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[Chem. Pharm. Bull. 36(12)4971—4975(1988)]

Low-Angle Laser Light Scattering Measurements on Highly Purified Sodium Hyaluronate from Rooster Comb

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(Received May 13, 1988)

Using highly purified sodium hyaluronate from rooster comb, we studied the relationship between the intrinsic viscosity and the molecular weight obtained by the low-angle laser light scattering technique. The equation of the relationship between the intrinsic viscosity and the molecular weight is $[\eta]=3.9\times10^{-4}\times M_w^{0.77}$. Gel permeation chromatography of the hyaluronate preparations was also performed.

Keywords——hyaluronic acid; sodium hyaluronate; hyaluronate; rooster comb; molecular weight; light scattering; viscosity; gel permeation chromatography

Hyaluronic acid is a naturally occurring highly viscous glycosaminoglycan composed of unbranched repeating disaccharide units of D-glucuronic acid and *N*-acetyl-D-glucosamine with alternating $\beta(1\rightarrow3)$ and $\beta(1\rightarrow4)$ linkages. The molecular weight of hyaluronic acid is generally in the range of 5×10^4 to 8×10^6 (although there are reports of hyaluronic acid having a molecular weight as high as 1.3×10^7) depending on the source, the method of isolation and the method of molecular weight determination. Hyaluronic acid is found in animal tissues, *e.g.*, in umbilical cord, vitreous humor, synovial fluid, rooster combs and skin, and in culture medium of group A and C hemolytic streptococci. The isolation and characterization of hyaluronic acid have been described by Meyer *et al.*,[1,2] Balazs,[3] Laurent,[4] and Varga,[5] and its chemical structure was elucidated by Blix and Snellman,[6] Meyer and Fellig,[7] and Weissmann and Meyer.[8]

The relationship between the intrinsic viscosity and the molecular weight obtained by light scattering measurement of hyaluronic acid, has already been reported by Laurent *et al.*,[9] Cleland and Wang,[10] and Balazs.[3] However, the origin of hyaluronic acid used in their studies was bovine vitreous body and the samples are thought to have been of low purity, since protein was present at levels of a few per cent.

The present report describes the relationship between the intrinsic viscosity and the molecular weight of highly purified sodium hyaluronate from rooster comb, using low-angle laser light scattering, as well as the molecular weight distribution obtained by gel permeation chromatography.

Experimental

Materials——Sodium hyaluronate was isolated from rooster comb by an industrial method.[11] The concentrations of hyaluronate were determined by the modified carbazole method[12] using D-glucuronolactone as a standard. Protein was measured according to the method of Lowry *et al.*,[13] using bovine serum albumin as a standard. Total nitrogen values were obtained by a semi-micro Kjeldahl analysis.[14] Eight preparations varying in

molecular weight were used in the present investigation. Analytical data are presented in Table I. All samples contained more than 99% hyaluronate, less than 0.02% protein and less than 0.2% sodium chloride and showed little absorption due to nucleic acid at 260 nm.

Viscosity——The intrinsic viscosity $[\eta]$ was measured in the concentration range from 0.01 to 0.1% (w/v) in 0.2 M NaCl, using Ubhelohde viscometers and a Rigo VMC-052 Auto Viscosimeter instrument at 30 ± 0.1 °C.

Refractive Index Increment——The refractive index increments of sodium hyaluronate were measured in 0.2 M NaCl solution, using a Union Giken differential refractometer at 25 °C and a wavelength of 633 nm.

Light Scattering——Measurements were performed in the concentration range from 1.5×10^{-5} to 1×10^{-2} g/ml, using 0.2 M NaCl as a solvent and a Chromatix KMX-6 low-angle laser light scattering instrument at 25 °C and a wavelength of 633 nm with a scattering angle of 6—7°. Sample solutions were filtered through a 0.22 μm membrane (Millipore Corporation) and transferred to a cell at a constant speed (about 0.3 ml/min) with an infusion pump. There was no change in the concentration of sodium hyaluronate before and after filtration.

Gel Permeation Chromatography——The measurement was performed using a Toyo Soda HLC-803D high-speed liquid chromatograph with a TSK-GEL G6000PW column (60 cm, × 2) on the basis of the method reported by Beaty *et al.*[15] The column was eluted with 0.2 M NaCl at flow rate of 1.0 ml/min. The elution pattern was detected with an RI-8 differential refractometer (Toyo Soda Co., Ltd., Tokyo) at 40 °C. The retention time and peak integration were recorded on CHROMATOPAC C-R1B (Shimadzu Co., Ltd., Kyoto).

Results and Discussion

The intrinsic viscosity $[\eta]$ values of sodium hyaluronate preparations used in this investigation were found to be 5.28 to 24.6 dl/g (Table I). The value of refractive index increment (dn/dc) in 0.2 M NaCl was 0.162 ± 0.004 for experimental samples and was independent of viscosity (molecular weight). This value is in agreement with the data reported earlier for hyaluronic acid.[9,16] Thus, this value was used for calculation of the molecular weight of sodium hyaluronate.

