Studies on the Structure of the Vitreous Body

V. SOLUBLE PROTEIN CONTENT*

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Since Mörner (1), the proteins of the vitreous body have been generally classified as residual and soluble proteins. The former includes those elements of the gel which can be collected as a residue after filtration or as a sediment after high speed centrifugation (more than 30,000 × g). The latter refers to the proteins in the liquid vitreous, the viscous fluid remaining after removal of the structural elements of the gel, viz. fibers, filaments, and cells.

The soluable protein content of the cattle vitreous body has been studied by several authors and was reported to be between 0.02 and 0.2% (2). The albumin-globulin ratio, based on ammonium sulfate precipitation, was found to be much lower than that of blood serum (2). Ultraviolet absorption studies on the proteins of the vitreous body also indicate a low albumin-globulin ratio (3).

Electrophoretic studies suggest the presence of two protein components with mobilities similar to those of serum albumin and γ-globulin (4, 5). However, it was found that γ-globulin added to the soluable proteins forms a separate boundary in the course of electrophoresis (6) and ultracentrifugation (5).

All available data indicate that the protein composition of the vitreous body is different from that of plasma. Since this is the only connective tissue in the mammalian body in which the composition of intercellular substance, uncontaminated by blood, lymph, or cells, can be studied, a closer investigation of proteins, with special regard to glycoproteins, appeared to be of great interest. Previous investigations have shown that certain macromolecular components, such as hyaluronic acid and collagen, are unevenly distributed in the vitreous bodies of various animal species (7, 8). Therefore, the distribution of soluable proteins and glycoproteins was studied in the gel. The investigations reported herein are based on sialic acid, hexosamine and nitrogen analyses, on the separation of the perchloric acid-soluble protein fraction and on electrophoretic analyses. A preliminary report has been given (9).

While these studies were in progress a brief report was given by Dische et al. (10) on the isolation of a glycoprotein fraction from the bovine vitreous body representing 15% of the total soluable proteins and containing 12% carbohydrates: viz. galactose, mannose, fucose, glucose, and hexosamine.

MATERIALS

Vitreous bodies from the eyes of adult steers (2 to 3 years old), cows (5 to 7 years old), and young calves (½ to 2 months old) were obtained from the slaughterhouse. The eyes were dissected within 1 hour after the death of the animals. The preparation of samples from different parts of the vitreous body is described elsewhere. Care was taken to avoid contamination with blood and other ocular tissues. Unless otherwise stated, all determinations were made on samples centrifuged at 105,000 × g for 1½ hours or at 78,000 × g for 4 hours at 4°. The supernatants ("liquid vitreous") were dialyzed for 24 to 48 hours at 4° against several changes of large volumes of 0.15 x NaCl.

Aqueous humor was collected within 10 minutes after slaughter by puncturing the anterior chamber of the steer eye through the cornea with a needle without applying suction. In some cases, pressure was applied to the eyeball on withdrawal of the aqeous humor. The corneas were used for the cornea analysis, as described elsewhere.

Synovial fluid was obtained by puncture of the astragalotibial joints of steers immediately after they were slaughtered. The fluid was centrifuged and dialized in the same manner as the vitreous body.

Serum—Pooled bovine serum* was used after dialysis against several changes of large volumes of 0.15 x NaCl at 4° for 24 hours.

METHODS

Hexosamine, hexuronic acid, and nitrogen were determined as described elsewhere.†

Protein nitrogen was calculated from the nondialyzable nitrogen content corrected for hyaluronic acid present in the samples. The hyaluronic acid content was determined from hexuronic acid analyses.

Protein-bound hexose was determined as galactose-mannose by the orcinol reaction, as described by Winzler (11).

Sialic acid was determined on dialyzed samples by Bial's re-
action, as described by Werner and Odin (12). A crystalline preparation of N-acetyleneuraminic acid (O-sialic acid) was used as standard. In a preparation of human α-acid glycoprotein, 11.0% sialic acid was found with the use of this standard. The molecular extinction coefficient of different sialic acids varies somewhat (maximum 20%) in Bial's reaction (13). It is known that at least two sialic acids are present in the ox serum glycoproteins (14). Since the sialic acids present in bovine glycoproteins have not been completely characterized, all values reported in this paper are as N-acetyleneuraminic acid.

