FORM AND DIMENSIONS OF ISOLATED HYALURONIC ACID

by

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To date the biologic role of hyaluronic acid (HA) remains obscure. Since many investigations[1] have suggested the possibility that its degradation may be an important factor in the joint and collagenous diseases, several laboratories have undertaken physical studies upon this important molecule. The results of the early streaming birefringence work of SNELLMAN AND BLIX[2] were compatible with the concept that the undegraded HA isolated from either umbilical cords or vitreous humor was asymmetric. The results of other physical studies[3,4,5] appeared to be in agreement with this picture.

Recently BLUMBERG AND OSTER[6] working with an umbilical cord HA made light scattering measurements which they interpreted in terms of a sphere. These authors note that their deduction is compatible with conclusions[9] drawn from studies of the hydrodynamic properties of synovial fluid which contains appreciable amounts of protein in addition to HA. The chemical analyses reported by BLUMBERG AND OSTER suggest that their sample contains chondroitin sulfate and possibly other impurities. Their conclusions that HA is spherical is a variance with the recent light-scattering observations and conclusions of LAURENT AND GERGELY[7]. The latters' data on an umbilical cord HA, free of protein but contaminated with 15% chondroitin sulfate showed that the particle scattering factors fell intermediate between those of the rod and coil models. They interpreted their data in terms of a rigid chain.

The above apparent discrepancy may be due, in part, to contamination of the HA with low concentrations of protein, chondroitin sulfate, or other polysaccharide. These electrically charged macromolecules may, under appropriate conditions, complex with HA to form aggregates with different molecular dimensions and shapes. To circumvent this possibility we have examined the size and shape of HA employing a preparation free of chondroitin sulfate, protein and other polysaccharides. To facilitate this purification the source of HA chosen was vitreous humor, a tissue containing little, if any, chondroitin sulfate, other contaminating polysaccharides or nucleic acid. This approach was taken with the possibility in mind that the values of the molecular parameters of HA isolated from vitreous might indeed be quite different from HA isolated from other sources. However, it was hoped that the size and shape of vitreous HA might be well defined and unambiguous when studied in this system, and that this study might serve to throw light on the above apparent discrepancy. We have studied the sample under controlled conditions with the following techniques: sedimentation, streaming birefringence, light scattering and

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electron microscopy. The data obtained suggest that the chondroitin sulfate and protein-free HA molecules are asymmetrical, not spherical. The purpose of this communication is to report the results of these studies.

Isolation of sodium hyaluronate

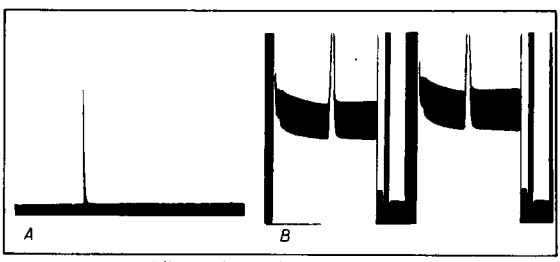
The HA was isolated as the sodium salt by applying the detergent method of SIMMONS[10] to the vitreous humor[11]. The dried material was analyzed for C, N, H, Na, acetyl content, glucosamine, sulfur and phosphorous. The analyses seen in Table I indicated the product to be pure. The HA displayed a single sharp peak in both the Tiselius apparatus and the ultracentrifuge (see Fig. 1). The excessively fine and sharp boundary persists to low concentrations; this behavior is characteristic of desoxyribonucleic acid (DNA), tobacco mosaic virus (TMV) and other asymmetric macromolecules.

TABLE I

ANALYSES OF SODIUM HYALURONATE FROM VITREOUS HUMOR*

	% *Experimental*	% *Theoretical*	*Atoms/Atom of N*	
			Experimental	*Theoretical*
N	3.46	3.49	1.0	1.0
C	40.09	41.80	13.5	14.0
H	5.06	5.00	20.5	20.0
Na	5.74	5.74	1.0	1.0
Acetyl	9.98	10.70	0.94	1.0
Glucosamine	43.5	44.60	0.98	1.0
S	0.03	0.00	—	—
P	0.022	0.00	—	—

* Analyses (except glucosamine) performed by Dr. ADALBERT ELEK, Elek Microanalytical Laboratory, Los Angeles, California. Nitrogen values obtained in our laboratory agreed with the above value.



