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STUDIES ON HYALURONIC ACID

I. THE INFLUENCE OF IONIC STRENGTH ON THE SEDIMENTATION AND DIFFUSION PROPERTIES*

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SUMMARY

Hyaluronic acid was prepared by an electrophoretic separation technique. This was considered to be the mildest possible method of preparation and the method least likely to cause any changes during preparation.

The following physicochemical parameters were obtained: Sedimentation constant, $s_{20} = 3.1$ S; diffusion constant, $D_{20} = 1.25 \cdot 10^{-7}$ cm²/sec; intrinsic viscosity, $[\eta] = 7.0$. From these values we were able to calculate a molecular weight of M = 178,000 and a hydrodynamically effective volume of $V_e = 1.0$ ml/g, indicating a hydration of about 30 %. On this basis and on the assumption that the shape of the molecule approximates the shape of a prolate ellipsoid of revolution, the molecular length was calculated to be $L = 1900$ Å and the diameter of maximal circumference, $d = 17.5$ Å.

A frequency distribution coefficient of $\gamma = 0.44$ was calculated from the spread of the sedimentation patterns. It was found that hyaluronic acid satisfies the Huggins equation when the constant $k' = 0.275$.

The sedimentation and diffusion characteristics of hyaluronic acid have been studied as a function of ionic strength. It was found that these properties are very sensitive to changes in ionic strength, with the dependence most marked as zero ionic strength is approached. However, the changes induced by varying the ionic strength are perfectly reversible. Changes in temperature had no effect within the range investigated.

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INTRODUCTION

Since some of the physical theories describing the molecular kinetic behavior of macromolecules are based on the assumption that the particles are electrically neutral, it is important to study their molecular parameters in solutions of high ionic strength. In a solution of sufficiently high ionic strength the electrically charged groups of macromolecules can be shielded to such a degree that their physicochemical behavior will be similar to that of neutral particles. The smaller the shielding influence of the ionic milieu, the greater will be the effect of the charged groups and, therefore, the deviation from theoretical behavior. In physiological salt solutions with an ionic strength of about 0.14 hyaluronic acid does not fulfil this condition, since it still carries a considerable effective charge[1]. In view of the fact that hyaluronic acid is one of the main extracellular components of many connective tissues it was of interest to study the influence of ionic strength on this macromolecule, even though a theoretical explanation cannot always be given for experimental findings.

EXPERIMENTAL

Preparation of hyaluronic acid

Hyaluronic acid was prepared electrophoretically from bovine vitreous bodies. The observation that the hyaluronic acid moves ahead of the proteins during an electrophoretic analysis serves as the basis for the preparation. The technical details of this method have already been described in an earlier paper[2], where the chemical analysis of the preparation was also reported. A protein impurity of not more than 3.5 % was calculated on the basis of nonhexosamine nitrogen. An electrophoretic pattern of a liquefied and centrifuged vitreous solution is shown in Fig. 1. The area under the hyaluronic acid peak is proportional to the average hyaluronic acid content

Fig. 1. Electrophoretic pattern of vitreous solution dialyzed against 0.006 *M* phosphate buffer at pH 5.5, ionic strength 0.08, regulated by NaCl. Migration is from left to right in the ascending channel of the cell. The distance between the forward hyaluronic acid peak[3] and the protein peak has been enhanced by a countercurrent flow of the buffer.

of the vitreous based on chemical analysis[3]. Separation of the hyaluronic acid peak from the other peaks was enhanced by the application of a countercurrent flow of the buffer. The final yield, after one recycling, was about 15 %. It should be pointed out that although the term hyaluronic acid is used throughout this paper, actually the sodium salt was used for this study.

In the present work, the liquefied vitreous was concentrated prior to separation by dialysis against a 40 % solution of Plasdone (Polyvinylpyrrolidone, Type NP-K-30, General Aniline and Film Co., N. Y., N. Y.). This method materially reduced the preparation time as compared with the evaporation technique used earlier. The Plasdone solution was tested for the presence of dialyzable components. Nitrogen could not be detected in distilled water that had been dialyzed against Plasdone solutions.

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Instruments

For sedimentation and diffusion measurements a Spinco-Beckman analytical ultracentrifuge equipped with a phase plate and with a Philpot-Svensson cylindrical lens optical system was used.

