Penetration of horseradish peroxidase into the optic nerve after vitreal or vascular injections in the developing chick

Henry B. Kistler, Jr., and Jennifer H. LaVail

In order to determine whether a possible barrier exists to diffusion of tracer into the optic nerve during development and to provide a basis for later studies of retrograde axonal transport in embryonic nerves, we studied the diffusion of horseradish peroxidase (HRP) into the nerve after vitreal injections in chicks ranging in age from embryonic day 6 to 3 days after hatching. We found that HRP may reach the periaxonal spaces of the retrobulbar optic nerve after vitreal injection, vitreal injection into the opposite eye, or vascular injection. These and other observations suggest that vitreally injected HRP may reach the periaxonal spaces of the optic nerve by at least two routes: (1) by the obvious diffusion of marker from the vitreal surface into the optic nerve head and (2) by vascular leakage from fenestrated capillaries of the choriocapillaris into the pericapillary spaces of these and other capillaries that feed into the optic nerve parenchyma. There is a breakdown in the blood-brain barrier to HRP in the optic nerve head of the chick at embryonic day 13. The development of the breakdown depends at least in part on the maturation of vasculature in the nerve and the establishment of anastomotic branches between these vessels and those of the choriocapillaris. Our results further suggest that the limited diffusion of HRP into the retrobulbar nerve of fetal and newly hatched chicks is a function of uptake of tracer by glial cells within the nerve. Investigators of axonal transport who use this visual pathway as a model should be reminded of the potential artifact involved in this access of vascularly circulating label into the region of the lamina cribrosa.

Key words: Optic nerve, diffusion barrier, Gallus domesticus, development, horseradish peroxidase, retina, blood-brain barrier

One reason the retinal ganglion cell has been used so widely as the model of choice for studies of axonal transport lies in the ease with which isotopes or electron-dense tracers may be supplied to the cell body with limited trauma and yet excluded from most of the axonal surface. Although in many species the
extracellular spaces of the optic nerve head are continuous with the vitreal space of the eye as determined by the diffusion of extracellular tracers, the concentration of markers such as horseradish peroxidase (HRP) is markedly reduced with distance from the lamina cribrosa to more distal points along the nerve.\textsuperscript{1,4}

Several explanations might account for the reduction in concentration. Either a physical barrier resulting from the compactness of the nerve fibers or a network of membrane appositions between glial cells might impede the extracellular movement of HRP, as seen by Tso et al.\textsuperscript{5} in the border tissue of Kuhnt in the optic disc of primates and by Quigley\textsuperscript{6} in the optic nerve. Alternatively, in the absence of a physical barrier, a physiological barrier resulting from the rapid endocytosis and turnover of HRP by glial or vascular elements in the lamina cribrosa might serve to reduce the level of HRP at more distal points.\textsuperscript{3}

Whatever the basis for the barrier in the mature animal, it may affect the concentration of retinally synthesized extracellular molecules within the optic nerve in a region that is particularly susceptible to the transfer of toxic agents via the blood supply.\textsuperscript{1} During development, diffusible trophic substances might also be controlled and thus might affect the timing or coordination of differentiation in the retina or optic nerve fibers. Finally, the presence of the barrier has been considered a practical advantage for the study of axonal transport because the vitreal chamber is isolated from the nerve and serves as a closed compartment for the introduction of radiolabeled or histochemically identifiable
Table 1. Summary of results of HRP localization in the right optic nerve of the developing chick after an intravitreal injection into the right eye or after intravascular injection

