PROTEINS AND HYALURONIC ACID OF BEEF VITREOUS HUMOR*

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IN CONSIDERING the contents of the aqueous and vitreous cavities, we are challenged to explain why two substances, aqueous and vitreous humor, so similar in chemical composition, differ so drastically in physical structure. With regard to the vitreous humor, the problem is to explain the stability of the structure of a substance which contains so low a concentration of those constituents—namely protein and hyaluronic acid—which might contribute to its physical state. Evidently the minimal protein and polysaccharide contents are of such a molecular nature and physical organization as to be able to bind relatively large quantities of water to form a gel-like structure.

An understanding of how this structure liquefies, collapses, and retracts may be a key to some of the many clinical conditions related to pathologic states of the vitreous, such as liquefaction of the vitreous with age, separated retina, and traction sequelae consequent to loss of vitreous at operation. In this regard Meyer (1) has suggested that simple glaucoma may well be explained on the basis of an abnormal hyaluronic acid content due to inhibition of the enzyme, hyaluronidase. With the goal of attaining such understanding the following basic research was undertaken. At present this work is limited to study of the macromolecules of soluble

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protein and hyaluronic acid. The following represents a progress report on the work commenced during the past year.

PREPARATION

There are approximately 40 to 50 mg. of protein in 100 c.c. of vitreous humor, whereas in serum there are about 6,000 mg. in the same volume. As much protein is contained in a 1 percent serum contamination as in the remaining 99 percent vitreous sample. The very low concentration of protein in the vitreous humor means, also, that large volumes must be collected, pooled, and concentrated in order to carry out all microchemical procedures except those requiring the most minute amounts of material. Consequently, to study the protein of the vitreous humor it is necessary to develop techniques for dissecting out many eyes quickly and with no serum or tissue contamination. Two procedures have been used: one involves dissection of beef eyes stored at 0°C.; the other employs tissue which has been quick-frozen and maintained at —50°C. The technique for preparing vitreous samples from eyes maintained at 0°C. was developed with the aid of Mr. John Sinclair, Department of Surgery, University of California at Los Angeles Medical School, and consists essentially of the following steps:

TECHNIQUE FOR PREPARING VITREOUS SAMPLES. The cornea is removed. The ciliary body is then gently dialyzed so that a cut of the sclera down to the equator of the globe can be made. The scissors are turned at the equator, a complete circumferential incision of the sclera is made, and the anterior half of the uveal tract exposed. An incision is then made in the choroid, taking care to avoid the retina, and the choroid is incised around the equator. With an applicator, the choroidal tissue is then peeled forward to the ciliary body. A small spatula is then passed between the retina and the vitreous at the equator, and the retina is peeled forward to the pars plana. A suction cup is then placed on the lens and, with gentle traction, the vitreous body attached to lens and anterior uvea is lifted from the posterior half of the globe. While the lens is held with the vitreous body still suspended from it, the vitreous is cut from its base with a scissors, great care being taken to cut behind the areas of pigment and retina which are adherent to the vitreous at the ora serrata. This produces a sample of vitreous humor free from contamination by retina or pigment.

Approximately 10 minutes per eye is required with the above technique. Since the number of eyes which can be dissected per day is limited with this procedure, it became expeditious, when larger quantities of material were needed, to freeze the eyes quickly in a mixture of dry ice and acetone and to store them at −50°C. until they could be dissected. This technique of quick-freezing produces small uniform ice crystals which damage the tissues less than do the large crystals formed by slow freezing in a deep-freeze unit. Dissection is effected easily by placing the eyes in warm water and allowing the outer membranes to thaw while the vitreous humor remains frozen. The outer tissues are then cut meridionally over the anterior hemisphere, the incision starting just over the ciliary body. The vitreous can then be expressed from the globe free of surrounding tissues except for the lens and the ciliary process. The anterior layers containing these structures are then cut away. The vitreous is then crushed and allowed to thaw prior to filtration. This procedure was impossible to perform upon the slow-frozen eyes, since the breakdown of tissue during freezing was such that bits of retina frequently penetrated quite deeply into the vitreous mass.

In order to measure the integrity of the vitreous samples prepared by each technique, viscosity measurements and protein nitrogen determinations were undertaken at various intervals after storage of the eyes prior to dissection. The resu'ts are shown in Figure 1. The most striking change noted was the rapid increase in protein with storage at 0°C. There was some indication of a minor increase after only 24 hours, while after two days the level had more than doubled. This indicates that although the vitreous samples had been procured without evident contamination, there must have been serious contamination from external sources when the eyes stood intact for more than 24 hours. In spite of this change in protein nitrogen content, the viscosity values were essentially unchanged. Therefore, viscosity is an inadequate criterion of vitreous integrity. In general, both the viscosity and the protein nitrogen content of the frozen eyes were equal to or slightly lower than the values for the eyes stored at 0°C., and the values remained almost constant. The second technique, quick-freezing, was prefer-

able because of the constancy of composition over a longer period of time, and this technique was used for most of the studies to be reported.