The concentration of sodium hyaluronate should be low to calculate the true molecular weight, particularly for higher values of molecular weight, and therefore measurements were performed in the concentration range from 1.5×10^{-5} to 2.5×10^{-4} g/ml for hyaluronate having a molecular weight of more than 5×10^5. An example of the relationship between the concentration and the value of K_c/R_θ for high-molecular-weight hyaluronate (9.6×10^5) is shown in Fig. 1.

Figure 1 shows that the experimental concentration range remarkably influences the values of K_c/T_θ. Scattering intensity was sufficient for the measurement of K_c/R_θ at these low sample concentrations, even below 2.5×10^{-4} g/ml. The value of the weight-average molecular weight was determined from the reciprocal of K_c/R_θ extrapolated to zero concentration (Fig. 2).

The second virial coefficients (A_2) of hyaluronate preparations decreased with increasing molecular weight of hyaluronate and the values were in the range from 3.2×10^{-3} to 2.0×10^{-3}. The results are consistent with the data of Cleland and Wang.[10]

TABLE I. Analytical Data for Sodium Hyaluronate

Sample No.	Uronic acid (%)	Nitrogen (%)	Protein (%)	$[\eta]$ (dl/g)
1	46.8	3.26	0.015	5.28
2	46.8	3.13	0.020	9.4
3	49.3	3.39	0.018	13.6
4	46.0	3.34	0.019	14.7
5	48.7	3.40	0.010	15.7
6	48.1	3.38	0.020	17.8
7	49.1	3.48	0.019	18.8
8	48.2	3.37	0.017	24.6

Samples were extracted from rooster comb. The values of $[\eta]$ were determined at 30 ± 0.1 C from the reduced viscosity η_{red} extrapolated to zero concentration by a linear least-squares method.

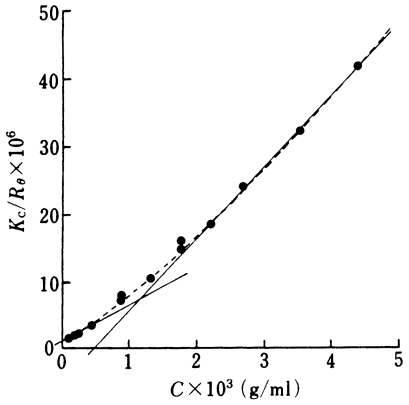

Fig. 1. Influence of Concentration Range on Molecular Weight Determination

Concentration range greatly influences the determination of molecular weight for high-molecular-weight hyaluronate, because molecular weight is determined from the reciprocal of K_c/R_θ extrapolated to zero concentration. The case of sodium hyaluronate having a molecular weight of 9.6×10^5 is shown in this figure as an example.

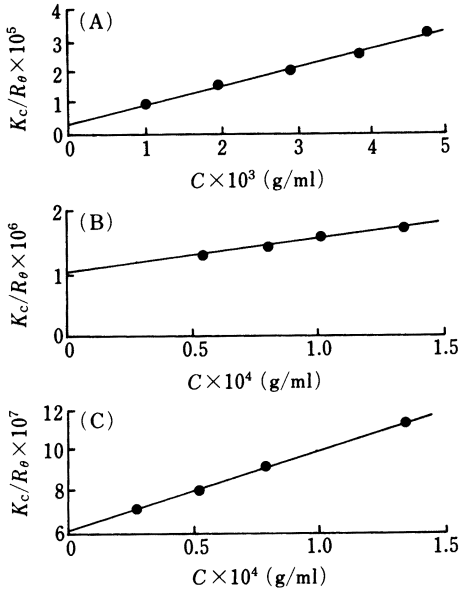

Fig. 2. Determination of Molecular Weight

The value of molecular weight was determined from the reciprocal of K_c/R_θ extrapolated to zero concentration. (A) sample No. 1; (B) sample No. 5; (C) sample No. 8.

TABLE II. Physical Properties of Sodium Hyaluronate

Sample No.	$[\eta]$ (dl/g)	$M_w \times 10^{-5}$	$A_2 \times 10^3$	M_w/M_n
1	5.28	2.5	3.0	2.05
2	9.4	4.6	3.2	2.13
3	13.6	7.6	2.9	2.14
4	14.7	8.4	2.7	1.97
5	15.7	9.6	2.8	1.82
6	17.8	11.9	2.8	1.96
7	18.8	13.7	2.7	2.10
8	24.6	16.3	2.0	1.82

Light scattering data (M_w, A_2) were obtained using a low-angle laser light scattering instrument, at 25 °C and at a wavelength of 633 nm. The values of M_w/M_n were determined on the basis of gel permeation chromatography.