Vitreous humor hyaluronic acid

Fig. 1. Vitreous humor hyaluronic acid. A. Electrophoresis of a 0.3% HA solution in barbital buffer, pH 8.6, $\mu = 0.1$. B. Sedimentation of a 0.3% HA solution in 0.3 *M* KCl, pH 7.28.

Streaming birefringence

Our measurements were performed with an apparatus similar in design to EDSALL's[12] and is described elsewhere[13]. Confidence in the calibration and alignment of our streaming birefringence apparatus was obtained by concurrent measurements on ethyl cinnamate and TMV as standardizing materials. Excellent agreement with electron microsopy was obtained for specimens of TMV from the χ-G relationships via SADRON's[14] equation:

$$\text{Tan } 2\chi = \frac{\sum_i \delta_i \sin 2\chi_i}{\sum_i \delta_i \cos 2\chi_i} \qquad (2)$$

Where χ_i is the extinction angle of the ith species and δ_i the birefringence of the ith species. The purified HA was investigated as a function of concentration, over the tenfold range of 0.03 to 0.30%, at ionic strengths of zero and 0.3 and pH 6.5. It was

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noted that the birefringence was markedly decreased when the ionic strength was increased from zero to 0.3. During this ionic strength change the birefringence-gradient curve changed shape from that of a typical "rigid" rod curve to the "S" shaped curved frequently observed with flexible molecules[15]. This flexibility was reflected again in the value of 1.57 (at $\mu = 0.3$) for the constant K of Peterlin's equation[16]:

$$\text{Cot } 2\chi = \frac{KM}{RT} \frac{(\eta - \eta_0)\, G}{C} \tag{1}$$

Where χ is the extinction angle, M the molecular weight (in this case, $1.27 \cdot 10^6$, our light-scattering value), R the gas constant, C the concentration, η the viscosity at the gradient G at which χ was observed and η_0 the solvent viscosity. K has been shown to range from 1.0 for a "soft" or flexible molecule to 3.0 for a "hard" or stiff molecule. The shape of the χ versus $(\eta - \eta_0)G/C$ plots at $\mu = 0$, and $\mu = 0.3$ and their relative positions support the hypothesis that the molecule (or particle) at $\mu = 0$ is considerably less flexible than at $\mu = 0.3$. The value of $K = 1.57$ places the molecule intermediate between the "soft" and "hard" models[16].

The dimensions of the particle were estimated from the values of the χ-G curves obtained by the extrapolation of the data to the infinitely dilute state. The estimation of the dimension employs the theory of PETERLIN AND STUART[17] which assumes an ellipsoid as the molecular model. Plots of the χ-G curves at $C = 0$ are shown in Fig. 2. It is noted that the material is polydisperse and it appears to contract from an average value of 5,500 A to approximately 2,500 A.

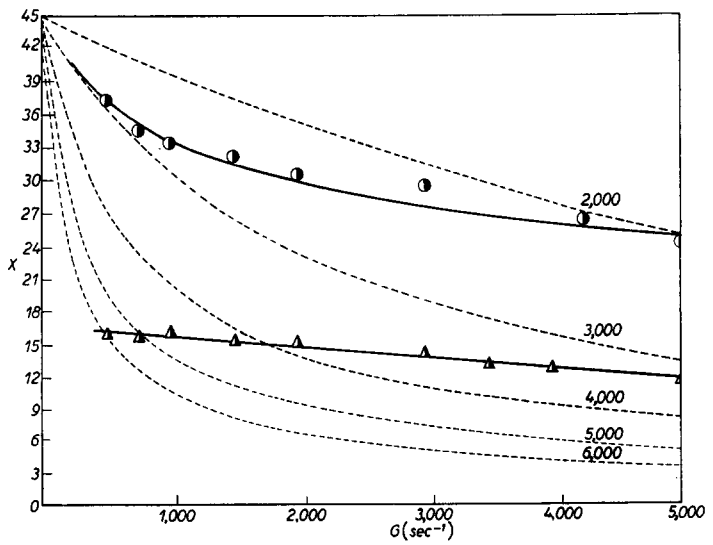

Fig. 2. Extinction angle χ versus gradient for the infinitely dilutesolutions ($c = 0$) at ionic strength $\mu = 0$, ◭ and $\mu = 0.3$ ◑.

