Studies on the Structure of the Vitreous Body*

VI. BIOCHEMICAL CHANGES DURING DEVELOPMENT

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Only a few studies have been reported on the biochemistry of the vitreous body in the developing eye. Bernbridge and Pirie (1) determined the total hexosamine and the total nitrogen content of the filtered vitreous body of rabbits after birth and found that during the first three weeks there was a decrease in both the nitrogen and the hexosamine concentration. Schweer and Sudhof (2) confirmed our earlier findings (3) that the hexosamine content of the vitreous body of cattle varies with the age of the animal. Boyer et al. (4) found that in growing calves the increase in the amount of “residual proteins,” determined as the weight of the sediment after high speed centrifugation, is proportional to the weight of the vitreous body.

Studies on the ascorbic acid, calcium, hyaluronic acid and protein content of the bovine vitreous body in the course of embryonic and postnatal development are reported in this paper. Some of this work has been presented previously in a short communication (3). Studies on the ascorbic acid, hyaluronic acid and protein content of the vitreous body of adult steers have been reported elsewhere (5).1

MATERIALS AND METHODS

The eyes of adult cattle, calves and embryos of Aberdeen-Angus, Hereford and Jersey breeds were collected at the slaughterhouse. The age of steers varied between 1 and 3 years, but they are shown in the figures as 3 years old. The age of the embryos could be determined with an accuracy of one-half month. The eyes of embryos younger than two months were not used, since the amount of vitreous body was not sufficient for chemical analysis. On the average, the vitreous body was removed from the eye two hours after the death of the animal.

The eye was divided by an equatorial cut behind the lens, and all of the gel in the posterior segment was removed without contamination by the retina and placed in a centrifuge tube. This part of the vitreous body is termed the “occipital sample.” The preparation method has been described elsewhere in greater detail (3). When the eyes of embryos 2 to 1 months of age were used, separation of the gel from the retina was more difficult and adhering tissues were removed with forceps before centrifugation. Some eyes were frozen in Dry Ice-acetone. The sclera, the choroid, and the retina were peeled from the frozen eyeball, and the frozen lens and ciliary body were also removed. The still-frozen vitreous body was then divided by an equatorial cut behind the lens into an “anterior” and a “posterior” sample (3). The anterior sample contained the gel between the lens and the ciliary body; the posterior sample contained the rest of the vitreous body, but some fragments of the cortical layer of the gel may have been removed with the frozen retina. Anterior and posterior samples prepared together are called “total vitreous.” All of the gel samples were centrifuged in a Spinco preparative ultracentrifuge at 4° until completely liquefied; this required 2 to 3 hours of centrifugation at 105,000 X g. Samples used for calcium determination were liquefied by pressing the gel through a 25-gauge needle with a syringe. For ascorbic acid and calcium determinations the liquefied vitreous body itself was used, but for other analyses it was dialyzed against a 0.02 M phosphate buffer (pH 7.0) containing 0.1 M sodium chloride for 24 hours at 4°. The volume change during dialysis was less than 1 per cent. For some determinations the vitreous body samples taken from several eyes were pooled.

Determination of Volume of Eyeball and Vitreous Body—The eyes were carefully cleaned of connective tissue and muscle and the eyeballs were measured by dipping them in a graduate containing water. The eyes were then frozen in Dry Ice-acetone, the tissues around the vitreous body, including the lens, were removed while still frozen and the volume of the frozen vitreous body was measured as described above.

Ascorbic acid, dehydroascorbic acid, and diketogulonic acid contents were determined by the 2,4-dinitrophenylhydrazine method of Roe et al. (6). Three ml. of 10 per cent SnCl2 in 5 per cent metaphosphoric acid was added to 2 ml. of liquefied, undialyzed vitreous fluid immediately after centrifugation. The samples were then stored at 4° overnight, and the determinations were made the following day after dilution of the samples to 60 ml. with 5 per cent metaphosphoric acid. Controls showed that no oxidation of ascorbic acid occurred during storage.