This investigation indicates that vitreous samples obtained clinically or from experimental animals should be used immediately, or the eyes should be preserved by prompt quick-freezing.

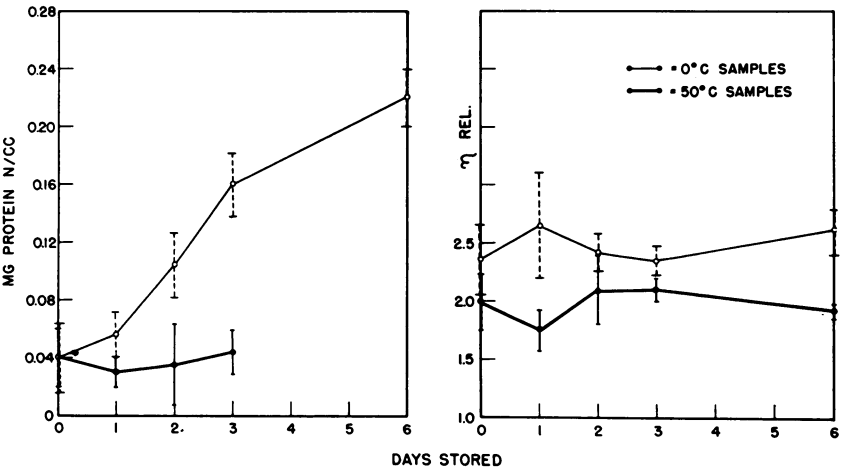

FIGURE 1. EFFECT OF STORAGE ON PROTEIN CONTENT AND VISCOSITY OF BEEF VITREOUS HUMOR

During the course of making the viscosity determinations it was found that the viscosity of the vitreous humor samples decreased at a measurable rate at 36°C. Consequently the relative viscosities reported in Figure 1 were determined at 0°C.

HYALURONIC ACID DEPOLYMERIZATION

The changing viscosity at 36°C. was investigated further in order to ascertain whether hyaluronidase, the degradative enzyme, was present in the vitreous filtrate or whether a nonenzymatic oxidation of the hyaluronic acid was taking place. For this purpose vitreous humor was used as an enzyme system acting upon umbilical hyaluronate. The rate of decrease in viscosity of a standard hyaluronic substrate, prepared from umbilical cord, was followed by incubation at 36°C., in a 0.1 M acetate buffer, $_{p}$H 6.0, containing 0.15 M NaCl (1). Vitreous humor, when allowed to act on umbili-

cal hyaluronate, showed depolymerization activity equivalent to that of 1 to 2 viscosity units (Searle's "Alidase") per c.c. This value is an approximation, since it is difficult to assay such small amounts of enzyme. Since the depolymerization of hyaluronate by oxidation through the action of ascorbic acid and copper ion has been reported (2), the effect of copper and ascorbate upon the vitreous humor depolymerization system and isolated hyaluronidase was tested. The time for reduction of the relative viscosity of the umbilical-cord hyaluronate substrate to one half the initial value is given in Table 1.

TABLE 1. HYALURONIDASE ACTIVITY (HALF TIME)

Added to Umbilical Cord Hyaluronate Substrate	*Minutes to Reduce the Substrate Viscosity to One Half*
(1) 2 mg. % Cu^{++} plus 10 mg. % ascorbate	5
(2) Hyaluronidase, 2 viscosity units/c.c.	50
(3) Hyaluronidase, 2 V.U./c.c. containing 2 mg. % Cu^{++}	104
(4) Vitreous humor containing 2 mg. % Cu^{++}	not reduced to $\frac{1}{2}$ viscosity in 120 minutes
(5) Vitreous humor	99

The hyaluronate substrate alone was perfectly stable at 36°C., and neither copper nor ascorbic acid alone affected its viscosity. The vitreous humor contains ascorbic acid, and the addition of copper to it should have resulted in a rapid breakdown of the hyaluronic acid (see Table 1, Item 1). Since it did not do this and appeared to decrease the activity of the vitreous humor (Table 1, Item 4) as well as that of the hyaluronidase standard (Table 1, Item 3), and since copper is known to inhibit this enzyme (3), this would suggest that the active principle of the vitreous humor is hyaluronidase. In this connection it might be pointed out that hyaluronidase has been detected in the ciliary body, iris, and aqueous humor (4). To confirm its presence in the vitreous it would be necessary to isolate the enzyme in pure form. Difficulty has been encountered in this respect due to the extremely small quantity of total protein and the even smaller amount of this particular com-

ponent in the vitreous. The extreme instability of hyaluronidase in dilute solution is an additional deterrent. The copper may have been without effect due to interaction and combination with hyaluronidase or other proteins present. The relatively high catalase content of the vitreous humor would also tend to vitiate the oxidative activity of ascorbic acid plus copper which is due, in part, to the formation of hydrogen peroxide (2).

