Studies on the incorporation of U-\(^{14}\)C-glucose into vitreous polymers in vitro and in vivo

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The incorporation of U-\(^{14}\)C-glucose into the nondialyzable polymers of the vitreous was studied both in vitro, with bovine tissue preparations, and in vivo, after the infusion of radioactive glucose through the lateral long ciliary artery of the cat. The radioactivity present in glucosamine isolated after Dowex 50 chromatography was taken as a measure of hyaluronic acid synthesis. It was found that cortical layer vitreous from young (4-week-old) calves was more active in converting glucose to glucosamine than equivalent samples taken from adult animals. No striking differences in biosynthetic activity were observed between the vitreous alone or with attached ciliary body, suggesting that under these experimental conditions the ciliary body does not contribute to the production of vitreous hyaluronic acid. The central (acellular) regions of the vitreous were nearly as active as the peripheral regions in incorporating labeled glucose into nondialyzable polymers, a finding which could be ascribed to "extracellular enzymes" in the vitreous. Studies of the distribution of radioactivity in purified fractions showed that most of the glucose had been utilized for the synthesis of proteins (and glycoproteins). Incorporation of radioactive glucose into nondialyzable polymers of the vitreous was inhibited by approximately 80 per cent in the presence of puromycin, due mainly to the depression of protein and glycoprotein synthesis. Very little incorporation of U-\(^{14}\)C-glucose into hyaluronic acid occurred in vivo, although under the same experimental conditions appreciable amounts of radioactivity were found in the amino sugar moieties of the corneal mucopolysaccharides.

Key words: vitreous, glucose, glucosamine, hyaluronic acid, protein synthesis, glycoproteins, mucopolysaccharides, cornea, puromycin, pars plana, cats.

The metabolism of the vitreous body is of particular interest not only because the tissue occupies a major portion of the globe in nearly all vertebrate species but also because the degenerative changes that occur in it during lifetime (and especially in senescence) appear to be essentially irreversible. Nevertheless, our knowledge of its metabolic patterns is very meager; the biosynthesis of its macromolecular components appears to be limited in nature and occurs mainly during periods of active growth and development. These and other aspects of vitreous chemistry and metabolism have been recently reviewed.\(^1\) Both synthetic\(^2\), \(^3\) and hydrolytic\(^4\) enzymes have been detected in high-speed sediments of vitreous pooled from approximately 100 calf eyes. The latter enzymes are localized mainly in the "granular" fraction of the vitreous cells (hyalocytes).
and resemble in several respects lysosomal enzymes isolated from other tissues. The histochemical and morphological properties of the cortical layer cells have been studied extensively and it has been proposed that they play an important role in the biosynthesis of vitreous hyaluronic acid. That the ciliary body may also take part in its production may be inferred from histochemical studies in the developing rabbit eye. Light and electron microscopic evidence suggests that the acid mucopolysaccharides found in the intercellular spaces of the nonpigmented epithelium of the ciliary body may be transported into adjacent areas of the vitreous through narrow channels or "intercellular canaliculi." This hypothesis was strengthened by the observation that hyaluronidase-sensitive mucopolysaccharides not only accumulate within cysts and neoplastic growths of the ciliary epithelium but also extruded into the surrounding extracellular spaces.

In mammalian tissues and in bacteria both the glucuronic acid and the glucosamine moieties of hyaluronic acid are derived directly from glucose. The assumption that a similar pathway exists in the vitreous is based mainly on a report describing the incorporation of glucose into hyaluronic acid in vitro and after incubation of portions of cortical tissue layer. Nevertheless, parenterally administered glucose is not incorporated into the hyaluronic acid of owl monkey vitreous, nor does it appear to be an effective precursor of any nondialyzable macromolecular components in the cat vitreous.

The present investigations were prompted mainly by the scarcity of precise information on the extent to which (radioactive) glucose is utilized for hyaluronic acid synthesis by the vitreous body. In addition, attempts have been made to establish the principal site(s) of metabolic activity in the vitreous itself and to determine whether the ciliary body participates in the production of vitreous hyaluronic acid.

