Hyaluronic Acid

VIII. A Preliminary Diffusion and Sedimentation Study of Potassium Hyaluronate Including a Determination of the Partial Specific Volume *

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Measurements on a 0.1 % solution of potassium hyaluronate gave a diffusion coefficient of 1.0×10^{-7} cm²sec⁻¹, a sedimentation coefficient of 3.0 S, and a partial specific volume of 0.86. From Svedberg's formula the molecular weight was found to be 5.2×10^5, and a calculation of the molar frictional ratio gave a value of 3.8.

Substances giving highly viscous solutions are not ideal for diffusion and sedimentation studies. Hyaluronic acid is known to give very viscous solutions, and the present substance, described in a recent paper by one of the authors [1] has a relative viscosity of about 12 when dissolved in redistilled water at a concentration of 1 g per l.

For a thorough investigation in such a case a series of experiments with decreasing concentration of the solute is normally performed, and the diffusion or sedimentation coefficients found are plotted against the concentration. The values obtained when extrapolated to zero concentration are then assumed to be the best values that can be given for the diffusion — or sedimentation coefficients.

In this preliminary study we have chosen a concentration of about 0.1 % — which is about the lowest that will give reasonable accuracy in the apparatus used for the experiments, and it is assumed to give values which are not too far from those which would be obtained if the above mentioned method of extrapolation was applied.

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** For earlier publications by the latter author, see Robert Jensen.

DIFFUSION EXPERIMENT

The general equation for free diffusion was adapted by Fick [2] in 1855 from the equation for the conduction of heat. The equation can be written:

$$J = -D\frac{dc}{dx} \quad \text{(Fick's first law)}$$

where J = amount of substance passing through unit area in unit time
t = time after the beginning of diffusion
D = diffusion coefficient (cm^2/sec)
c = concentration
x = distance in the direction of diffusion.
The minus sign means that the solute diffuses in the direction of decreasing concentration.

This equation combined with $\frac{dJ}{dx} = -\frac{dc}{dt}$ yields at constant D:

$$\frac{dc}{dt} = D\ \frac{d^2c}{dx^2} \quad \text{(Fick's second law)}$$

These equations are derived under the condition that the gas-law is valid for the substance in question.

When Fick's second law is integrated with the assumption of the boundary-condition which corresponds to the experimental arrangement, the result is

$$c = \frac{c_0}{\sqrt{\pi}} \int_{-\infty}^{\beta} e^{-\beta^2} d\beta; \quad \beta = \frac{x}{2\sqrt{Dt}}$$

and

$$\frac{dc}{dx} = \frac{c_0}{\sqrt{\pi}}\ e^{-\beta^2}\ \frac{d\beta}{dx}$$

or

$$\frac{dc}{dx} = \frac{c_0}{2\ \sqrt{\pi Dt}}\ \exp(-x^2/4Dt) \tag{1}$$

This equation conforms with the well known normal — or Gaussian distribution. Consequently several plots of the concentration gradient $\frac{dc}{dx}$ (or the refractive index gradient $\frac{dn}{dx}$) against x at different times will form a family of smooth Gaussian curves, provided, of course, that the diffusion is ideal. In our experiments the diffusion coefficient is calculated from equation (1) by the "area method" as

$$D_A = \frac{A^2}{4\pi t H^2} \tag{2}$$

where A = area between the diffusion curve, $\frac{dc}{dx} = f(x)$, and the x axis; H = maximum height of the curve.
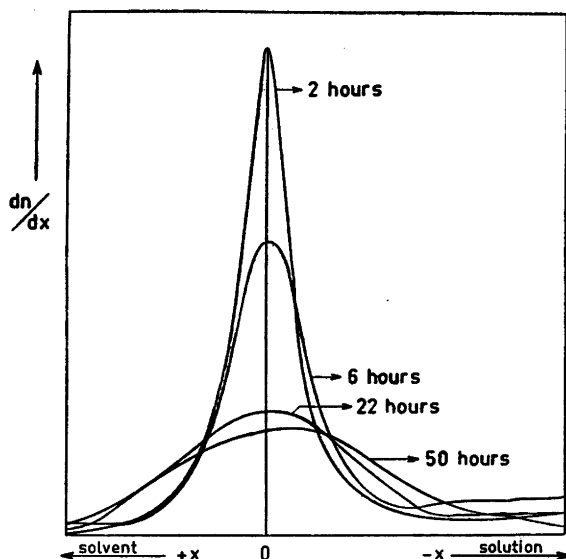
Fig. 1. Diffusion curves of potassium hyaluronate.

