DOUBLE REFRACTION OF FLOW STUDIES ON HYALURONIC ACID PREPARED FROM THE VITREOUS BODY*

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The size of the hyaluronic acid molecule appears to vary considerably, depending on the source of the material and the method of preparation. It is evident that the milder the method of preparation, the less likely it is that the hyaluronic acid molecule will be degraded in the process of isolation.

Earlier double refraction of flow studies by BLIX AND SNELLMAN were performed on a material obtained by treatment with organic solvents[1]. The length of the molecule was between 1000 A–4000 A. In the evaluation of the data the hyaluronic acid molecules were considered to be rigid bodies. BRUNISH, ROWEN AND IRVINE[2] have recently reported molecular lengths of 7–10,000 A, on a sample containing an unidentified component of high molecular weight.

This report describes double refraction of flow studies on hyaluronic acid prepared by means of an electrophoretic separation technique. The results are interpreted in terms of more recent theoretical developments, taking into account the polydispersity of the sample and the flexibility of the molecule.

EXPERIMENTAL

Preparation

Hyaluronic acid was prepared from the vitreous bodies of one- to two-year-old steers. The eyes were removed within one hour after killing the animal and were stored until use at 5°. The posterior portions of the vitreous body were collected after careful removal of adhering tissues. The gel pieces were dialyzed at 5° for 24 hours against 4 liters of buffer solution (0.006 *M* phosphate, 0.12 *M* NaCl, pH 7.00) per 100 eyes. After dialysis the material was centrifuged for two hours in the preparative ultracentrifuge at *ca.* 80,000 *g*. The supernatant solution was placed in cellophane tubings and concentrated to 1/8 of its original volume in a stream of air at 4°. The concentrated material was again dialyzed against the buffer. This dialyzed solution was placed in a standard preparative electrophoresis cell of the Tiselius type with a capacity of 50 ml, and the hyaluronic acid was then separated electrophoretically at 1°***.

Since hyaluronic acid has a higher mobility than the protein components in this solution[3,4,5], it moves in the ascending limb towards the anode as a distinct peak ahead of the proteins. About

* This investigation was supported in part by research grant No. B-907 (C2) from the National Institutes of Neurological Diseases and Blindness and No. 1066(C–3) from the National Heart Institute, Public Health Service. Paper No. 40, Retina Foundation.

** This work was carried out during the tenure of an established investigatorship of the American Heart Association, Inc.

*** An American Instrument Company portable electrophoretic instrument equipped with the Philpot-Svensson optical system was used.

References p. 6.

3.5 ml of hyaluronic acid solution could be safely removed after each run. On re-electrophoresis of a 0.6% solution, thus removed, only one peak was found. The purity of the preparation was checked by chemical analysis. The results of chemical analysis were based on total nitrogen, glucosamine, and glucuronic acid determinations after acidic hydrolysis of the hyaluronic acid[6,7]. Analytical data are given in Table I. The results indicate that not more than 3.5% protein impurity was present. The method of preparation described here does not exclude the presence of a small amount of chondroitin-sulfuric acid. One preparation was analyzed for sulfur, and the result indicates the presence of less than 1% chondroitin sulfuric acid impurity.

TABLE I

CHEMICAL ANALYSIS OF HYALURONIC ACID

*Hexosamine** / *Hexuronic acid*	*Hexosamine** / *Nitrogen*	*Protein impurity*** %
—	0.88	3.2
0.96	0.89	2.7
1.01	0.91	3.5

* Ratios are expressed in gram equivalents.

** It was assumed that the only nitrogen-containing substances in the preparation are hyaluronic acid and proteins, and the protein impurity was expressed as per cent of hyaluronic acid. The nitrogen content of the protein was assumed to be 16.0%. The nitrogen content of hexosamine is 7.75%, and the theoretical nitrogen content of hyaluronic acid is 3.74%.

Measurement of double refraction of flow

The double refraction of flow instrument was of the Edsall type, manufactured by the Rao Instrument Company, Brooklyn, New York, with a rotating outer cylinder. The gap between the inner and outer cylinder was 0.4 mm, and the maximum speed 3340 r.p.m., corresponding to a maximum velocity gradient of 22,000 sec^{-1}. The birefringence (Δn) was measured by means of a quarter wave mica plate compensator, according to the Sénarmont method. The extinction angle and Δn were measured at 23–25°.