Materials and methods

In vitro experiments. Cattle eyes from the following three age groups were used: (1) 4-week-old calves, (2) 3- to 6-month-old calves, and (3) 1- to 2-year-old cattle. They were brought from the slaughterhouse on ice and dissected immediately. After the cornea, iris, and lens were removed, the exposed vitreous was cut circularly at a point 3 to 4 mm. from the peripheral edge of the tissue to a depth of approximately 5 mm. In this way it was possible to separate the vitreous lying directly adjacent to the ciliary body from the rest of the vitreous tissue. An incision 5 mm. deep was then made (posteriorly) across the ciliary body and sclera, and the tissues were then cut around the entire circumference. The resulting strip of tissue, measuring 7 to 10 cm. in length (depending on the age of the animal), was held firmly at one end with a small forceps and the ciliary body (together with the vitreous adhering to it) was gently pulled away from the sclera. This preparation, consisting of intact ciliary body together with 2 to 3 ml. of cortical layer (pars plana) vitreous, has been designated Preparation A. Four identical strips were used for each experiment. Another set of four cattle eyes was dissected in the same manner except that the ciliary body-vitreous preparation was pinned to a small cork board and the vitreous was carefully removed. This vitreous tissue, originating mainly in the pars plana region, has been designated Preparation B. Preparation C consisted of equivalent amounts of vitreous gel taken only from the central region of the tissue. These preparations were then incubated with U-14C-glucose (Amersham, England) for 6 hours unless otherwise indicated.

In vivo experiments. Cats weighing 2 1/2 to 3 kilograms were used in all experiments. The U-14C-glucose (100 μc), dissolved in 6.5 ml. of 0.9 per cent NaCl, was infused through the lateral long ciliary artery for 4 hours at a rate of 1.6 ml. per hour. At the end of the experiment both the cornea and the vitreous were removed and frozen at -25° C. Five identical experiments were carried out and the vitreous and cornes from each were then pooled.

Measurement of total radioactivity incorporated.

In vitro experiments. After incubation of the vitreous Preparations A, B, and C, each was centrifuged at 35,000 × g for 1 hour and dialyzed overnight in a Perspex cylinder against 5 to 6 L. of continuously running deionized water. Control experiments with the use of approximately the same amount of radioactivity had shown that this method was effective in removing all of the free (nonincorporated) radioactive glucose. Therefore, the amount of radioactivity remaining after dialysis under these conditions was considered a valid estimation of the extent of incorporation into
nondialyzable polymers. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer as described previously.\(^\text{16}\)

**In vivo experiments.** The pooled preparations obtained from the infusion experiments were treated as follows. Ten "carrier" corneas from (enucleated) cats' eyes were added to the corneas from the five perfused eyes and all of them extracted twice with water and twice with 0.1M K\(^2\)CO\(_3\) in a VirTis homogenizer. The extracts were combined, centrifuged at 37,000 \(\times\) g for 2 hours, and then dialyzed exhaustively in a Perspex cylinder. Radioactivity was measured as described for the in vitro preparations.

"Carrier" vitreous from 10 cats' eyes was added to the vitreous from the five perfused eyes. After centrifugation at 35,000 \(\times\) g for 1 hour, the supernatant was dialyzed exhaustively and counted.

**Ethanol precipitation.** Hyaluronic acid was precipitated from the dialyzed vitreous samples by the addition of 3 volumes of ethanol in the presence of sodium acetate (25 mg. per milliliter). After standing overnight at 4\(^\circ\)C, the solution was centrifuged at 20,000 \(\times\) g for 20 minutes and the residue taken up in water. Insoluble materials (mainly denatured proteins) were removed by centrifugation and the hyaluronic acid was again precipitated with ethanol, dissolved in water, and dialyzed.

**Separation of hyaluronic acid and proteins.** Vitreous preparations that had been incubated with radioactive glucose were centrifuged and dialyzed, first against water (as described above) and then against 0.005M phosphate buffer, pH 7. The sample was applied to a column (1 by 10 cm.) of DEAE-Sephadex that had previously been equilibrated with the same buffer; the fractions were eluted in a closed system gradient of NaCl.\(^\text{16, 17}\)

**Separation of mucopolysaccharides and peptides.** Centrifuged vitreous or corneal extracts were incubated for 16 hours at 37\(^\circ\)C. with Promase (Calbiochem AG, Luzern, Switzerland) in the presence of 2 to 3 drops of toluene, using 1 \(\mu\)g of enzyme for each 50 \(\mu\)g of protein. The digested preparations were concentrated to a volume of 2 to 3 ml. and chromatographed on columns of Sephadex G-25 (2.5 by 40 cm.) previously equilibrated with 0.02M phosphate buffer, pH 7. The fractions were eluted in the same buffer at a flow rate of 20 ml. per hour.

