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

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ABSTRACT

On the basis of available volumes (K_{av}) of a chondroitin sulphate fraction and some proteins with known Stokes radii (R) in Sepharose (6B, 4B and 2B) gel chromatography, it was found that Ogston's equation correlating K_{av} with R was also applicable to Sepharose gel in addition to the Sephadex gel previously examined. Therefore, R values for sodium hyaluronate samples were determined in a similar manner to that already described for R values for hyaluronate oligosaccharides by Sephadex gel chromatography, using bovine serum albumin as a standard of known size and the radius (2.5 nm) of an agarose chain forming the gel networks. The molecular masses (M) of these samples, estimated from their intrinsic viscosities according to Mark–Houwink equations, were in the range $3.9 \cdot 10^4$–$3.0 \cdot 10^5$. Their Stokes radii determined by gel chromatography were in the range 5.9–14.4 nm. A simple relationship of $R = 0.030\,M^{1/2}$ was maintained for the hyaluronates with M below $1.3 \cdot 10^5$ (R = 11 nm). The plots of R against $M^{1/2}$ gave $R = 4.82 + 0.0175\,M^{1/2}$ for M above $1.3 \cdot 10^5$. By use of these relationships, the molecular mass of hyaluronate can be determined from its Stokes radius under the experimental conditions.

INTRODUCTION

In a previous paper [1] we presented a method for determining the Stokes radius (R) of a substance by use of its available volume (K_{av}) in Sephadex gel chromatography. The method is based on Ogston's equation [2]. Taking the natural logarithm of the equation gives

$$\log K_{av} = -\pi L (R + r)^2 \quad (1)$$

where, for Sephadex gel, L is the total concentration of the dextran chain expressed in length per unit volume and r is the radius of a dextran chain. K_{av} was calculated from the relationship determined by Laurent and Killander [3]:

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (2)$$

where V_e is elution volume, V_0 is void volume and V_t is total bed volume. The term K_{av} is hereafter denoted K.

When substances A and B are chromatographed on the same Sephadex gel, the following relationship is derived from eqn.1:

$$\log K(B)/\log K(A) = \{[R(B) + r]/[R(A) + r]\}^2 \quad (3)$$

where K(A), K(B) and R(A), R(B) are available volumes and Stokes radii of A and B, respectively. It is emphasized that a complicated factor, L, in eqn. 1 is eliminated in the above ratio. If r is independent of the type of Sephadex, log K(B)/log K(A) will be constant for any Sephadex gel.

When a substance A is chromatographed on two types of Sephadex (m and n), another relationship is derived from eqn. 1 on the assumption that R(A) and r in gel m are identical with those in gel n:

$$\log K_n(A)/\log K_m(A) = L_n/L_m \quad (4)$$

This equation suggests that the ratio log K_m(A)/log K_n(A) is determined by the dextran concentrations in gels m and n.

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The validity of eqns. 3 and 4, as described previously [1], was verified by gel chromatography of a series of even-numbered hyaluronate oligosaccharides on Sephadex G-25, -50 and -75. Therefore, we could determine the Stokes radius of hyaluronate oligosaccharide, R(B), using an equation obtained directly from eqn. 3:

$$R(\mathrm{B}) = R(\mathrm{A})\,[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} + r\{[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} - 1\} \quad (5)$$

where A is a substance with known Stokes radius such bovine serum albumin, and r is 0.2 nm [1,4].

We are interested in the conformation of hyaluronic acid of high molecular mass, which is generally excluded from Sephadex gels, and we therefore tried to examine whether Ogston's model is applicable to Sepharose gels, which can include the glycosaminoglycan of larger molecular size. As the usefulness of Ogston's equation was also evidenced by means of Sepharose (6B, 4B and 2B) gel filtration of some materials with known molecular sizes, R values for hyaluronate fractions covering a molecular mass range from $4 \cdot 10^4$ to $3 \cdot 10^5$ were calculated according to eqn. 5, using 2.5 nm as the radius of a straight agarose chain constituting Sepharose gel.