Hyaluronic acid was always present as its Na-salt dissolved in 0.006 M phosphate buffer, 0.12 M NaCl, pH 7.00. In some experiments, a varying amount of glycerol was added in order to increase the viscosity of the medium. The solvent alone, without glycerol, had a viscosity of 0.01 poise.

THEORY

Recent reviews[8,9] give an excellent account of the double refraction of flow theory and its applications to macromolecules. A very brief summary of the main conclusions should suffice here.

For rigid ellipsoids of revolution

$$\chi = \frac{\pi}{4} - \frac{G}{12\Theta}$$

for small values of G/Θ, where χ = extinction angle, G = velocity gradient, Θ = rotary diffusion constant. For larger values of G/Θ the relation between χ and G/Θ depends also on the higher powers of G/Θ. The dependence of χ on G/Θ for ideally behaving systems of rigid ellipsoids having various axial ratios has been tabulated by SCHERAGA, EDSALL AND GADD[10]. If the axial ratio of a molecule is known with reasonable certainty, the length (in A) of prolate ellipsoids can be calculated according to the formula[11]

$$L = 404\, Q^{1/3} \left(\frac{\Theta \eta}{T}\right)^{1/3}$$

where $Q = (-1 + 2 \ln 2a/b)$, T = absolute temperature, a and b are the major and minor axes of the ellipsoid, respectively, η = viscosity of the medium in poise.

Deviations from the ideal behaviour do occur in experimentally observed systems. One important factor in bringing about deviations is the polydispersity of the material. Θ would then increase with G, owing to the gradual orientation of the small particles at high velocity gradients.

Another important factor is the deformability of the particles. Under the influence of shearing forces, particles with low internal viscosity may be reversibly elongated[12–16]. The quantitative aspects of deformability will be discussed under RESULTS.

References p. 6.

RESULTS

The dependence of the extinction angle on the velocity gradient at various concentrations is represented in Fig. 1. It can be seen that at a sufficiently low concentration χ becomes independent of the concentration; this indicates the disappearance of molecular interactions.

The dependence of Θ on the velocity gradient is shown in Fig. 2. For a monodisperse system of rigid ellipsoids, Θ should be independent of G. The data indicate a certain amount of polydispersity, since Θ increases with G. This is due to the fact that shorter particles with a higher Θ become oriented only at higher velocity gradients. Lacking a precise knowledge of the distribution function it is difficult to arrive at an average particle length. However, if one calculates the length corresponding to the lowest gradient where extinction can be experimentally observed, as well as that corresponding to the highest gradient used, where the extinction curve flattens out, one can obtain a fair estimate of the range of spread. Thus the molecular length ranges from 1550 A to 1750 A.

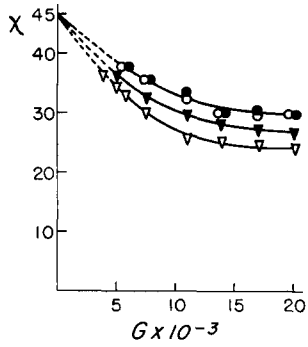

Fig. 1. Extinction angle (χ) of hyaluronic acid solutions as a function of velocity gradient (G sec^{-1}) at various concentrations. ▽ = 0.6 g %, ▼ = 0.3 g %, ○ = 0.15 g %, ● = 0.075 g % hyaluronic acid. Solvent: 0.006 M phosphate buffer, pH 7.00, ionic strength 0.12, adjusted with NaCl.

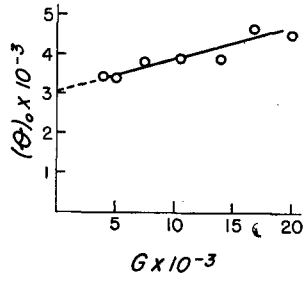

Fig. 2. Rotary diffusion constant extrapolated to zero concentration $(\Theta)_0$ as a function of velocity gradient (G, sec^{-1}). Solvent, same as for Fig. 1.

The birefringence (Δn) of hyaluronic acid, measured with a first-order red plate, was found to be positive with respect to the longitudinal axis of the particles. In Fig. 3 Δn is plotted against the velocity gradient at different concentrations. In the lower concentration range Δn increases more than linearly with the velocity gradient. This can be considered as a sign of deformation of the molecules under the influence of shearing forces[9]. It would appear that the molecular interactions at higher concentrations interfere with the unfolding of the hyaluronic acid chains.