<table>
<thead>
<tr>
<th>Age</th>
<th>Zone</th>
<th>Injection site</th>
<th>Pericapillary site</th>
<th>Astrocytic end space</th>
<th>Periaxonal space</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1 hr</td>
<td>4 hr</td>
<td>1 hr</td>
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<tr>
<td>E6</td>
<td>1</td>
<td>Vitreous</td>
<td>+</td>
<td>+</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<td></td>
<td>3</td>
<td></td>
<td>−</td>
<td>−</td>
<td>NA</td>
</tr>
<tr>
<td>E13</td>
<td>1</td>
<td>Vitreous</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td></td>
<td>+</td>
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<td>3</td>
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<tr>
<td>P3</td>
<td>1</td>
<td>Vitreous</td>
<td>+</td>
<td>+</td>
<td>NA</td>
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<tr>
<td></td>
<td>2</td>
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<td>3</td>
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<td>+</td>
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</tr>
<tr>
<td>E14</td>
<td>1</td>
<td>Vitelline vein</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>2</td>
<td></td>
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<td>3</td>
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<tr>
<td>P3</td>
<td>1</td>
<td>Left ventricle</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
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<td>3</td>
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</table>

+ = HRP present in a majority of the fields examined; ** = not examined; NA = not applicable since astrocytes have not yet formed end-feet around the developing capillaries; − = no HRP observed; +/− = HRP present in a minority of the fields examined.

Each value is based on observations of at least 10 fields, each 178 μm², from at least two animals.

The avascular retina of the chick has further encouraged its use as an experimental model for studies of axonal transport. The avascular retina of the chick has further encouraged its use as an experimental model for studies of axonal transport. Thus far, the diffusion of extracellular markers from the eye has not been examined in embryos. This information would be important not only for understanding the differentiation of the retina and optic nerve but also for the interpretation of axonal transport studies in embryonic systems. Therefore we have studied the extracellular spread of HRP from the vitreous to the optic nerve in developing chick embryos by light and electron microscopy. We have also examined the spread of HRP into the nerve after vascular injections. At those times when we found the extracellular passage of marker was limited, we examined the optic nerve for specialized glial membrane junctions. We also paid attention to the development of the blood-brain barrier to HRP in the chick optic nerve.

Material and methods

Animals. White leghorn chicks were used for all experiments. Six-day chick embryos (E6; stage 28), 14 13-day embryos (E13; stage 39), or 3-day-old newly hatched chicks (P3) were examined after HRP intravitreal injections. E13 and P3 chicks were used for vascular injections of HRP, and E8 through E16 embryos received intravascular injections of India ink.

Extracellular markers. For the intravitreal injections into embryos, solutions of 30% HRP (Sigma Chemical Co., type VI) in phosphate-buffered saline (PBS, pH 7.4), in a volume of 1 to 5 μl, were used for each eye. For the intravitreal injections into P3 animals, 25 μl of a 20% solution in PBS were placed in each eye. For the intracardiac injections of HRP, 100 to 150 μl of a 4% solution of HRP in PBS were used for each embryo, and 150 to 200 μl of a 4% solution of HRP in PBS were injected into each P3 chick. For the intracardiac injections of India ink (Higgins), we used 200 to 500 μl of undiluted ink.

Injections. Embryonic chicks received HRP or India ink through an opening made in the egg shell and extraembryonic membranes. For the intravitreal injections, the loose tissue surrounding the forming cornea was grasped with forceps, and a hole was made in the right eye with a 27-gauge needle. Through this hole, the beveled tip of a 26-gauge needle on a 10 μl Hamilton syringe was inserted toward the temporoinferior quadrant of the orbit. For the intravascular injections, a syringe adapted with a pulled glass micropipet was inserted into a vitelline vein of the embryo. The eggs were sealed with transparent tape and returned to a forced-draft incubator, the temperature of which was kept at 38°C. The animals were allowed to survive for 1 or 4 hr after intravitreal or intravascular injections of HRP. The animals that

Fig. 2. Electron micrograph from zone 2 of an E6 chick embryo 4 hr after intravitreal injection of HRP. The astrocyte (A) contains numerous HRP-positive inclusions (larger arrow). The HRP reaction product appears adsorbed to the surface of axonal and glial profiles (smaller arrows). N, Nucleus. (Bar = 0.75 \mu m.)

received India ink injections were perfused with fixative immediately.

P3 chicks were first anesthetized with ether and then they received intravitreal injections through a previously opened hole in the cornea. One or 4 hr later they were reanesthetized and perfused with fixative. The intravascular injections of HRP were made directly into the left ventricle of P3 chicks anesthetized with tribromoethanol.\textsuperscript{16} They were reanesthetized 1 or 4 hr later and perfused intracardially with fixative.

