PARTICLE INTERACTION IN SOLUTIONS DERIVED FROM OX VITREOUS HUMOR

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The vitreous humor is a transparent, non-cellular, non-vascular gel that by various manipulations can be separated into two phases, a fibrous solid and a viscous solution. The former, known as the residual protein, has been shown to have many features in common with collagen (1, 2). Matoltsy, Gross, and Grignolo (3) found in residual protein preparations some fibers which showed in electron micrographs some of the fine structure characteristic of collagen. Other fibers lacked this fine structure either as a consequence of a different molecular organization or because of a chemical difference from collagen.

The viscous solution which is obtained when the residual protein is removed from the vitreous body contains hyaluronic acid and soluble protein. The hyaluronic acid has been well characterized by Meyer (4), although complete characterization of the combined carbohydrate by chromatographic means has not been attempted. Blix (5) was able to isolate hyaluronic acid from vitreous humor by electrophoresis and considered it not to be combined with protein. There is little satisfactory information on the nature of the soluble protein.

Connective tissues characteristically contain collagen, acid mucopolysaccharides, and soluble protein, and the interaction of these components is relevant to the problem of the structure of such tissues. Because of the ease with which it can be converted to a form suitable for physical measurements, the vitreous humor is an ideal material with which to study this interaction.

Methods

Preparation of Material.—Ox vitreous humor was taken from eyes dissected from the heads of cattle 15 minutes after death. Batches of vitreous removed from the heads 2 hours after the death of the animal have not given different results from these. Adhering tissue was cut from the sclera, the eyes washed under the tap, and then blotted free from liquid. An equatorial cut was made through the eye and the vitreous cut free from attachments to the ciliary body and collected in a watch glass which had been washed with a solution of 0.1 per cent sodium diethyldithiocarbamate and dried. Any adhering pigment was dissected away and the gel poured into a measuring cylinder containing 10 ml. 0.1 per cent diethyl-

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dithiocarbamate for about 250 ml. of gel. Diethyldithiocarbamate was incorporated at this stage to prevent oxidative depolymerization of the hyaluronic acid by copper ions and ascorbic acid (6).

Preparation of Solutions from the Vitreous Body.—In some cases the vitreous isolated in this way was immediately homogenized at 0° in a blendor[1]. The solution and foam were transferred to a dialysis sac and dialyzed against the standard buffer for 3 days. During this period the foam subsided. The solution was centrifuged at 2500 R.P.M. for 15 minutes and the sediment discarded. The supernatant will be known as the "homogenate."

Some batches of vitreous humor with added diethyldithiocarbamate were placed directly in dialysis sacs and dialyzed against the standard buffer for 3 days. The gel structure was preserved during this period. Subsequent homogenization in the blendor gave a solution and deposited fibers around the blades of the blendor. The solution was centrifuged at 2500 R.P.M. for 15 minutes and the sediment discarded. The supernatant will be known as the "homogenate from dialyzed gels."

The solution obtained by passing the homogenate through a sintered glass filter, pore size 5 to 10 microns will be known as the "porosity 4 filtrate." A solution could also be obtained by centrifugation of the vitreous gels at high speed.

The standard buffer had the composition NaCl (0.2 M) Na_2HPO_4(0.016 M) NaH_2PO_4 (0.034 M), pH 6.9.

Dialyzed solutions were diluted to the volume occupied by the original vitreous. Unless otherwise stated all analytical figures in this paper will refer to unconcentrated vitreous humor.