Light-scattering studies

The light-scattering method[18,19] in contrast to the streaming birefringence technique is a static one. In the hydrodynamic method shearing forces operate on the particle and may possibly distort a flexible particle. In the light scattering procedure there

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is no applied force on the suspended molecule or particle and hence this system is free of this potential source of distortion. On the other hand, the light scattering method has another possible source of error which effects molecular weight determinations. The molecular weight is a "weight" average and hence extremely sensitive to relatively small concentrations of large aggregates or foreign particles.

A modified Speiser-Brice light scattering apparatus[18] was used for these studies. It was calibrated in several ways, which included the Ludox technique[20], the method deduced by Brice et al[18], the "standard" Debye polystyrene[19] and a sample of polystyrene (sample III) distributed by the Commission on Macromolecules of the International Congress of Pure and Applied Chemistry[21]. The general agreement obtained (including an accurate value for bovine serum albumin) indicated that the calibration was correct. Since the HA particle was greater than 1/15 the wave length of light used in this investigation (λ = 4360 A) the simple Debye equation[19]:

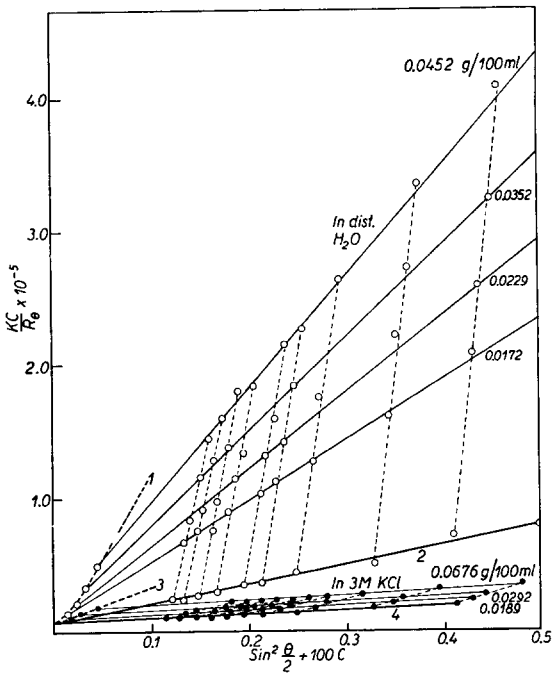

Fig. 3. Zimm plots of HA at ionic strength μ = 0 and μ = 0.3. Ordinate = KC/R_θ, abscissa = $\sin^2 \theta/2 + KC$.

$$\frac{H\,C}{\tau} = \frac{1}{M} + B\,C \tag{3}$$

could not be used. Here H is the refractive constant, τ the turbidity, C the concentration, M the molecular weight and B the much discussed first virial coefficient. The value of dn/dc found for the HA was $0.168 \cdot 10^3$ g/ml. This value was used to calculate H. Our data were plotted by the method suggested by ZIMM[22] in accordance with the equation:

$$\frac{K\,C}{R_\theta} = \frac{1}{M}\,\frac{1}{P_{(\theta)}} + B\,C \tag{4}$$

where $K = 16/3\,\pi H$, R_θ is the reduced intensity and $P\,(\theta)$ is the particle scattering factor[22,19] and is given by

$$P_{(\theta)} = 1 - \frac{C}{3} D^2 \sin^2 \frac{\theta}{2} + \frac{C^2}{12} D^4 \sin^4 \frac{\theta}{2} + \cdots \tag{5}$$

where D is the radius of gyration and $C = 8/9\,(\pi/\lambda)^2$. In this procedure a molecular dimension is obtained from D by assuming a model. The value of D is obtained from the Zimm plots shown in Figs. 3 and 4; it is the ratio of the initial slope divided by the intercept.

Plots of Z, the dissymetry (R_{45}/R_{135}), and the ratios of the intrinsic dissymetries at μ = 0 and μ = 0.3 and the data of Fig. 3 once more suggested the possibility of marked changes in molecular shape or molecular interaction. Assuming, first relatively little interaction, as indicated by the low dependence of the χ-G curve on HA concentration, we have tentatively interpreted the changes in the initial slope of the Zimm

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plot as dimensional changes. Assuming the molecule (or particle) to be rod-like we obtain a length change of 6,700 A to 2,900 A. These values are in fair agreement