**Isolation of amino sugars.** Samples were hydrolyzed in 2N HCl for 12 hours at 100\(^\circ\)C. and the HCl was evaporated in vacuo over P\(_2\)O\(_5\) and NaOH. The dried samples were dissolved in 0.3N HCl and chromatographed on columns (1 by 25 cm.) of Dowex 50 according to Cardell.\(^\text{14}\)

**Chemical analyses.** Uronic acid and protein were measured as described previously.\(^\text{19}\) Hexosamine was assayed by a modification\(^\text{19}\) of Elson and Morgan’s procedure and neutral sugar by a modification of the anthrone reaction.\(^\text{21}\)

**Results.**

**In vitro experiments.**

**Incorporation into glucosamine.** The experimental conditions leading to maximum incorporation of radioactive glucose were determined by examining the influence of (1) the amount of radioactivity in the incubation medium, (2) the age of the animal, and (3) the length of time of the incubation. Moreover, in order to gain some information on the principal biosynthetic site(s) in the vitreous and also to assess the role of the ciliary body in the

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**Table I. Incorporation of \(^{14}\)C-glucose into nondialyzable polymers of calf vitreous**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Initial radioactivity (mc)</th>
<th>Uronic acid (mg)</th>
<th>Protein (mg)</th>
<th>Radioactivity incorporated (counts/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitreous (ciliary) A</td>
<td>10</td>
<td>746</td>
<td>36.2</td>
<td>19,600</td>
</tr>
<tr>
<td>Vitreous A</td>
<td>10</td>
<td>600</td>
<td>6.2</td>
<td>17,400</td>
</tr>
<tr>
<td>Vitreous B</td>
<td>10</td>
<td>1,100</td>
<td>3.8</td>
<td>14,160</td>
</tr>
<tr>
<td>Vitreous (ciliary) A</td>
<td>20</td>
<td>1,040</td>
<td>49.4</td>
<td>18,000</td>
</tr>
<tr>
<td>Vitreous B</td>
<td>20</td>
<td>975</td>
<td>3.8</td>
<td>25,000</td>
</tr>
<tr>
<td>Vitreous C</td>
<td>20</td>
<td>1,520</td>
<td>4.1</td>
<td>15,000</td>
</tr>
<tr>
<td>Vitreous (ciliary) A</td>
<td>50</td>
<td>694</td>
<td>33.2</td>
<td>50,000</td>
</tr>
<tr>
<td>Vitreous B</td>
<td>50</td>
<td>782</td>
<td>5.4</td>
<td>52,700</td>
</tr>
<tr>
<td>Vitreous C</td>
<td>50</td>
<td>1,020</td>
<td>3.4</td>
<td>37,500</td>
</tr>
</tbody>
</table>

*The vitreous samples were prepared from 3- to 6-month-old calf eyes as described in Materials and methods. Each flask contained approximately 10 ml. of the designated preparation plus glutamine (1 mg. per milliliter) and unlabeled glucose (0.5 mg. per milliliter). Incubations were carried out in an atmosphere of 95 per cent oxygen and 5 per cent CO\(_2\) for 6 hours at 37\(^\circ\)C.
Table II. Distribution of radioactivity after ethanol precipitation and Dowex 50 chromatography

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Ethanol precipitation</th>
<th>Dowex 50 chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucosamine (µg)</td>
<td>Protein (mg.)</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>A</td>
<td>630</td>
<td>405</td>
</tr>
<tr>
<td>B</td>
<td>710</td>
<td>493</td>
</tr>
<tr>
<td>C</td>
<td>984</td>
<td>585</td>
</tr>
</tbody>
</table>

*Vitreous-ciliary preparations from 3- to 6-month-old calf eyes were incubated for 6 hours at 37° C. with 50 µc of U-14C-glucose. Other conditions were the same as those described in Table I.

†Before and after ethanol precipitation.

The production of hyaluronic acid, three different tissue preparations were used (see Materials and methods).

The results of experiments with 3- to 6-month-old calf eyes are summarized in Table I. Although increasing the amount of radioactive glucose in the medium from 10 to 20 µc did not result in a corresponding increase in radioactivity incorporated, 50 µc of labeled glucose caused a substantial rise. However, regardless of the amount of radioactivity initially present, there was no clear indication of where the principal biosynthetic sites were located. In most cases it appeared that the central (acellular) portion of the vitreous (Preparation C) was somewhat less active than the pars plana region (Preparation B), which has the highest concentration of vitreous cells. However, the finding that so much radioactivity was incorporated by the acellular portions at all was unexpected since most of the metabolic activity of the tissue is believed to reside in the cortical layer cells. It should also be noted (Table I) that there was relatively little difference in the amount of labeled glucose incorporated by Preparation A (pars plana vitreous plus ciliary body) and Preparation B (pars plana vitreous alone), implying that the ciliary body, under these experimental conditions, does not play an active role in the biosynthesis of the nondialyzable vitreous polymers.

