a small shoulder corresponding to a 75-ml elution volume.

2.3. Analytical methods

Hyaluronate in column eluates was detected by the method of Bitter and Muir [9], using glucuronolactone as a standard. Its contents in solution were calculated from the glucuronolactone value by multiplication by an experimental factor of 2.39 [5]. Bovine serum albumin and blue dextran were determined by absorbance measurements at 230 and 620 nm, respectively.

2.4. Intrinsic viscosity and molecular mass

For the determination of the mass-average molecular mass (M) of hyaluronic acid, a simple procedure is to measure its intrinsic viscosity ($[\eta]$), whose measurement was done in the same way as described previously [1], and then utilize a Mark–Houwink equation established for the polymer, $[\eta] = KM^{\alpha}$, where K and α are empirical constants. In a previous paper [5], we presented double logarithmic plots of $[\eta]$ and M of hyaluronate samples with M from 10^4 to $1.2 \cdot 10^6$: $[\eta]$ was measured in sodium phosphate buffer (pH 7.3) at 37°C and M was determined by sedimentation equilibrium. This graph indicated two linear regions below and above $M =$

$1.5 \cdot 10^5$. The former was represented by $[\eta] = 3.0 \cdot 10^{-4} M^{1.20}$ and the latter by $[\eta] = 5.7 \cdot 10^{-2} M^{0.76}$, in which $[\eta]$ is here expressed in ml/g instead of 100 ml/g as in the previous paper [5]. Cleland and Wang [4] also suggested such a tendency and obtained $[\eta] = 2.28 \cdot 10^{-2} M^{0.816}$ for $M > 10^5$: $[\eta]$ was measured in 0.2 M sodium chloride at 25°C and M was determined by light scattering. Cleland [10] proposed $[\eta] = 2.8 \cdot 10^{-3} M$ for low-molecular-mass samples of hyaluronate below $M = 10^5$ under the same conditions as described above. In this experiment, M was determined by using the Mark–Houwink coefficients by Cleland [10] and by Cleland and Wang [4] for hyaluronate samples below and above $M = 10^5$, respectively.

3. Results and discussion

3.1. Relationship between intrinsic viscosity and molecular mass

With the aim of examining the reliability of Mark–Houwink equations proposed for hyaluronic acid by several investigators, four equations [4–7] obtained from the various combinations of solvent and temperature were compared. Molecular masses of six hyaluronate samples above $M = 10^5$ (a–f), whose gel chromatograms demonstrated different molecular size distributions, were calculated from their intrinsic viscosities measured under various conditions. The results are summarized in Table 1. The molecular masses calculated from four Mark–Houwink equations differed from each other. Standard deviations (S.D.), also shown in Table 1, were in the range 13–19% on the basis of each mean value. The unexpected difference may be due to the difficulty in measuring the molecular mass of these polymers, the degree of polydispersity of the samples examined, etc. As a matter of convenience, the average molecular mass of these equations will be taken as the polymer's molecular mass. It should be noted in Table 1 that the molecular masses estimated from the Mark–Houwink equation (0.2 M, 25°C) by Cleland and Wang [4] agreed well with the mean values. Based on this finding, the average molecular mass of hyaluronate above $M = 10^5$ was approximated by the mass determined by using the Mark–Houwink coefficients in 0.2 M NaCl at 25°C.

For hyaluronic acid with $M < 10^5$, there are two Mark–Houwink equations by Shimada and Matsumura [5] and Cleland [10], as described

Table 1
Molecular masses (above 10^5) of hyaluronate samples from four Mark–Houwink equations

Sample	Conditions				
	0.2 M NaP[a], 37°C	0.2 M NaCl[b], 30°C	0.2 M NaCl[c], 25°C	0.15 M NaCl[d], 25°C	Mean ± S.D.
a	96	126	120	148	123 ± 19
b	83	107	102	120	103 ± 13
c	59	83	80	96	80 ± 13
d	37	58	55	65	54 ± 10
e	24	39	36	40	35 ± 6
f	17	28	25	27	24 ± 4

Molecular masses (M) of samples were calculated from their intrinsic viscosities according to Mark–Houwink equations and expressed as $M \cdot 10^{-4}$.
[a] Sodium phosphate buffer (pH 7.3), $[\eta] = 0.057 M^{0.76}$ [5].
[b] $[\eta] = 0.039 M^{0.77}$ [6].
[c] $[\eta] = 0.0228 M^{0.816}$ [4].
[d] $[\eta] = 0.0346 M^{0.779}$ [7].