The intraocular pressure of E13 and P3 chicks was measured before and after injections of the appropriate volume of saline into the vitreous. Before the injection the pressures were about 3 and 10 mm Hg, respectively; after injection, they were about 2.5 and 8 mm Hg, respectively. Several E13 and P3 birds were also used for controls for the vascular transfer of HRP. Two of each received 25 \mu l injections of 20% HRP in the anterior chamber. Uninjured corneas were simply bathed with the same solutions in two additional animals of each age. All these animals were allowed to survive for 1 hr, and then they were treated according to the same procedures used for the experimental animals.

**Fixation.** All embryos were sacrificed by decapitation, the eyes were slit open, and the head was immersed for 4 hr in 0.1M sodium cacodylate—buffered (pH 7.4) fixative containing 2.5% paraformaldehyde, 3% glutaraldehyde, 0.12% calcium chloride, and 10% sucrose. The eyes, including a 1 cm\(^3\) region containing the optic nerve, optic disc, and retina, were then dissected and immersed in fresh fixative. Two hours later the optic nerves were bisected along the vertical meridian and rinsed overnight in the same buffer with 10% sucrose.

All P3 chicks were anesthetized with ether and perfused with fixative according to the standard procedure described previously.\textsuperscript{16} In this case, the buffer-rinsed optic nerves, optic discs, and retinas were either bisected along the vertical meridian or cut longitudinally into slabs 200 \mu m thick by use of a Vibratome.

**Histologic procedures.** All tissues prepared for HRP histochemistry were incubated, dehydrated, and embedded in plastic according to standard procedures.\textsuperscript{16} Serial 10 \mu m thick sections were cut from the optic nerves with glass knives. These were examined to determine the presence or absence of HRP throughout the nerve and to be sure that we were examining regions where the substrate had fully penetrated, e.g., in Fig. 4. Selected sections were reembedded in Epon-Araldite and thin-sectioned for electron microscopy. Some, but not all, of the thin sections were stained with uranyl acetate and lead citrate before examination in a Zeiss EM 10 microscope.

The India ink embryos were fixed by immersion overnight. The heads were rinsed in 0.1M cacodylate buffer (pH 7.4) with 5% sucrose and sectioned at 150 to 200 \mu m on a freezing microtome. Serial sections were mounted on gelatin-coated slides and stained with thionin.

**Results**

The avascular chick retina is supplied with an optic pecten, a pleated, vascularized sheet of connective tissue that projects into the vitreal chamber and presumably supplies nutrition to the retina. The endothelial cells lining the vessels of the pecten and optic nerve are not fenestrated, however, and are tightly joined by excluding junctions.\textsuperscript{17} Surrounding the base of the retrobulbar portion of the nerve is a cartilagenous cuff that lies embedded in peripapillary connective and glial tis-
Fig. 3. Electron micrograph of zone 2 of an E13 embryo 4 hr after intravitreal injection of HRP. The marker appears within the pericapillary space (P) and surrounding axons in the nerve parenchyma (arrows). Astrocytic processes (A) that form the boundary of the pericapillary space also contain HRP-positive inclusions (I). The junction of membranes (smaller arrows) of the developing endothelial cells (E) creates an irregular capillary lumen (L). (Bar = 1 μm.)

Intravitreal HRP injections. A summary of results of experiments in which HRP was injected intravitreally or intravascularly is presented in Table I. Since differences generally were not found between 1 and 4 hr survivals, we shall concentrate on the 1 hr results and note in passing any exceptions at 4 hr. After intravitreal injections of HRP in E6 embryos, the reaction product was located at the inner limiting membrane and in the extracellular spaces of the neural retina. The concentration diminished with distance from the vitreal surface until only isolated pockets of
Fig. 4. Photomicrograph of the optic nerve head of an E13 embryo 4 hr after intravitreal HRP injection. Diffuse reaction product extends from the vitreal surface into zones 1 and 2. HRP-filled vascular septa (arrows) extend from a region of diffuse extracellular staining into zone 3. An unstained region of zone 1 can be seen in the lower left corner where the substrate failed to penetrate. (Non-counterstained, 10 μm thick plastic section; Bar = 400 μm.)

marker were seen surrounding axons in zone 2 (Fig. 2). In both zones 1 and 2 glial cells contained HRP-positive inclusions ranging in size from 64 by 64 nm$^2$ to 0.4 by 0.5 μm$^2$ (Fig. 2). However, periaxonal HRP was absent in both zone 3 and the un.injected left eyes and nerves of E6 embryos.