The data given in Table I represent the total radioactivity incorporated into both hyaluronic acid and proteins, the principal soluble nondialyzable polymers of the vitreous. The amount specifically incorporated into the hyaluronic acid in the six vitreous samples that had been incubated with either 10 or 20 µc of labeled glucose was determined after Dowex 50 chromatography. Even though the amino sugars were recovered in yields of 60 to 70 per cent, no radioactivity could be detected in any of the six samples.

Examination of the samples incubated with 50 µc of U-14C-glucose showed that after ethanol precipitation there was a marked shift in the distribution of radioactivity (Table II). Although 60 to 70 per cent of the hyaluronic acid was recovered, only about 10 to 20 per cent of the radioactivity was associated with it. The rest of the radioactivity was lost, together with approximately 75 per cent of the protein. When these samples were hydrolyzed and chromatographed on Dowex 50, the glucosamine isolated contained approximately 100 c.p.m., representing 1 to 2 per cent of the total counts applied to the Dowex 50 column.

Thus, although very little glucose had been incorporated into the hyaluronic acid under the experimental conditions described above, it was still necessary to determine whether the age of the animal or the length of time of the incubation could influence the extent of conversion to glucosamine.
Table III. Effect of age and time of incubation on the incorporation of U-14C-glucose into vitreous hyaluronic acid*

<table>
<thead>
<tr>
<th>Tissue preparation</th>
<th>Time of incubation (hr.)</th>
<th>Uronic acid (µg)</th>
<th>Radioactivity (counts/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>In nondialyzable polymers</td>
</tr>
<tr>
<td>4-week-old calves</td>
<td>6</td>
<td>610</td>
<td>98,150</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>680</td>
<td>90,400</td>
</tr>
<tr>
<td>3- to 6-month-old calves</td>
<td>6</td>
<td>830</td>
<td>56,700</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>740</td>
<td>43,100</td>
</tr>
<tr>
<td>1- to 2-year-old cattle</td>
<td>6</td>
<td>1,020</td>
<td>30,200</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>950</td>
<td>38,800</td>
</tr>
</tbody>
</table>

*Each incubation flask contained 50 µg of U-14C-glucose in a total volume of approximately 10 ml. In the experiments with 3- to 6-month-old calves and 1- to 2-year-old cattle, vitreous with attached ciliary body (preparation A) from 4 eyes was used; but for 4-week-old calves, 8 eyes were required in order to obtain equivalent amounts of hyaluronic acid.

Penicillin (100 units per milliliter) and streptomycin (50 µg per milliliter) were added to these preparations.

It has been shown by Balazs and co-workers that not only is the increase in the concentration of hyaluronic acid greatest in the early postnatal period of growth but also the number of cells in the peripheral layer of the tissue (especially in the pars plana region) is higher in calves than in adult cattle. Preparation A (pars plana vitreous with attached ciliary body) was used in all subsequent in vitro experiments even though the data in Tables I and II do not support the view that the ciliary body plays a direct role in the biosynthesis of vitreous hyaluronic acid. However, it may be assumed that in Preparation A little or no damage to the vitreous cells had taken place, whereas in Preparation B, which requires stripping the vitreous from the ciliary body, some damage or loss of cells may have occurred.

The results of these experiments (Table III) show that, although the time of incubation does not appear to be an important factor, the age of the animal is. The radioactivity incorporated was 2 to 3 times greater for 4-week-old calves than for older animals. Differences in biosynthetic activity between the age groups are even more marked when considering the synthesis of radioactive glucosamine (Table III, last column). In this case there was 5 to 6 times more radioactivity in the amino sugar isolated from 4-week-old calf vitreous than from older animals.