The experiment was performed in a Lamm-Polson [3] diffusion cell made from stainless steel, and the Lamm [4] scale method was used for the optical registration. The temperature in the thermostat was 20.0° C and the experiment was run for about 50 hours. The concentration was 0.1 %.

The diffusion curves obtained are seen in Fig. 1. The ordinate is given as $\frac{dn}{dx}$, where n is the refractive index of the solution. During the 50 hours of the experiment the area enclosed by the diffusion curve diminished by nearly 25 % which indicates a corresponding degradation of the preparation during the run, or the presence in the solution of a substance with low molecular weight that diffuses very quickly. The latter alternative is not very probable because the
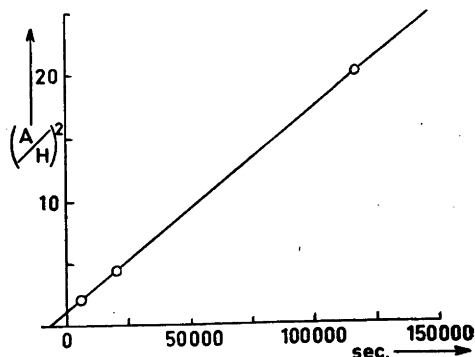

Fig. 2. The graph used for the computation of the diffusion coefficient.

solution was dialyzed for five days against the buffer (0.2 N NaCl, 0.025 N Na$_2$HPO$_4$, 0.025 N NaH$_2$PO$_4$, pH 6.6) at 5° C before the start of the experiment. In case a degradation has taken place only the segments unable to pass the dialyzing membrane will be left.

In Fig. 2 values of $\left(\dfrac{A}{H}\right)^2$ are plotted against t. From equation (2) it is seen that in the case of ideal diffusion a linear relationship must be expected. In our experiment a certain mixing of solute and solvent seems to have taken place at the start of the experiment, and therefore the graph does not pass through the zero point. However, when only the slope of the curve is used in the calculation of D this has no influence upon the result. The diffusion coefficient was found to be: $D = 1.0 \times 10^{-7}$ cm^2 sec^{-1}.

The skewness of the diffusion curves in Fig. 1 also indicates that the diffusion coefficient depends on the concentration, and therefore, the value given above should only be taken as an approximate value.

ULTRACENTRIFUGE EXPERIMENT

This experiment was performed on the same dialysed solution used for the diffusion experiment. The inner part of the centrifuge cell is made from "Tufnol" (lined bakelite resin), thereby avoiding contact with heavy metals. As shown by one of the present authors [5] ferrous and ferric ions cause a continuous decrease of the viscosity even at a concentration of about 2.5×10^{-5} M. The oil driven ultracentrifuge was run at 1 000 r.p.s. for about 2 hours, and the rotor temperature was 28° C. A "Schlieren" system was used for the optical recording of the experiment.

The sedimentation coefficient — the sedimentation rate per unit field of force — is defined by the expression

$$s = \frac{\mathrm{d}x/\mathrm{d}t}{\omega^2 x}$$

where ω is the angular velocity in radians per second, and x the distance from the axis of rotation.