By embryonic day 6, developing capillaries similar to those described by Delorme et al. could be recognized in the developing choriocapillaris in the back of the eye and in zone 1. These capillaries could be followed in serial, thick plastic sections to a cluster of cells, Bergmeister's papilla, that would later form the optic pecten. Zones 2 and 3 of the optic nerve were free of capillaries at this age, and no astrocytic end-feet could be identified in these zones.

One week later, at embryonic day 13, numerous capillaries had invaded all three zones of the nerve. The capillary walls were formed by the immature endothelial cells and the tight junctions between endothelial cells (Fig. 3). The endothelial cells were enclosed within a pericapillary space defined by a series of astrocytic foot processes. In addition to the endothelial cells, the space also included presumptive fibroblasts, pericytes in some cases, bundles of collagen, and a discontinuous basal lamina located just subjacent to the glial foot processes. Although the plasmalemmas of adjacent glial processes often came into close apposition, the extracellular spaces between them were not consistently occluded. The foot processes were often separated by gaps, sometimes as large as 0.5 μm, that connected the periaxonal space with the pericapillary space.

As in younger embryos after intravitreal injections, at embryonic day 13, the HRP appeared most densely distributed along the vitreal surface, and its concentration appeared to diminish with distance into zone 1. The region of dark-brown staining seen in the light microscope extended about 340 μm into the optic nerve and approached the boundary of zones 2 and 3 (Fig. 4). In addition, longitudinally running vascular septa that were traced, in some cases, in continuity with the spurs of scleral trabeculae at the lamina cribrosa also contained HRP. These septa could be followed into the proximal portions of zone 3 (Fig. 4).

One hour after injection of HRP into E13 embryos, we identified the marker in the pericapillary spaces of zone 3 and also around some of the axons in the region (Table I). The amount of HRP adhering to these membranes was less than that seen in the periaxonal spaces of zones 1 or 2, however. On rare occasions, we also found it in inclusions in glial cells and in the glial end-feet that incompletely surrounded the invading capillaries (Fig. 3). By contrast, the uptake of HRP by glial cells and processes was much more evident 3 hr later, but the periaxonal spaces were essentially free of label.

By day 3 after hatching the vascular invasion of the nerve was extensive in all three zones. Three features ultrastructurally distinguished the capillaries and surrounding vascular spaces from those of E13 embryos. (1) Endothelial cells were displaced laterally in the now open lumina of the capillaries and
Fig. 5. Electron micrograph of zone 3 of the optic nerve of a P3 chick 1 hr after intravitreal HRP injection. The pericapillary space (P) contains the marker. Reaction product also has collected outside the boundary of astrocytic end-feet (A) in the periaxonal space (arrow). A few dense inclusions also are present within astrocytic processes. L, Capillary lumen. (Bar = 1 μm.)

their apposed membranes formed occluding junctions; (2) adjacent glial end-feet were uninmrupted by the large gaps seen at embryonic day 13, and in some cases clear gap junctions could be identified between the processes; (3) a basal lamina could now be traced around the capillary endothelial cells and along glial processes, but its continuity was sometimes obscured by the dense investment of collagen within the pericapillary spaces. In summary, these vessels resembled mature mammalian capillaries in the central nervous system.