Viscosity measurements were made in a Couette viscosimeter (7). The annular gap was 2 mm. The inner cylinder, diameter 12 mm., was immersed to a depth of 10 cm. in 10 ml. of solution. The shear rate could be varied from 0.5 to 150 $sec.^{-1}$ In general deflections were measured in the shear rate range 0.5 to 50 $sec.^{-1}$. The relative viscosity ($N_{rel.}$) for both dialyzed and non-dialyzed solutions was measured relative to the deflections produced by the standard buffer. $\frac{1}{c}(N_{rel.} - 1)$ will be called the "specific viscosity" and $\frac{1}{c}(N_{rel.} - 1)$ $c \rightarrow o$ the "intrinsic viscosity" in which c is the concentration in gm./100 ml. solution. ($N_{rel.} - 1$) is the "reduced viscosity."[2]

Filtration through sintered glass filters was under negative pressure, through gradocol membranes under a positive pressure of air or nitrogen. The gradocol membranes were obtained from the Wright Fleming Institute.

Ultraviolet absorption was measured in a Unicam spectrophotometer. Optical densities were measured in the range 350 to 240 mμ. The points in the range 350 to 320 mμ were extrapolated to shorter wave lengths to obtain a line from which the absorption corrected for scatter could be calculated.

Crystalline trypsin was from the Armour Laboratories. It was dialyzed against N/1000 HCl and dried by sublimation before use. *The hyaluronidase preparation* was rondase.[3]

Analytical Methods

Nitrogen was determined by Kjeldahl digestion with a selenium catalyst for 5 hours followed by distillation in a Markam still.

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[1] Measuring and Scientific Equipment, Ltd.

[2] The intrinsic viscosity is a measure of the effective volume of the particles in solution. The specific viscosity depends on this and also on the interaction between the particles. The reduced viscosity is a measure of the fractional increase in the resistance to flow of the solvent due to the presence of the solute.

[3] Evans medical supplies.

Hexosamine was determined by the Elson-Morgan reaction (8) on samples of solution containing 10 to 100 μg. hexosamine in 0.5 ml. The vitreous was hydrolyzed in 5.8 N HCl for 6 hours, standards of glucosamine hydrochloride being heated at the same time. Fivefold dilutions of vitreous were suitable for assay.

Hexuronic acids were determined by the carbazole method (9). The blank in the analysis of the vitreous was taken as the sum of the optical densities developed by water put through the whole procedure and vitreous to which the carbazole reagent was not added after heating with H_2SO_4. Fivefold dilutions of vitreous were suitable for assay.

Hydroxyproline was determined by the method of Neuman and Logan (10). The same color intensity was obtained from solutions of vitreous which had been hydrolyzed in 5.8 N HCl for 6 or 24 hours; quantitative recovery of added hydroxyproline was obtained after 20 hours' hydrolysis, and as a routine method hydrolysis was carried out overnight. The blank in the colorimetric assay of vitreous was taken as the optical density developed by hydrolyzed vitreous put through the whole procedure except the final heating at 70°. Solutions of vitreous concentrated two- to threefold were suitable for assay.

TABLE I

Viscosity and Combined Hydroxyproline Content of Solutions Derived from Vitreous Humor

Material	$N_{rel.}$ at β = 30 sec.$^{-1}$	Flow behavior at low shear rate	Hydroxyproline content
			μg./ml.
Solutions derived from fresh gels			
(1) Homogenate	1.82–2.0	Anomalous	6.0–8.75
(2) Porosity 4 filtrate	1.55–1.70	Newtonian	1.0–2.25
(3) Supernatant from high speed centrifugation of (1)	1.75	Newtonian	3.5
Solutions derived from dialyzed gels			
(4) Homogenate	1.68	Newtonian	2.5
(5) Porosity 4 filtrate	1.52	Newtonian	Less than 0.5
(6) Supernatant from high speed centrifugation of dialyzed gels	1.58	Newtonian	1.5

Tyrosine was determined by the method of Udenfriend and Cooper (11). Samples of vitreous were hydrolyzed for 6 hours.

Glycine was determined by colorimetric assay of the dinitrophenyl derivative isolated chromatographically on celite columns (12). Samples of vitreous were hydrolyzed 12 to 18 hours.

