Distributions of $^{35}$S-sulfate and $^3$H-glucosamine in the Angular Region of the Hamster: Light and Electron Microscopic Autoradiography

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The distribution of $^{35}$S-sulfate and $^3$H-glucosamine in the angular region of the hamster was studied by light and electron microscopic autoradiography following intraperitoneal injection of these compounds to hamsters. Exposed silver grains of $^{35}$S-sulfate were concentrated in the trabecular meshwork, sclera, and cornea, and grains of $^3$H-glucosamine were localized in the trabecular region. The radioactivity of both isotopes was observed in the Golgi apparatuses of the endothelial cells of the angular aqueous plexus and the trabecular meshwork. The grains were noted over the entire cytoplasm, except for the nucleus, and then were incorporated into the amorphous substance and collagen fibers in the region adjacent to the angular aqueous sinus. These results suggest that endothelial cells in the angular region synthesize and secrete the sulfated glycosaminoglycans and hyaluronic acid. Invest Ophthalmol Vis Sci 24:697-703, 1983

It is generally accepted that the aqueous humor flows out from the anterior chamber mainly into the aqueous vein through Schlemm's canal via the trabecular meshwork in primates. The outflow resistance occurring in this pathway may be the main cause of glaucoma. There was a reduction in the outflow resistance of the aqueous humor, when hyaluronidase was instilled into the anterior chamber in cattle eyes, and it was suggested that glycosaminoglycans (GAG) may be a barrier against the outflow of aqueous humor.1,2 Zimmerman provided histochemical evidence of the presence of hyaluronidase-sensitive GAG in the trabecular meshwork.3 The existence of GAG in the aqueous outflow apparatus was also later confirmed by histochemistry and electron microscopy.4-8

Although the existence of GAG in the angular region was demonstrated in these studies, the possible synthesis of GAG by the cells in this area remains unknown. Autoradiography using a precursor for GAG is one method that allows for morphologic confirmation of GAG production by the cells in the angular region. Autoradiographic studies on the angular region have been reported rarely, and we were able to find only two light microscopic autoradiographs of the ciliary body as related to the use of $^{35}$S-sulfate and $^3$H-galactose in the literature.9

This work determined if $^{35}$S-sulfate and $^3$H-glucosamine would localize in the angular region. Light and electron microscopic autoradiographies (LMARG and EMARG) were used to elucidate the metabolism of GAG in this region.

Materials and Methods

Twelve-day-old and 3-month-old hamsters, weighing 12 g and 120 g, respectively, were used in the sulfate experiments. Sixteen-day-old and other three-month-old animals, weighing 14 g and 120 g, respectively, were used for the glucosamine experiment. $\text{Na}_2^{35}\text{SO}_4$ (specific activity 787 mCi per millimole) and $^3$H-glucosamine hydrochloride (SA 21 Ci/mmoll) were used. $^3$H-glucosamine in ethanol-water solution (9:1) was evaporated to dryness. Sterile physiologic saline solution was added to the concentrated isotope. Under anesthesia, the hamsters were given 40 $\mu$Ci per gram of body weight of $^{35}$S-sulfate and 25 $\mu$Ci/g body weight of $^3$H-glucosamine intraperitoneally. These animals were killed at 2, 5, 10, 15, 30, 60, 180 min, and 24 hrs after the injection, and the eyes were enucleated and cut horizontally.

The upper halves were fixed in 10% neutral formaldehyde, dehydrated in a graded series of ethyl alcohol, and then embedded in methacyrile for LMARG. Horizontal sections were cut at 3 $\mu$m with glass knives on a Porter-Blum MT-2 ultramicrotome, collected on glass microscopic slides, coated with Sak-
Figs. 1–6. Light microscopic autoradiographs of the angular region and its adjacent tissues from 14-day-old hamsters injected with 35S-sulfate (hematoxylin-eosin, ×360). Fig. 1. Ten minutes after injection a few grains are seen over the specimen. Figs. 2, 3. Fifteen and 30 min after injection, respectively, the silver grains are localized near the nuclei in the trabecular region, the adjacent cornea and sclera. Figs. 4, 5. Sixty and 180 min after injection, respectively, numerous grains are seen over the specimen from the anterior segment. Fig. 6. Twenty-four hours after injection heavy labeling is seen over the angular region and its adjacent tissues, so that individual cells cannot be identified.

ura NR-M2 emulsion (Konishiroku, Tokyo) diluted 2:3 with water, and maintained at 42 C, by the dipping method. Sections were exposed for 1 to 13 weeks, developed using Sakura Konidol X for 5 min at 20 C, stained with hematoxylin-eosin, and examined under light microscopy.