In this paper, we describe a method for determining the Stokes radii of sodium hyaluronates by Sepharose gel chromatography and discuss the relationships between their molecular sizes and masses.

EXPERIMENTAL

Materials

For the determination of the Stokes radius of hyaluronate by gel chromatography, it is necessary to obtain a fraction characterized by limited molecular size or mass ranges. These fractions were prepared in the following way from purified sodium hyaluronate (rooster comb) provided by Seikagaku Kogyo. The given hyaluronate had a relatively low molecular mass and was included in Sepharose 2B gel as shown by the dashed curve in Fig. 1.

Part (0.2 g) of the hyaluronate was dissolved in 20 ml of 0.2 M sodium chloride and 2-ml aliquots were chromatographed on a column (100 × 2 cm I.D.) of Sepharose 2B equilibrated with the above salt solution. The eluate was divided into four fractions (1, 2, 3 and 4), as indicated in Fig. 1, in ten identical runs. The molecular size distributions of the four

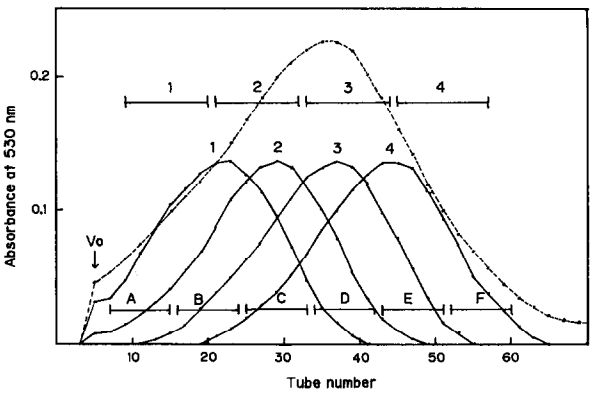

Fig. 1. Gel chromatograms of a hyaluronate preparation and several fractions obtained from it on Sepharose 2B. Samples of 20 mg of a sodium hyaluronate preparation were dissolved in 2 ml of 0.2 M sodium chloride solution and fractionated on a column (100 x 2 cm I.D.) of Sepharose 2B (dashed curve). The column eluents were divided into four fractions (1–4) as indicated. Each rechromatographic pattern of fractions 1–4 on the same column is also given (solid curve). In this instance, samples of 10 mg of a fraction were applied. These column eluents were divided into six fractions (A–F) on the basis of elution volume. All the hyaluronate samples were chromatographed with 0.2 M sodium chloride solution and fractions of 3 ml were analysed for uronic acid. The elution volume of Blue Dextran 2000, V_0, was 86 ml.

fractions were checked by rechromatography on the same column. Their elution profiles, illustrated by solid curves in Fig. 1, were still broad, so these hyaluronates were further fractionated into six fractions (A, B, C, D, E and F in Fig. 1) after rechromatography. Fractions B and E were further rechromatographed under the same conditions mentioned above; B was subdivided into three fractions (B-1, -2 and -3) and E into four fractions (E-1, -2, -3 and -4). Finally, each pooled fraction was lyophilized after dialysis against distilled water. Fraction F was not used in this experiment because of its trace amounts.

In order to obtain a chondroitin sulphate fraction with limited molecular sizes, commercial chondroitin sulphate A (Seikagaku Kogyo) was fractionated with a Sephadex G-200 column (57 x 2 cm I.D.), essentially according to the procedure of Wasteson [5]. The sulphated glcosaminoglycan (150 mg) was dissolved in 9 ml of 0.2 M sodium chloride solution and each 1.5 ml portion was applied to the column, which was eluted with the above salt solution. The eluate was divided into five groups. The corresponding fractions from six separate runs were

pooled, dialysed against distilled water and then lyophilized. The major fraction among them was employed to examine its Stokes radius.