Sialic acid determination was also made on dialyzed samples by the thiorbituric acid method (15). The sialic acid content of the same α-acid glycoprotein preparation as used above was 11.2%, with the method. When the N-acetyleneuraminic acid content of the vitreous body and the aqueous humor was determined both by Bial's method and by the thiorbituric acid method, the latter method gave up to 18% lower figures in some cases. Unless otherwise stated, the figures given in this paper are those determined by Bial's method.

Hyaluronic acid, in the amount present in the samples, did not significantly influence the determinations. The absorption spectra obtained for Bial's reaction showed identical absorption bands with N-acetyleneuraminic acid and vitreous body proteins. Therefore, no correction was necessary for the color contribution of other nondialyzable carbohydrates present in the samples.

Removal of Hyaluronic Acid—This was done by hyaluronidase digestion and dialysis. Highly purified tests hyaluronidase, 100 to 200 U.S.P. units (31,000 U.S.P. units per mg nitrogen) was used per ml of sample. Digestion was carried out for 2 days at 37° under agitation and continuous dialysis against several changes of acetate-NaCl buffer (pH 5.5, P/2 0.16). Merthiolate in 1:10,000 dilution or tolune was used to prevent growth of microorganisms. This treatment removed 82 to 93% of the hyaluronic acid in the liquid vitreous and 95 to 100% of that in synovial fluid.

Enzymatic Hydrolysis of Proteins—Twice crystallized pepsin was used. Enzyme, 0.1 mg, was added to 1 ml of liquid vitreous, and the solutions were incubated for 48 hours at 37° under agitation and continuous dialysis against P/15 phosphate buffer (pH 7.1), with 0.12 N NaCl added, in the case of trypsin, and in the case of pepsin, against 0.1 N HCl. Control samples were dialyzed against 0.1 N HCl under the same conditions. Thymol crystals were added to prevent growth of microorganisms. The nitrogen and sialic acid contents were determined before and after digestion. Corrections were made for the nitrogen and sialic acid contained in the enzyme.

Percoll acid-soluble proteins were separated essentially as described by Winacor et al. (16). Hyaluronic acid was removed from the liquid vitreous or from synovial fluid by hyaluronidase digestion, as described above. Percoll acid (1 volume, 3.6 N) was then added to 50 times diluted serum or synovial fluid, or to undiluted liquid vitreous or aqueous humor. The samples were allowed to stand for 10 minutes and were subsequently centrifuged at 78,000 × g for 30 minutes. The supernatants were immediately transferred to dialysis bags and dialyzed against repeated changes of distilled water. The entire procedure was carried out at 4° in order to preclude the removal of sialic acid by percoll acid treatment, which may occur at higher temperature (17). In control experiments no loss of sialic acid during percoll acid treatment and dialysis could be demonstrated.

In order to determine the nitrogen and the hexosamine content of the percoll acid-soluble protein fraction in the liquid vitreous, it was necessary to make corrections for the nitrogen and hexosamine content of the remaining hyaluronic acid. This was done on the basis of hexuronic acid analysis of the percoll acid-soluble protein fraction, assuming that all of the hexuronic acid present is due to the hyaluronic acid content. Such corrections were not necessary for the percoll acid-soluble protein fraction of synovial fluid in which practically no hyaluronic acid remained after treatment with hyaluronidase.

Electrophoretic experiments were carried out in an Amine portable electrophoresis apparatus in Veronal, acetate, or glycine buffers of 0.1 ionic strength. Mobility and conductivity measurements were made at 0.75°.

Pooled liquid vitreous samples were concentrated about 20 times by dialysis against 20% polyvinylpyrrolidone. Hyaluronic acid was removed by hyaluronidase digestion and dialysis as described above. Before electrophoresis the concentrated liquid vitreous samples were dialyzed against repeated changes of the buffer. During these procedures a slight precipitate formed, representing 2 to 3% of the total protein nitrogen and 0.6 to 0.9% of the sialic acid, which was removed by centrifugation. Further precipitate formation occurred when the concentrated solution was brought to pH 4.5 with acetate buffer. The precipitate represented approximately 6% of the total protein nitrogen and 6% of the sialic acid in the original preparation, and it readily dissolved at higher or lower pH values.