RESULTS

Preparation and Viscosity of Solutions Derived from Vitreous Humor

To study the interaction of the components of the vitreous humor by viscosity measurements it was necessary to destroy the gel structure but retain the components in solution. Several procedures were investigated and Table I describes the properties of solutions derived in various ways. Homogenization of fresh gels deposited no fibers on the blades of the blendor and little sediment on low speed centrifugation and gave a solution with non-Newtonian viscosity at low shear rates and a high combined hydroxyproline content. Homogenization of gels which had been dialyzed against the standard buffer caused deposi-

tion of fibers on the blades of the blendor and gave a solution with no anomalous flow properties and a low hydroxyproline content.

The homogenates of either origin when filtered through a porosity 4 glass filter or centrifuged at high speed gave solutions with a smaller hydroxyproline content and no anomalous viscosity.

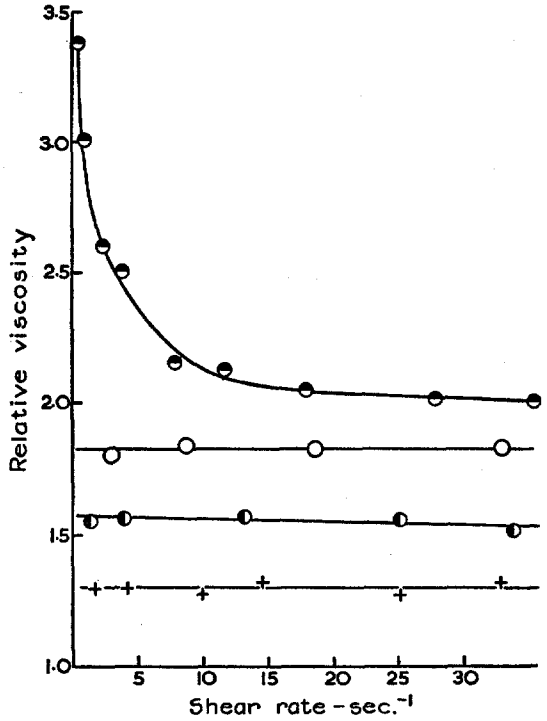

FIG. 1. Effect of filtration on the viscosity of homogenates from vitreous humor. -●—●-, homogenate; -○—○-, porosity 4 filtrate; -◐—◐-, 230 mμ membrane filtrate; -+—+-, 170 mμ membrane filtrate.

The viscosity of the homogenates or the porosity 4 filtrates was stable to incubation at 30° in the presence of toluene for 6 hours. The relationship between shear rate and relative viscosity of some of the solutions is described in Fig. 1.

It thus appeared that the anomalous viscosity depended on the presence of a hydroxyproline-containing material large enough to be retained by a porosity 4 filter and that certain treatments before homogenization could prevent its solubilization and simultaneously induce the formation of macroscopic fibers. Some experiments were carried out to investigate the stability of solutions of the hydroxypropline-containing material, and Table II describes the effects

of homogenization, dialysis, and storage on the solubilization. A smaller amount of hydroxyproline-containing material is brought into solution from gels in the ionic environment of the standard buffer than from normal gels; the amount solubilized is dependent on the speed of homogenization and the stability of solubilized material is slightly decreased by dialysis.

TABLE II

Combined Hydroxyproline Content of Homogenized Vitreous Body

Material	Conditions of homogenization	Hydroxyproline content
		μg./ml.
Homogenate after 3 days at 0°	14,000 R. P. M. 15 min.	6.10
Homogenate after 3 days' dialysis	14,000 R. P. M. 15 min.	5.25
Homogenate from fresh gels	1,000 R. P. M. 15 min.	4.8
Homogenate from gels which had stood 3 days at 0°	1,000 R. P. M. 15 min.	5.1
Homogenate from gels which had been dialyzed 3 days	1,000 R. P. M. 15 min.	2.2
Homogenate from gels which had stood 3 days at 0°	14,000 R. P. M. 15 min.	5.9
Homogenate from gels which had been dialyzed 3 days	14,000 R. P. M. 15 min.	4.0

In all cases the solutions were centrifuged at low speed before analysis. Dialysis was against the standard buffer. Whole vitreous body before homogenization contained 8 to 10 μg./ml. combined hydroxyproline.