Hexosamines were determined by a modification of the method of Elson and Morgan (7). Dialyzed samples were hydrolyzed in 6 N hydrochloric acid for 16 hours at 90°. Pooled samples of vitreous body hydrolysates were chromatographed in acid resin columns (Dowex 50, H cycle) to separate glucosamine and galactosamine (8). For this purpose the dialyzed vitreous body was concentrated about five times in dialysis bags before a fan at 4° and then hydrolyzed as above. The recovery was
calculated from the total hexosamine determination carried out on the sample before chromatography.

Hexosamine determinations carried out on the hydrolysate of dialyzed or undialyzed vitreous body indicated that no interfering substances were present (9). The amount of hexosamine recovered on column from the vitreous body hydrolysate was in good agreement with the analysis values made directly on the hydrolysate.

Hexuronic acid was determined on dialyzed vitreous body samples by the carbazole method (10). Glucuronic acid was used as standard.

Ester sulfate was determined spectrophotometrically as benzidine sulfate (11) after hydrolysis of pooled dialyzed occipital samples of vitreous body in 6 N hydrochloric acid.

Protein Nitrogen—Nitrogen was determined by the micro-Kjeldahl method (12) on centrifuged and dialyzed samples. The hexosamine nitrogen content was subtracted from the values obtained and the remaining nitrogen value is regarded as that originating from the soluble protein fraction of the vitreous body.

Hydroxyproline and Collagen—Hydroxyproline was determined in the sediment of dialyzed vitreous body samples following centrifugation of anterior, posterior and occipital parts of the vitreous body for 2 hours at 105,000 × g. The method of Neuman and Logan (13), modified by Martin and Axelrod (14), was used after hydrolysis of the proteins with 6 N hydrochloric acid for 3 hours at 140°. Collagen was estimated by multiplying the hydroxyproline values by a factor of 7.46 (13).

Calcium was determined on undialyzed occipital samples according to Denson (15). 3 ml. of liquefied vitreous body were shaken with 1 gm. of Amberlite IR-120 cation resin (H cycle). After several washings with distilled water the cations were eluted from the resin with 10 ml. of 6 N hydrochloric acid and the calcium content of the eluate was determined in a Beckman flame spectrophotometer.

Electrophoretic experiments were performed in an Aminco electrophoresis apparatus in Veronal buffer at pH 8.0 to 9.0, ionic strength 0.12. Pooled vitreous samples were concentrated 2 to 14 times by dialysis against a 20 per cent high-polymer dextran solution in the same buffer. The concentrated samples were then redialyzed against pure buffer before the runs were made.

RESULTS

To compare the biochemical with the anatomical development of the vitreous body, the size of the eyeball and the vitreous body in animals of different ages was measured. The results are given in Fig. 1.

Hexosamines—The concentration of nondialyzable hexosamine in the occipital sample of individual vitreous bodies was plotted against age (Fig. 2). The concentration was high in embryos less than five months of age (15 to 35 mg./100 ml.) but decreased during the last month of embryonic development (6 to 12 mg./100 ml.). In calves the concentration was low, but showed a gradual increase, reaching adult level at the end of the first year. In the vitreous body of cows and bulls the hexosamine value is, in general, lower than the values in the steer vitreous body reported earlier.1

Column chromatography was applied to pooled occipital samples of steers, calves, and embryos of different ages. The

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**Fig. 1.** Volume of the eyeball and of the vitreous body in animals of different age. Vertical lines represent the range of determinations made on individual eyes.
Fig. 2. Hexosamine concentration in the occipital part of individual vitreous bodies (●, cow; ●, bull; ●, embryo, calf or steer). Vertical line represents the standard deviation of individual determinations made on 3-year-old steers.

TABLE I
Chromatographic separation of glucosamine and galactosamine in pooled occipital samples of vitreous body from cattle of different age groups