ELECTROPHORETIC ANALYSES

In 1939 Hesselvik published results obtained from electrophoresis of vitreous humor (5). In these studies he found three components: hyaluronic acid, and two proteins which he reported were similar to serum albumin and gamma globulin. In order to analyze vitreous humor electrophoretically it is necessary to concentrate the fluid some thirtyfold. Freeze-drying proved undesirable since the residue so obtained was largely insoluble. By placing the sample in dialyzing casings of small diameter, placed in front of a fan in a room maintained at 1-2°C., the necessary concentration could be

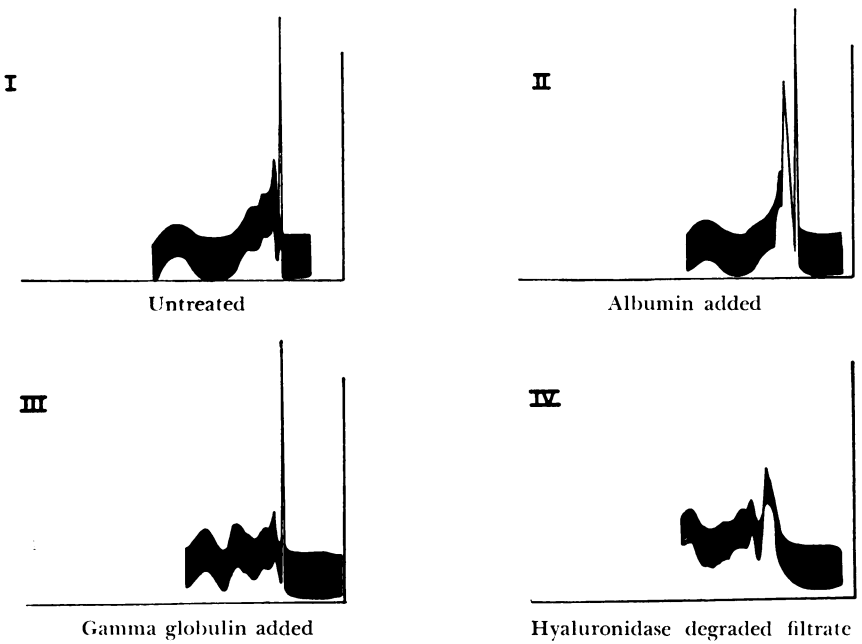

FIGURE 2. ELECTROPHORESIS OF BEEF VITREOUS FILTRATE. ($_{p}$H 8.6, μ=0.1)

completed overnight. Figure 2 shows the electrophoretic pattern obtained from such a concentrated solution at $_{p}$H 8.6, ionic strength equal to 0.1. The components, as seen in Pattern I, were tentatively identified as hyaluronic acid, present as a sharp spike, followed by three protein components of lower mobility. The prominent bump on the left represents the salt anomaly. Since Hesselvik had reported the presence of albumin and gamma globulin in vitreous humor, these proteins were added to the concentrated filtrate. Addition of serum albumin enhanced the first protein peak, as seen in Pattern II. Addition of bovine gamma globulin, on the other hand, caused an additional peak between the existing protein components and the initial boundary (Pattern III). This study differs from the earlier work of Hesselvik, therefore, in demonstrating the presence of at least three protein components, which appear to have mobilities similar to albumin, alpha globulin, and beta globulin. When the percent distribution of the three protein components was calculated by the method of Tiselius and Kabat (6), the albumin was shown to be 32 percent of the total. This value, which is the average of six runs, is, of course, lower than that seen in serum or aqueous humor, indicating that the protein components probably did not arise as a unit from serum through a simple filtration mechanism. If this were the case the albumin, which is the smaller molecule, would be present in a relatively higher concentration.

In order to determine if the leading component were hyaluronic acid, hyaluronidase equal to approximately 1/40 the weight of the substrate was added to a sample of vitreous filtrate. The sample was then incubated at $_{p}$H 6 for eight hours. Upon concentration, this solution did not become viscous, as did untreated samples. The pattern obtained after extended dialysis against several buffer changes is shown in Pattern IV of Figure 2. It was anticipated that the hyaluronic acid would disappear. It is noted that a broad peak replaces the sharp spike. In order to determine if this remaining peak were due to a protein moiety associated with the hyaluronic acid, rather than to a polysaccharide fraction resistant to the enzyme, this treated filtrate was separated electrophoretically upon paper and the fractions stained by bromphenol blue, the conventional protein stain. Three bands were seen, again corresponding

to albumin, alpha globulin, and beta globulin. No band was seen in either the gamma globulin region or in the region where the hyaluronic material would be found.