For diffusion measurements the synthetic boundary cell[4] was used. The solvent was layered over the solution at about 12,000 rev./min. The speed was then cut back to either 2,500 or 4,000 rev./min and kept constant without the use of a low-speed attachment.

Glucose, sucrose and crystallized serum albumin solutions were used to test the applicability of the instrument for diffusion measurements (Crystallized bovine serum albumin, Armour and Company, Chicago, Illinois). Diffusion constants of $D_{20} = 6.7 \cdot 10^{-6}$ for glucose, $5.3 \cdot 10^{-6}$ for sucrose and $5.9 \cdot 10^{-7}$ cm^2/sec for serum albumin were obtained. These data are in agreement with those found in the literature[5-7].

In order to calculate the sedimentation and diffusion constants, a negative of a fine co-ordinate graph was superimposed on the ultracentrifuge plates and a photographic print was made of the two. With this technique we obtained an over-all magnification of about 15 ×, which allowed us to read a distance corresponding in the cell to 1/20 mm. Areas under the diffusion curves were measured on the photographic prints with an Ott compensating planimeter (Ott precision differential planimeter, Jena, Germany).

Electrophoretic mobility measurements were made with an Aminco portable electrophoresis apparatus which was also equipped with the Philpot-Svensson cylindrical lens optical system (American Instrument Co., Silver Springs, Md.). The standard analytical cell was used for mobility measurements. The conductivity of the buffers and the solutions was determined by a conductivity bridge (Industrial Instruments, Inc., Jersey City, N. J.). Viscosity measurements were made with Ostwald viscometers with a capillary length of about 12 cm and varying bore diameters which gave outflow times for water of 27 to 32 sec at 37°. The measurements were carried out at 25°.

RESULTS

Molecular weight and size measurements

The molecular weight of the electrophoretically prepared hyaluronic acid, determined by sedimentation and diffusion measurements at an ionic strength of 0.16, was found to be 178,000. The experimentally determined values of $s_{20} = 3.1$ S and $D_{20} = 1.25 \cdot 10^{-7}$ cm^2/sec were used to arrive at this value. For partial specific volume 0.66 cm^3/g was substituted[1] into the well-known Svedberg formulas for molecular weight[8].

Fig. 2 shows the experimentally determined sedimentation coefficient plotted against concentration; in Fig. 3 the diffusion coefficient is plotted against concentration. While no difficulties were encountered in extrapolating the diffusion coefficient to zero concentration because of the linear relationship, it was necessary to base the extrapolation of the sedimentation coefficient on the best possible estimate. A plot of $s \cdot \eta$ versus concentration gave the same value as indicated in Fig. 2. The difficulties involved in the extrapolation of such nonlinear sedimentation curves are discussed in a recent article by OGSTON[9].

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The data obtained from sedimentation and diffusion analyses, together with the value for the partial specific volume[8], permit the calculation of other molecular parameters such as the frictional ratio, molecular asymmetry and molecular dimensions, in addition to molecular weight. The frictional ratio (f/f_0) was found to be 4.5, indicating a molecular asymmetry of 145. Considering these values and assuming that the particles approximate the shape of a prolate ellipsoid of revolution, the length of the molecule was calculated to be 2100 Å, and the diameter of maximal circumference to be 14.5 Å.

It should be noted that these calculations do not take into consideration the effect of hydration of the molecules, since the Svedberg formula for molecular weight neglects hydration. This question will be discussed later.

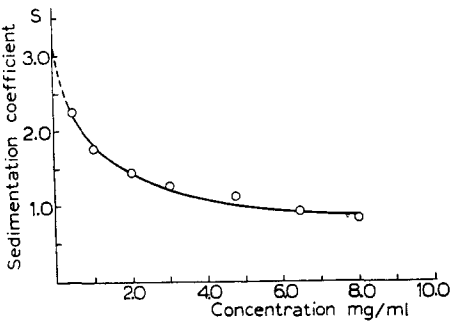

Fig. 2. Sedimentation coefficient of electrophoretically prepared hyaluronic acid as a function of concentration. Medium, 0.006 *M* phosphate buffer at pH 7.0, ionic strength 0.16, regulated by NaCl.