Several proteins with known Stokes radii were also used, namely bovine serum albumin (BSA) (Sigma), soybean trypsin inhibitor and 75% clottable bovine fibrinogen (Miles Labs.), cytochrome *c* (Boehringer) and γ-globulin (United States Biochemical); their molecular radii are 3.49 [6], 2.26 [3], 10.6 [7], 1.64 [3] and 5.55 [3] nm, respectively.

Gel chromatography

Gel filtration for the determination of K was performed on Sephadex G-200 and Sepharose 6B, 4B and 2B (Pharmacia) at room temperature, using 0.01 M sodium phosphate buffer (pH 7.4) containing 0.15 M sodium chloride as the eluent, which was chosen by taking into account Wasteson's comment: on Sephadex G-200 or Sepharose 6B gel chromatography of chondroitin sulphate fractions, he emphasized that the ionic strength of the eluent was an important factor affecting their elution positions and patterns especially at low ionic strength, and recommended 0.2 M sodium chloride solution as an adequate eluent. Chromatographic columns (54–57 × 2 cm I.D.) were fitted with a sintered-glass filter disc at the bottom to facilitate the measurement of the total bed volume, V_t. The samples (1 mg for proteins and 2 mg for glycosaminoglycans) were applied in 1 ml of the buffer described above. Column eluents were collected in 2-ml fractions.

Blue Dextran 2000 (Pharmacia) of concentration 10 mg/ml was used to determine the void volume, V_0, of a given column.

Analytical methods

Hyaluronate and chondroitin sulphate samples in column eluents were detected by the method of Bitter and Muir [8]. Proteins and blue dextran were determined by absorbance measurements at 230 and 620 nm, respectively.

Molecular mass

The mass-average molecular masses (M) of hyaluronate samples were calculated from their intrinsic viscosities, $[\eta]$ (100 ml/g), according to the Mark–Houwink relationships based on the mass-average molecular mass of hyaluronic acid determined by sedimentation equilibrium described in a previous paper [9], in which the plots of log $[\eta]$ against log M suggested two linear regions below and above $[\eta] = 4.9$ corresponding to $M = 1.5 \cdot 10^5$, and therefore $[\eta] = 3.0 \cdot 10^{-6} \cdot M^{1.20}$ was used for lower $[\eta]$ and $[\eta] = 5.7 \cdot 10^{-4} \cdot M^{0.76}$ for higher values. Viscosity measurements were performed at 37°C on the glycosaminoglycan samples (E-1–4, D, C, B-1–3 and A) dialysed against 0.2 M sodium phosphate buffer (pH 7.3) with a Cannon–Manning semi-micro viscometer using 0.5 ml of the sample solution. Hyaluronic acid concentrations were calculated from the uronic acid value, using glucuronolactone as a standard, by multiplication by an experimental factor of 2.39. In order to determine intrinsic viscosities, one concentration analysis [9] using the equation $[\eta] = [2(\eta_{sp} - \log \eta_{rel})]^{1/2}/c$ derived from the combination of both equations of Huggins and Kraemer was employed. Mean $[\eta]$ values of the two concentrations (2 and 1 mg/ml for E series, 0.7 and 0.35 mg/ml for D and C, 0.5 and 0.25 mg/ml for the other fractions) are listed in Table II. Each error was less than 1%.

RESULTS AND DISCUSSION

Gel chromatography of glycosaminoglycans and proteins

Representative elution profiles of various substances on a Sepharose 2B column are given in Fig. 2. In spite of repeated fractionation by rechromatography, a series of fractions of hyaluronate still covered a wide range of molecular sizes compared with the chondroitin sulphate fraction (ChS) and commercial proteins. However, further fractionation of these hyaluronate samples (A, B-1–3, C, D and E-1–4) was not done.