To characterize carefully the sodium hyaluronate from vitreous humor and also to study this seemingly enzyme-resistant fraction, sodium hyaluronate was isolated by the detergent technique developed by Dr. Norman W. Simmons, of the Atomic Energy Project, University of California at Los Angeles (7). Approximately 1,000 beef eyes were required to prepare each Gm. of purified sodium hyaluronate. Table 2 shows the analyses obtained from a representative sample of this material.[1] The theoretical values are derived by assuming that the hyaluronic acid is composed of equimolar quantities of acetyl-glaucosamine and glucuronic acid (8). The experimental values obtained agree very closely with such a structure. The nitrogen value agrees well with theory suggesting no contamination by protein, glycogen, salts, or water. The small amounts of sulfur and phosphorus, which may represent sulfated

TABLE 2. COMPOSITION OF VITREOUS HUMOR SODIUM HYALURONATE

	PERCENT COMPOSITION	
	Experimental	*Calculated*
Carbon	39.64	41.80
Hydrogen	5.46	5.00
Sodium	5.56	5.74
Nitrogen	3.44	3.49
Phosphorus	0.07	0.00
Sulfur	0.06	0.00
Acetyl	9.86	10.70

polysaccharides or nucleic acid, indicate that the hyaluronic acid is 98 percent pure.

Electrophoresis of this hyaluronic acid and of the material left after enzyme action upon it is shown in Figure 3. Here again, a broad peak is seen after hyaluronidase action. Extending the enzymatic action to 24 hours and adding 3 aliquots of enzyme at 4-hour intervals did not alter this observation. When the isolated hyaluronic acid was examined with the ultracentrifuge the same

[1] Analyses performed by Dr. Adalbert Elek, Elek Microanalytical Laboratory, Los Angeles, California.

sharp spike was seen before enzyme action and a similar broad peak was observed after enzyme digestion. Elementary analyses of the non-dialyzable material left after enzyme action showed the same distribution of carbon, hydrogen, nitrogen, and acetyl as in the original material. This suggests that the glucosamine and glucuronic acid residues must alternate throughout the molecule

FIGURE 3. VITREOUS HUMOR SODIUM HYALURONATE. ($_{p}$H 8.6, $\mu = 0.1$)

without there being a local concentration of one type of residue, with peripheral areas containing a predominance of the other residue. If this were the case, partial degradation should yield a "core" with an appreciably different chemical composition from that of the molecule as a whole. This work, therefore, is in disagreement with that of Kaye and Stacey, who postulated a core consisting primarily of glucosamine residues (9). If the theory of a perfectly uniform structure throughout the molecule is correct, degradation should proceed to a disaccharide stage, or to some other basic unit. This resistance to complete degradation has been seen with hyaluronate preparations from various sources in several laboratories in addition to our own (10, 11), while other groups have obtained the theoretical maximum hydrolysis (3, 12). Alkali degradation of vitreous hyaluronic acid to a specific macromolecular size has also been reported (13). The inability to degrade completely the hyaluronate molecule with hyaluronidase may have been due to having nonoptimal conditions for enzyme action. If this is the case, the interesting fact that enzymatic degradation, and also alkali treatment, tends to stop at a macromolecular level must still be examined.

MOLECULAR SIZE

To determine the length of the isolated sodium hyaluronate molecule, streaming birefringence measurements were undertaken; these studies have been briefly reported elsewhere (14). Basically, the principle of streaming birefringence is as follows: If a particle with a large axial ratio is placed in a solution which is flowing, the particle will tend to line up with the stream lines, just as logs orient as they move downstream. This is shown in Figure 4,

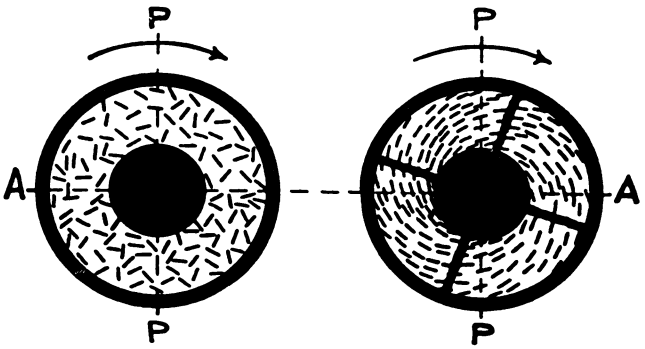

FIGURE 4. STREAMING BIREFRINGENCE
Left: Particles, each schematically represented by a line indicating its optic axis, at rest. Right: Orientation caused by motion of external cylinder.