Chemical Properties of the Hydroxyproline-Containing Material

To obtain further information on the material responsible for the anomalous viscosity, samples of the homogenate and the corresponding porosity 4 filtrate or supernatant from high speed centrifugation were analyzed for hydroxyproline, glycine, tyrosine, and uronic acids. The ultraviolet absorption was also noted. The results (Table III) reveal that the material removed from the homogenate contained no acid mucopolysaccharide, little aromatic amino acid, and had the glycine/hydroxyproline ratio of a collagenous protein.

The glycine and hydroxyproline contents so calculated are small differences of larger quantities. To obtain more accurate values gelatins were prepared

from the material removed from the homogenate. The washed filter residue, or sediment from centrifugation, was heated with distilled water in a sealed tube at 150° for 8 hours. The suspension was centrifuged and the supernatant dialyzed and analyzed. The glycine/hydroxyproline ratio was 1.75–1.89. Other samples were prepared similarly and after dialysis dried in weighed ampules. One sample had 11.5 per cent N and 9.1 per cent hydroxyproline, the other 13.7 per cent N and 10 per cent hydroxyproline. If all the nitrogen is combined in a protein with 15 per cent N then the gelatins contain 11.8 per cent and 11.0 per cent hydroxyproline respectively. The yields in the preparation of all samples of gelatin, calculated from the hydroxyproline contents of the homogenate, filtrate, or supernatant and gelatin were 25 to 30 per cent.

TABLE III

Effect of Filtration and Centrifugation on the Composition of Homogenates of Vitreous Body

Material	Hydroxyproline	Glycine	Uronic acid	Tyrosine	E_{280}	Glycine/hydroxyproline ratio of material removed from solution
	μg./ml.	μg./ml.	μg./ml.	μg./ml.		
Homogenate	8.75	42	280	23	0.76	2.3
Filtrate	2.25	26.9	280	24	0.70	
Homogenate	7.3	33				2.5
Supernatant	4.5	26				

The filtrate was from a porosity 4 sintered glass filter. The supernatant is from centrifugation at 20,000 *g* at 4°C. for 2 hours. All solutions were centrifuged at low speed before analysis

The composition of these materials is very similar to that of the vitrosin isolated from vitreous humor by Gross, Matoltsy, and Cohen (13).

Action of Hyaluronidase and Trypsin on the Viscosity of Solutions from Vitreous Humor

The relative viscosity of solutions of the porosity 4 filtrates was completely abolished by incubation with rondase, 80 μg./ml. at 30° for 30 minutes. Simultaneously there was an increase in the concentration of reducing sugars.

Fig. 2 illustrates the action of rondase on the viscosity of the homogenate. This sample had been concentrated twofold by pressure dialysis. Incubation with rondase decreased, but did not abolish, the relative viscosity at high shear rates. At low shear rates the relative viscosity is also decreased; it is still dependent on the shear rate but to a smaller degree than before incubation.

Trypsin had no effect on the viscosity although after incubation with 2.4 mg. enzyme/ml. solution for 6 hours 85 per cent of the nitrogen was diffusible through cellophane.

Ultrafiltration of Solutions Derived from Vitreous Humor

The possibility of fractionation by ultrafiltration was investigated. Fig. 1 illustrates the effect of ultrafiltration on the viscosity and Table IV, the composition of one sample of ultrafiltrates.