Table 2
Molecular masses (below 10^5) of hyaluronate samples from two Mark–Houwink equations

Sample	0.2 *M* NaP, 37°C [a]		0.25 *M* NaCl, 25°C [b]	
	$[\eta]$	*M*	$[\eta]$	*M*
E-1	372	13	425	15
E-2	256	8.8	280	10
E-3	167	6.1	177	6.3
E-4	96	3.9	109	3.9

Molecular mass (M) is expressed as $M \cdot 10^{-4}$ and intrinsic viscosity, $[\eta]$, is given in ml/g.
[a] Sodium phosphate buffer (pH 7.3), $[\eta] = 3.0 \cdot 10^{-4} M^{1.20}$ [5].
[b] $[\eta] = 2.8 \cdot 10^{-3} M$ [10].

under Experimental. The intrinsic viscosities of hyaluronate samples with such low molecular masses were measured under each given conditions and are listed in Table 2 together with the molecular masses calculated by using the Mark–Houwink relationships mentioned above. Table 2 indicates that the M values for the same sample agree well with each other and therefore suggests that both equations are suitable. For the determination of molecular masses of hyaluronate samples below $M = 10^5$, Cleland's equation was used because of the same conditions (0.2 M, 25°C) as those for $M > 10^5$.

3.2. Radii of the fibres in Sephacryl gels

An averaged radius of the fibres in Sephacryl networks was determined in a similar manner to that for agarose fibres forming Sepharose gels [1].

Six hyaluronate fractions and BSA with known Stokes radii were separately chromatographed on both columns of Sephacryl S-500 and S-1000. Their available volumes (K) on both gels are given in Table 3 and their Stokes radii (R) are given in square brackets. The values for $(-\log K)^{1/2}$ were plotted against R as shown in Fig. 1. These plots suggested two straight lines crossing at a point of −5.8 nm on the abscissa. As can be seen in Table 3, the log K ratios based on BSA are in good agreement for Sephacryl S-500 and S-1000, and the ratios of log $K_{S\text{-}1000}$ to log $K_{S\text{-}500}$ [0.60 ± 0.02 (mean ± S.D.)] are considered to be constant. The observations

Table 3
Relationships between log K values on Sephacryl S-500 and S-1000 and physico-chemical data for sodium hyaluronates

Sample	K	Ratio[a]	Log $K_{S\text{-}1000}$ / log $K_{S\text{-}500}$	R (nm)	$[\eta]$ (ml/g)	M ($\times 10^{-4}$)
I	0.19			19.6	1611	88
II	0.26			17.1	1280	66
III	0.34			14.7	915	44
IV	0.38			13.5	732	33
B-1	0.39 (0.19)	4.2 (4.5)	0.57	13.3 [13.4]	723	33 [23.6]
B-3	0.43 (0.25)	3.8 (3.7)	0.61	12.2 [12.0]	504	21 [16.8]
V	0.46			11.6	554	24
E-1	0.48 (0.31)	3.3 (3.2)	0.63	11.0 [11.1]	425	17
E-2	0.56 (0.37)	2.6 (2.7)	0.58	9.2 [8.9]	280	10
E-3	0.63 (0.47)	2.1 (2.0)	0.61	7.6 [7.5]	177	6.3
E-4	0.70 (0.54)	1.6 (1.7)	0.58	5.9 [5.9]	109	3.9
BSA	0.80 (0.69)	1.0 (1.0)	0.60			

Chromatographic data for seven samples on a column of Sephacryl S-500 are given in parentheses. Some R and M values obtained from our previous experiments [1] are given in square brackets for comparison.
[a] Log K ratio based on that of BSA.