which is a cross-sectional view of an annular space, containing the solution to be investigated, between two cylindrical surfaces. The solution to the left is at rest and the molecules are disoriented. When one of the cylinders is rotated, circular flow of the solution results and the molecules begin to orient under the shear force. The rate of flow can be governed by the speed with which the cylindrical wall is rotated and the width of the annular space. When streaming stops the organized array of molecules becomes disorganized and they point in all directions. The disorientation force is the well-known Brownian motion of molecules. When the opposing forces in such a system, i.e., the shear force of the moving solution and Brownian motion, or rotational diffusion, are balanced in an equilibrium situation, the degree of orientation, measured as the "extinction angle," is governed by the length of the particle. The extinction angle is the angle which the particle

makes with the stream lines. The orientation of the molecules can be measured by taking advantage of their streaming birefringence properties, which are analogous to the birefringence of polaroid crystals.

The tendency for the particle to orient is governed by its rotational diffusion constant which is perfectly analogous to the well-known translational diffusion constant. The relationship between the rotational diffusion constant and particle length was first given by Perrin in 1934 (15), and is as follows:

$$\Theta = \frac{3\,k\,T}{16\pi\,\eta\,a^3}\left[\ln\frac{2a}{b} - 1\right]$$

where Θ = rotational diffusion constant
k = the Boltzman constant
T = the absolute temperature
η = the viscosity of the solvent
a = the half length of the particle, and
b = the half diameter of the particle

The rotational diffusion constant Θ is sensitive to variations in length *(a)* and insensitive to variations in axial ratio $(\frac{a}{b})$, because of the presence of the logarithmic term. The larger a is, the smaller Θ will be. Since Θ is functionally related to the extinction angle, the larger a is, the smaller the extinction angle will be. With the aid of Perrin's relationship and additional relationships between Θ and the extinction angle (16, 17), it is possible to calculate the extinction angle for a particle of a particular length. Variation in extinction angle, χ, with shear gradient (stream force) for the particles varying in length from 6,000 to 12,000 A° are shown in Figure 5. If all the molecules present are the same size their χ versus gradient curves will follow the theoretical length curve.

In Figure 5 are plotted also the experimental points obtained in our laboratory on the sample of purified sodium hyaluronate from the vitreous humor and, for comparison, the points obtained on a comparable sample from umbilical cord. The patterns shown are representative of some eight to ten runs which were made. It is noted that the points for both samples cut across the theoretical curves, indicating that the hyaluronic acid molecules are polydis-

perse, not monodisperse—i.e., there is absence of uniformity in the size of these molecules. Proteins, in general, are monodisperse, whereas this is not the case with polysaccharides. The values observed were not markedly dependent upon concentration or ionic

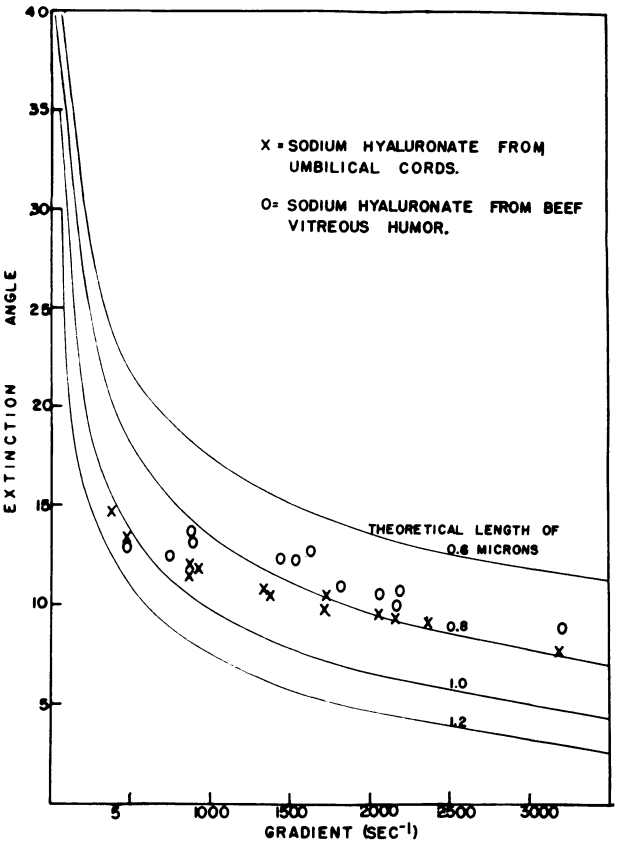

FIGURE 5. STREAMING BIREFRINGENCE OF SODIUM HYALURONATE

strength of the solution. Most polyelectrolytes show dependence upon both. It is noted that the lengths of the sodium hyaluronate molecules appear to lie between 7,000 and 10,000 A°. It is of interest that the lengths of the molecules derived from umbilical cord are approximately 10 percent greater than those derived from the vitreous humor. These values are larger than those reported by Blix and Snellman (13), and may be due in part to the difference in the newer method of preparation.