Relationships between log K values for glycosaminoglycans and proteins on three types of Sepharose

Hyaluronate fractions (E-1, -2 and -3), a chondroitin sulphate fraction (ChS) and five proteins (fibrinogen, γ-globulin, BSA, trypsin inhibitor and cytochrome *c*) were separately chromatographed on Sepharose 6B, 4B and 2B, respectively. Available volumes (K) of the nine substances on each Sepharose type and log K ratios based on BSA are presented in Table I. The ratios of log K to log K(BSA) for a given substance on the three Sepharose gels

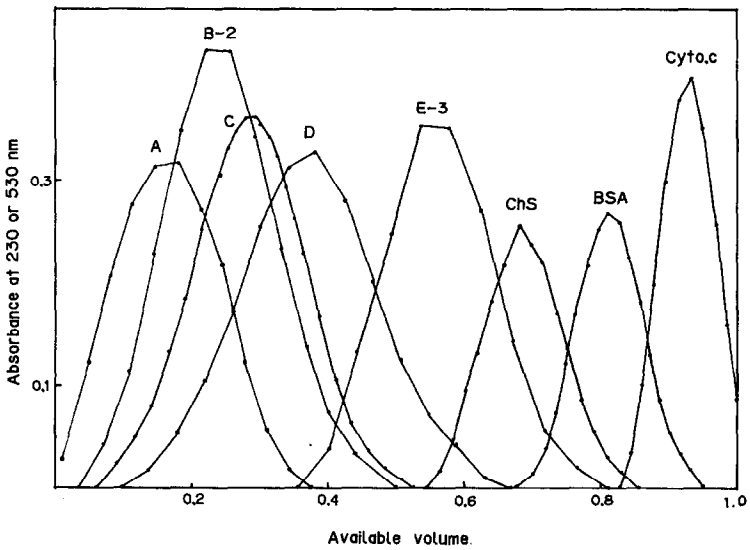

Fig. 2. Available volumes (K) of glycosaminoglycans and proteins on Sepharose 2B. Hyaluronate samples (A, B-2, C, D and E-3), a chondroitin sulphate fraction (ChS), BSA and cytochrome c (Cyto.c) were separately chromatographed on a column (54–57 × 2 cm I.D.) of Sepharose 2B. Fractions of 2 ml per tube were collected. Glycosaminoglycans were detected by the carbazole reaction and proteins by the absorbance at 230 nm.

agreed well with each other but were clearly distinct from those on Sephadex G-200. The results suggest a difference between the values of r(agarose) and r(dextran). Log K ratios of a given substance on Sepharose 2B, 4B and 6B are also shown in Table I. Mean values ± S.D. for log K_{2B}/log K_{4B}, log K_{2B}/log K_{6B} and log K_{4B}/log K_{6B} were 0.74 ± 0.02, 0.46 ± 0.01 and 0.62 ± 0.02, respectively. The constancy of the various log K ratios listed in Table I indicates that Ogston's model is applicable to Sepharose gel chromatography.

On the basis of eqn. 1, we obtain

$$(-\log K)^{1/2} = (\pi L)^{1/2} (R + r) \quad (6)$$

This equation implies that the plots of $(-\log K)^{1/2}$ against Stokes radius (R) should give a straight line for a given Sepharose gel and these lines reflecting agarose concentrations of the gels should intersect at one point on the abscissa, the absolute value of the intersection representing the radius of the agarose chain (r). We have already demonstrated the validity of Ogston's model in Sephadex gel chromatography. Therefore, the values of $(-\log K)^{1/2}$ from the data on Sephadex G-200 in Table I were plotted against the Stokes radii obtained from the literature (see Experimental); 5.5 nm as R(ChS) was determined according to eqn. 5, by a combination of 2.3 = log K(ChS)/log K(BSA) in Table I, R(BSA) = 3.49 nm and r(dextran) = 0.20 nm. As can be seen in Fig. 3, these plots suggested a straight line crossing at a point −0.2 nm on the abscissa, with the exception of γ-globulin. On the other hand, the data for six samples with known Stokes radii on Sepharose gels (Table I) were similarly plotted in Fig. 3, where we utilized 2.5 nm for r(agarose) estimated by Laurent [10]. Three points corresponding to chondroitin sulphate, BSA and trypsin inhibitor appeared to lie on a straight line, which intersected at a point −2.5 nm on the abscissa, for any Sepharose gel examined. Judging from the linearity of these points and the satisfactory value of the intercept, it seems reasonable to conclude that Ogston's equation is applicable to substances that do not interact with Sepharose gel grains. Fig. 3 shows that the estimated Stokes radii of cytochrome c, γ-globulin and fibrinogen are smaller than their reference values. Although the reason for this deviation is unknown, there is a possibility that they interact with the gel grains to retard their elution.