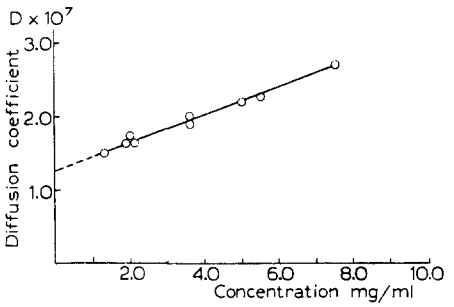

Fig. 3. Diffusion coefficient of hyaluronic acid as a function of concentration. Same medium as in Fig. 2.

The effect of ionic strength and temperature

The effect of the ionic strength of the solvent on the sedimentation and on the diffusion properties of our electrophoretic preparation is shown in Figs. 4 and 5. As the ionic strength is lowered, sedimentation gradually decreases, reaching its minimum value in solutions of zero ionic strength. In these experiments zero ionic strength means that the preparation was thoroughly dialyzed against distilled water. The diffusion coefficient in distilled water increases sharply to a value approximately 40 times the value in a salt solution of 0.2 ionic strength (Fig. 5). It exceeds even the diffusion coefficient of uncharged monosaccharides such as glucose. A possible explanation for this is that as fewer and fewer carboxyl groups on the hyaluronic acid molecule are shielded, the electrostatic repulsion between neighboring molecules will be such that the rate of diffusion will be tremendously increased.

The sedimentation and diffusion patterns also show a marked change with ionic strength. Fig. 6 illustrates the difference in the sedimentation pattern in media of varying ionic strength. The upper series of photographs shows the pattern in a neutral solution of 0.1 ionic strength, regulated by NaCl, and the lower series of photographs shows the pattern in a neutral solution at an ionic strength of zero.

In the sedimentation pattern at the lower ionic strength, an increased skewness and a large piling-up at the bottom of the cell can be observed. This increased

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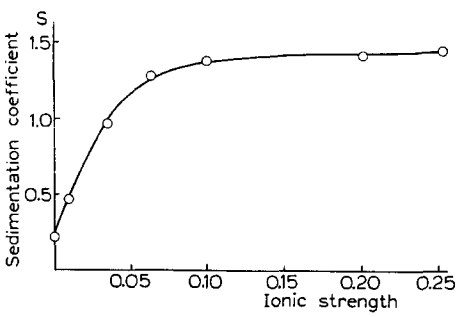

Fig. 4. Sedimentation coefficient of hyaluronic acid as a function of ionic strength. Concentration 2.0 mg/ml.

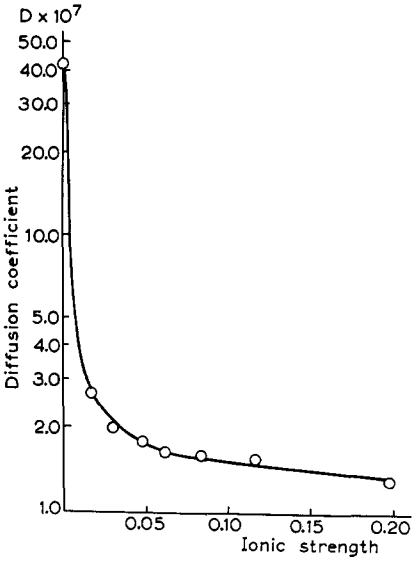

Fig. 5. Diffusion coefficient of hyaluronic acid as a function of ionic strength. Ordinate is given in logarithmic scale. Concentration 2.0 mg/ml.

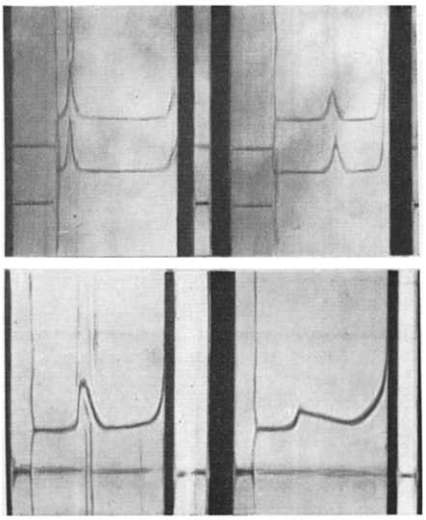

Fig. 6. Sedimentation patterns of 2.0 mg/ml hyaluronic acid solutions. Upper row: NaCl, ionic strength = 0.10 in the normal cell and 0.06 in the wedge cell. Time interval between pictures is 256 min. Lower row: distilled water, ionic strength = 0. Time interval between pictures is 80 min.