The sedimentation coefficient was determined at certain intervals of time. Corrected to 20° C the average value for the sedimentation coefficient was found to be $s_{20} = 3.0$ S. The sedimentation coefficient is measured in Svedberg units, S, one Svedberg unit being 10^{-13} cm sec^{-1}. Fig. 3 gives the sedimentation diagrams 0.5 h, 1 h, and 1.5 h after the beginning of the experiment. These diagrammes show that the main peak was skew during the first hour of sedimentation and then broadened gradually. Possibly a slower moving component is also present. In another experiment an undialysed 0.3 % solution of the same preparation was investigated. In this case the peak behaved as a "Schliere" during all three hours of the experiment. After two hours the diagram was as seen in Fig. 4, and practically no component was visible after the main component. (The sedimentation coefficient was calculated to be $s_{20} = 2.3$ S in this case).
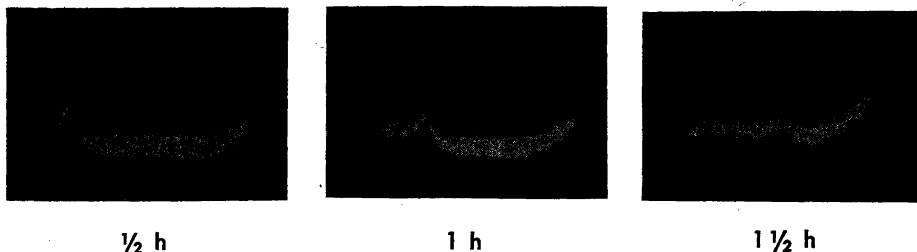
½ h   1 h   1 ½ h

Fig. 3. Sedimentation digrams of 0.1 % potassium hyaluronate. Sedimentation from left to right.

Fig. 4. Sedimentation di-
agram of 0.3 % potassium
hyaluronate.

DETERMINATION OF THE PARTIAL SPECIFIC VOLUME

Besides a knowledge of the diffusion and sedimentation coefficients it is necessary to know the value of the partial specific volume, V, of the substance in order to calculate the molecular weight by Svedberg's formula: $M = \dfrac{RT s}{D(1 - V\varrho)}$

The partial specific volume of potassium hyaluronate was determined from density measurements with two Sprengel-Ostwald pycnometers of about 9 and 5 ml capacity, respectively. The concentration of hyaluronate was about 50 mg dissolved in 10 ml of 0.2 M potassium chloride. All measurements were at 20.0° C (Variations 0.005° C). The mean value of three determinations was 0.86 (0.862, 0.859 and 0.860). This value is surprisingly high compared to the specific volume of other substances containing a carbohydrate skeleton where V normally is between 0.5 and 0.6 (*cf.* R. Jensen *et al.*[6]). However, we controlled our method of measurement and calculation by measuring V for a commercial sample of urea and found a value deviating only 2—3 % from the value for urea ($V = 0.738$) given by Drucker [7]. We have assumed that the partial specific volume is independent of the concentration in dilute solution, and therefore, strictly speaking, we have only determined the so-called apparent specific volume.

Insertion of the data given above into Svedberg's formula shows that the molecular weight of our sample of potassium hyaluronate is about 5.2 × 10^{5}. This value corresponds closely to that obtained recently by Christiansen and one of the present authors [8] from osmotic pressure measurements, but this

very close correspondence must be taken as accidental, since hyaluronic acid, as has been shown, is not ideal for diffusion and sedimentation analysis. Besides, the molecular weight given by the ultracentrifugation method is a weight-average ($\overline{M}_w = \dfrac{\Sigma N_i \cdot M_i^2}{\Sigma N_i \cdot M_i}$), while the molecular weight given by the osmotic method is a number-average ($\overline{M}_n = \dfrac{\Sigma N_i \cdot M_i}{\Sigma N_i}$) — and our hyaluronic acid must be considered a heterogenous, polydisperse substance.

CALCULATION OF MOLAR FRICTIONAL RATIO

The molar frictional ratio f/f_0 is defined as the ratio of the frictional coefficient of the molecule in question to that of a spherical, anhydrous molecule of the same molecular weight. When diffusion and sedimentation analyses are involved this ratio can, according to Svedberg [9], be expressed by

$$\frac{f}{f_0} = \left[\frac{(1-V\varrho)}{D^2 s V}\right]^{1/3} \times 10^{-8}$$

Inserting the experimental data found in this investigation, we find f/f_0 equal to 3.8. Assuming only negligible hydration this indicates threadlike molecules which also corresponds to other findings described in the literature on this substance. (Blix [10]. C. E. Jensen [11,12]).

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