RESULTS

Dialyzable Nitrogen Content—The total nitrogen content of pooled central and cortical samples of the vitreous body of steers was determined after removal of the collagen filaments and fibers and the cellular elements by high speed centrifugation. The same pooled samples were dialyzed against 0.15 N NaCl until equilibrium was reached, and the nitrogen content was determined. Table I shows that almost half of the nitrogen can be removed by dialysis. The dialyzable nitrogen is the same in the different parts of the vitreous body, but the nondialyzable nitrogen is significantly higher in the cortical region.

Protein Nitrogen and Sialic Acid Content—The protein nitrogen and sialic acid contents were determined in pooled dialyzed liquid vitreous samples prepared from different parts of the gel (Fig. 1). The protein nitrogen content is highest in the cortical layer, next to the retina, and decreases toward the central and anterior portions of the vitreous. It is lowest in the anterior part, next to the ciliary body, where it is not much higher than the protein nitrogen content of the aqueous humor. The sialic acid distribution follows the same pattern as the protein nitrogen. In the aqueous humor, however, its content is only 3% of the lowest value found in the anterior segments of the vitreous body.

The sialic acid content of pooled occipital samples obtained from the vitreous bodies of cows, steers and calves was found to be 27.3, 36.5, and 41.0 μg per ml, respectively. Other pooled oc-
TABLE I

Dialyzable and undialyzable nitrogen content of pooled central and cortical samples

<table>
<thead>
<tr>
<th></th>
<th>Total nitrogen</th>
<th>Undialyzable nitrogen</th>
<th>Dialyzable nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>µg/ml</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Central</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>910 ± 3</td>
<td>115 ± 1</td>
<td>104</td>
</tr>
<tr>
<td>Cortical</td>
<td>248 ± 2</td>
<td>145 ± 2</td>
<td>103</td>
</tr>
</tbody>
</table>

Table II shows that the value in the latter is about three times higher than those found in the body fluids (Table II).

The sialic acid content of the vitreous body was compared before and after centrifugation at 105,000 × g for 2 hours. Centrifugation at this speed removes the collagen filaments and fibers and the cells. It was found that the sialic acid content in the anterior, central, and cortical samples did not change significantly after high speed centrifugation. The cortical layer sample was centrifuged for 2 hours at 105,000 × g, and the sediment was washed several times in distilled water, lyophilized, and dried to constant weight. The sialic acid content of two different preparations was found to be less than 0.05%.

The sialic acid content of the vitreous body is much lower than that of serum or synovial fluid (Table II). However, the protein content of the vitreous body is about 30% that of serum and about 70% that of synovial fluid. Comparison of the sialic acid-protein ratios in serum, synovial fluid, aqueous humor, and vitreous body shows that the value in the latter is about three times higher than those found in the body fluids (Table II).

Perchloric Acid-soluble Proteins—The nitrogen content of the perchloric acid-soluble protein fraction was determined in aqueous humor, vitreous body, synovial fluid, and serum (Table II). In liquid vitreous more than 20% of the total protein nitrogen is not precipitated by perchloric acid. The corresponding percentages in serum and synovial fluid are about ten times lower.

The concentration of the perchloric acid-soluble protein fraction, as measured by its sialic acid content, was determined in different parts of the vitreous body. 50 to 60% of the total sialic acid remains in solution after perchloric acid precipitation of the proteins in the vitreous body (Table III). The distribution of the perchloric acid-soluble protein fraction is seen to be similar.
Table IV
Analysis of perchloric acid-soluble protein fractions

<table>
<thead>
<tr>
<th>Perchloric acid-soluble protein fractions</th>
<th>Hexosamine Nitrogen</th>
<th>Hexosamine Nitrogen</th>
<th>Sialic acid Nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreous body*</td>
<td>1.06</td>
<td>0.89</td>
<td>0.77</td>
</tr>
<tr>
<td>Synovial fluid</td>
<td>0.87</td>
<td>0.75</td>
<td>0.92</td>
</tr>
<tr>
<td>Serum</td>
<td>0.75</td>
<td>0.71</td>
<td>0.90</td>
</tr>
<tr>
<td>α-Acid glycoprotein from serum (18)</td>
<td>1.17</td>
<td>0.66</td>
<td>0.92</td>
</tr>
</tbody>
</table>

* Occipital samples of steer eyes.