The notation 2B′ in Fig. 3 indicates a different lot of Sepharose 2B. The available volumes of a sample on the two lots differed from each other but the log

TABLE I

RELATIONSHIPS BETWEEN LOG K VALUES FOR GLYCOSAMINOGLYCANS AND PROTEINS ON SEPHAROSE 6B, 4B AND 2B

K values for a chondroitin sulphate fraction (ChS) and four proteins on Sephadex G-200 are also given for comparison.

Sample	2B		4B		6B		G-200		$\log K_{2B}/\log K_{4B}$	$\log K_{2B}/\log K_{6B}$	$\log K_{4B}/\log K_{6B}$
	K	Ratio[a]	K	Ratio	K	Ratio	K	Ratio			
E-1	0.32	5.1	0.21	5.2	0.08	5.3			0.73	0.45	0.62
E-2	0.45	3.6	0.35	3.5	0.17	3.7			0.76	0.45	0.59
E-3	0.54	2.8	0.43	2.8	0.25	2.9			0.73	0.44	0.61
Fibrinogen	0.62	2.1	0.51	2.2	0.35	2.2			0.71	0.44	0.64
ChS	0.68	1.7	0.60	1.7	0.43	1.8	0.08	2.3	0.75	0.46	0.61
γ-Globulin	0.76	1.2	0.69	1.2	0.56	1.2	0.15	1.8	0.74	0.47	0.64
BSA	0.80	1.0	0.74	1.0	0.62	1.0	0.34	1.0	0.74	0.47	0.63
Trypsin inhibitor	0.87	0.62	0.83	0.62	0.73	0.66	0.63	0.43	0.75	0.44	0.59
Cytochrome c	0.93	0.33	0.91	0.31	0.86	0.32	0.75	0.27	0.77	0.48	0.63

[a] Log K ratio based on that of BSA.