<table>
<thead>
<tr>
<th>Age groups</th>
<th>Concentration</th>
<th>Recovery</th>
<th>Glucosamine-galactosamine ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucosamine</td>
<td>Galactosamine</td>
<td>%</td>
</tr>
<tr>
<td>Embryos</td>
<td>mg./100 ml.</td>
<td>mg./100 ml.</td>
<td></td>
</tr>
<tr>
<td>4 to 5 months</td>
<td>4.05</td>
<td>1.35</td>
<td>85</td>
</tr>
<tr>
<td>6 months</td>
<td>4.41</td>
<td>1.63</td>
<td>111</td>
</tr>
<tr>
<td>8 to 8.5 months</td>
<td>4.45</td>
<td>1.96</td>
<td>85</td>
</tr>
<tr>
<td>Calves</td>
<td>6.47</td>
<td>1.84</td>
<td>99</td>
</tr>
<tr>
<td>1 to 2 months</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steers</td>
<td>24.5</td>
<td>1.4</td>
<td>95</td>
</tr>
</tbody>
</table>

results are tabulated in Table I. The galactosamine concentration of the vitreous body is fairly constant in animals of varying age; viz. 1 to 2 mg./100 ml. The differences between the amount of hexosamine recovered on the column and the values obtained from direct hexosamine determination were within the error of the method. This indicates that in the vitreous body of embryos and calves, just as in that of adults, the direct hexosamine determination gives reliable results. The distribution of hexosamine in the vitreous body is not homogeneous. In the anterior part of the steer vitreous body the hexosamine content is approximately half the concentration in the posterior part. To check this difference similar experi-

TABLE II
Hexosamine and protein nitrogen content of anterior and posterior samples of vitreous body from cattle of different age groups

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hexosamine</th>
<th>Protein nitrogen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior</td>
<td>Posterior</td>
</tr>
<tr>
<td></td>
<td>mg./100 ml.</td>
<td>mg./100 ml.</td>
</tr>
<tr>
<td>Embryos</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5 months</td>
<td>13.9</td>
<td>13.4</td>
</tr>
<tr>
<td>4.5 months</td>
<td>5.3</td>
<td>4.6</td>
</tr>
<tr>
<td>5 months</td>
<td>7.8</td>
<td>10.5</td>
</tr>
<tr>
<td>7 months</td>
<td>4.9</td>
<td>4.7</td>
</tr>
<tr>
<td>8 months</td>
<td>3.1</td>
<td>5.5</td>
</tr>
<tr>
<td>9 months</td>
<td>4.7</td>
<td>6.6</td>
</tr>
<tr>
<td>Calves</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. 1</td>
<td>4.3</td>
<td>8.8</td>
</tr>
<tr>
<td>No. 2</td>
<td>3.0</td>
<td>5.4</td>
</tr>
<tr>
<td>No. 3</td>
<td>3.7</td>
<td>6.5</td>
</tr>
<tr>
<td>No. 4</td>
<td>4.1</td>
<td>7.3</td>
</tr>
<tr>
<td>No. 5</td>
<td>4.5</td>
<td>5.7</td>
</tr>
<tr>
<td>No. 6</td>
<td>6.8</td>
<td>14.1</td>
</tr>
<tr>
<td>Average</td>
<td>4.4</td>
<td>7.8</td>
</tr>
<tr>
<td>Steer</td>
<td>13.6</td>
<td>25.0</td>
</tr>
</tbody>
</table>

ments were performed on calf and embryo eyes. The results are given in Table II. In 4- to 7-month-old embryos there was no significant difference in the hexosamine concentration of the
two samples, but in 8- to 9-month-old embryos and in calves the difference became more and more marked, with a higher hexosamine concentration always in the posterior part.

**Hexuronic Acid**—Hexuronic acid analyses were made on some of the individual and pooled samples on which hexosamine was also determined. The hexuronic acid concentration in the occipital part of the vitreous body is given in Fig. 3. For comparison, analysis values for other parts of the vitreous body are also included. In embryos the hexuronic acid concentration is very low but increases after birth. The concentration was always higher in the posterior than in the anterior part of the vitreous body.

**Ester sulfate**—Seven pooled occipital samples of vitreous body from animals varying in age from 6-month-old embryos to adult steers gave sulfur values between 0.2 to 0.4 mg./100 ml. of vitreous body. The concentration did not vary with the age of the animal, and both the lowest and the highest values were found in steers.