After vitreal injections in P3 chicks, reaction product was dense throughout the retina and zone 1 of the nerve. Although the density of the reaction diminished with distance from the vitreal surface, by 4 hr it was still conspicuous 0.75 mm from the surface through zones 1 and 2 and into the proximal portions of zone 3. Moreover, in each of the three zones and in the optic chiasm, the marker could be identified in the spaces of vascular septa (e.g., Fig. 5). In general, our observations confirmed those of Peyman and Apple, who examined the diffusion of HRP into the optic nerve of mature primates after intravitreal injections, and similar observations by Okinami et al. following intravitreal injections of lanthanum in mature rats.

Extracellular HRP was never identified in the left optic nerves, i.e., in the side opposite the intravitreal injection, of E13 embryos. However, at day 3 after hatching the choroid was stained brown, and the pericapillary spaces contained the marker in both zones 1 and 2 and in the most proximal portions of zone 3. We could recognize the product in zone 2 near the basal lamina adjacent to glial foot processes and surrounding axons near vascular septa (Fig. 6), but in zone 3, we failed to identify the marker in the periaxonal spaces outside the limits of the glial end-feet.

In summary (Table 1), in E6 embryos, capillaries had invaded only the first zone of the optic nerve, and HRP was found within the pericapillary spaces of this zone. In E13 embryos and P3 chicks in which capillary invasion was more complete, HRP appeared in the pericapillary spaces throughout all three zones, although at embryonic day 13 it was clearly present periaxonally only in zones 1 and 2 and the most proximal regions of zone 3. In P3 chicks, HRP could be found enveloping axons of all three zones. The presence of HRP-positive inclusions in glial end-feet...
Fig. 6. Electron micrograph of zone 2 of the un.injected, control left optic nerve of a P3 chick 4 hr after injection of HRP into the opposite eye. The marker (smaller arrow) has collected near the astrocytic processes (A) that bound the pericapillary space (P). Reaction product also is present between the axonal surfaces and the astrocytic processes (larger arrow). a, Unmyelinated axon. (Bar = 0.5 μm.)

closely paralleled the presence of extraaxonal HRP in E13 and P3 chicks. Moreover, although we observed desmosomal and gap junctions between glial elements throughout the nerve, we found no suggestion of tight junctions that might serve as a barrier to the free diffusion of extracellular HRP.

It was unclear to us from the results obtained in E13 and P3 chicks whether the HRP identified periaxionally might have traveled to zone 3 via an extra-axonal diffusion route from the vitreous or within the capillaries and pericapillary spaces. The HRP present periaxionally in zone 2 of the P3 control nerves was also surprising. To test for a possible vascular route, we inspected optic nerves after injecting the HRP intravascularly.

Intravascular injections of HRP. In E14 embryos, after intravascular HRP injection, tracer was distributed throughout the periaxonal spaces of all zones of the optic nerve (Table I). Densely filled pericapillary spaces were also obvious in all three zones. In contrast to results after intravitreal injections, the concentration of periaxonal HRP increased with distance from the vitreal surface, such that the region of nerve closest to the vitreal...
surface had less extracellular HRP than was observed in zone 2 and proximal zone 3. Both glial cell bodies and perivascular end-feet were found to contain HRP-positive inclusions throughout all three zones by 1 hr after intravascular injection. By 4 hr, the distribution of labeled glial processes and cell bodies in the nerve was the same as that after 1 hr, but the number of organelles in glial cells was increased.

In P3 chicks intravascular injection of HRP resulted in labeled pericapillary spaces in vascular septa in all three zones. Since the ultrastructural appearance of tracer in the optic nerve after intravenous injections in mature mammals has been described fully by others\(^5\) and since these results in P3 chicks confirm those earlier findings of a defect in the blood—optic nerve barrier in the optic nerve head, no further description is necessary.

We also investigated the possibility that the HRP might reflux from the eye and distribute to the optic nerve by way of orbital veins or might fill the anterior chamber and gain access to the venous system by way of Schlemm's canal or a uvea-vortex pathway.\(^22\) In the chicks receiving injections into the anterior chamber of the eye rather than the vitreal space, we found that the marker leaked from the anterior chamber into the posterior and vitreal chambers as evidenced by the brown stain of the inner limiting membrane of the optic nerve head. Posteriorly, HRP could be identified in the meninges of the optic nerve, but no HRP was found in either control or experimental optic nerves. Similarly, dripping HRP onto the cornea of E13 or P3 birds resulted in brown stain throughout the muscles and meninges of the eye, but no HRP was visible within either optic nerve.