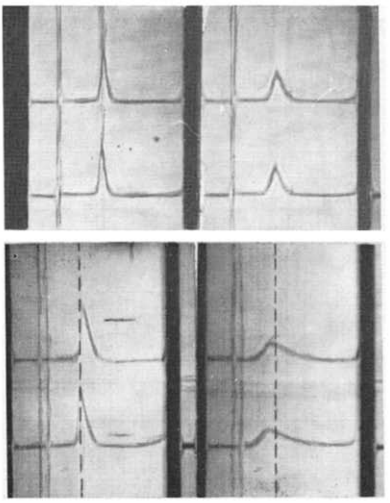

Fig. 7. Diffusion patterns of 2.0 mg/ml hyaluronic acid solutions. Upper row: NaCl, ionic strength = 0.06 in the normal cell and 0.03 in the wedge cell. Time interval between pictures is 56 min. Lower row: NaCl, ionic strength = 0.01 in both cells. Time interval between pictures is 256 min. Dotted lines mark the initial position of the boundary in the normal cell.

skewness, together with the decreasing sedimentation rate, gives the impression that the sedimenting particles cannot leave their original boundary. This phenomenon has been described by SVEDBERG AND PEDERSEN and can be interpreted in terms of charge effects[8].

The diffusion pattern shows a marked deviation from the ideal Gaussian shape in solutions of low ionic strength. Fig. 7 illustrates the diffusion pattern in a solution at an ionic strength of 0.06 (upper row) compared with the pattern at an ionic strength of 0.01 (lower row). The constituents of the media are the same as in Fig. 6. It can be seen that at low ionic strength the curve becomes skewed toward the solution side and steeper toward the solvent side, suggesting a discontinuous transition of concentration. It also has been observed, as already described by SAVERBORN in connection with gum arabic solutions[10], that the solvent–solution boundary interface moves away from its original position, probably under the influence of a diffusion potential set up in the system. It was of interest to calculate the velocity of this displacement at various ionic strengths. The results are given in Table I. A negative value was assigned to the calculated velocities to indicate that the potential responsible for this effect works toward the solvent side of the boundary, thus opposing the centrifugal force. Sedimentation constants can be corrected for this effect in the range of lower ionic strengths. Such a correction, whenever applicable, always increases the calculated value of the sedimentation constant. In Fig. 2 the sedimentation constant is shown without such a correction. The lower the ionic strength and the lower the hyaluronic acid concentration, the more difficult it is to determine this correction because of various technical difficulties, as for example, a rapidly spreading boundary.

TABLE I

BOUNDARY DISPLACEMENT IN DIFFUSION EXPERIMENTS

2.0 mg/ml hyaluronic acid solution. The negative sign indicates that the displacement occurs toward the solvent side.

Ionic strength	*Displacement cm/sec*
0.00	$-68.0 \cdot 10^{-6}$
0.01	$-2.7 \cdot 10^{-6}$
0.03	$-1.8 \cdot 10^{-6}$
0.05	$-0.7 \cdot 10^{-6}$
0.08	$-0.3 \cdot 10^{-6}$
0.16	None
0.20	None

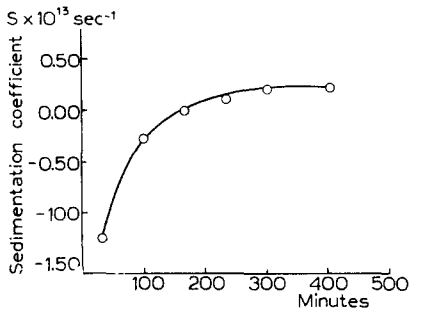

Fig. 8. Sedimentation behavior of hyaluronic acid in distilled water. After an initial negative boundary displacement, sedimentation reaches a stationary level in about 300 min. Hyaluronic acid concentration 4.0 mg/ml.