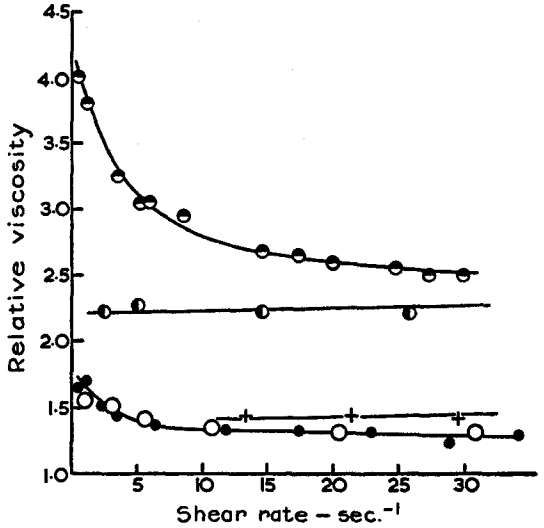

FIG. 2. Effect of hyaluronidase on the viscosity of homogenized vitreous humor. —●–●—, homogenate after twofold concentration; –◐—◐–, porosity 4 filtrate; –+—+–, homogenate after incubation with 63 μg/ml. rondase, 30 minutes; –○—○–, homogenate after incubation with an additional 60 μg./ml. rondase 30 minutes; –●—●–, homogenate after incubation with an additional 80 μg./ml. rondase for 30 minutes. The enzyme was added in successive amounts to the same sample of homogenate.

TABLE IV

Effect of Ultrafiltration on the Composition of a Solution Derived from Vitreous Humor

Filter	Nitrogen	Hexosamine	Uronic acid	E_{280}
	μg./ml.	μg./ml.	μg./ml.	
Porosity 4 glass filter	113	276	270	0.52
520 mμ gradocol membrane	77	250	270	0.41
230 mμ gradocol membrane	50	—	210	0.32
170 mμ gradocol membrane	25	180	190	0.20

Passage through membranes in the pore size range 1000 to 230 mμ had no effect on the viscosity but membranes with pore sizes below 230 mμ progressively reduced the viscosity. A membrane, 170 mμ pore size, gave a filtrate with a reduced viscosity about 60 per cent of that of the porosity 4 filtrate.

Material with a composition to be expected for an acid mucopolysaccharide is removed by membranes with pore sizes smaller than 230 mμ and about 20 to 45 per cent is removed by a 170 mμ membrane.

The protein content is decreased by passage through the membranes. This effect, which is probably due to absorption rather than ultrafiltration, provides further evidence that the soluble protein contributes little to the viscosity, for protein is removed by membranes which do not affect the viscosity.

Properties of Hyaluronic Acid from Vitreous Humor

It was of interest to determine the shear dependence of the viscosity of concentrated solutions of hyaluronic acid from vitreous humor and also the chemical relationship of hyaluronic acid to soluble protein.

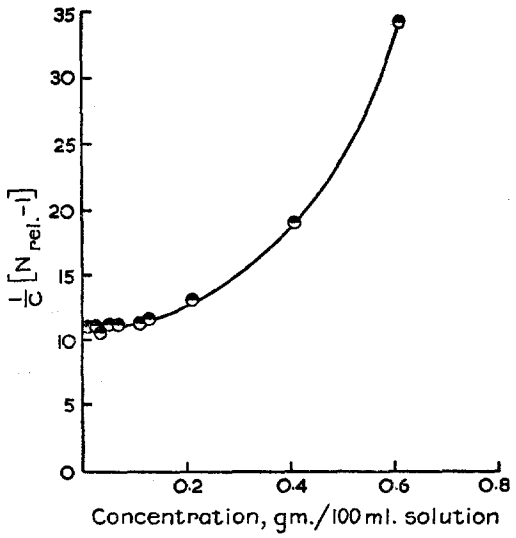

FIG. 3. Specific viscosity of hyaluronic acid in vitreous humor. The hyaluronic acid concentration was determined from the uronic acid concentration.