DEAE-Sephadex chromatography. Indirect evidence from the above experiments suggests that most of the incorporated radioactivity is associated with the proteins (and glycoproteins). In order to assess this more accurately, the experiments were repeated and the dialyzed samples chromatographed on DEAE-Sephadex. As shown in Fig. 1, two well-defined peaks appear in the elution profile, the first one consisting of proteins (and glycoproteins) and the second one of hyaluronic acid. Almost all of the radioactivity was eluted in the first peak, whereas only negligible amounts were found in the later (hyaluronic acid) fractions. In similar experiments on 4-week-old calf vitreous, although more radioactivity appeared in the second (hyaluronic acid) peak, the greater part of it was still found in the protein and glycoprotein fractions.

Gel filtration and puromycin. Further experiments to determine the extent of conversion of labeled glucose to protein components were carried out using calf eyes from both 4-week-old and 3- to 6-month-old animals, the age depending mainly on the availability of material at the slaughterhouse. After incubation with 50 µg of labeled glucose, the preparations were dialyzed, digested with Pronase, and applied to columns of Sephadex G-25. In the gel filtration diagram shown in Fig. 2, hyaluronic acid is eluted first as a single
Fig. 1. Fractionation of cattle vitreous on DEAE-Sephadex after incubation with U-\textsuperscript{14}C-glucose. The effluent fractions were analyzed for protein (\textbullet\textbullet\textbullet\textbullet\textbullet), uronic acid (\textxtextx\textx), and radioactivity (\textOtextO\textOtextO).

Fig. 2. Gel filtration on Sephadex G-25 of Pronase-digested cattle vitreous after incubation with U-\textsuperscript{14}C-glucose. The effluent fractions were analyzed for ninhydrin (\textbullet\textbullet), uronic acid (\textOtextO\textO\textO) and radioactivity (\textxtextx\textx).

Symmetrical peak with the void volume of the column. This fraction (I) also contained some ninhydrin- and anthrone-positive material, probably representing either high molecular weight glycopeptides or Pronase-resistant glycoproteins.\textsuperscript{23-25} Approximately one quarter of the total radioactivity eluted from the column was present in Fraction I. It should be noted, however, that the principal radioactive peak did not coincide with either the hyaluronic acid or the low molecular weight peptides.
and amino acids, which were eluted at the end of the chromatogram. The fractions were pooled as indicated in Fig. 2 and analyzed together with the puromycin-treated preparations described below.

Parallel experiments were carried out under identical conditions except that, prior to adding the radioactive glucose, the vitreous was preincubated with puromycin (100 μg per milliliter) for 15 minutes. Gel filtration of a puromycin-treated Pronase-digested preparation (Fig. 3) showed the same elution pattern for the hyaluronic acid and the ninhydrin-positive substances, but revealed striking quantitative differences in the radioactive peaks, both of which were markedly depressed. Inhibition of protein synthesis by this antibiotic is well known and similar effects on the biosynthesis of mucopolysaccharides and of galactosamine-containing proteinpolysaccharides have also been demonstrated. Hyaluronic acid represents a special case since the biosynthesis of this polysaccharide is less sensitive to puromycin than other substances, possibly because of its lower protein content.

Hydrolysis and (Dowex 50) chromatography of Fractions I and II obtained after gel filtration of the Pronase digests showed that two thirds of the total radioactivity recovered was present in the ninhydrin-positive fractions (Table IV). In absolute terms, the conversion of labeled glucose to amino acids and to neutral sugar was strongly inhibited (71 to 85 per cent) in the presence of puromycin, whereas conversion to glucosamine was depressed by only 28 per cent. The over-all inhibition by puromycin (Table IV, last column), therefore, reflects mainly a decrease in the conversion of labeled glucose to amino acids and neutral sugar (mainly galactose), thus providing further evidence that the glucose is being utilized mainly for the synthesis of proteins and glycoproteins and to only a limited extent for the synthesis of hyaluronic acid.

In vivo experiments. Previous investigations had shown that although considerable radioactivity entered the vitreous body after the infusion of labeled glucose
Table IV. Effect of puromycin on the distribution of radioactivity in fractions* isolated after gel filtration and Dowex 50 chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Neutral sugar</th>
<th>Glucosamine</th>
<th>Neutral sugar</th>
<th>Ninhydrin-positive fractions</th>
<th>Total radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (μg)</td>
<td>Radioactivity (c.p.m.)</td>
<td>Amount (μg)</td>
<td>Radioactivity (c.p.m.)</td>
<td>Amount (μg)</td>
</tr>
<tr>
<td>Control</td>
<td>87</td>
<td>785</td>
<td>113</td>
<td>132</td>
<td>28</td>
</tr>
<tr>
<td>Puromycin-treated</td>
<td>73</td>
<td>120</td>
<td>102</td>
<td>95</td>
<td>32</td>
</tr>
</tbody>
</table>

