# FRACTIONATION OF HYALURONIC ACID

## THE POLYDISPERSITY OF HYALURONIC ACID FROM THE BOVINE VITREOUS BODY*

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SUMMARY

The polydispersity of hyaluronic acid from the bovine vitreous body has been demonstrated by the successful separation of the polysaccharide into fractions with molecular weights ranging from $7.7 \cdot 10^4$ to $1.7 \cdot 10^6$. The results of light-scattering, ultracentrifuge and viscosity studies have been compared; it has been found that, where discrepancies occur, the limiting viscosity numbers correspond more closely to the molecular weights obtained by light-scattering than to those evaluated from ultracentrifuge data. The extent to which light-scattering and ultracentrifuge molecular weights are at variance appears to be a function of the degree of polydispersity of each individual sample.

The relationship between the limiting viscosity number and molecular weight, $[\eta] = 0.036 \cdot M^{0.78}$ is expected for a coil configuration for hyaluronic acid.

## INTRODUCTION

While there is general agreement that hyaluronic acid from the bovine vitreous body has a lower molecular weight than that from most other sources, the molecular weight as reported in the literature varies between $5.7 \cdot 10^4$ and $1.3 \cdot 10^6$ (see ref.1–9). There is usually a large discrepancy between values obtained by sedimentation and diffusion[4,8,9] and light-scattering[2,3,6–8], the former being lower than the latter. The rather large variability of data has been ascribed, among other things, to methods of preparation, to the use of several physicochemical methods in the determination of molecular weight, and finally, and perhaps most importantly, to the effects of polydispersity of the polymer. Viscosity studies on ammonium sulfate fractionated material[10] and on material isolated from different parts of the bovine vitreous body[11], as well as light-scattering[3], diffusion[12] and streaming birefringence studies[1,6], and sedimentation boundary analysis[4] on unfractionated material, have indeed indicated that hyaluronic acid from the bovine vitreous body is highly polydisperse.

This paper describes the fractionation of hyaluronic acid, obtained in high

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yield, into fractions of different molecular weight. The fractions obtained have been studied by light-scattering, sedimentation and diffusion analysis, and viscosity.

## METHODS

### *Refractive increment*

The refractive increment of sodium hyaluronate was determined in a calibrated Rayleigh-type Zeiss interferometer at a wavelength of 546 mμ at 25°. Measurements were made at several concentrations on unfractionated sodium hyaluronate that had been carefully dialyzed against 0.1 *M* sodium chloride. The concentrations of the samples on which readings were taken were determined on a dry-weight basis. Equal volumes of dialysate and sodium hyaluronate solutions were dried over sulfuric acid at 60° for 48 h and the dry weight was obtained as the weight difference.

### *Light-scattering*

Measurements were made at room temperature in a BRICE-SPEISER instrument[13] (Pheonix Precision Instrument Co.) at a wavelength of 436 mμ, and the angular distribution of scattered light was measured at angles between 35° and 135° with respect to the incident beam. The specimens were centrifuged in calibrated glass cells similar to those described by DANDLIKER AND KRAUT[14] for 2 h at 24,800 × *g*. In order to minimize the effects of the concentration gradients obtained during centrifugation, the cells were allowed to stand in the cold for 24 h before readings were taken. Blank readings were obtained from centrifuged sodium chloride solutions. The concentration range employed in each series of experiments was dictated by the molecular weight of the particular fraction under study.

### *Sedimentation*

Determination of sedimentation velocities using the moving boundary method were made at 52,640 rev./min at room temperature in a Spinco Model E analytical ultracentrifuge equipped with a Philpot-Svensson cylindrical lens optical system. Sedimentation coefficients ($S_{20}$) were obtained at five concentrations for each fraction. Extrapolation to infinite dilution was obtained by plotting $1/S$ against concentration[15].

### *Diffusion*

Diffusion experiments were conducted in the analytical ultracentrifuge with valve-type synthetic-boundary cells. Solvent was layered over sodium hyaluronate solutions at speeds of 7 to 10,000 rev./min, and the speed was then reduced to 5,000 rev./min. Diffusion coefficients ($D_{20}$) were calculated by the height–area method[16] from photographic enlargements using a planimeter for area measurements. 6 to 8 determinations sufficed to permit a linear extrapolation to zero concentration.