The values for the molecular weight and second virial coefficient calculated from the light scattering data and for the intrinsic viscosity are summarized in Table II. When the logarithm of the intrinsic viscosity was plotted against the logarithm of molecular weight, a linear correlation was found (Fig. 3). The equation calculated from the linear relation is $[\eta] = 3.9 \times 10^{-4} \times M_w^{0.77}$. The equation is in good agreement with the relationship between the intrinsic viscosity and the molecular weight described earlier.[9] Protein content did not affect the molecular weight obtained by the light scattering method.

Elution patterns of the samples in gel permeation chromatography are shown in Fig. 4. A calibration curve was calculated from the logarithm of molecular weight and elution time, and

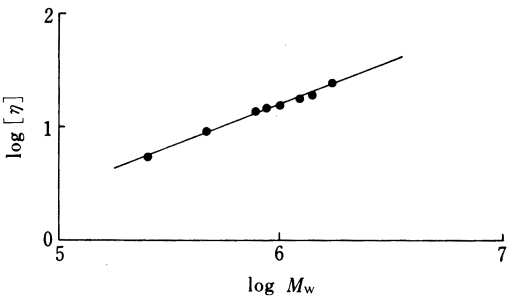

Fig. 3. Relationship between Intrinsic Viscosity and Molecular Weight

Coefficient of correlation: 0.993.

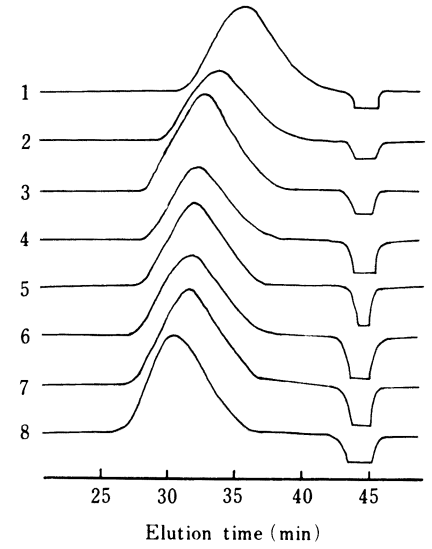

Fig. 4. Elution Patterns on TSK-GEL G6000 PW (60 cm, × 2)

Elution with 0.2 M NaCl was performed at a flow rate of 1.0 ml/min. The elution pattern was detected with a differential refractometer at 40 C. The numbers in this figure indicate the sample number in Table I.

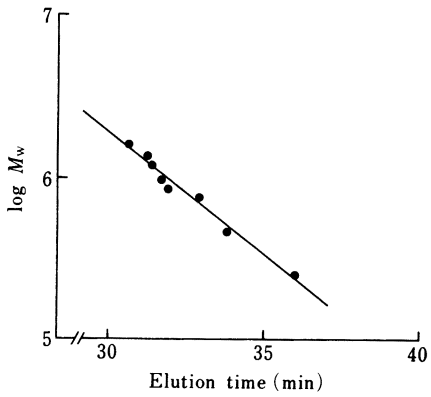

Fig. 5. Correlation between Molecular Weight and Elution Time on TSK-GEL G6000PW (60 cm, × 2)

Conditions of gel permeation chromatography were the same as in Fig. 4. Coefficient of correlation: 0.986.

a linear relationship was observed between them (Fig. 5). The values of the number-average molecular weight (M_n) and the weight-average molecular weight (M_w) were calculated on the basis of the calibration curve determined by a linear least-squares method[17] and each M_w/M_n ratio was approximately 2. The results are given in Table II. These values suggest that the hyaluronate preparations used in this study, prepared by an industrial procedure, are not polydisperse.

The relationship between the intrinsic viscosity and the molecular weight, obtained by light scattering measurement of hyaluronic acid from bovine vitreous body, has already been reported by Laurent *et al.*,[9] Cleland and Wang,[10] and Balazs.[3] The relationship between the intrinsic viscosity and the molecular weight determined by sedimentation equilibrium of hyaluronate from human umbilical cord was described by Shimada and Matsumura.[18] As the hyaluronate preparations used in those studies were not of adequate purity, we studied this relationship using highly purified sodium hyaluronate, containing less than 0.02% protein, by low-angle laser light scattering techniques. The refractive index increment of hyaluronate was

constant independently of molecular weight. The equation of the relationship between the intrinsic viscosity and the molecular weight of hyaluronate from rooster comb is $[\eta] = 3.9 \times 10^{-4} \times M_w^{0.77}$.

Acknowledgment We are greatly indebted to Prof. Yuuko Kambe of Showa College of Pharmaceutical Sciences, Tokyo, Japan, for the use of the light scattering instrument and the differential refractometer in her laboratory and for her helpful suggestions.

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