Sedimentation studies made in a synthetic boundary cell at zero ionic strength revealed that the rate of sedimentation could vary from an initial negative value, gradually increase, and finally attain a constant positive level. This is shown in Fig. 8 for a solution of 4.0 mg/ml hyaluronic acid in distilled water. In a newly-formed boundary in a medium of zero ionic strength, diffusion effects predominate initially. This results in a boundary displacement toward the solvent side, which has been considered a negative sedimentation. Since sedimentation and diffusion occur simultaneously, this boundary displacement also causes very fast spreading, and thus dilution,

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in the boundary region. However, because sedimentation is greater in the lower concentration range, as shown in Fig. 2, the effect of sedimentation overcomes the effect of boundary displacement and true sedimentation soon reaches a stationary level.

Diffusion as a function of concentration was measured at ionic strengths of 0.05 and 0.14. Interestingly, the extrapolation of the diffusion coefficients to zero concentration in both cases was found to arrive at the same value, $1.25 \cdot 10^{-7}$ cm²/sec.

Sedimentation and diffusion properties were not affected by temperature changes within a range of 2 to 30°. The measured values gave the same results when corrected to 20°, indicating that no molecular association or dissociation occurs under the influence of temperature in this range. It should be pointed out that the effect of change in ionic strength was found to be reversible throughout all of the reported experiments.

Measurements of diffusion in various solvents

It has been reported[11] that the diffusion pattern of certain polymeric substances frequently varies from solvent to solvent. The diffusion pattern in solvents in which the polymeric substance under investigation is just barely soluble is closer to the Gaussian shape than the pattern in more perfect solvents. We have tested the effect of methyl cellosolve and methyl alcohol on the symmetry of the diffusion curves of hyaluronic acid. Because hyaluronic acid is not soluble in these solvents alone, various aqueous mixtures were used. Even with the addition of salt, it was found that the patterns were still skewed. The diffusion coefficients measured on 2 mg/ml hyaluronic acid in these solvents are shown in Table II.

TABLE II

DIFFUSION OF HYALURONIC ACID IN VARIOUS SOLVENTS

Solvent	*Concentration mg/ml*	*Ionic strength*	$D_{20} \cdot 10^7$ *cm²/sec*
40 % CH_3OH–NaCl	3.0	0.16	1.05
40 % CH_3OH	3.0	0.00	16.30
50 % Methyl Cellosolve–NaCl	2.0	0.16	28.2
50 % Methyl Cellosolve	2.0	0.00	67.0

Measurements of intrinsic viscosity

The intrinsic viscosity of hyaluronic acid prepared electrophoretically from bovine vitreous bodies was found to be 7.0. This value was measured in a solution containing 0.006 *M* phosphate buffer at a pH of 7.0 and an ionic strength of 0.16 regulated by NaCl. This high ionic strength was chosen in order to avoid the well-known polyelectrolyte effect of hyaluronic acid[12].

DISCUSSION

The molecular dimensions reported in this paper were calculated without considering the possible hydration of the macromolecule. The hydration of macromolecules is a complex question which has aroused much controversial discussion, especially in

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connection with proteins. A new approach to this question recently was proposed by SCHERAGA AND MANDELKERN[13], who introduced the concept of a "hydrodynamically equivalent ellipsoid" or "effective volume" (V_e) defined as an ellipsoid of revolution which exhibits the same hydrodynamic behavior as the hydrated molecule in solution. From simultaneous determinations of the unhydrated molecular weight, sedimentation constant, intrinsic viscosity and dry partial specific volume, a function (β) and, from this, V_e can be calculated. β depends only on the frictional ratio of the hydrated molecule, which can finally be related to the axial ratio of the hydrated particles according to PERRIN's equation[14]. SCHERAGA AND MANDELKERN derive the following equation for β:

$$\beta = \frac{Ns\,[\eta]^{1/3}\,\eta}{M^{2/3}\,(1 - V\varrho)}$$

In this formula N is the Avogadro number; s, the sedimentation constant; $[\eta]$, the intrinsic viscosity; η, the viscosity of the solvent; M, the molecular weight; V, the partial specific volume; ϱ, the density of the medium.