A sample of the porosity 4 filtrate was passed through a 170 mμ membrane using a new membrane for each 25 ml. of filtrate and the resultant solution concentrated and dialyzed against the standard buffer. The concentrate had a relative viscosity of 22 and this was independent of shear rate in the range 0.5 to 12.0 sec.$^{-1}$. Fig. 3 describes the relationship between the specific viscosity and concentration. A concentrated filtrate from a 800 mμ glass filter had an identical specific viscosity-concentration relationship. The intrinsic viscosity was 10.5 deciliters/gm.

A further sample of a 170 mμ membrane filtrate was prepared, concentrated, dialyzed against NaCl (0.18 M), Na_2HPO_4 (0.0066 M), NaH_2PO_4 (0.0006 M) pH 8.2, and examined in the Tiselius apparatus. After 24 hours' electrophoresis with compensation of the boundaries to keep only the fastest component in the

ascending limb, that component was isolated. It was dialyzed against N/1000 HCl, centrifuged, and the supernatant dried in weighed ampules. The solid contained 4.01 per cent N and 47 per cent hexosamine, and a solution containing 1.32 mg. solids/ml. had no specific absorption in the range 260 mμ to 300 mμ at pH 7. We are thus able to confirm the finding of Blix that the hyaluronic acid of vitreous humor is not in combination with protein.

DISCUSSION

The hydroxyproline-containing material of our homogenates has a ratio of hydroxyproline, glycine, and aromatic amino acids similar to that of a collagen and can be gelatinized by hot water. Pirie, Schmidt, and Waters (1) prepared residual protein from vitreous humor by filtration and showed that it had many properties similar to those of collagen. Their yield was 11 to 16 mg. protein from 100 ml. vitreous humor, and similarly Friedenwald and Stiehler (14) report a yield of 17 to 20 mg./100 ml. vitreous humor. The hydroxyproline content of the vitreous humor samples used by us corresponds to a collagen content of 9 mg./100 ml. vitreous. The difference may be due to different sampling techniques; for example we have not included all the vitreous attached to the ciliary body. However, while it is possible to assume that the hydroxyproline-containing material of homogenized vitreous humor forms part of the residual protein it is not permissible to assume that the residual protein of earlier workers is composed only of the hydroxyproline-containing material. However, the yield and composition of our material is almost identical with those of the vitrosin of Gross, Matoltsy, and Cohen (13).

The anomalous viscosity of the homogenates derived from vitreous humor is due to the presence of a hydroxyproline-containing material of considerable particle size and its magnitude depends on the presence of hyaluronic acid. The evidence for the latter is that the difference between the viscosity at high and low shear rates is reduced by incubation with hyaluronidase. If the hydroxyproline-containing material and the hyaluronic acid were not interacting then the viscosity at low shear rates should be the sum of the contributions due to hyaluronic acid and the hydroxyproline-containing material separately, which is not the case. The anomaly is only present in solutions subjected to very small shear stress; above a shear rate of 15 $sec.^{-1}$ the relative viscosity is the sum of the contributions made by the hydroxyproline-containing material and the porosity 4 filtrate.

Digestion with proteolytic enzymes and ultrafiltration are consistent with the soluble protein contributing little to the viscosity under all the conditions used in this work. Also we are able to confirm the finding of Blix (5) that the soluble protein is not in combination with hyaluronic acid. This is in contrast to the condition in synovial fluid and may be related to the very small albumin concentration of vitreous humor.

We ascribe the flow properties of the porosity 4 filtrate to hyaluronic acid. It has been observed that a concentrated filtrate freed from hydroxyproline-containing material, has a viscosity independent of shear rate. The intrinsic viscosity is very high, which requires the solution to contain particles which are either very asymmetric or voluminous. The lack of shear dependence favors the latter and we conclude that the hyaluronic acid of vitreous humor is coiled in such a way that the over-all shape is not greatly asymmetric. Such a conclusion is in keeping with the properties of other polyelectrolytes in salt solution (15), but is contrary to the conclusion drawn by Blix and Snellman (16) and repeated by Brunish, Rowen, and Irvine (17) from the flow birefringence of hyaluronic acid. At the very high shear rate and unspecified concentration employed by these authors linear aggregation or disruption of coiled particles may arise. The hyaluronic acid–protein complex of synovial fluid does not exhibit flow birefringence (18).