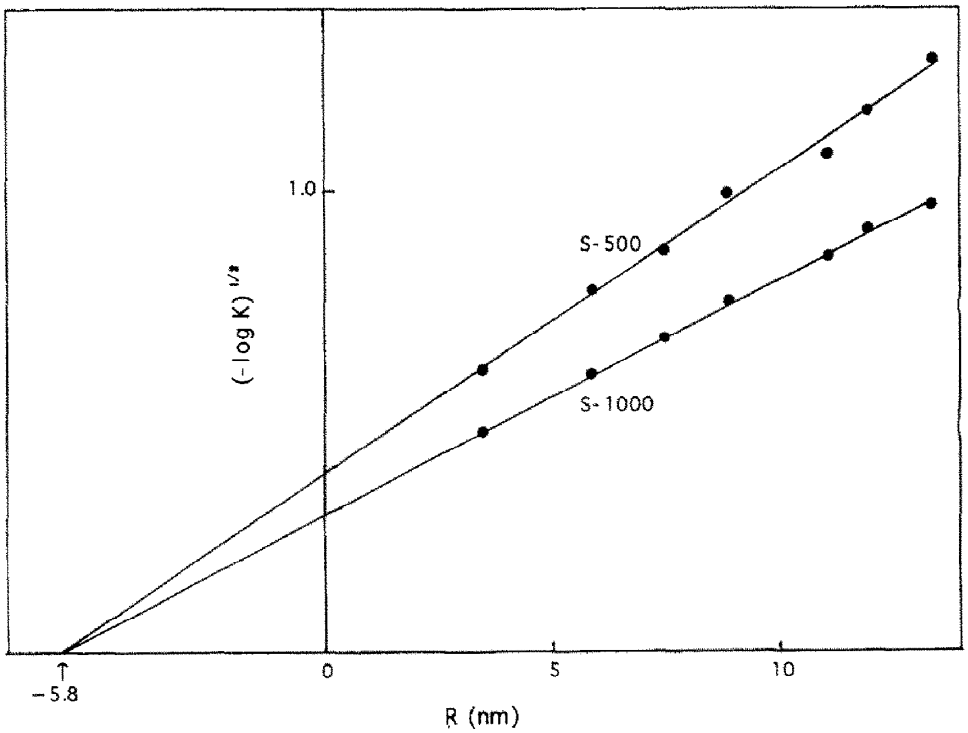

Fig. 1. Relationship between available volume (K) and Stokes radius (R) of six hyaluronate samples and BSA by gel chromatography on Sephacryl S-500 or S-1000. The plots of $(-\log K)^{1/2}$ against R are shown with the use of the K values (B-1, B-3, E-1 to 4 and BSA) in Table 3. These lines were determined by a linear least-squares method.

indicate that Eq. 1 is applicable to both types of Sephacryl gels, S-500 and S-1000.

3.3. Stokes radius and molecular mass of hyaluronic acid

Sodium hyaluronate samples were chromatographed on a column of Sephacryl S-1000 as described. Their Stokes radii (R) were calculated from Eq. 1 by use of the K values, R(BSA) = 3.5 nm and r = 5.8 nm. The molecular masses (M) of hyaluronates were calculated using the Mark–Houwink equations given by Cleland and Wang [4] and Cleland [10]; the latter equation was used for samples E-2 to E-4. The R and M values are summarized in Table 3, together with intrinsic viscosities ($[\eta]$). R was plotted against $M^{1/2}$ as shown in Fig. 2. The open circles are our previous results from Sepharose gel chromatography [1]. It is reasonable that the open and closed circles overlap in the range of low molecular masses, because the two Mark–Houwink equations give similar values, as seen in Table 2. The equation for each line of closed circles was calculated by a linear least-squares method: $R = 0.030\ M^{1/2}$ for the lower range and $R = 0.016\ M^{1/2} + 4.3$ for the higher range. A line of open circles with $M > 10^5$, $R = 0.0175\ M^{1/2} + 4.82$ [1], is also shown in Fig. 2 for comparison. This different line is due to the lower values of the molecular mass of hyaluronic acid calculated using the Mark–Houwink equation by Shimada and Matsumura [5].

The present method for determining the molecular mass of hyaluronic acid by use of a Sephacryl S-1000 column has the advantage that it needs only a standard of known size such as BSA. However, it is not applicable to the glycos-

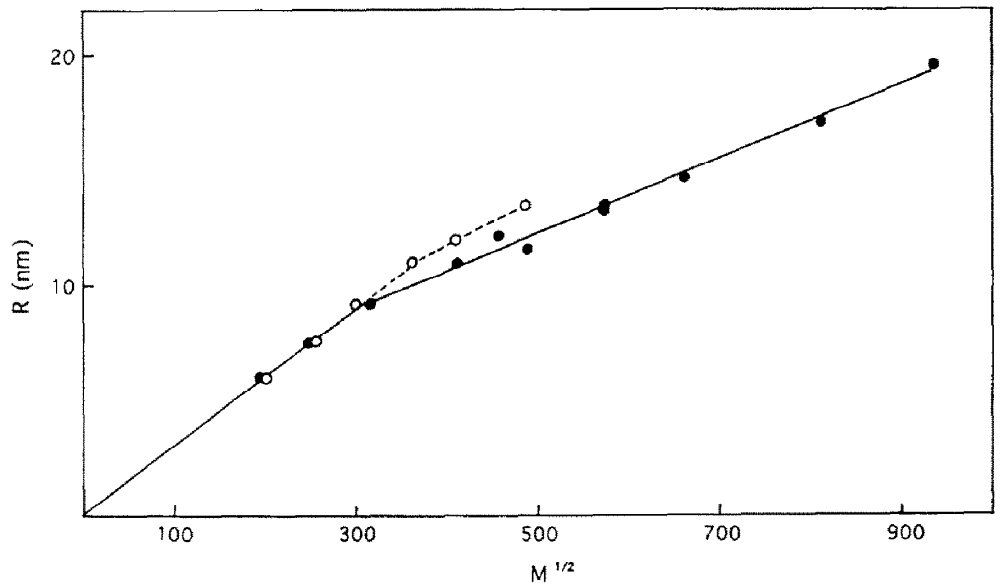

Fig. 2. Stokes radius (R) and molecular mass (M) of hyaluronate samples. The open circles are the data from Sepharose gel chromatography in a previous paper [1].

aminoglycan fraction with a very large size. An upper limit of M may be $1.5 \cdot 10^6$, by assuming K(hyaluronate) = 0.10 and K(BSA) = 0.80 in gel chromatography on Sephacryl S-1000.

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