*After incubation with U-14C-glucose, the vitreous samples were digested with Pronase. Two principal fractions (I and II) were then isolated after Sephadex G-25 chromatography (see Figs. 2 and 3). Each was hydrolyzed and chromatographed on Dowex 50. The ninhydrin-positive fractions (neutral sugar) appear in the first Dowex 50 eluants. The glucosamine in Fraction I and the ninhydrin-positive substances (peptides and amino acids) in Fraction II appear in the effluent fractions eluted afterward with HCl.

through the lateral long ciliary artery of the cat, very little remained after dialysis. It has subsequently been found that in order to study the in vivo metabolism of labeled glucose in greater detail, larger amounts of radioactive glucose had to be administered. Moreover, it was necessary to pool the vitreous from 5 separate experiments and to add “carrier” vitreous so that sufficient hyaluronic acid would be available for subsequent purification. As a check on the in vivo technique it-
self, the incorporation of glucose into the mucopolysaccharides of the cornea under the same experimental conditions was also examined.

After infusion of the radioactive glucose and exhaustive dialysis of the pooled samples (see Materials and methods) the vitreous preparation contained 31,600 c.p.m. and the corneal extract, 25,300 c.p.m. Each was digested with Pronase and chromatographed on Sephadex G-25. In the cornea (Fig. 4) most of the radioactivity was eluted in the first peak together with all of the chondroitin sulfate and keratosulfate. Relatively little radioactivity was found in the ninhydrin-positive effluents. It should also be noted that in the gel filtration chromatogram of the corneal extract there was only one major ninhydrin peak; it appeared in the elution profile after the mucopolysaccharide peak. Although some ninhydrin-positive materials were also eluted in the void volume of the column together with the mucopolysaccharides, they did not form a well-defined peak and apparently were not related to any of the components appearing in the first effluents, i.e., the mucopolysaccharides. In contrast, two distinct ninhydrin peaks were present in the vitreous chromatogram (Fig. 5), the first one coinciding in elution position with the hyaluronic acid and the second (major) one appearing in the later effluents. It was also apparent that in the vitreous only a small fraction of the total radioactivity was found in the first (hyaluronic acid) fractions, whereas in the corneal digests most of the radioactivity was eluted in this position.

The mucopolysaccharide fractions from both the cornea and the vitreous were hydrolyzed and chromatographed on Dowex 50. These analyses (Table V) showed that both the glucosamine and the galactosamine isolated from the corneal digests contained significant amounts of radioactivity, whereas the glucosamine isolated from the vitreous contained only 35 c.p.m. Hence, during the 4 hour period of infusion of radioactive glucose in five consecutive experiments only a minimal amount of hyaluronic acid synthesis had taken place in the vitreous. The main purpose of studying the incorporation into the corneal mucopolysaccharides was to test the experimental technique itself.
Table V. Distribution of radioactivity in the mucopolysaccharides of the cornea and the vitreous after infusion of U-14C-glucose through the lateral long ciliary artery*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Neutral sugar</th>
<th>Galactosamine</th>
<th>Glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (µg)</td>
<td>Radioactivity (c.p.m.)</td>
<td>Amount (µg)</td>
</tr>
<tr>
<td>Cornea</td>
<td>550</td>
<td>864</td>
<td>284</td>
</tr>
<tr>
<td>Vitreous</td>
<td>190</td>
<td>327</td>
<td></td>
</tr>
</tbody>
</table>

*After gel filtration of Pronase-digested vitreous and cornea (Figs. 4 and 5, respectively), the first peak, which contained all of the mucopolysaccharides, was hydrolyzed and chromatographed on Dowex 50. The anthrone-positive material (neutral sugar) was found in the effluent fractions not retained on the Dowex 50 column; the amino sugars were eluted afterward with HCl.

no radioactivity been found in the corneal mucopolysaccharides either, it would have cast considerable doubt on the adequacy of this in vivo technique. Notwithstanding the differences in the mucopolysaccharide content of the two tissues,* the amount of radioactivity incorporated into amino sugars by the cornea was about 40 times greater than in the vitreous.