Substituting our experimental values, it was found that $\beta = 3.2$. In accordance with the proposed theory, this value rules out the question of an oblate ellipsoid and also gives an axial ratio of 108, from which a viscosity increment ($\nu = 700$) can be calculated, as described by MEHL, ONCLEY AND SIMHA[15]. Finally, V_e, the effective hydrodynamic volume, was calculated to be 1.0 ml/g from the formula $V = 100\,[\eta]/\nu$. In view of the dry partial specific volume of 0.66 ml/g, this amounts to about 30% hydration. This, of course, effects a small decrease in the asymmetry of the molecule, giving a length $L = 1900$ Å and a width $d = 17.5$ Å, considering the hyaluronic acid molecule a prolate ellipsoid of revolution. The hydration calculated in this way has about the same magnitude as that of most proteins of similar molecular weight[6].

HUGGINS[16] has found that the concentration dependence of the viscosity in solutions of high polymeric substances generally follows the equation:

$$\eta_s/c = [\eta]\,(1 + k'\,[\eta]\,c)$$

where k' is a characteristic constant of the specific polymer independent of its molecular weight, η_s is the relative viscosity of the solution, and c is the concentration in g/100 ml. Fig. 9 shows a logarithmic plot in the form suggested by KINELL AND RÅNBY[17] for the calculation of k' for hyaluronic acid. k' has been found to equal 0.275. This value is in the order of magnitude characteristic of several cellulose derivatives listed by MOSIMANN[18].

In an earlier paper by one of the authors[1] the molecular weight of hyaluronic acid prepared from the vitreous body was reported to be only $9 \cdot 10^4$. This was determined by the same sedimentation-diffusion method which gave us our present value of $17.8 \cdot 10^4$. The hyaluronic acid reported in that paper was prepared by precipitation of the proteins with an ion exchange resin as opposed to the electrophoretic method applied in the present work. The question of whether or not the hyaluronic acid may have depolymerized in the course of preparation under the influence of various treatments has frequently been raised. In this connection, one might also question whether it is possible for the different methods to have a selective effect on hyaluronic acid so that with one method the smaller molecular weight fractions are represented while with another the larger fractions are obtained. Since all preparations are polydisperse

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and have considerably less than a 100 % yield, the likelihood of these suggestions is enhanced. We believe that our electrophoretic technique provides a truly representative sample despite the rather low yield (15–20 %), since the present preparation is more polydisperse than the hyaluronic acid prepared with an ion exchange resin. It seems that the lower molecular weight of the resin-treated hyaluronic acid is not due to depolymerization, for depolymerization usually results in increased polydispersity.

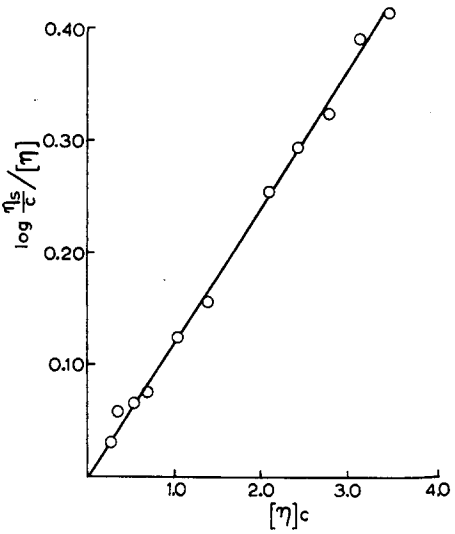

Fig. 9. Test of Huggins' equation according to a plot suggested by KINELL AND RÅNBY[17]. The straight line indicates that hyaluronic acid satisfies the equation. Same medium as in Fig. 2.

It is more likely that resin treatment removes some of the components of larger molecular weight. The polydispersity distribution was calculated according to the method of GRALÉN[19]:

$$\left(\frac{dB}{dx}\right)_0 = \sqrt{\pi}\,\gamma e^{\frac{1}{4}\gamma^2}$$

where γ is a characteristic parameter of the polydispersity, B is the ratio of area to height under the curve during ultracentrifugation, x is the distance from the center of rotation, and subscript o denotes that dB/dx is extrapolated to zero concentration. The value for γ is directly related to polydispersity; the larger its value, the more disperse the material.