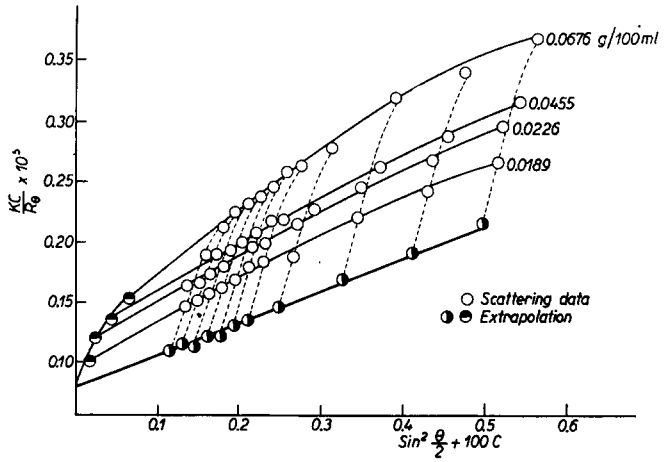

Fig. 4. Zimm plot. Data of Fig. 3 for HA at $\mu = 0.3$ on an expanded scale.

with the values deduced above from streaming birefringence measurements. Since the latter is a hydrodynamic method, in which stresses could deform the molecule, it is particularly noteworthy that the stress-free static light scattering procedure yields comparable values. The nonconformity of the particle scattering factors with the standard models is illustrated in Fig. 5. Attention is directed to the fact that the experimental points were furthest from the sphere curve. These data are in agreement with the shape data and conclusions presented by LAURENT AND GERGELEY[7] on umbilical cord HA, but they are not in agreement with the conclusions of BLUMBERG AND OSTER that HA is a sphere.

It should be pointed out that deductions concerning shape from the data treatment of BLUMBERG AND OSTER[6] on the one hand and LAURENT AND GERGELEY[7] and the present study on the other hand are somewhat different. The latter workers employed the method described by DEBYE AND ZIMM[22,19] while BLUMBERG AND OSTER used essentially the method of GUINIER[23]. In the procedure of GUINIER the dissymmetry, $(90° - \theta)/(90° + \theta)$ is compared with the ratio of the particle dimension to the wave

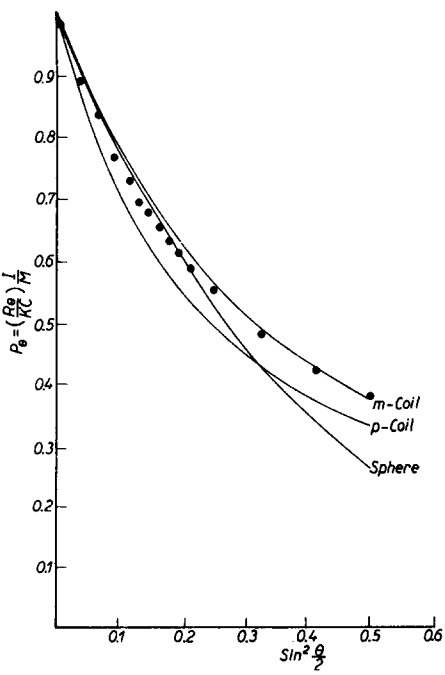

Fig. 5. Particle scattering factor, $P(\theta)$ versus $\sin^2 \theta/2$. Solid points represent experimental values. Solid curves theoretical curves for sphere, polydisperse coil and monodisperse coil for radius of gyration of $1.2 \cdot 10^3$ A at ionic strength $\mu = 0.3$.

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length of light. This procedure appears to be less sensitive than the other method in the 2000 A range. The deductions from the two procedures should be compared using a single set of experimental data in this molecular size range.

The weight average molecular weight obtained on our sample was $1.27 \cdot 10^6$, considerably lower than the values obtained for umbilical HA by both of the above groups. Nevertheless it was greater than the number average molecular weight recently reported by CHRISTIANSEN AND JENSEN[24] for their better preparations (3.3% nitrogen). LAURENT has found[25] that HA from vitreous humor, as determined by light scattering measurements, is smaller (approximately $4.5 \cdot 10^5$) than his previously reported value of $2.4\text{–}4.31 \cdot 10^6$ for umbilical cord HA[7]. This finding suggests the possibility that HA in the vitreous may have a lower molecular weight than comparable samples from umbilical cords.

Mention should be made of the possibility that HA particles or molecules may associate and aggregate in salt solution upon standing. We have not investigated aggregation quantitatively but have removed the small amount of extremely large aggregates, when present, before making light-scattering measurements. It is possible that the higher values reported, $3\text{–}8 \cdot 10^6$, represent weight averages of samples containing a fair proportion of aggregates (either HA or HA-contaminant aggregates). The weight average value will, of course, be very sensitive to relatively small amounts of such giant aggregates. We have obtained some evidence of such aggregation in our electron microscope studies summarized below.