### *Viscosity*

Limiting viscosity numbers were calculated for each fraction at 25°. The measurements were made over a concentration range of 0.15 to $5.6 \cdot 10^{-3}$ g/ml, using Ostwald viscometers with outflow times of 40 to 50 sec/ml and shear gradients of the order of 1,000 $cm^{-1}$.

*Chemical methods*

Hexuronic acid was measured by the carbazole method[17], using glucuronic acid as a standard, and sodium hyaluronate concentrations were then calculated from hexuronic acid values by multiplication with a factor of 1.9 found empirically from dry-weight determinations. A modification of the Elson-Morgan method[18,19] was used to determine hexosamine. Total nitrogen values were obtained by a micro Kjeldahl analysis[20] and the protein content was calculated from non-hyaluronic acid nitrogen (protein = N × 6.25).

*Preparation and fractionation techniques*

*Preparation.* The occipital part of the vitreous body collected from fresh steer and cow eyes as described earlier[3] was liquified by centrifugation for 2 h at 65,000 × *g* and dialyzed against several changes of distilled water at 4°. Hyaluronic acid was isolated as cetylpyridinium hyaluronate by the addition of 0.11 g of cetylpyridinium chloride (Kebo Co., Stockholm) per 100 ml of liquid vitreous[21]. The cetylpyridinium hyaluronate precipitate was collected by centrifugation and suspended in 2 *M* sodium chloride. Constant stirring for 2 days at room temperature dissolved all but a small part of the precipitate. The insoluble matter, although finely dispersed, resisted all efforts to bring it into solution. Micro-cel E (Johns Manville Co.) was now added at the rate of 3.5 g/100 ml to remove both cetylpyridinium chloride and the remaining protein impurities[22]. After having been stirred for 2 h, the suspension was centrifuged for 20 min at 35,000 × *g* and the supernatant was collected. To minimize the loss of hyaluronic acid it was necessary to resuspend the Micro-cel E in an equal volume of 2 *M* sodium chloride, centrifuge again, and collect the supernatant. The supernatants were combined and the solution was analyzed.

At this stage the sodium hyaluronate was dialyzed against 0.2 *M* sodium chloride and precipitated from solution by the addition of 3 volumes of ethanol. The material was stored in the cold in the aqueous ethanol. The sodium hyaluronate was then redissolved in concentrations suitable for the work to be performed.

*Fractionation.* A solution of 1.77 g of cetylpyridinium chloride in 50 ml of 0.25 *N* $Na_2SO_4$ was added to 1.24 g of sodium hyaluronate dissolved in 205 ml of the same medium. The slightly turbid solution was centrifuged for 15 min at 75,000 × *g* and the small amount of precipitate obtained was labeled fraction I. The supernatant was then diluted with distilled water in successive stages to $Na_2SO_4$ concentrations of 0.190, 0.174, 0.165, 0.155, 0.130 and 0.060 *N* and after each dilution the solution was centrifuged. The sediments collected constituted fractions II through VII. Each precipitate was dissolved in 2.5 *N* $Na_2SO_4$ and treated with Micro-cel E to remove cetylpyridinium chloride. This was followed by dialysis against 0.2 *M* sodium chloride, chemical analysis and reprecipitation with 3 volumes of ethanol.

The solution remaining after separation of fraction VII was treated with Micro-cel E. Subsequent addition of 3 volumes of ethanol precipitated a large amount of salt as well as sodium hyaluronate (fraction VIII). This precipitate was then dissolved in distilled water and dialyzed at + 4°. A precipitate formed on addition of 3 volumes of ethanol to the dialyzed solution was collected as fraction VIII A. Storage of the turbid supernatant in the cold and the addition of a small amount of sodium chloride gave a second precipitate (fraction VIII B).

All physicochemical studies were made on sodium hyaluronate dialyzed against

0.2 *M* sodium chloride unless otherwise stated. Fractions VI and VII were combined before the physicochemical studies.

## RESULTS

### *Fractionation*

Sodium hyaluronate was isolated from bovine vitreous body in 89% yield with a protein contamination of less than 2%. Table I summarizes the results of the preparative procedure. The data tabulated represent the combined material obtained from three separate preparations.