With the present preparation we obtained $\gamma = 0.44$ compared with $\gamma = 0.29$ for the resin-treated preparation. From γ the dc/ds, and subsequently ds/dM, can be calculated because of the following relations[19]:

$$\frac{dc}{ds} = Ke^{-y^2} \qquad \text{and} \qquad y = \frac{1}{\gamma}\log\frac{s}{s_0}$$

This mathematical treatment assumes a logarithmic distribution of polydispersity, which yields a valid approximation in the case of a sedimentation pattern with positive skewness. K is a mathematical constant transforming the ordinates into percentage

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values; s_0 is the sedimentation constant corresponding to the maximum of the distribution curve. The dc/ds distribution curve calculated for the present hyaluronic acid preparation is given in Fig. 10. Since the dependence of M on s is unknown, the frequency distribution curve cannot be transformed into a dc/dM curve. From this distribution the average $\bar{s}$ obtained is 3.28 S, which gives a ratio of $s_{max}/\bar{s} = 1.086$.

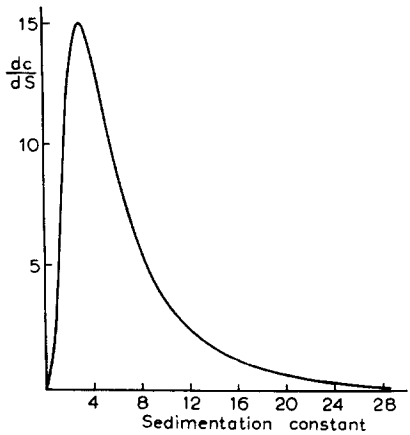

Fig. 10. Frequency distribution curve of the sedimentation of hyaluronic acid. The distribution was calculated according to the method suggested by GRALÉN[19].

In Fig. 3, representing diffusion experiments, we have calculated the diffusion constant from the maximum ordinate and the area under the curve ($D_{H,A}$). Theoretically, the statistical method of calculation from the second moment of the curve (D_σ) should give higher values, and the ratio of the two is usually considered a measure of polydispersity. However, like GRALÉN in his study of the diffusion of some cellulose derivatives[19] and SAVERBORN in his investigation of the diffusion of pectinic acid substances[10], we found just the opposite, $D_\sigma/D_{H,A} > 1$. A decrease in the ionic strength of the medium shifted this ratio even farther from the theoretical; *e.g.*, in a solution of 0.16 ionic strength $D_\sigma/D_{H,A}$ was found to be 0.95, while at an ionic strength of zero $D_\sigma/D_{H,A}$ was equal to 0.58 when they were measured in a 2 mg/ml hyaluronic acid solution.

It has been mentioned that a decrease in the ionic strength of the medium results in an increased asymmetry of the diffusion and sedimentation patterns and that this effect is reversible. While this change in the asymmetry of the sedimentation and diffusion patterns could mean a shift in the polydispersity distribution, if this variation were to be thus interpreted, a reversible association of the hyaluronic acid molecules and, consequently, a reversible redistribution of particle sizes dependent on ionic strength would have to be postulated. Although we do not entirely exclude this possibility, we do think that a change in the shape of the diffusion and sedimentation curves can be explained by the charge effect[8] between solvent and solute. Most probably this effect is also responsible for a change in the ratio of D_σ to $D_{H,A}$.

It has also been mentioned that the boundary displacement, which is apparently the result of a diffusion potential set up at the boundary, increases considerably as the ionic strength is lowered. This force also operates during sedimentation at low

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ionic strength, and because its direction is opposite to that of the centrifugal force, the sedimentation at the boundary differs from that in other parts of the solution. This would explain why the sedimentation patterns at low ionic strengths sometimes exhibit two maxima and a very great piling-up at the bottom despite the fact that the solution is homogenous. In an electrophoretic experiment with a 3.0 mg/ml hyaluronic acid solution at an ionic strength of 0.05, such displacement would be caused by a potential gradient of $4.5 \cdot 10^{-3}$ V/cm. If we consider, as a first approximation, that only the diffusion potential set up in the boundary is responsible for this displacement, we can perhaps calculate the diffusion potential from the displacement and evaluate the valency of the polyacid ion. A subsequent paper will deal with this problem.

ACKNOWLEDGEMENTS

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