The behavior of the extinction angles of the hyaluronate samples in the low-shear region attracted attention because they were quite different from the values observed in our laboratory with purified samples of tobacco mosaic virus and deoxyribonucleic acid. The extinction angles of these molecules approach 45° at low shear gradients, which indicates zero orientation of the molecules. It seemed, therefore, desirable to investigate the behavior of the sodium hyaluronate samples at low shear rates.

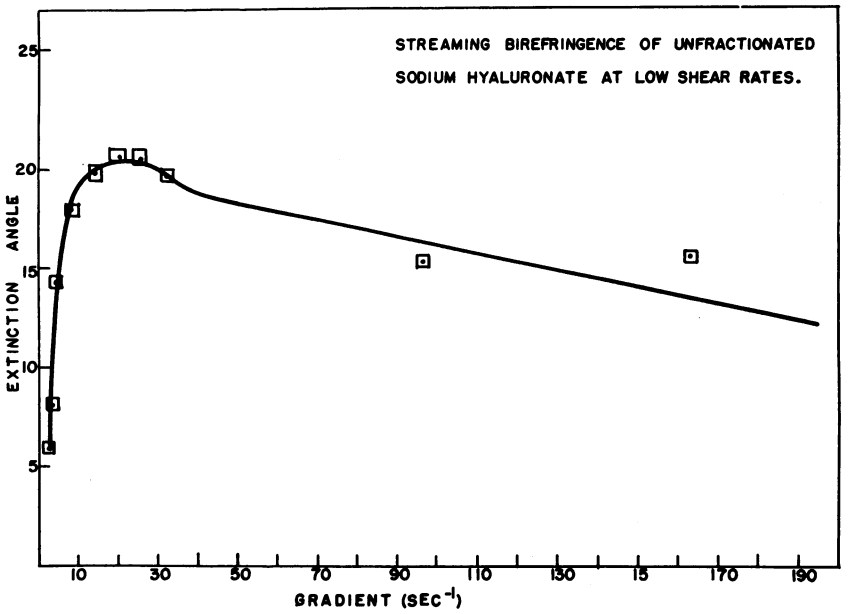

FIGURE 6. STREAMING BIREFRINGENCE OF UNFRACTIONATED SODIUM HYALURONATE AT LOW SHEAR RATES

In Figure 6 we see the variations in χ for the vitreous sample at low shear rates. It is observed that instead of approaching 45° the values go through a maximum and then decrease. The same type of curve was seen with umbilical hyaluronate. To explain this phenomenon we considered a recent study of Foster and Zucker (18); they observed similar unexpected behavior when small amounts of amylopectin, the branched component of starch, were added to amylose, the straight chained component. The data obtained by these workers are shown in Figure 7. The maximum value fol-