TABLE I

THE ISOLATION OF SODIUM HYALURONATE

| | *Original vitreous humor* | *Preparation* | *Recovery* |
|---|---|---|---|
| Sodium hyaluronate | 1.359 g | 1.210 g | 89.2% |
| Protein | 1.415 g | 0.0185 g | 1.3% |
| Ratio sodium hyaluronate: protein | 0.96:1 | 65.5:1 | |

Table II outlines the results obtained in the fractionation procedure. The recovery was 79%, which gave an overall yield of 70% for preparation and fractionation. There seems to be no indication of any difference in the chemical composition of the various fractions. The discrepancy often encountered between the methods we used for hexosamine and hexuronic acid analysis[17,23] was observed.

TABLE II

ANALYTICAL DATA

| | *Normality of $Na_2SO_4$ at precipitation* | *Size of fraction mg* | *Percent of starting material* | *Hexosamine: hexuronic acid: nitrogen molar ratio* |
|---|---|---|---|---|
| Unfractionated sodium hyaluronate, sample A* | 0.00 | | | 0.85:0.94:1.00 |
| Unfractionated sodium hyaluronate, sample B* | 0.00 | | | 0.90:1.05:1.00 |
| Fraction I | 0.250 | — | — | |
| II | 0.190 | 11 | 0.9 | |
| III | 0.174 | 58 | 4.7 | 0.88:0.96:1.00 |
| IV | 0.165 | 135 | 10.9 | 0.85:0.93:1.00 |
| V | 0.155 | 260 | 20.9 | 0.81:0.99:1.00 |
| VI | 0.130 | 152 | 12.3 | 0.82:0.94:1.00 |
| VII | 0.060 | 28 | 2.2 | |
| VIIIA | — | 144 | 11.6 | 0.81:0.99:1.00 |
| VIIIB | — | 192 | 15.5 | 0.88:0.96:1.00 |
| Recovery | | 980 mg | 79.0% | |

* Samples A and B and a third preparation were pooled for the fractionation experiment.

*Refractive increment*

The refractive increment of sodium hyaluronate was found to be 0.166 at 546 mμ. Since this is substantially in agreement with the refractive increments reported earlier[24], there was no need to recalculate the constant $K$ of the light-scattering equation.

*Light-scattering*

The data were treated and evaluated according to ZIMM[25]. $K \cdot c/R_\theta$ was plotted against $\sin^2 \theta/2 + kc$, where $K$ is a constant, $c$ the concentration, $R_\theta$ the reduced angular intensity of the scattering, $\theta$ the observation angle and $k$ an arbitrary constant. The value $4.58 \cdot 10^{-7}$ was used for the constant $K$ (see ref. 24). A double extrapolation to zero angle and zero concentration gave the reciprocal molecular weight. The radius of gyration was calculated from the slope of the initial angular scattering envelope and its intercept on the $K \cdot c/R_\theta$ axis. Representative ZIMM-plots are given in Figs. 1 and 2.

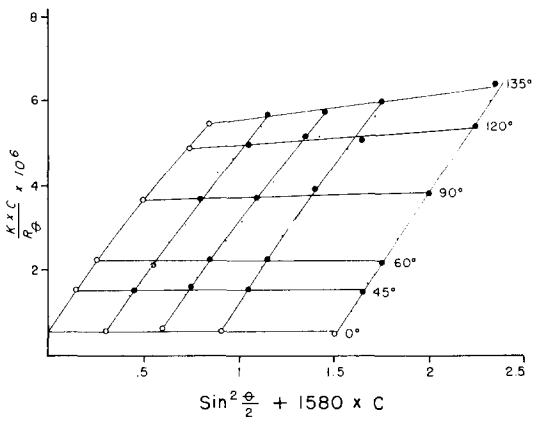



Fig. 1. ZIMM-plot of fraction III. Mol. wt. $1.7 \cdot 10^6$. Radius of gyration 1430 Å.

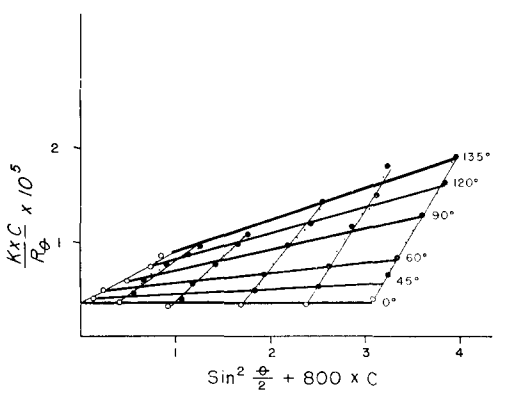



Fig. 2. ZIMM-plot of fraction VI-VII. Mol. wt. $2.8 \cdot 10^5$. Radius of gyration 540 Å.