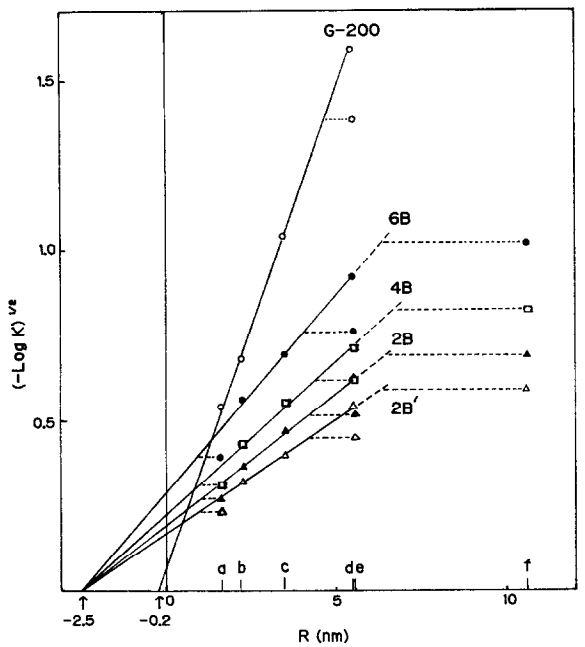

Fig. 3. Relationship between available volume (K) and Stokes radius (R) of chondroitin sulphate and protein samples on Sephadex or Sepharose gel chromatography. The plots of $(-\log K)^{1/2}$ against R are shown with the use of K values in Table I. On the abscissa, a (1.64 nm), b (2.26), c (3.49), d (5.5), e (5.55) and f (10.6) indicate R values in the literature for cytochrome c, trypsin inhibitor, BSA, a chondroitin sulphate fraction (ChS), γ-globulin and fibrinogen, respectively. Sephadex G-200 and Sepharose 6B, 4B and 2B were employed; 2B′ indicates a different lot.

K ratios based on BSA agreed well. The mean ± S.D. for $\log K_{2B}/\log K'_{2B}$ of the six samples in Fig. 3 was 1.37 ± 0.04, indicating a constant value. The observations seem to reflect the difference in agarose concentration. Similar results for log K ratios had been obtained in previous work by use of different lots of Sephadex G-15 [1].

Determination of Stokes radii of hyaluronates

Sodium hyaluronate fractions were chromatographed on a Sepharose gel column as described in the text and their Stokes radii (R) were estimated from eqn. 5 with a combination of R(BSA) = 3.49 nm and r(agarose) = 2.5 nm. R values for these fractions are summarized in Table II, together with data for the available volume (K), the intrinsic viscosity ($[\eta]$), the molecular mass (M) and the number of repeating disaccharide units (N).

Relationship of Stokes radius with molecular mass or viscosity

In order to evaluate the relationship between molecular size and chain length, the ratio of R to $N^{1/2}$ was useful (Table II). As can be seen in Table II, a constant ratio (0.60) of R to $N^{1/2}$ is observed, indicating that the Stokes radius is proportional to the square root of the number of disaccharide repeating units. In other words, R is proportional to $M^{1/2}$. This relationship is illustrated in Fig. 4 (closed circles). These plots are approximated by two linear regions below and above R = 11 nm corresponding to $M = 1.3 \cdot 10^5$. Such a downward tendency appears to reflect the downward break observed in the double logarithmic plot of $[\eta]$ against M for hyaluronic acid [9,11,12]. Cleland and Wang [11] explained the conformational change of hyaluronic acid at $M \approx 10^5$ in terms of a worm-like chain or non-Gaussian behaviour of short chains at lower molecular masses and a flexible chain or Gaussian behaviour of long chains at higher molecular masses. The equation for each line (Fig. 4) calculated by a linear least-squares method was $R = 0.030\ M^{1/2}$ for the former and $R = 0.0175\ M^{1/2} + 4.82$ for the latter. The results can be used to determine the molecular mass of hyaluronic acid from its Stokes radius estimated by Sephadex or Sepharose gel chromatography. This method has the advantage that calibration with hyaluronic acid of known molecular mass is not necessary. Fraction A had the highest molecular mass of $3 \cdot 10^5$ among the hyaluronate fractions examined and its K value was 0.17 on Sepharose 2B. Under the experimental conditions, an upper limit of M for hyaluronate measurable by gel filtration on Sepharose 2B may be $4 \cdot 10^5$, by assuming K(hyaluronate) = 0.10 and K(BSA) = 0.80.

Using the data in Table II, the relationship between R and $[\eta]$ of hyaluronic acid was investigated. The open circles in Fig. 4 are for R plotted against $[\eta]^{1/3}$. The reason for the choice of the exponent 1/3 is that intrinsic viscosity is related to the hydrodynamic volume (V_h) for spheres by $[\eta]$ = constant · (V_h/M), in which V_h is proportional to R^3. These plots gave a straight line with a correlation coefficient of 0.999. Although the molecular mass of hyaluronic acid with an appropriate size can be estimated from the R value determined by the method described in the text, the relationship among R, $[\eta]$ and M seems not to be simple for the glycosaminoglycan and remains to be elucidated.