Thus, from our observations of the spread of HRP after intravascular injections, it was apparent that HRP circulating through the vasculature had access to the optic nerve parenchyma of E13 and older chicks. The enzyme may have reached the pericapillary spaces of vascular septa either via immature capillaries within the nerve in the case of E13 chicks or via fenestrations in the endothelial walls of the choriocapillaries in E13 or P3 chicks, since these patent capillaries were continuous with those in the scleral trabeculae at either age. It is unlikely that the HRP reached the pericapillary spaces directly from the capillary lumina, since these vessels were neither fenestrated nor apparently involved in significant vesicular transport. At present the actual route by which the marker gained access to the control optic nerve remains unknown.

In addition, after demonstrating that circulating HRP could enter the nerve, we wondered whether vitreally injected tracer might reach the leaky choroid capillaries via anastomotic connections between the two vascular nets. We injected India ink intravascu-
larly in 8 to 16 day embryos and examined the optic nerve head vasculature to determine whether an anastomotic connection existed between the vessels supplying the highly vascular optic pecten and the fenestrated choroidal branches of the short posterior ciliary arteries.

Examination of embryos sacrificed immediately after intracardiac perfusion with India ink revealed that the vessels supplying the optic pecten, the choroid, and the scleral trabeculae at the lamina cribrosa interconnected in an anastomotic system. By following ink-filled vessels in sequential sections we traced the vascular supply of the optic pecten directly to the plexus of vessels supplying the choroid. This same plexus was found to anastomose with vessels that continued into the trabeculae and spurs of glial and connective tissues that invaded the nerve.

Discussion

HRP that is injected into the vitreous of fetal or newly hatched chicks may reach axons of the optic nerve by at least two routes. The first and most obvious route involves direct diffusion from the vitreal surface, past the inner limiting membrane and around the axons and glial and vascular cells of the nerve (large arrows, Fig. 8). A second possible route involves the vascular system with entrance of HRP into the pericapillary spaces of choriocapillaris vessels. From this point it moves into the pericapillary connective tissue surrounding the retrobulbar portion of the nerve and into scleral trabeculae in the lamina cribrosa (smaller arrows, Fig. 7). From there the marker diffuses into connective tissue and glial septa in zones 2 and 3 and escapes the glial end-feet boundary to reach the periaxonal space of the nerve (inset, Fig. 7).

Three indirect lines of evidence support the existence of the second route. First, using India ink infusion of the vessels, we traced anastomotic connections between the arterioles and capillaries of the optic pecten and optic nerve head to the choriocapillaries. Thus there is a potential pericapillary conduit from the vitreous to the choriocapillaris and the optic nerve. Second, after vascular injections of HRP the marker could be identified surrounding axons of all three zones of the nerve. In this case we propose the marker traverses the fenestrated choriocapillaris to reach (1) the pericapillary spaces of these vessels, (2) those of zone 2, and (3) zones 1 and 3. It escapes the glial end-feet boundary to reach the periaxonal space. A similar route of access of vascularly injected markers has been reported earlier for mature mammals.5, 19–21, 23, 24 Third, we identified HRP peripapially in zone 2 of the control nerves after intravitreal injections into the opposite eye. In this case, the marker may have reached capillary lumina in the optic nerve head or pecten of the injected eye and may have been transferred in some way to the fenestrated capillaries of the opposite choriocapillaris before passage through the pericapillary channels into zone 2 of the nerve.

Once the HRP reaches the periaxonal spaces of the optic nerve, what might limit its further diffusion in the P3 chick? The possibility that some sort of physical occlusion of the extracellular space might exist encouraged us to examine embryos; for example, in early developmental stages, junctions might not yet exist and no barrier would be detected. To our surprise, however, the spread of HRP was restricted at embryonic day 6, the earliest stage examined. We now believe that this was due to the immature state of the vasculature in the embryonic nerve, i.e., the second route described above was poorly developed and thereby limited the spread of HRP.