The values for molecular weight and radius of gyration obtained from the light-scattering data are tabulated in Table III. The errors of the determinations are estimated to be of the order of ± 10 %[24]. In the case of the fractions of lowest molecular weight the data were not reliable enough to warrant calculation of the radius of gyration.

TABLE III

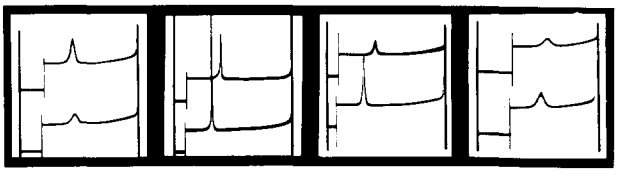
PHYSICOCHEMICAL DATA

| *Fractions* | *Ultracentrifuge data* | | | *Light-scattering data* | | *Limiting viscosity number ml/g* |
|---|---|---|---|---|---|---|
| | *Sedimentation constant* $S_{20,w} \times 10^{13}$ | *Diffusion constant* $D_{20,w} \times 10^{7}$ | *Molecular weight* | *Molecular weight* | *Radius of gyration Å* | |
| Unfractionated sodium hyaluronate, sample A | 2.98 | 0.99 | $2.2 \cdot 10^5$ | $3.7 \cdot 10^5$ | 640 | 945 |
| Unfractionated sodium hyaluronate, sample B | 3.13 | 0.80 | $2.8 \cdot 10^5$ | $4.5 \cdot 10^5$ | 710 | 970 |
| Fraction III | 7.15 | 0.34 | $1.5 \cdot 10^6$ | $1.7 \cdot 10^6$ | 1430 | 2450 |
| IV | 6.67 | 0.38 | $1.3 \cdot 10^6$ | $1.3 \cdot 10^6$ | 1570 | 2340 |
| V | 3.76 | 0.65 | $4.1 \cdot 10^5$ | $3.8 \cdot 10^5$ | 630 | 875 |
| VI–VII | 3.12 | 1.16 | $1.9 \cdot 10^5$ | $2.8 \cdot 10^5$ | 540 | 700 |
| VIIIA | 2.52 | 1.89 | $9.5 \cdot 10^4$ | $1.1 \cdot 10^5$ | — | 295 |
| VIIIB | 2.46 | 1.74 | $1.0 \cdot 10^5$ | $7.7 \cdot 10^4$ | — | 250 |

## *Sedimentation*

Fig. 3 depicts the sedimentation patterns of unfractionated hyaluronic acid, and of samples of high, medium and low molecular weight. It can be seen that the sharpness of the boundary varies directly with molecular weight and concentration.

Fig. 4 shows the concentration dependence of the reciprocal sedimentation coefficient. The sedimentation velocity is independent of molecular weight at concentrations above $2 \cdot 10^{-3}$ g/ml. The sedimentation constants are listed in Table III.



| *Fraction* | *Unfractionated NaHyaluronate B* | *III* | *V* | *VIII-A* |
|---|---|---|---|---|
| Concn., (mg/ml) | 1.40 | 0.88 | 0.70 | 0.96 |
| | 0.90 | 1.33 | 1.41 | 1.44 |
| Time (min) | 140 | 141 | 144 | 152 |
| Bar angle | 60° | 60° | 60° | 55° |

Fig. 3. Sedimentation patterns.

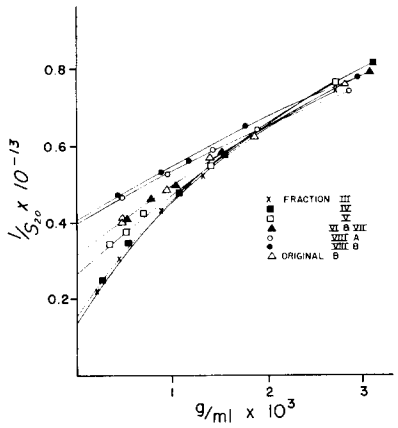



Fig. 4. Reciprocal sedimentation velocity as a function of concentration.