It will also be useful to discuss our data from the standpoint of the resolution concept in size-exclusion chromatography (SEC) of polymers [13], by the use of measurable column parameters. On the assumption that each peak in Fig. 2 is approximated by a Gaussian function, standard deviations (σ) calculated for cytochrome *c*, BSA, and nine hyaluronate fractions including four (E-1, E-2, B-1 and B-3) not shown in Fig. 2 were 5.0, 6.1, and 10.0 ± 0.9 ml (mean ± S.D.), respectively. Higher values of σ for the glycosaminoglycan fractions than those for single molecular species such as protein standards are due to broad molecular mass distributions of the polymer. For BSA given in Fig. 2, the number of theoretical plates, $(V_R/\sigma)^2$, where V_R (peak retention volume) in ref. 13 corresponds to V_e (elution volume) in the text, was about 600, which was very low compared with, for instance, the plate numbers of toluene (2700–24 500) obtained using high-speed SEC by use of various types of column packings [14]. The low value appears to reflect an inherent property in a soft gel such as Sepharose. The relationship between the molecular mass of a polymer (M) and its elution volumes (V_R or V_e) is known as the SEC calibration graph [13], and this is expressed by

$$\log_{10} M = \log_{10} D_1 - (D_2 / 2.303) V_R \quad (7)$$

where D_1 and D_2 are constants. Therefore, the molecular masses of sodium hyaluronates (Table II) were plotted against their elution volumes, which were estimated by use of K values, except for E-4 (Table II) and V_0 = 52 ml found for a Sepharose 2B column (55.1 × 2 cm I.D., V_t = 173 ml). These plots were approximated by a straight line. The equation calculated by a linear least-squares method was $\log_{10} M = 6.52 - 0.015 V_R$, with a correlation coefficient 0.990. Hence, D_2 was 0.035. This small value can be ascribed to the narrow molecular mass range of hyaluronic acids separable on a Sepharose 2B column. The specific resolution R_{sp} ($= 0.58/\sigma D_2$) of the above column using D_2 = 0.035 and σ = 10.0 ml was 1.66, which means that the difference in elution volume is about 6.8 σ (68 ml) for a pair of peaks having a decade of molecular mass difference. The molecular mass accuracy (M^*), that is, the error averaged for $\overline{M}_n^*$ {$= \exp[-(\sigma D_2)^2/2] - 1$} and $\overline{M}_w^*$ {$= \exp[(\sigma D_2)^2/2 - 1$} was 6.1%, where $\overline{M}_n^*$ ($\overline{M}_w^*$) is defined as the relative number- (mass-) average molecular mass error [14]. As the error is caused by column dispersion alone, it is expected to decrease with the decrease in the molecular mass distribution of each polymer fraction.

Apart from the resolution concept of SEC, we speculate here on an error in M originating from the assumed error in the measurement of V_e. If V_e to give K in Table II has an error of 2 ml (volume per tube), the error for each K becomes about ± 0.02. On the basis of the resulting K values and a fixed K (0.80) of BSA, R and then M were recalculated. The

TABLE II

PHYSICO-CHEMICAL DATA FOR SODIUM HYALURONATES

Sample	K	$[\eta]$ (100 ml/g)	M^b ($\times 10^{-4}$)	N^c	R (nm)	$R/N^{1/2}$
E-4	0.39[a]	0.96	3.9	97	5.9	0.60
E-3	0.54	1.67	6.1	152	7.5	0.61
E-2	0.45	2.56	8.8	219	8.9	0.60
D	0.36	3.57	11.6	289	10.4	0.61
E-1	0.32	4.13	13.1	327	11.1	0.61
C	0.28	5.08	15.8	394	11.8	0.60
B-3	0.27	5.33	16.8	419	12.0	0.59
B-2	0.23	6.44	21.5	536	12.9	0.56
B-1	0.21	6.91	23.6	589	13.4	0.55
A	0.17	8.34	30.2	753	14.4	0.52

[a] K(E-4) was obtained by gel chromatography on Sepharose 6B and R(E-4) was calculated by use of K(BSA) = 0.62 given in Table I. The other K values were obtained by gel filtration on Sepharose 2B and their R values were calculated with the use of K(BSA) = 0.80.