**Protein Nitrogen**—The level of the “soluble protein” nitrogen was fairly constant: 8 mg./100 ml. from birth throughout life (Fig. 4). The individual variation was smaller than for hexosamine. The protein nitrogen concentration was highest in embryos less than four months old and decreased, gradually reaching, in 8-month-old embryos, the same level as in calves. The highest nitrogen values in 3- to 4-month-old embryos (30 to 80 mg./100 ml.) are not recorded in Fig. 4. Protein nitrogen values from anterior and posterior parts of the vitreous body are given in Table II. The nitrogen of the soluble protein fraction is lower in the anterior part than in the posterior, as is the case for hexosamine and hexuronic acid.

**Hydroxyproline**—Another protein fraction of the vitreous body can be collected as the sediment after high speed centrifugation. The collagen content of the sediment can be estimated from the hydroxyproline determination. Fig. 5 gives the concentration of collagen in the occipital sample of the vitreous body of animals of different ages. Data are also given on the concentration of collagen in the anterior and posterior vitreous body, with the higher values always in the anterior samples. Because of the small volume of vitreous available, we were not able to determine the collagen content in the anterior samples of embryos.

**Electrophoretic pattern** observed in the occipital sample of the vitreous body of young animals is shown in Fig. 6. It has been shown previously that the sharp, fast-moving peak in the steer vitreous body (Fig. 6 a) is hyaluronic acid (16). The area of this peak is much smaller in the calf vitreous body (Fig. 6 b) and could only be traced as a small, broad peak in embryos (Figs. 6 c, d). The protein components dominate the electrophoretic pattern of the vitreous body of young animals. The first large peak after the hyaluronic acid has the same mobility as albumin. The mobility of the peak after albumin corresponds approximately to that of the α-globulins and is most prominent in the vitreous body of embryos and young calves.

**Ascorbic Acid**—The amounts of oxidized forms of ascorbic acid, e.g. dehydroascorbic acid and diketogulonic acid, were always found to be low and in no case did they constitute more than 10 per cent of the total ascorbic acid. This level is close to...
Fig. 4. Protein nitrogen concentration in the occipital part of individual vitreous bodies (♀, cow; ♂, bull; ♣, embryo, calf or steer). Solid line represents the standard deviation of determinations made on steer eyes.

Fig. 5. Collagen concentration in different parts of the vitreous body (○, individual occipital samples; ●, pooled occipital samples; △, pooled posterior samples; and ○, pooled anterior samples). Solid vertical line represents the standard deviation of determinations made on individual steer eyes.
the margin of error for the determination method when samples of low concentration are used and therefore cannot be measured with accuracy in those samples. It is also possible that a small amount of ascorbic acid is oxidized during the preparation procedure. All values given below are the sum of reduced and oxidized ascorbic acid.

61 individual steer eyes gave an average of 13.7 mg. of ascorbic acid in 100 ml. of vitreous body with a standard deviation of 1.6 mg. and a spreading of the values from 9.2 to 22.0 mg./100 ml. Fig. 7 shows that, from birth throughout life, the ascorbic acid concentration of the vitreous body is within the same range but with a large individual variation. From the second month of embryonic life until birth, there is a gradual increase in the ascorbic acid concentration, which reaches the adult level immediately after birth (Fig. 7).

Calcium—The calcium values showed the least individual variation. The level in newborn animals and in adults was about 4.5 to 5 m.eq. per liter, but it was higher in embryos (Fig. 8). The calcium concentration in the serum of embryos 3 to 5 months old was found to be about 8 m.eq. per liter, and in 7- to 9-month-old embryos, 7 m.eq. per liter.

DISCUSSION

Morphological studies indicate that the vitreous body undergoes various changes during its embryonic development (17). The first important change is the transformation of a vascular and cellular vitreous body into a cell-free, avascular tissue; only Cloquet's canal, a vascular strand connecting the optic nervehead with the posterior lens pole, remains. This stage is reached in cattle in approximately the third month of development. In addition to the cells in Cloquet's canal, the only other cells present are in the cortical layer of the vitreous body, where they remain throughout life (18). The next stage of

Fig. 6. Electrophoretic patterns of occipital samples of the vitreous body of steers, calves and embryos. Verona1 buffer, ionic strength 0.12; current 10 ma. Ascending side; arrows indicate migration toward the anode. (a) Steer (1 to 3 years old): original vitreous body concentrated 13.5 X, pH 8.15, after 278 minutes, bar angle 40°. (b) Calf (4 to 1 month old): original vitreous body concentrated 1.8 X, pH 8.90, after 348 minutes, bar angle 70°. (c) Embryo (6 to 8 months old): original vitreous body concentrated 2.6 X, pH 8.88, after 263 minutes, bar angle 75°. (d) Embryo (3 to 4 months old): original vitreous body concentrated 2.5 X, pH 8.75, after 257 minutes, bar angle 81°.