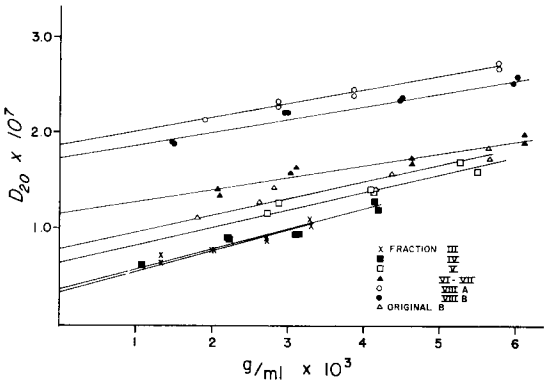
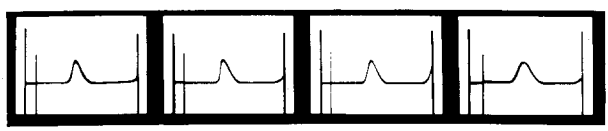



Fig. 5. The diffusion coefficient as a function of concentration.

*Diffusion*

Diffusion has been studied as a function of concentration (Fig. 5). A linear relationship was observed in each case, and the concentration dependence was found to increase with the molecular weight. The marked differences in concentration dependence of the diffusion characteristics of the individual fractions were manifested



| *Fraction* | *Unfractionated NaHyaluronate B* | *III* | *V* | *VIII-A* |
|---|---|---|---|---|
| Concn., (mg/ml) | 2.71 | 3.30 | 2.80 | 2.87 |
| Time (min) | 154 | 159 | 153 | 152 |
| Bar angle | 60° | 65° | 60° | 55° |

Fig. 6. Diffusion patterns.

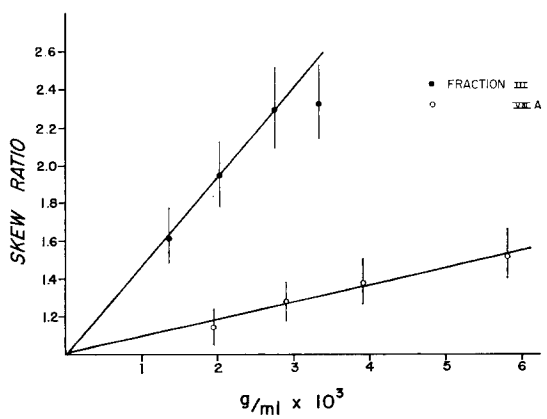



Fig. 7. The skew ratio (see text) of diffusion patterns as a function of concentration. The vertical lines represent the effects of a theoretical 2 % error in the area determinations.

as a variation in the skewness of the corresponding diffusion patterns (Fig. 6). GOSTING[16] has reviewed this interrelationship from a theoretical standpoint. The ratio of the areas on either side of the maximum was used as a measure of skewness (Fig. 7). In accordance with the theoretical expectations, extrapolation of the skew ratios to infinite dilution gave a value of one.

Molecular weights were calculated from sedimentation and diffusion data according to the Svedberg formula with use of the value 0.66 $cm^3/g$ for the partial specific volume of hyaluronic acid[4]. Owing to the difficulties in the extrapolation procedures, the molecular weight values (Table III) have errors of the same magnitude as those obtained from light-scattering measurements.

*Viscosity*

The viscosity data plotted as a function of concentration are shown in Fig. 8 and the extrapolated limiting viscosity numbers are given in Table III.

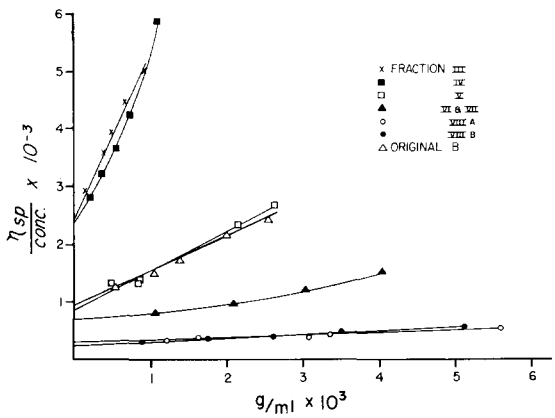



Fig. 8. Viscosity data.