It is of interest to consider the nature of the interaction between the hyaluronic acid and the hydroxyproline-containing material. The interaction is abolished by a gravitational field or by a small shear stress so that only weak bonds can be involved. Consideration of an interaction due to restriction by one component of the volume available to the other necessitates assumptions about the molecular size of the components. Jensen (19) has data relating the specific viscosity to molecular weight of hyaluronic acid. The viscosity we have observed for hyaluronic acid from vitreous humor corresponds to a molecular weight of 0.5×10^6. This is also the value found by Brunish, Rowen, and Irvine (17) from turbidity measurements.

A flexible chain polymer, molecular weight 0.5×10^6, composed of segments 10 A long and equivalent weight 180 would have a mean radius of 330 A according to the theory of Kirkwood and Riseman (20). The equation of Flory and Fox (21) relating the molecular weight, intrinsic viscosity, and mean square separation of the ends gives a similar value. At a concentration of 0.04 per cent, molecules of this sort would occupy 8 per cent of the total volume of the solution. It is difficult therefore to account for the interaction between the hyaluronic acid and the hydroxyproline-containing material in terms of restriction by the former of the volume available to the latter. Data are not available to permit a precise assessment of the effective volume of the hydroxyproline-containing material in solution. The concentration of the hydroxyproline-containing material in solutions showing anomalous viscosity is 0.005 to 0.01 per cent. At this level the particles must be very asymmetric or voluminous to occupy a significant proportion of the volume. Such particles should give very viscous solutions. However, incubation of a homogenate with hyaluronidase does not leave a very viscous solution. Similarly filtration of the hydroxyproline-containing material from a homogenate decreases the reduced viscosity at high shear rate by only 20 to 30 per cent. It is unlikely that the volume occupied

by the hydroxyproline-containing material in an unperturbed state is much greater than that of the hyaluronic acid. The interaction producing the anomalous viscosity is not to be explained in terms of the effective volumes of the unperturbed particles. It appears necessary therefore to postulate the existence in solutions subject to very low shear stress of forces weak enough to be overcome by a gravitational field but permitting departure from the shapes adopted by the particles separately. Coulombic forces between positively charged centers of the hydroxyproline-containing material and the negatively charged hyaluronic acid could be of the required nature for, if only a few positively charged sites are involved, in the presence of the salt concentration found physiologically, the interaction would be weak.

As the anomalous viscosity of solutions derived from the vitreous humor is due to interaction between the hyaluronic acid and the hydroxyproline-containing material it is probable that the structure of the intact gel will have the same cause. The homogenization may decrease the particle length of the hydroxyproline-containing material and may also disorganize a favorable orientation of the particles with respect to each other. Comparison of the conditions of homogenization suggests that macroscopic fibers with diameters much greater than those observed by Matoltsy, Gross, and Grignolo (3), are not present in the intact vitreous. We have only obtained macroscopic fibers on homogenization in the unphysiological condition of the environment of the standard buffer.

SUMMARY

Solutions made by homogenization of vitreous humor exhibit anomalous viscosity at low shear rates due to interaction between hyaluronic acid and a hydroxyproline-containing material similar to collagen. The latter can be removed by filtration or centrifugation to give a solution whose viscosity is due to hyaluronic acid. On concentration the viscosity of the latter remains independent of shear rate and it is concluded that hyaluronic acid in the vitreous humor is not greatly asymmetric. There is no evidence that the soluble protein of vitreous humor contributes to the viscosity. Hyaluronic acid in vitreous humor is not in chemical combination with protein. The interaction between the hyaluronic acid and the collagen-like material is ascribed to weak, coulombic forces.

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