Fig. 7. Ascorbic acid concentration in the occipital sample of individual vitreous bodies (•, cow; ♀, bull; ♂, embryo, calf or steer). Vertical line represents the standard deviation of determinations made on 61 steer eyes. The sum of oxidized and reduced ascorbic acid is recorded.
development is characterized by a considerable increase in the volume of the cell-free gel. During this phase Cloquet's canal slowly disappears and the retinal vessels begin to develop. This stage is not completed until the eye reaches adult size. The present biochemical study was carried out on eyes in the second stage of development, with special regard to some components of the vitreous body which may be involved in the formation and maintenance of the gel.

Since the amount of organic materials in the vitreous gel is very low, the slightest contamination by blood or cellular material from neighboring tissues will cause erroneous analysis values. In embryos younger than 4 to 5 months it was impossible to avoid contamination by microscopic pieces of retinal tissue. The analysis values for younger embryos must be considered with this in mind. There is an uneven distribution of macromolecular components in the vitreous body, which makes it difficult to undertake a comparative study of the vitreous body of animals of various ages. The occipital sample, however, offered for such a study material which is most easily reproducible. Although small variations in the cutting technique may still lead to some differences in the analysis values, the concentration changes in the course of development are far greater than these variations could account for.

Compounds Containing Hexosamine—The greater part of the nondialyzable hexosamine in the bovine vitreous body is glucosamine. Its concentration increases gradually from the sixth embryonic month (Fig. 2). Part of it belongs to hyaluronic acid (19), which gives a well defined peak in the electrophoretic pattern. The amount of hyaluronic acid is small in the vitreous body of embryos and increases in calves and adults as the concentration of hexuronic acid increases (Fig. 6).

In some cases, hexosamine and hexuronic acid were determined in the same vitreous samples of embryos, calves, and steers. The molar concentration of total hexosamine plotted against hexuronic acid is shown in Fig. 9. The molar ratio is close to 1:1 throughout the whole concentration range except in embryos. However, the hexosamine in the vitreous body of young calves, and especially in that of embryos, has a much higher concentration than the hexuronic acid. It is most probable that some of the hexosamine is a glycoprotein moiety.

There is no significant variation in the galactosamine concentration of the vitreous body during the course of development. In embryos and in calves it represents 20 to 30 per cent of the total hexosamine, but in adult steers it is only 5 per cent. The galactosamine-containing compound has not been isolated and identified. It might be a sulfated polysaccharide, as the molar concentration of ester sulfate seems to be approximately equivalent to that of galactosamine.

There is an even distribution of hexosamine in the vitreous body of very young embryos (Table II). However, hexuronic acid determinations indicate that the hyaluronic acid concentration is higher in the posterior part, as in adult animals. The nonhyaluronic acid-hexosamine may therefore have a more even distribution pattern than the hyaluronic acid.

Proteins—In these studies the proteins of the vitreous body were divided into a soluble and an insoluble fraction, and the two fractions were collected as the supernatant and the sediment after centrifugation at high speed. The small molecular, dialyzable, nitrogen-containing compounds were not included in these studies. The distribution of collagen is uneven in the vitreous body of calves and steers, with the highest concentration in the frontal area. The collagen concentration in the occipital part of the gel.
Fig. 9. Molar concentration of hexosamine and hexuronic acid determined in the same individual vitreous bodies. Occipital samples of embryos (Δ), of calves (Δ), and of steers (○); total vitreous samples of steers (○). Solid line represents the 1:1 hexosamine-hexuronic acid ratio.

is fairly constant from the third month of embryonic life to adulthood. This agrees with the observation of Boyer et al. (4) on residual proteins.