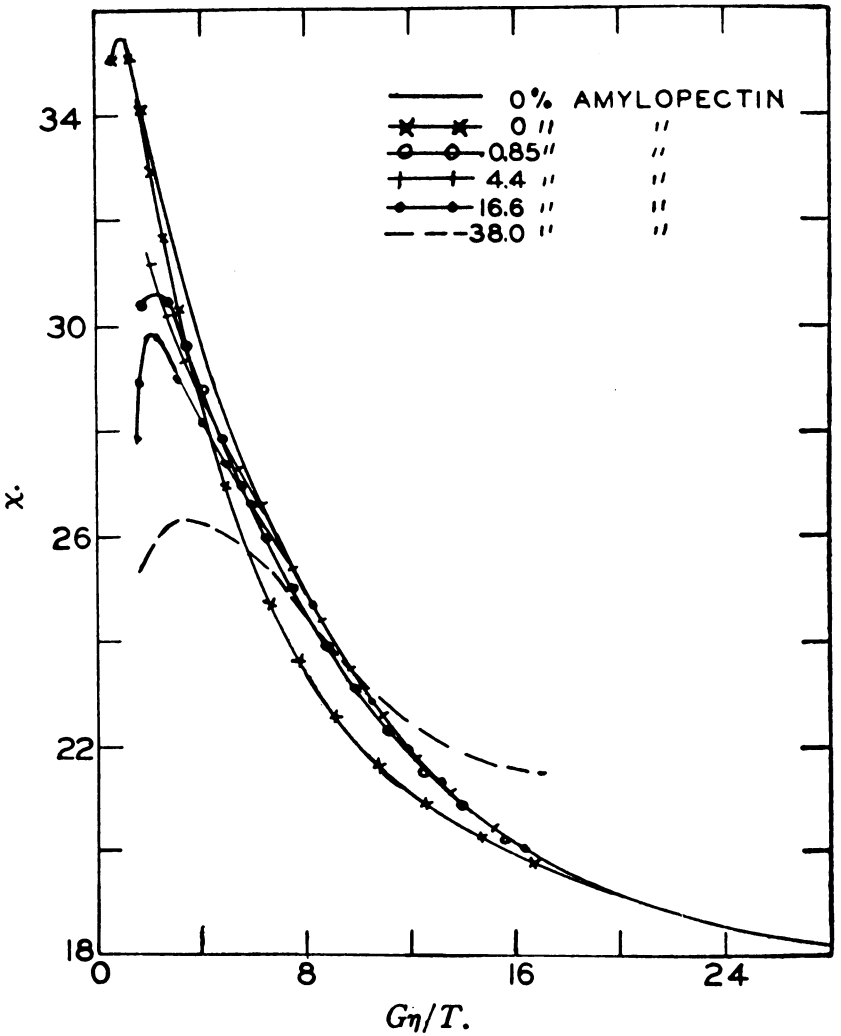

FIGURE 7. EXPERIMENTAL χ *vs.* G η/T CURVES FOR THE PURIFIED AMYLOSE PREPARATION OF HASSID AND THE EFFECT OF ADDITION OF VARYING PROPORTIONS OF AMYLOPECTIN

lowed by the decrease in the lower shear region indicates the presence of a readily oriented fraction. It is noted that the height of the maximum decreases and shifts to the right as the percentage of amylopectin increases.

After adopting the working hypothesis that both samples of

sodium hyaluronate contained a heretofore unidentified component of high molecular weight, some preliminary fractionation experiments were undertaken. When the sodium hyaluronate samples were centrifuged for three hours at 30,000 rpm the remaining supernatant solution showed a shift in the maximum upward and to the left. This suggests that a small amount of a component of high molecular weight was removed during centrifugation. Preliminary attempts to isolate the two components, using isopropyl alcohol fractionation from sodium acetate solutions, appear promising.

Light-scattering experiments (19) were undertaken in order to obtain estimates of the molecular weight of the purified sodium hyaluronate. The method for determining the size and weight of macromolecules from the intensity of scattered light is relatively new. The technique and theory of light scattering have been described adequately in several review articles (19-21). When a light wave encounters a molecule, the electric field associated with the light and that associated with the molecule interact in such a manner that light is scattered in all directions rather than being transmitted as a single beam. The total amount of light scattered by a dilute solution will be directly proportional to the number of dissolved molecules, each of which will scatter light independently. It was Debye (22) who showed first that the scattering (τ) is related to the molecular weight according to the expression:

$$H\,(C/\tau) = 1/M + 2\,BC$$

where H is a constant composed of factors which include the refractive index of the liquid, the wave length of light, and the refractive increment of the solution. C is the concentration of the macromolecule, M is the molecular weight, and B is a second constant related to interaction forces between the liquid and the macromolecule. In practice one measures τ for a series of solutions at different concentrations. Then C is plotted against $H\,(C/\tau)$, obtaining a line whose intercept is $1/M$.

When the macromolecule under investigation is larger than one twentieth of the wave length of light used, the first term of the right-hand member of the above equation must be multiplied by an additional factor: $[1/P\ (\ominus)]$. This factor may be calculated

from the data obtained experimentally from the ratio of light scattered in a forward direction to that in the backward direction (23).

Preliminary studies yielded a molecular weight for the vitreous sodium hyaluronate on the order of 400,000 to 1,000,000. These weight values, together with the length values obtained in streaming birefringence, lead to the concept that the sodium hyaluronate is essentially an unbranched molecule which is not appreciably coiled. A molecule 7,000 A° in length, composed of repeating disaccharide units, each of which is 10 A° in length and has a formula weight of 401 (13), would have a molecular weight of about 300,000 if it were a single, unbranched, unfolded chain. Therefore much branching or folding of a single chain, or, alternatively, many parallel unbranched chains within the molecule, are inconsistent with the molecular weight as determined by light scattering.