The lower halves of the eyes were fixed with 4% glutaraldehyde solution in 0.15 M phosphate buffer solution. The anterior segment was cut into wedges that included the trabecular meshwork, cornea, sclera, iris, and ciliary body. These dissected specimens were postfixed in 1% osmium tetroxide, in the same buffer, dehydrated in graded ethanol, and embedded in an epoxy resin. Ultrathin sections were transferred to celloidin-coated glass slides, stained with uranyl acetate and lead citrate, coated with carbon, and dipped
Figs. 7–8. Light microscopic autoradiographs of the angular region and its adjacent tissues from adult hamsters injected with $^{35}$S-sulfate. There are no striking differences in the localization of the grains between the trabecular and sclerocorneal regions (hematoxylin-eosin, ×210). Fig. 7. Thirty minutes after injection; Fig. 8. Sixty minutes after injection.

into Ilford L4 nuclear track emulsion (Ilford, England) diluted 1:6 with water, and maintained at 40 C. The coated slides were exposed in dark boxes at 4 C for 2 to 8 months, then developed in Phenidonic-containing developer for 1 min at 15 C. After fixation, celloidin membranes were separated from the slides by floating on water, and grids were placed on the sections. These sections were viewed in HU-11Ds (Hitachi, Tokyo) or JEOL 100CX (Nihondenshi, Tokyo) electron microscopes.

**Results**

The structures in the angular region of hamsters were composed of the trabecular meshwork, the angular aqueous plexus/sinus, and the exit channels.

![Angular aqueous sinus](image)

Fig. 9. Electron microscopic autoradiograph of the endothelial cell in the inner wall of the angular aqueous sinus from an adult hamster 60 min after the injection of $^{35}$S-sulfate. Accumulations of the grains are observed in the Golgi apparatus near the nucleus and in the cell process (arrows) (×8,400).
Figs. 10, 11. Light microscopic autoradiographs of the angular region and its adjacent tissues from adult hamsters injected with \(^{3}H\)-glucosamine. Specific localization of grains is seen over the angular region between the two arrows (×330). Fig. 10. Sixty minutes after injection; Fig. 11. One-hundred eighty minutes after injection.

The trabecular meshwork formed a reticular network, and the angular aqueous sinus was lined with a layer of endothelium with giant vacuoles. The angular region in a newborn hamster was basically the same as that in the adult except for cell number. These configurations in hamsters are similar to findings in rats.

1. \(^{35}S\)-sulfate

In 12-day-old hamsters given \(^{35}S\)-sulfate intraperitoneally, there was no evidence of a localized uptake of exposed silver grains in the angular region at 5 min after the injection. However, a gradual accumulation of silver grains near the nuclei in the angular region, sclera, and cornea was observed by LMARG, with the lapse of time (Figs. 1–3). One and 3 hrs after the injection, the silver grains tended to locate over the entire region of the trabecula, sclera, and cornea (Figs. 4, 5). Twenty-four hours after the injection, the grain concentration in these regions was predominant to the extent that the cells could hardly be seen (Fig. 6).

While the accumulation of \(^{35}S\) was thus conspic-
Figs. 13, 14. Electron microscopic autoradiograph of the endothelial cell in the trabecular meshwork from adult hamster 60 min after the injection of \(^3\)H-glucosamine. The radioactive material is associated with the Golgi apparatus and its adjacent cytoplasm. Figures 13 and 14 are photographs taken from the subserial sections (×25,400).

uous in newborn hamsters, the silver grains were slightly less dense in tissues from adult animals (Figs. 7, 8). There were no differences in the accumulation of grains among the trabecular, scleral, and corneal regions.

The EMARG revealed the distribution of grains...
in the cytoplasm of the endothelial cells of the angular aqueous plexus and the trabecular meshwork, 15 min after the injection. One hour after the injection, the grains were accumulated in the Golgi apparatus near the nucleus of the endothelial cells, in the inner wall of the angular aqueous sinus (Fig. 9).

2. \(^3\text{H}\)-glucosamine

Light microscopic autoradiography confirmed a small amount of the exposed silver grains in the angular region, sclera, and cornea of 16-day-old and adult hamsters, 15 min after the intraperitoneal administration of \(^3\text{H}\)-glucosamine. At 1 hour after the injection, the grains accumulated in the angular region (Fig. 10), and 3 and 24 hrs later, localization of the grains, particularly in the angular region was evident (Fig. 11).