Another indication of the deformation is reflected in the $(\mathrm{tg}\alpha)_0$ *vs.* η_0 plot[12,13]. $(\mathrm{tg}\alpha)_0$ is the initial slope of the extinction angle *vs.* velocity gradient curve extrapolated to zero concentration and η_0 is the viscosity of the medium.

η_0 was changed by adding varying amounts of glycerol to the solution. For rigid particles the $(\mathrm{tg}\alpha)_0$ *vs.* η_0 curve should go through the origin[12,13]. The viscosity ranged from 0.01 to 0.05 poise. For each viscosity value, χ as a function of G was measured at various concentrations and the initial slope of the curve was extrapolated

References p. 6.

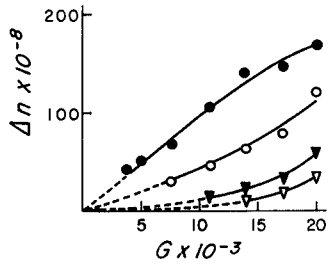

Fig. 3. Birefringence (Δn) of hyaluronic acid solutions as a function of velocity gradient (G sec^{-1}) at various concentrations. ● = 0.6 g %, ○ = 0.3 g %, ▼ = 0.15 g %, ▽ = 0.075 g % hyaluronic acid. Solvent, same as for Fig. 1.

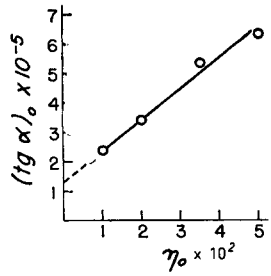

Fig. 4. Initial slope of the extinction angle *vs.* velocity gradient curves extrapolated to zero concentration $(tg\alpha)_0$ as a function of the viscosity of the solvent (η_0). The viscosity was varied by adding different amounts of glycerol to the same solvent as described for Fig. 1.

to zero concentration (Fig. 4). If the particles are deformable, there is a positive intercept on the ordinate. This appears to be the case for hyaluronic acid. This criterion is valid even if the sample is polydisperse[12 13]. If we consider the hyaluronic acid molecules as being hydrodynamically equivalent to an elastic sphere, the internal viscosity (η_i) and the shearing coefficient (μ) can be calculated from the equation

$$(tg\alpha)_0 = \frac{1.25}{\mu}(\eta_0 + 0.4\,\eta_i)$$

From the plot in Fig. 4, one finds $\mu = 1.4 \cdot 10^3$ dyne/cm^2, $\eta_i = 4 \cdot 10^{-2}$,

At present, these parameters have only a phenomenological value and cannot be interpreted unequivocally in terms of actual molecular structure[12 13]. This is due mainly to the lack of experimental data against which existing theories can be checked.

DISCUSSION

The hyaluronic acid used in this work has been prepared by an electrophoretic method of separation based on the difference in the mobility, at pH 7, of the hyaluronic acid and protein components of the vitreous. This method of preparation would appear to be the mildest possible one, and the hyaluronic acid so obtained is more likely to resemble the native form than hyaluronic acid obtained by repeated precipitations or extraction with organic solvents. Moreover, in principle, it is possible to recover 100% of the total hyaluronic acid present in the vitreous, whereas the yield with other methods is much lower. Now, in view of the polydispersity of hyaluronic acid in the vitreous, it is rather likely that methods of isolation having a low yield lead to a non-representative hyaluronic acid preparation; that is, a preparation in which one or another part of the hyaluronic acid spectrum predominates. It is rather reassuring, and confirms our initial assumption, that little degradation occurs in the course of the isolation procedure, for the polydispersity of the preparation is not very great. We have found that at low velocity gradients the length of the molecule would appear to be 1550 A, whereas at high velocity gradients, 1750 A. At low velocity gradients, the double refraction of flow is mainly due to larger molecules, whereas the contribution of the shorter ones becomes noticeable only at higher values of G. The small difference between the two figures seems to exclude a very high degree of polydispersity. How-

References p. 6.

ever, the flexibility of the molecules, to be discussed below, may lead one to underestimate the degree of polydispersity. The purity of the preparation has been checked both chemically and electrophoretically. The chemical analysis puts an upper limit of 3.5% by weight on possible protein impurities. Electrophoresis shows only one component, but the sensitivity of the instrument would not reveal any impurity below 1–2% by weight, under the conditions of our run. Whether the protein "impurity" is an impurity in the proper sense, or whether there might be a stoichiometric combination between hyaluronic acid and a protein of low molecular weight is at the moment an open question.