## DISCUSSION

The conclusions that can be deduced from the data gathered in this investigation are of particular interest if the hyaluronic acid studied is representative of that existing in the tissue from which it was isolated. That this is the case is supported not only by the high yields that were obtained, but also by the fact that the molecular weight of unfractionated vitreous hyaluronic acid prepared by cetylpyridinium chloride precipitation—calculated from either light-scattering or sedimentation-diffusion data—agrees with the values similarly calculated for material prepared by other methods[3,4,7–9].

The results show the molecular weight of unfractionated hyaluronic acid to be $4 \cdot 10^5$, when evaluated from light-scattering measurements, and $2.5 \cdot 10^5$, when determined from sedimentation and diffusion data. This discrepancy, which has also been reported in earlier work, might be explained by the demonstrated polydispersity of bovine vitreous hyaluronic acid[8] (Fig. 9). MOORE AND GREEAR[26], in recent studies on fractionated polyethylenes, have found that large molecules, which exert a great influence on light-scattering measurements, in low concentration escape detection in the ultracentrifuge.

It is apparent then that the weight-average molecular weights of polydisperse materials tend to be underestimated by ultracentrifugal methods, whereas light-scattering methods yield values that are more correct. The agreement between the two methods improves the more homogeneous is the system. The calculated weight-average molecular weight of unfractionated hyaluronic acid obtained from the molecular weights of the fractions is $4.5 \cdot 10^5$ and is in close agreement with the light-scattering value. Similarly, the limiting viscosity number of unfractionated hyaluronic acid corresponds to a molecular weight which is closer to that found by light-scattering than to that obtained from ultracentrifuge results.

The data seem to indicate that there may be two species of hyaluronic acid in the vitreous, one with a molecular weight of the order of $1.5 \cdot 10^6$, the other with a molecular weight below $4 \cdot 10^5$ (Fig. 9). These have been isolated as fractions III and IV, and fractions V–VIII, respectively. With the exception of fractions VI–VII, the fractions were judged to be relatively homogeneous on the basis of the agreement between light-scattering and sedimentation-diffusion molecular weights.

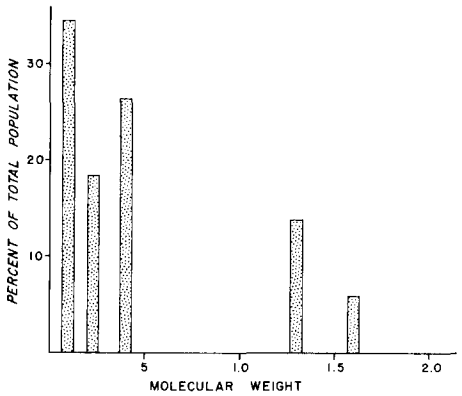



Fig. 9. The molecular weight distribution in hyaluronic acid from the bovine vitreous body.

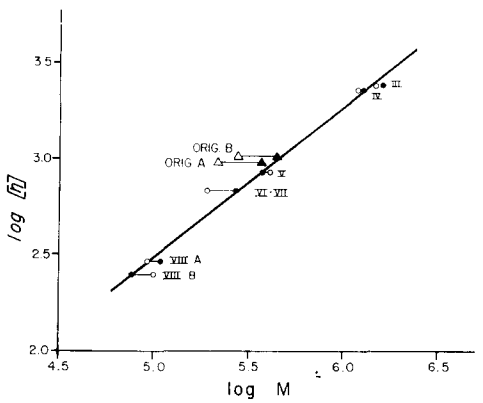



Fig. 10. The limiting viscosity number as a function of molecular weight. Open symbols, ultracentrifuge data, shaded symbols, data from light-scattering.

When the logarithm of the limiting viscosity number is plotted against the logarithm of molecular weight (Fig. 10), the light-scattering molecular weights are seen to fall on a straight line. The equation calculated for the line is $[\eta] = 0.036\ M^{0.78}$. The equation agrees with the relationships found for polymers with known coil configurations[27]. Similarly, the proportionality observed between the radius of gyration and the limiting viscosity number ($(\bar{r}^2)^{1/2} = k[\eta]^{0.85}$), as well as the functional relationship observed between radius of gyration and molecular weight ($(\bar{r}^2)^{1/2} = k \cdot M^{0.60}$), is close to that expected for a coil.