The concentration of the soluble protein fraction in the vitreous body is low and remains constant from birth throughout life (Fig. 4). During embryonic life, however, it is considerably higher. The very high protein nitrogen values in 2- to 3-month-old embryos is probably an indication that the formation of the avascular, cell-free vitreous body has not been completed.

The electrophoretic pattern of the embryo and the calf vitreous body differs from that of the adult not only in the hyaluronic acid peak but in other respects as well. In embryos there is a large peak corresponding to that of the α-globulins, which is not present in adults. It is interesting to note that the plasma of cattle embryos and calves contains a glycoprotein, fetuin, in the α-globulin fraction (20, 21). Fetuin makes up about 30 percent of the total protein in the embryonic plasma and contains 8 percent hexosamine (21). Although we have not isolated fetuin from the embryo or the calf vitreous body, both the electrophoretic pattern and the high nonmucopolysaccharide hexosamine content (Fig. 9) suggest its presence.

Ascorbic Acid—The concentration of ascorbic acid is much higher in the adult vitreous body than in serum (5, 22). At birth ascorbic acid has already reached its adult level, which was found to be 13 mg./100 ml. and is in good agreement with the concentrations found by spectrophotometric determination (5). The ascorbic acid concentration in the vitreous body of 2- to 3-month-old embryos is 3 to 4 mg./100 ml. and continues to increase until birth (Fig. 7).

Calcium It is known that polyuronic acids can form gels in the presence of calcium (23). Therefore, the calcium content was followed along with the other determinations made on the vitreous body. The adult concentration of 4.5 to 5 m.eq. per liter agrees very well with the values given by other authors (24). In embryos the values were generally higher, reaching a maximum at about 8.5 m.eq. per liter in 2.5-month-old embryos. It was reported recently that the calcium content in the serum of human and pig embryos is higher than in the mothers' serum (25). This agrees with our observation in cattle. In adult cattle the calcium content of the vitreous body is very similar to that of the serum (24). Therefore, one can conclude that the calcium content of the vitreous body and of serum changes with age and seems to be independent of the hyaluronic acid content of the gel.

The present investigation shows that the vitreous gel is not fully developed from a biochemical point of view until the animal reaches adulthood. The size of the eyeball continues to increase from early embryonic life to adult age, and the volume of the vitreous body closely follows this growth: its size at birth is about half the adult size. The volume increase of the avascular, cell-free vitreous gel is related not merely to a swelling of the tissue, but represents a net increase in the amount of the gel-forming material.

One must clearly differentiate between the concentration of such macromolecular components of the gel as collagen, hyaluronic acid and proteins, and the total amount of these components at any stage in the development of the vitreous body. Although the concentration of collagen and soluble proteins does not change after birth, the total collagen and soluble protein content increases as the volume increases. The concentration of small
molecular components such as ascorbic acid and calcium is also constant after birth. The only component which shows an increase in concentration after birth is hyaluronic acid. The concentration of another hexosamine-containing compound, possibly fetuin, decreases after birth.

SUMMARY

The biochemical changes in the developing cattle vitreous body (from 2-month-old embryos to 10-year-old adult animals) have been followed. It was found that:

1. The hyaluronic acid concentration is low in the embryo vitreous body and increases to the adult level during the first year after birth.
2. The concentration of nondialyzable galactosamine is constant (1 to 2 mg./100 ml.) during the whole age range studied. The molar concentration of the ester sulfate present is about equivalent to that of the galactosamine.
3. The vitreous body of young embryos contains hexosamine, but very little heparonic acid. It is suggested that most of the hexosamine belongs to fetuin, a glycoprotein also present in the α-globulin fraction of embryo and calf plasma. The electrophoretic picture of the embryo vitreous body indicates a large α-globulin fraction.
4. The collagen concentration in the occipital part of the vitreous body is rather constant during all stages of development.
5. The soluble protein concentration of the vitreous body does not change after birth, but it is higher in embryos.
6. The ascorbic acid concentration increases during the prenatal period, reaching its adult level after birth.
7. The calcium concentration remains constant after birth, but it is high during the prenatal period. In both cases it follows the concentration in serum.

The biochemical development of the vitreous body is not completed until adulthood.

REFERENCES