Discussion

Incorporation of labeled glucose into hyaluronic acid in vitro. These studies have revealed that the utilization of U-14C-glucose for hyaluronic acid synthesis in the vitreous can be demonstrated when relatively large amounts (50 µC) of labeled glucose are present in the incubating medium. When small amounts (10 or 20 µC) are used, none of the radioactivity incorporated into the nondialyzable polymers is present in the glucosamine. Although conversion to this amino sugar has been used as an "indicator" of hyaluronic acid synthesis, it should be pointed out that 5 to 10 per cent of the glucosamine in these preparations may have originated from glycoproteins. Neglecting the contribution from this source would not alter the interpretation of the findings reported here.

Considerably more radioactivity is incorporated into vitreous polymers using 50 µg of labeled glucose but, even under these conditions, the amount converted to glucosamine represented only 1 to 2 per cent of the total radioactivity applied to the Dowex 50 column (Table II). Moreover, longer periods of incubation did not result in any greater synthesis of labeled glucosamine (Table III).

Of all the factors investigated, only the age of the animal appears to influence the extent of hyaluronic acid synthesis. Nearly six times more radioactivity was found in the amino sugars isolated from young (4-week-old) calves than from adult (1- to 2-year-old) cattle. It should be pointed out, however, that these experiments may not be strictly comparable because twice as many vitreous-ciliary body preparations were required from 4-week-old calves than from adult animals in order to obtain equivalent amounts of uronic acid (Table III). It may therefore be argued that, in the experiments with 4-week-old calf vitreous, the amount of tissue or the number of cells actually incubated was at least twice as great as that in the older animals. In spite of this, the results of the present experiments are in good agreement with previous chemical studies which showed that net synthesis of hyaluronic acid occurs mainly during the early postnatal period of growth in the cattle eye. The amount of radioactivity found in the glucosamine isolated from vitreous of older animals was so low that its significance must be considered as questionable.

Glucose is a major precursor of hyaluronic acid in other biological systems and, by analogy, it had been anticipated

*The total content of hyaluronic acid in a single cat vitreous is approximately 75 µg; the total mucopolysaccharide content of a single cat cornea varies from 400 to 600 µg (Salitemik-Givant and Berman, unpublished observations).
that this would likewise hold true for the vitreous. Enzymes capable of transferring the labeled carbohydrate moieties of UDP-glucuronic acid-$^{14}C$ and UDP-N-acetylglucosamine-$^{14}C$ to endogenous hyaluronic acid acceptors have been demonstrated in high-speed sediments of calf vitreous. These findings do not necessarily contradict the present investigations because they represent the final steps in the synthesis of hyaluronic acid. Glucose is converted to uridine nucleotide sugars by enzymes found widely distributed in nature but to date only one of them, a transferase that converts 1-$^{14}C$-glucosamine to labeled UDP-N-acetylglucosamine, has been isolated from the vitreous. That others may also be present in this tissue may be inferred from the observation that after injection of labeled glucose into the aqueous of an enucleated calf eye, some radioactivity was found in alcohol-soluble oligosaccharides present in the central vitreous. Since whole eyes were used in these experiments, the actual site of conversion of glucose to nucleotide sugars could not be established.

**Fate of radioactive glucose in vitro.** The finding that only a small fraction of the incorporated radioactivity was associated with the glucosamine moiety of the hyaluronic acid prompted further investigations on the metabolic fate of the remainder of the glucose. Fractionation on DEAE-Sephadex of vitreous preparations that had been incubated with $U^{14}C$-glucose showed that most of the radioactivity was in the protein (and glycoprotein) fractions. Similarly, with Pronase-digested preparations subjected to gel filtration, most of the radioactivity was found in the low molecular weight peptide fractions eluted after the hyaluronic acid. Studies on the distribution of radioactivity in the two principal fractions obtained by gel filtration (Table IV) showed that in this experiment 4 per cent of the radioactivity recovered after Dowex 50 chromatography was associated with the glucosamine. The remainder was found in the neutral sugar and the ninhydrin-positive fractions. Although it was not anticipated that such an extensive conversion of glucose carbon to amino acids would occur in the vitreous, a similar phenomenon has been observed in brain and in surviving bone fragments where radioactive glucose is converted more rapidly to amino acids than to other monosaccharides.

The most convincing evidence supporting the view that, in the vitreous, glucose participates mainly in the synthesis of proteins (and glycoproteins) and to a lesser extent in the biosynthesis of hyaluronic acid has come from studies using puromycin. A comparison of the gel filtration patterns of Pronase-digested vitreous in the presence (Fig. 3) and absence (Fig. 2) of puromycin reveals the marked inhibitory effect of this antibiotic. Further examination of the distribution of the radioactivity showed that, on an absolute basis, the largest inhibition was in the neutral sugar and ninhydrin-positive fractions. In contrast, conversion to glucosamine was only slightly decreased in the presence of puromycin.