Our search for the presence of occluding junctions, however, was directed to a fairly broad region of the nerve near the limit of spread of HRP, for each of the three ages studied. The concentration of reaction product decreased gradually throughout the region of limit of spread, suggesting that any possible structure that acted as a physical barrier would not be confined to a narrow band within the nerve. Although junctional specializations, such as gap junctions and
desmosomes, similar to those described earlier by others in other species, were occasionally observed, no tight junctions (with the exception of those between endothelial cells at embryonic day 13 and day 3 after hatching) were recognized at any age. Assuming that sampling restrictions inherent in thin-section electron microscopy were not critical to our interpretation, the fact that not a single occluding junction was observed suggested that membrane junctional specializations were not a significant factor in impeding the passage of HRP and that the spread of marker was not principally restricted by a physical blockage of the extracellular space.

Furthermore, our observations supported the proposition that endocytic uptake of HRP from the extracellular space of the optic nerve by glial and vascular elements served to limit its spread. Frequently, HRP inclusions were seen in astrocytic profiles forming the end-feet around a capillary. The number of these HRP-filled profiles was directly related to the concentration of HRP reaction product in the extracellular space. Such clearing of the extracellular space of markers by glial cells has been seen in other regions of the central nervous system. Our findings are relevant to two broader aspects of developmental neurobiology. First, they bear directly on a variety of findings on the rates and components of macromolecules transported intraaxonally in the developing chick visual system. A major motivation for this study was to determine whether the eyes of developing chick embryos were similar to those of more mature birds whose vitreal chamber has been considered a relatively closed system for injection of tracers. Although the extracellular diffusion of HRP was restricted, in the main, to a region of nerve just distal to the lamina cribrosa, as described earlier, a second vascular channel for passage of HRP from the eye into the optic nerve is also present. Material introduced into the vitreous, in general, may move through this conduit and leak between adjacent glial processes to collect in the extracellular space surrounding axons in zones 2 and 3. This passage might account for (1) the unexplained observation of LaVail and LaVail of HRP accumulation in glial cells in the control optic nerves after restricted tectal injections and (2) the presence of fluorescent tracer at the site of the lamina cribrosa of rabbits after orbital injection of Evans blue albumin, as described by Olsson and Kristensson.

Second, the presence of HRP in the optic nerve and chiasm after intravitreal injections would have important consequences for the interpretation of results of axonal transport studies using HRP in embryonic optic nerves. At embryonic day 6 the vascular invasion of the optic nerve is incomplete, and the passage of HRP from the vitreal surface is the more important route of entry of HRP. By later stages (embryonic days 10 to 14), the capillaries, including their investment of pericapillary components, have invaded well into the optic nerve and optic chiasm. The growth cones of developing axons traveling through the nerve will be exposed to tracer in at least minimal quantities well outside the limits of zones 1 or 2 of our study. Furthermore, in biochemical studies of anterograde axonal transport the possibility of confusing extraaxonal diffusion with intraaxonal transport of radioactively labeled molecules must be considered.

The presence of HRP in the connective tissue surrounding the retrobulbar portion of the optic nerve coincides with the location of one of the important targets of retrograde transported label from the iris and ciliary body of the eye, the ciliary ganglion. This parasympathetic ganglion is embedded in the connective tissue in the area, and although immediately surrounded by a tough capsule of connective tissue, the capsule does not completely prevent the influx of diffusible substances (LaVail JH, LaVail MM, and DiCiamberadino L: unpublished observations). Therefore, in experiments involving retrograde transport to the ciliary ganglion, consideration must be given to control experiments such as those in which transection of the postganglionic branches of the ganglion blocks the retrograde transport of the marker.

In conclusion, investigators who use this
visual pathway for studies of axonal transport should be aware of the simultaneous limit to extraxonally moving label by astrocytic endocytosis and paradoxical access of vascularly circulating label into this region of the lamina cribrosa.

We thank Dr. Robert A. Lisenmeier for his help in measuring intraocular pressures and Ms. Alana Schilling for her secretarial assistance.

REFERENCES