Electron microscopy

The following precautions were observed during electron microscopy. The distilled water employed in dilution was cleaned by passage through a millipore filter[26]. When examination by light scattering showed the water to be free of particles it was used for dilution of the HA samples. The samples were shadowed at an angle of ten degrees and the particle diameters ($2R$) were obtained with the aid of the formula deduced by KAHLER AND LLOYD[27]:

$$2R = \frac{2A}{M} \frac{\tan \alpha/2}{1 - \tan \alpha/2}$$

where A is the shadow length from the edge of the particle and M is the magnification.

Typical results are shown in Figs. 6 and 7. The HA is seen to consist of asymmetric (anisometric) particles (*cf.*[28]). Figure 6 represents typical fields at three dilutions—from one to a million (c–f) and one to 200,000 (a). Fig. 7 is included to show a few of the larger aggregates. The large spheres are calibrated polyvinyltoluene spheres of diameter 1,380 A[29]. It is noted that in addition to rodlike fibers of varying lengths and diameters there are a few small symmetrical particles of diameter less than 500 A (Fig. 6a, b, d and e). It was not possible to establish whether these symmetrical particles were also HA.

In Fig. 7 are seen at higher dilution several examples of the longer fibers. These fibers varied in length from 3,000 to 6,000 A and were therefore comparable to the average lengths deduced from streaming birefringence and light scattering. The polydispersity of the lengths and the multiplicity of the diameters support the hypothesis, mentioned above, that there is a tendency for aggregation. Calculation shows that the diameter of a thread consisting of a single chain of anhydro-glucose

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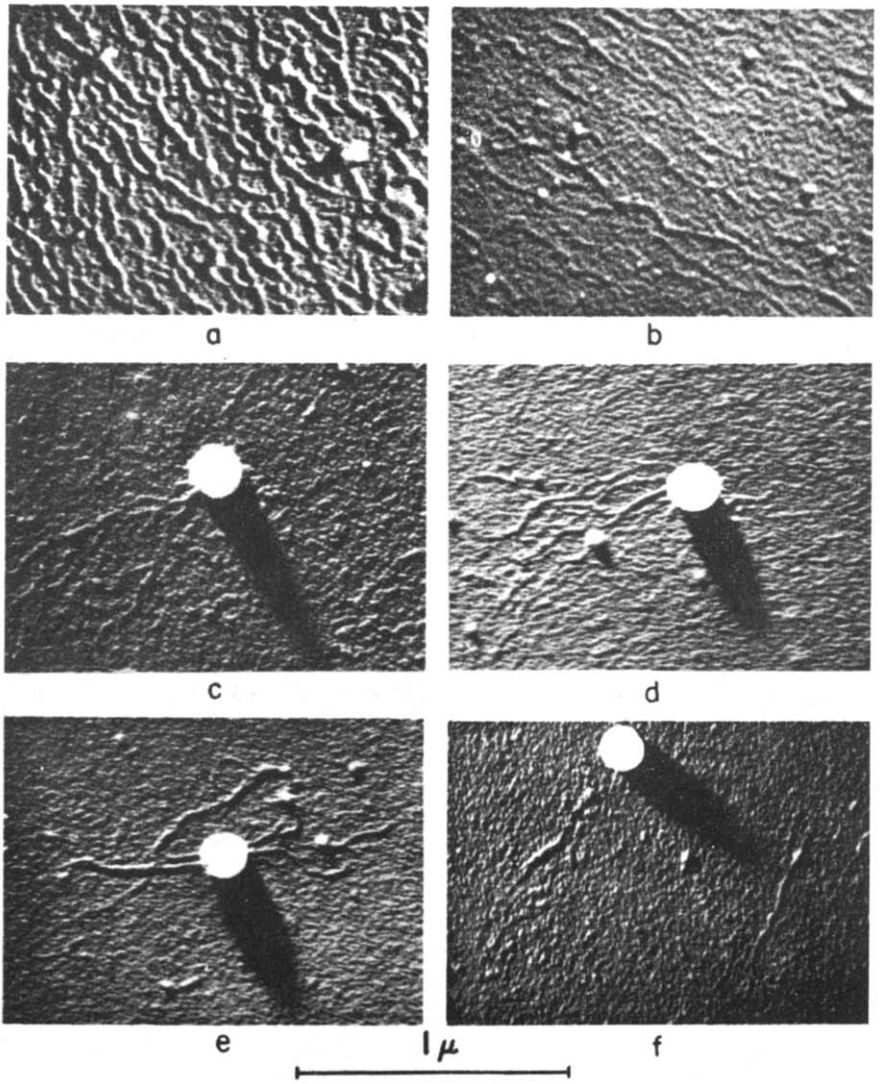