The concentration dependence of sedimentation velocity is greatest at low concentrations and this complicates the evaluation of sedimentation constants. The hazards of extrapolating the data to infinite dilution have been minimized by plotting the reciprocal sedimentation velocity as a function of concentration (Fig. 4). At high concentrations the sedimentation velocity is independent of molecular weight, indicating that no longer are single molecules sedimenting, but rather a continuous three-dimensional network[28].

The concentration dependence of the diffusion coefficients imparts a definite skewness to the diffusion patterns (Fig. 6). Under these circumstances, erroneous values are obtained when diffusion coefficients are calculated by standard methods. However, since the skewness disappears at infinite dilution (Fig. 7), diffusion constants obtained by extrapolation of data to zero concentration should be correct.

The wide variance in the values reported for the refractive increment of hyaluronic acid[6,24,29,30] prompted a reinvestigation. In order to avoid the errors inherent in chemical analysis, the concentration of the samples on which readings were taken were determined directly as dry-weights. It is felt that this procedure and the purity of the preparation enhance the reliability of our data. Actually, a value in agreement with our earlier results[24] has been obtained.

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REFERENCES

1 G. BLIX AND O. SNELLMAN, *Arkiv Kemi, Mineral. Geol.*, 19A (1945) 1.
2 R. BRUNISH, J. W. ROWEN AND S. R. IRVINE, *Trans. Am. Ophthalmol. Soc.*, 52 (1954) 369.
3 T. C. LAURENT, *J. Biol. Chem.*, 216 (1955) 263.
4 L. VARGA, *J. Biol. Chem.*, 217 (1955) 651.
5 J. A. CHRISTIANSEN AND E. C. JENSEN, *Acta Chem. Scand.*, 9 (1955) 1405.
6 J. W. ROWEN, R. BRUNISH AND F. W. BISHOP, *Biochim. Biophys. Acta*, 19 (1956) 480.
7 T. C. LAURENT, *Arkiv Kemi*, 11 (1957) 487.
8 E. A. BALAZS, *Federation Proc.*, 17 (1958) 1086.
9 L. VARGA, A. PIETRUSZKIEWICZ AND M. RYAN, *Biochim. Biophys. Acta*, 32 (1959) 155.
10 K. MEYER, *Physiol. Rev.*, 27 (1947) 335.
11 E. A. BALAZS AND L. SUNDBLAD, personal communication.
12 R. BRUNISH, M. VANGEROV AND W. IRVINE, *Federation Proc.*, 15 (1956) 226.
13 B. A. BRICE, M. HALWER AND R. SPEISER, *J. Opt. Soc. Am.*, 40 (1950) 768.
14 W. B. DANDLIKER AND J. KRAUT, *J. Am. Chem. Soc.*, 78 (1956) 2380.
15 H. K. SCHACHMAN, *Ultracentrifugation in Biochemistry*, Academic Press, New York, 1959.
16 L. J. GOSTING, *Advances in Protein Chem.*, 11 (1956) 429.
17 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
18 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
19 E. A. BALAZS, personal communication.
20 T. S. MA AND G. ZUAZAGA, *Ind. Eng. Chem., Anal. Ed.*, 14 (1942) 280.
21 J. E. SCOTT, *Biochim. Biophys. Acta*, 18 (1955) 428.
22 E. A. BALAZS AND L. SUNDBLAD, *Acta Soc. Med. Uppsaliensis*, 64 (1959) 137.
23 S. GARDELL, *Methods of Biochemical Analysis*, 6 (1958) 289.
24 T. C. LAURENT AND J. GERGELY, *J. Biol. Chem.*, 212 (1955) 325.
25 B. H. ZIMM, *J. Chem. Phys.*, 16 (1948) 1099.
26 L. D. MOORE, JR. AND G. R. GREEAR, *136th Meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959.*
27 P. J. FLORY AND T. G. FOX, JR., *J. Am. Chem. Soc.*, 73 (1951) 1904.
28 A. G. OGSTON AND E. F. WOODS, *Trans. Faraday Soc.*, 50 (1954) 635.
29 B. S. BLUMBERG AND G. OSTER, *Science*, 120 (1954) 432.
30 B. S. BLUMBERG, A. G. OGSTON, D. A. LOWTHER AND H. J. ROGERS, *Biochem. J.*, 70 (1958) 1.