The inhibitory effect of puromycin on protein synthesis at the ribosomal level is well documented and it is now known that this antibiotic also inhibits protein-polysaccharide-synthesizing systems in embryonic chick cartilage, in skin fibroblasts, and in sheep mucosal scrapings. It is, therefore, reasonable to assume that the formation of the protein or peptide backbone of the mucopolysaccharide not only precedes, but may actually be essential for, the subsequent incorporation of carbohydrate and sulfate residues. On the other hand, the requirement of a preformed peptide core for hyaluronic acid synthesis has not been clearly established. The fact that incorporation of radioactive precursors into hyaluronic acid by skin fibroblasts was only partially inhibited by puromycin suggests that its synthesis is less dependent upon protein synthesis than that of other polysaccharides or mucoproteins. In the
vitreous the synthesis of hyaluronic acid was only slightly (28 per cent) inhibited by puromycin whereas that of proteins and glycoproteins was depressed by approximately 80 per cent. Therefore, the over-all inhibition of 77 per cent in the incorporation of labeled glucose into vitreous polymers reflects mainly an inhibition of protein synthesis and, in this respect, the puromycin-sensitive protein-synthesizing pathways in the vitreous appear to be similar to those found in other tissues.

Glucose metabolism in vivo. It had previously been established that after the infusion of U-14C-glucose through the lateral long ciliary artery of the cat considerable amounts of radioactivity entered the vitreous. The use of this technique circumvents the problems inherent in the more conventional routes of administration (intravenous or peritoneal injection) which, because of the extensive dilution and rapid metabolism of the administered glucose, would not be expected to yield glucose of high specific activity in the vitreous. The problems inherent in the more conventional routes of administration (intravenous or peritoneal injection) which, because of the extensive dilution and rapid metabolism of the administered glucose, would not be expected to yield glucose of high specific activity in the vitreous. Even then, because of the low level of metabolic activity anticipated in the cat vitreous, 5 separate experiments were performed and the tissues (vitreous and cornea) were pooled for subsequent analyses. It was found that, although considerable radioactive activity was present in the amino sugar moieties of the corneal mucopolysaccharides, only borderline amounts (35 c.p.m.) could be detected in the glucosamine isolated from the vitreous of 5 pooled cats' eyes. This suggests that very little hyaluronic acid had been synthesized in vivo during the 4 hour period of continuous infusion of glucose through the lateral long ciliary artery. Parenteral injection of U-14C-glucose also resulted in very little incorporation into vitreous hyaluronic acid in the owl monkey. Labeled glucosamine is a far more efficient precursor of hyaluronic acid than glucose but, even then, maximum incorporation does not occur until 5 days after its administration. The slow turnover of hyaluronic acid is well known and it seems possible that the 4 hour period used in the present studies was not sufficient to demonstrate biosynthesis.

Site of metabolic activity. It has been proposed that most, or possibly all, of the metabolic activity of the vitreous resides in the cortical layer cells ("hyalocytes"). It was, therefore, surprising to find that the central, acellular, portion of the tissue (Preparation C) was nearly as active as the surface layer (Preparations A and B) in incorporating radioactive glucose into nondialyzable polymers. However, in view of the fact that soluble transferases, and possibly other enzymes, are present in the acellular regions of calf vitreous, the observation that Preparation C was so active may be explained in terms of "extracellular enzymes."

The present investigations do not support the view that the ciliary body plays a direct role in the production of vitreous hyaluronic acid. In all experiments where cortical layer vitreous was incubated either alone (Preparation B) or attached to the ciliary body (Preparation A), there was little difference in the total amount of radioactivity incorporated, or in the amount of labeled glucose converted to glucosamine. If one were to suppose that either hyaluronic acid, or some precursor of it, is normally present in the ciliary body, then it should be possible to detect such a substance by chemical methods. However, when the ciliary body was freed of all adhering vitreous and extracted in aqueous buffered media, no hyaluronic acid could be found (Berman, unpublished observations). The Alcian blue-staining "mucoids" detected histochemically in the tissue must then be another type of mucopolysaccharide and probably not one that is related, either chemically or metabolically, to the hyaluronic acid of the vitreous.

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REFERENCES
32. Deiss, W. P., Jr., Holms, L. B., and Johnston,