Table V
Effect of trypsin and pepsin digestion on soluble proteins of steer vitreous body

<table>
<thead>
<tr>
<th>Incubations at 37° under continuous dialysis.</th>
<th>0.1 N HCl</th>
<th>Pepsin</th>
<th>Trypsin</th>
<th>Pepsin and trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen removed (%)</td>
<td>7.5</td>
<td>69.4</td>
<td>36.4</td>
<td>84.2</td>
</tr>
<tr>
<td>Sialic acid removed (%)</td>
<td>61.0</td>
<td>70.3</td>
<td>9.7</td>
<td>73.6</td>
</tr>
<tr>
<td>Sialic acid-nitrogen ratio after digestion</td>
<td>0.12</td>
<td>0.28</td>
<td>0.41</td>
<td>0.40</td>
</tr>
</tbody>
</table>

The electrophoretic pattern of the perchloric acid-soluble protein fraction at pH 2.8 (glycine buffer) showed only one large boundary with a mobility of about $-0.6 \times 10^{-4}$ cm$^2$ volts$^{-1}$ sec$^{-1}$ (Fig. 2A, B, C).

Fig. 2. Electrophoretic patterns of the soluble proteins in the steer vitreous body (occipital samples). Ascending side, $\Gamma/2$ 0.1. Positions of initial boundaries indicated by vertical dotted lines. A, Soluble proteins, concentration 1.6 mg N per ml; Veronal buffer, pH 7.4; current 10 ma; after 168 minutes, bar angle 45°. B, Soluble proteins, concentration 3.5 mg N per ml; Acetate buffer, pH 5.03; current 10 ma; after 274 minutes, bar angle 30°. C, Soluble proteins, concentration 4.8 mg N per ml; Glycine buffer, pH 2.77; current 12.5 ma; after 120 minutes, bar angle 30°. D, Soluble proteins after trypsin digestion, concentration 3.2 mg N per ml; Glycine buffer, pH 2.80; current 10 ma; after 149 minutes, bar angle 30°.

to that found for total protein; viz. highest in the posterior cortical layer and lowest in the anterior part. The concentration of this fraction in the aqueous humor is nearly 10 times lower than the lowest value in the vitreous body.

The perchloric acid-soluble protein fraction of pooled steer ocipital vitreous body was further analyzed for hexosamine and hexose. The analytical values, expressed as carbohydrate-nitrogen ratios, are shown in Table IV. These values are of the same order as those reported for α-acid glycoprotein preparations from beef serum (18).

Treatment with Proteolytic Enzymes—Trypsin digestion removes only 36% of the total proteins and approximately 10% of the total sialic acid in the ocipital samples of steer liquid vitreous (Table V). The sialic acid-nitrogen ratio increases from 0.29 in the undigested sample to 0.41 in the digested vitreous. This indicates that trypsin removes only a very small amount of the sialic acid-containing proteins. The proteins, including those containing sialic acid, are removed more effectively by pepsin digestion. In 0.1 N HCl, without pepsin, $\frac{1}{2}$ of the total sialic acid is removed. After combined treatment with pepsin and trypsin, an undigested protein component still remains. The high sialic acid-nitrogen ratio indicates that this fraction is mainly composed of glycoproteins.

Electrophoretic Experiments—These experiments were carried out on ocipital samples after removal of the hyaluronic acid as described above. No hyaluronic acid boundary could be detected in solutions concentrated to a protein content of about 2%.

In pH 7.4 Veronal buffer three distinct boundaries are visible after 3 hours (Fig. 2A). In pH 5.0 acetate buffer only two boundaries can be observed after 4 hours (Fig. 2B). The best separation was obtained in pH 2.8 glycine buffer, where four boundaries became apparent within 1 hour and separated completely within 2 hours (Fig. 2C). The mobility of the three fastest components was found to be 10.0, 8.8, and $6.2 \times 10^{-5}$ cm$^2$ volts$^{-1}$ sec$^{-1}$, respectively. The mobility of added crystalline bovine albumin is similar to that of the fastest moving boundary; nevertheless, at pH 2.8 the two boundaries clearly separate. The fastest moving boundary at pH 2.8 (glycine buffer) showed a considerably decreased area after trypsin digestion (Fig. 2D). The slowest moving component, which, according to area measurements, constituted about 40% of the total, moved toward the anode at pH 2.8 with a mobility of about $-0.6 \times 10^{-4}$ cm$^2$ volts$^{-1}$ sec$^{-1}$ (Fig. 2A, B, C).
evenly distributed in the gel. The highest concentration is found in the cortical layer, next to the retina, and the lowest in the frontal area, next to the ciliary body and the lens. This distribution is very similar to that found for hyaluronic acid (7), whereas collagen shows quite a different distribution (7). It should be pointed out that the sialic acid content of the aqueous humor is considerably lower than that of the vitreous body in cattle of all age groups. The sialic acid content of the aqueous humor of cattle is somewhat lower than that reported in human cerebrospinal fluid (15).