Fig. 6. Hyaluronic acid from vitreous humor. a. 1:200,000, b. 1:500,000, c, d, e and f, 1:1,000,000. The thread-like structures varied from a minimum of 10 A to diameters greater than 100 A. Uranium shadowed-10 % polyvinyltoluene spheres 1,380 A.

rings could not possibly exceed 15 A. Because of lack of contrast it is very difficult to delineate particles of this order of magnitude but they may be detected in low angle shadow-cast preparations by the shadows they cast, and from these their dimensions may be deduced. Fibers of *ca.* 10 A thickness were seen in many of the fields examined. This finding is in agreement with diameters recently observed and described by JENSEN[30].

Enzyme experiments with three different preparations (Wyeth, Searle and Armour) demonstrated that substantially all the thread-like structures were sensitive

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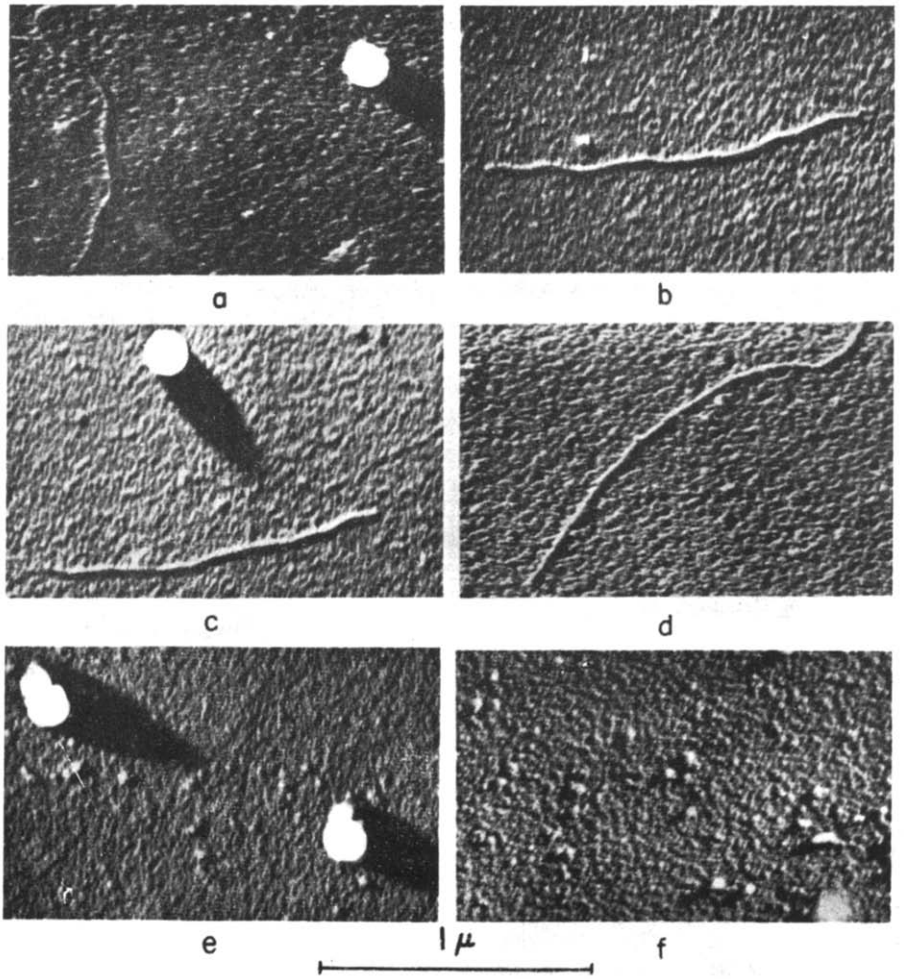

Fig. 7. Hyaluronic acid from vitreous humor. a, b, c and d 1:1,000,000 showing aggregated fibers sensitive to enzyme action. e and f same preparation following brief exposures to hyaluronidase showing the disappearance of aggregated threads and appearance of small globules.