It is of interest to compare the apparent length of hyaluronic acid from the vitreous body as determined by different methods. Light-scattering measurements[17] suggest a length of about 3000 A; calculations based on the molecular weight and axial ratio, determined from sedimentation and diffusion measurements[18], lead to a value of 1000 A. The present work suggests a length of about 1600 A*. The light-scattering and sedimentation-diffusion experiments were done on hyaluronic acid obtained by fractional precipitation, so that, in comparing those data with the present ones, the possible effect of the differences in the method of preparation must be borne in mind. The highest value was obtained from light-scattering measurements, and according to ZIMM AND STOCKMAYER'S theory[19], such measurements yield a "Z" type of average; that is, if the preparation is polydisperse, the contribution of the longer particles to the "Z" type of average is higher than to either a number or a weight type of average. For this reason we shall disregard the light-scattering data in what follows.

The present work shows that hyaluronic acid is a flexible molecule, and the length measured by this method would reflect some unfolding brought about by shearing forces. If the preparation is polydisperse, the effects of polydispersity and flexibility cancel each other to some extent: at higher shear rates, the elongation of the particles may mask the contribution of the smaller particles. Sedimentation and diffusion measurements indicate that the molecular weight of hyaluronic acid is of the order of 100,000[18]. If the molecule were a linear, stretched chain of N-acetyl-glucosamine-glucuronide units, its length would be about 3000 A. Thus, it is obvious that the length of 1000 A, as measured by sedimentation-diffusion methods, represents a fairly coiled state, and the value of 1600 A would be consistent with some unfolding under the shearing forces.

SUMMARY

Hyaluronic acid was prepared by a method of electrophoretic separation. The protein impurity of this preparation was 2–3%.

Double refraction of flow was measured at various concentrations and in media of varying viscosity, at velocity gradients ranging up to 22,000 sec^{-1}. The apparent molecular length varied between 1550 and 1750 A.

The sign of the double refraction was positive with respect to the longitudinal axis of the particle. There were indications of a slight degree of polydispersity. The hyaluronic acid appears to be somewhat flexible under the influence of shearing stress. This behaviour is characterized by the following parameters: $\eta_i = 4.2 \cdot 10^{-2}$ poise, shearing elasticity coefficient $\mu = 1.4 \cdot 10^{-3}$ dyne/cm^2.

* According to a recent publication by BRUNISH *et al.*, the length of the hyaluronic acid molecule, determined both by light scattering and by double refraction of flow techniques, is between 7 and 10,000 A. Their experiments suggest the presence of some unidentified high molecular weight components, possibly collagen. This fact and the lack of extrapolation to zero concentration in determining χ make the evaluation of their data somewhat difficult.

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Received April 24th, 1956

ON THE ENZYMIC FORMATION AND THE ISOLATION OF POLYPHOSPHATES OF ADENINE DEOXYRIBOSIDES*

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INTRODUCTION

In 1954 SABLE *et al.*[1] found that enzymes from muscle catalyzed the transfer of phosphate from ATP*** to deoxy-AMP. They found, likewise, that a suspension of liver mitochondria catalyzed the formation of acid-labile phosphate in the presence of deoxy-AMP. Evidence for the formation of the deoxyribose analogs of ADP and ATP under these conditions was obtained. Recently, LIEBERMAN *et al.*[2] have found that an enzyme from yeast catalyzes the transfer of phosphate from ATP to deoxy-AMP. The oxidative phosphorylation of thymidine monophosphate and deoxycytidine monophosphate by the "cytoplasmic" fraction of liver homogenate has been demonstrated by HECHT *et al.*[3]. The products were isolated and identified as di- and tri-

* Supported by grants from Nordisk Insulinfond and the Eli Lilly Research Foundation.
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*** The following abbreviations are used: AMP, ADP and ATP for adenosine mono-, di- and triphosphate; deoxy-AMP, deoxy-ADP and deoxy-ATP for deoxyadenosine mono-, di- and triphosphate; deoxy-GMP for deoxyguanosine monophosphate; deoxy-CMP for deoxycytidine monophosphate; TMP for thymidine monophosphate, Tris-buffer for tris(hydroxymethyl)aminomethane-HCl buffer and P for phosphate.

References p. 12.