Dische and Zelmanes (19) showed that a small amount of a glycoprotein containing glucose, galactose, and hexosamine is combined with the collagen fibers. In washed vitreous precipitate we found less than 15 µg of sialic acid per 100 ml of vitreous body (cortical layer), which is only 0.3% of the total sialic acid present in this part of the gel.

Sialic acid-containing glycoproteins constitute a much larger fraction of the soluble proteins of the vitreous body than those of serum, synovial fluid and aqueous humor. This is also shown by the presence of a very large perchorlic acid-soluble protein fraction in the vitreous body, representing more than 20% of the total protein nitrogen. The perchorlic acid-soluble protein fraction in cattle serum represents only about 2% of the total protein nitrogen. This fraction in serum is known to be a mixture of glycoproteins of high sialic acid content, the major fraction being the α1-acid glycoprotein (see reviews by Winzler (11, 20)). The carbohydrate content of the perchorlic acid-soluble protein fraction of the steer vitreous body was found to be very similar to that described for the α1-acid glycoprotein of bovine serum (18).

The high content of acidic glycoproteins explains the resistance of the vitreous body proteins to trypsin digestion. It has been shown that the resistance of α1-acid glycoprotein to trypsin and papain digestion is due to its sialic acid content, which can be cleaved by mild hydrolysis (21). The much better digestion obtained with pepsin in 0.1 N HCl is presumably due, in part, to the removal of sialic acid, occurring at this pH at 37°.

Electrophoretic experiments show that a large portion of the soluble proteins and of the perchloric acid-soluble protein fraction move as one boundary toward the anode at pH 2.8. Of the serum proteins, only the α1-acid glycoprotein is known to have a similar acidic character (18, 22, 23). Both electrophoretic and chemical analyses indicate that a relatively large portion of the soluble proteins in the vitreous body are glycoproteins, of which a prominent component appears to be similar to or identical with the α1-acid glycoprotein of serum.

The electrophoretic pattern of the liquid vitreous is quite different from that of serum. The presence of albumin seems to have been fairly well established in previous studies (4, 5). Our results indicate that albumin constitutes about 1/4 of the total soluble proteins. The fact that added crystalline albumin separates from the fastest moving boundary at pH 2.8 does not mean that albumin is not a component of the vitreous body proteins. Immunochemically, albumin was demonstrable in the same sample. Therefore, it is more likely that the albumin in the vitreous body interacts with other proteins or is somewhat altered in the course of acidification.

There are several possible explanations for the quantitative differences in the protein composition of the vitreous body and serum. One is that the proteins are derived from the plasma but are prevented by a filtration mechanism from freely diffusing into the vitreous body. It has been suggested that the hyaluronic acid accumulated in the cortical layer of the gel may act as a barrier against the diffusion of high-molecular plasma components. Such a mechanism would explain the absence of large protein molecules like γ-globulin and the preponderance of small-molecular proteins such as acidic glycoproteins and albumin. Another possibility is that some proteins are produced by the metabolically very active cells present in the surface layer of the vitreous body (24).

**SUMMARY**

The soluble proteins of the cattle vitreous body, an intercellular substance of connective tissue, have been studied. Although dialyzable nitrogen is evenly distributed in the gel, the protein nitrogen and sialic acid content are highest in the cortical layer next to the retina and lowest in the anterior part next to the lens.

The sialic acid-nitrogen ratio is about three times higher in the vitreous body than in the aqueous humor, synovial fluid and serum. The perchorlic acid-soluble protein fraction contains about 50% of the total sialic acid. The content of hexosamine and sialic acid in this fraction is very similar to that of the α1-acid glycoprotein of serum. Electrophoretic studies also indicate the presence of a large acidic glycoprotein fraction in the vitreous body.

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