to the specific enzyme action of hyaluronidase and were therefore aggregates of the HA molecule. All solutions examined after brief exposure to low concentrations of the enzyme (less than three turbidity reducing units per ml of 0.01% HA solution) were completely free of the threads. (Fig. 7e and f). No quantitative change in the number of the small spherical particles was noted; if there was any change at all it was an increase during the enzymatic depolymerization. These small spherical particles may be seen, in Fig. 7e and 7f, to be less than 500 A in diameter and identical with or very similar to the ones in Fig. 6a, b, d and e. We believe, therefore, that the specific action of the hyaluronidase establishes the fact that the thread-like particles, ranging in diameter from 10 A to 100 A and from several hundred angstroms in length to many thousands are the HA particles.

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We have never observed the 2,100 A spheres postulated by BLUMBERG AND OSTER. Our preparations, from which nearly 100 electron-micrographs were made, covered a large number of specimens of two different HA samples under a variety of experimental conditions. If our preparations had consisted of HA in the form of 2,100 A diameter spheres, they could not have been missed.

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SUMMARY

1. A highly purified sample of the sodium salt of hyaluronic acid (HA) was prepared free of contaminants from the vitreous humor.

2. The HA was studied by ultracentrifugation, light scattering, streaming birefringence and electron microscopy. All of the data could be interpreted in terms of an asymmetric molecule of particle approximately 6,000 A in length which contracted to approximately one half this value in the presence of ions.

3. The data indicate a polydisperse material of weight average molecular weight $1.27 \cdot 10^6$.

4. Electron microscopy revealed thread-like particles which were sensitive to the depolymerizing action of hyaluronidase. The particles were intermediate in form between a stiff rod and a flexible coil. There were no large spheres present and the dimensions and form seen in electron microscopy were in agreement with similar values deduced by light scattering and streaming birefringence studies.

RÉSUMÉ

1. Un échantillon très purifié du sel de sodium de l'acide hyaluronique (HA), débarrassé des contaminants provenant de l'humeur vitrée, a été préparé.

2. Le HA a été étudié par ultracentrifugation, diffraction de la lumière, biréfringence d'écoulement et microscopie électronique. Tous les résultats obtenus peuvent s'interpréter en fonction d'une asymétrie de la molécule, qui mesurait 6,000 A environ de longueur et qui se contracterait de moitié environ en présence d'ions.

3. Les résultats indiquent un matériel polydispersé de poids moléculaire moyen $1.27 \cdot 10^6$.

4. La microscopie électronique révèle l'existence de particules filiformes, sensibles à l'action dépolymérisante de la hyaluronidase. Les particules ont une forme intermédiaire entre celle d'un bâtonnet rigide et celle d'un enroulement irrégulier flexible. On n'observe pas de sphères importantes et les dimensions et la forme vues au microscope électronique sont en accord avec les valeurs correspondantes déduites des études de diffraction et de biréfringence d'écoulement.

ZUSAMMENFASSUNG

1. Ein weitgehend gereinigtes Muster des Natriumsalzes von Hyaluronsäure (HS), ohne Verunreinigungen aus dem Glaskörper, wurde hergestellt.

2. Die HS wurde durch Ultrazentrifugation, Licht-, Strömungsdoppelbrechung und Elektronenmikroskopie untersucht. Alle Ergebnisse konnten durch die Hypothese eines asymmetrischen, ungefähr 6,000 A langen Moleküls erklärt werden, welches sich in Anwesenheit von Ionen bis etwa auf die Hälfte dieses Wertes zusammenziehen würde.

3. Die Ergebnisse deuten auf ein polydispergiertes Material mit durchschnittlichem Molekulargewicht von $1.27 \cdot 10^6$.

3. Die Untersuchung mit Hilfe des Elektronenmikroskopes zeigte fadenförmige, auf die depolymerisierende Wirkung von Hyaluronidase empfindliche Partikeln. Die Partikel zeigten alle Übergänge zwischen der steifen Stabform und der biegsamen, der Zufallsanordnung überlassenen Spulenform. Grosse Kugeln waren nicht anwesend, und die mit Hilfe des Elektronenmikroskopes gefundenen Ausmasse und Formen stimmten mit den durch Lichtdispersion und Strömungsdoppelbrechung erhaltenen ähnlichen Werten überein.

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