From these studies we derive our present concept of the hyaluronic acid molecule as that of a long, threadlike molecule containing many hydroxyl groups and, at 10 A° intervals, a charged carboxyl group. By virtue of these easily accessible hydrophilic groups, the molecule is able to bind extremely large quantities of water, thereby maintaining the turgor of the vitreous humor. The nature of the molecules lends itself to the formation of a three-dimensional network which traps water and results in gel formation. An altered state of this molecule, such as coiling or contraction, then, could explain liquefaction of the vitreous humor, with the molecule binding less water than normally, and could also be related to retraction and collapse of the vitreous humor. Studies are under way to determine the effect of $_{n}H$, ions, and protein concentration upon the physical state of this molecule. Concurrent investigations of vitreous filtrate, which contains hyaluronate under physiological conditions, are also in progress and are designed to supply a reference point for elucidation of pathologic states in terms of chemical and physical changes.

SUMMARY

1. Preparation of vitreous samples.—Two techniques were described. Viscosity and protein concentration determinations sug-

gested that the quick-freezing technique with eyes stored at —50°C. was preferable if the eyes could not be used immediately.

2. *Hyaluronidase assay* demonstrated the presence of small amounts of a hyaluronic acid depolymerization system in vitreous humor. The data suggest that hyaluronidase, rather than a non-enzymatic oxidation, is the agent.

3. *Electrophoretic studies* showed the protein composition of the vitreous humor to be essentially different from that reported by Hesselvik. No gamma globulin was present. The albumin-to-globulin ratio was found to be lower than that of serum or aqueous humor, suggesting that these proteins do not arise as a body from serum through a simple diffusion mechanism.

4. *Enzymatic degradation* of sodium hyaluronate by the enzyme hyaluronidase appears to leave a resistant non-dialyzable fraction which has the same elemental composition as the total molecule.

5. *Streaming birefringence measurements* showed hyaluronic acid from vitreous humor and umbilical cord to be polydisperse, having lengths of 7,000 to 10,000 A°. These studies, plus *light-scattering measurements,* indicate that the hyaluronic acid has a molecular weight of 400,000 to 1,000,000 and exists as an elongated molecule which is probably not appreciably coiled. The presence of relatively small amounts of a second component of high molecular weight, possibly branched, is suggested.

6. The above observations suggest a molecular structure capable of binding quantities of water very large relative to the colloid concentration of the system.

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DISCUSSION

DR. KENNETH C. SWAN (read in his absence by Dr. Francis H. Adler). There is much that must be learned about the nature of the normal vitreous before many pathologic processes in this structure can be investigated properly; therefore an understanding of vitreous disease processes can be reached only after additional comprehensive and fundamental investigations of this type.

The authors have stated aptly the crux of the vitreous problem; that is, how is it possible for the vitreous to maintain a stable gel-form based on such small concentrations of colloidal constituents? The total concentration of vitreous protein which is considered to be a collagen is

in the neighborhood of 50 mg. percent, and the hyaluronic acid contributes an even smaller portion in terms of weight. That these constituents of vitreous can gelatinize is quite surprising when one considers that to gel, ordinary gelatin must be present in a concentration of at least 250 mg. percent.

As the major phase of their initial study the authors have examined the nature of the hyaluronic acid in the vitreous. As a result of studies with a diversity of exacting techniques, they have concluded that the molecule is an elongated one that does not tend to coil on itself as do many large molecules. Their estimation of the molecular weight agrees reasonably well with that reported by others. They suggest that such a long polysaccharide molecule exposes many hydrophilic groups (hydroxyl and carboxyl groups) and would be capable of binding considerable water. This is an interesting and reasonable explanation for the contribution that this polysaccharide makes to the formation and stability of the vitreous.

In this connection I would like to emphasize an aspect on which the authors have not dwelled. In his teaching of biochemistry in the Lancaster Course, my associate, Dr. John E. Harris, has emphasized that both colloidal constituents unquestionably contribute to the stability of the gel-like structure of the vitreous. Evidence to this effect has been obtained by Pirie and her co-workers as well as others. Dr. Harris has pointed out that the vitreous of various animals differs in stability. Whereas in the rabbit it can be liquefied with hyaluronidase preparations, the vitreous of other animals may remain less stable but nevertheless a gel, even when this hyaluronic acid is depolymerized. In most animals, however, the vitreous can be liquefied by the addition of the collagenase. This and other data indicate that the major protein contributing to the gel formation of the vitreous is a collagen. It would seem likely, therefore, that the major stability of the vitreous is due to both the polysaccharide and protein portions.

I sincerely hope that the authors will continue their investigations. The unique structure of the vitreous, as Karl Meyer has shown so well, permits the investigator an opportunity to contribute to general knowledge of biologic processes as well as to advance a clinical field.

DR. ROBERT BRUNISH. One point came to my mind regarding the action of collagenase. Preparation of collagenase without contamination with hyaluronidase is a major problem, and it is then possible that collagenase preparations may break down some hyaluronic acid while acting on collagen. I do not mean to underestimate the importance of collagen.