[b] M is the mass-average molecular mass determined by sedimentation equilibrium.

[c] Number of repeating disaccharide units. N was obtained by dividing M by the average molecular mass of the unit (401 g/mol).

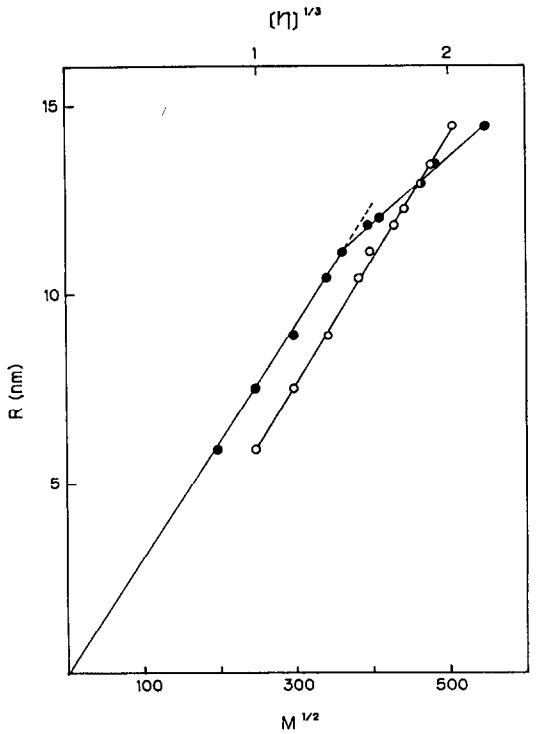

Fig. 4. Relationship between Stokes radius (R) and (●) molecular mass (M) and (○) intrinsic viscosity ([η]) of hyaluronate samples.

assumed error in the molecular mass increased with increase in M. It was approximately ± 6% and ± 10% for samples E-3 and A, respectively.

The most suitable terms (σ, D_2, R_{sp} and M^*) to express the column resolution in SEC of polymers have mainly been discussed in the field of high-speed or high-performance SEC. Therefore, it is interesting that these terms can be estimated from the chromatograms obtained using Sepharose gel filtration with a low flow-rate. These factors established in rapid SEC could also be utilized in the field of slow SEC such as Sephadex or Sepharose gel chromatography to obtain numerous data from SEC.

REFERENCES

1 E. Shimada and G. Matsumura, *J. Chromatogr.*, 472 (1989) 145.
2 A. G. Ogston, *Trans. Faraday Soc.*, 54 (1958) 1754.
3 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.
4 L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta*, 112 (1966) 346.
5 A. Wasteson, *Biochem. J.*, 122 (1971) 477.
6 J. M. Creeth, *Biochemistry*, 51 (1952) 10.
7 S. Shulman, *J. Am. Chem. Soc.*, 75 (1953) 5846.
8 J. Bitter and H. Muir, *Anal. Biochem.*, 4 (1962) 330.
9 E. Shimada and G. Matsumura, *J. Biochem. (Tokyo)*, 78 (1975) 513.
10 T. C. Laurent, *Biochim. Biophys. Acta*, 136 (1967) 199.
11 R. L. Cleland and J. L. Wang, *Biopolymers*, 9 (1970) 799.
12 R. L. Cleland, *Biopolymers*, 23 (1984) 647.
13 W. W. Yau, J. J. Kirkland and D. D. Bly, *Modern Size-Exclusion Liquid Chromatography*, Wiley-Interscience, New York, 1979, Ch. 4.
14 W. W. Yau, J. J. Kirkland, D. D. Bly and H. J. Stoklosa, *J. Chromatogr.*, 125 (1976) 219.