Electron microscopic autoradiography showed grains distributed in the cytoplasm of the endothelial cells of the angular aqueous plexus and the trabecular meshwork. The uptake of grains into the Golgi apparatus in the trabecular meshwork was observed 30 min after the injection (Fig. 12). An even greater increase was noted 60 min after the injection (Figs. 13, 14). Three hours after the injection, the grains were distributed over the entire area of the cytoplasm, except for the nucleus (Fig. 15). Twenty-four hours after the injection, the grains were incorporated into both the amorphous substance and the collagen fibers in the region adjacent to the angular aqueous sinus.

**Discussion**

The structure of the aqueous outflow apparatus differs with the species.\(^{11,12}\) The nonprimate placen- tals have the angular aqueous plexus/sinus instead of Schlemm's canal seen in primates. The trabecular meshwork interposed between the anterior chamber and Schlemm's canal in primates is composed of the uveal meshwork, corneoscleral meshwork, and jux-tacanalicular connective tissue. In human and rhesus monkey eyes, the uveal meshwork is made up of two to three layers of trabecular cords, the corneoscleral meshwork with 8 to 12 layers of trabecular sheets and the juxtacanalicular connective tissue with two to six layers. In contrast, the trabecular meshwork and the angular aqueous plexus in hamsters are formed of six to nine layers of sheets with the pectinate ligament. Therefore, monkey eyes may be better suited for the investigation of glaucoma research.\(^5\) In the present experiment, in which radioactive isotopes were used as precursors for GAG, small animals were chosen for reasons of economy, management, and facilities.
Morphologic studies on GAG in the aqueous outflow apparatus have so far been achieved using histochemical approaches. These procedures, in which the GAG are stained with colloidal iron, alcian blue, and ruthenium red and digested with hyaluronidase, allow for observation of the GAG, under both light and electron microscopes. However, histochemical analysis does not always verify the presence of GAG, even in specimens from the same tissue.

Glycosaminoglycans in the form of intercellular materials in vertebrates include chondroitin, various chondroitin sulfates, keratosulfate, and hyaluronic acid. As chondroitin sulfates and keratosulfate contain sulfate, and hyaluronic acid and keratosulfate contain glucosamine, 35S- or 3H-glucosamine can be used as a biosynthetic precursor for the autoradiographic study of GAG. LMARG with these isotopes should enable a serial observation of behavior into which cells these materials are incorporated, how they migrate, and to where they are transported. The present experiment demonstrated that the isotopes were incorporated into the endothelial cells of the angular aqueous plexus and the trabecular meshwork, and were synthesized and excreted into the intercellular space. However, due to probable loss of isotopes during fixation and dehydration of the tissue, we did not verify the presence of the silver grains in the cavity of the angular aqueous sinus or in the intertrabecular space. It should be noted that this inorganic sulfate is also incorporated into glycoproteins and glycolipids in addition to GAG, and these sulfated macromolecules are used in the formation of ground substance, basal lamina, plasma membrane, collagen fiber, and elastic fiber.

Electron microscopic autoradiography is required for a precise analysis of the site of intracellular synthesis of GAG, and this approach involves more complicated procedures with an emulsion of finer grain size for a thinner film and a longer exposure time than LMARG. Photographic development with a developer containing Phenidon allows for a presentation of exposed silver grains in a fine grain instead of a filament form. Incorporation of inorganic 35S is generally considered to be initiated into the Golgi apparatus within the cell and this did occur in our experiment. 3H-glucosamine was also incorporated into the Golgi apparatus and presumably contributes to GAG synthesis. The possible transfer of 3H-glucosamine to hyaluronic acid and the specialized localization of 3H-glucosamine in the trabecular region is noteworthy.

Schachtschabel et al incubated the isolated trabecular meshwork tissue and found biochemical evidence of secretion of GAG from these cells, using 35S- and 14C-glucosamine. They concluded that the cells in the trabecular meshwork synthesized chondroitin-4-sulfate, dermatan sulfate, and hyaluronic acid. Their data plus the findings in our present study confirm biochemically as well as morphologically that GAG are synthesized in and secreted from the trabecular meshwork.

Key words: autoradiography, 35S-sulfate, 3H-glucosamine, glycosaminoglycans, hyaluronic acid, trabecular meshwork, angular aqueous plexus/sinus, hamster, electron microscopy

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