Peroxidase diffusion in the normal and photocoagulated retina

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To study diffusion pathways in normal and photocoagulated mammalian retina, xenon photocoagulation was applied to one eye in each of a group of rabbits; three weeks later, peroxidase was injected in both eyes; thereafter, the animals were killed at intervals and the tissues were studied with electron microscopy. In the normal eyes, peroxidase diffused rapidly through the intercellular spaces in all layers of the sensory retina, but diffusion stopped abruptly at the zonulae occludentes in the intercellular spaces of the pigment epithelium. Three weeks after photocoagulation, peroxidase diffused rapidly through all layers of the scar to come in contact with Bruch's membrane. Consideration has been given to the anatomical basis for these observations, and to the mechanism of the action of photocoagulation in certain retinal diseases.

Key words: peroxidase diffusion, diffusion pathways, diffusion barriers, retinal diffusion, photocoagulation scar, tracer techniques, tracer materials.

In the metabolism of the retina, the importance of diffusion is increasingly recognized. Therefore, diffusion pathways in the retina and adjacent tissues have been studied with the introduction of tracer materials that are visible with electron microscopy.

Tracer materials include, among others, thorotrast (molecular size 90 to 150 Å), ferritin (molecular size 90 to 110 Å), and peroxidase (molecular size 25 to 30 Å). Advantages of peroxidase relate to its small size, considerably less than that of the intercellular spaces of the retina which are approximately 150 Å wide, and its enzymatic catalysis of colorless nonelectron-dense benzidine to brown electron-dense benzopurpurine. As a result of this small molecular size and enzymatic conversion, the peroxidase benzidine system is a very sensitive tracer technique.

Studies with various tracer materials have revealed interesting and significant information. Smelser and associates, for

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example, injected thorotrast and ferritin into the vitreous of live cats. One hour later, the tracer was present in the intercellular spaces of the innermost retinal layers, but even 24 hours after injection there was no diffusion external to the outer limiting membrane. Lasansky and Wald, however, using toads, described the passage of intravitreally injected peroxidase through the external limiting membrane to the rod and cone layer; they reported no data concerning the time required for this process.

External to the retina, electron-dense tracer materials have been shown to pass readily through the walls of the choroidal capillaries and Bruch's membrane. Passage through the pigment epithelium, however, is somewhat slower and is achieved by transport through pinocytotic vesicles rather than by diffusion. A combination of diffusion and pinocytotic vesicle transfer may enable tracer material to move from choroidal capillaries to the intercellular spaces of the retina.

Recognizing the significance of diffusion pathways, this study utilizes the peroxidase tracer technique and electron microscopy to investigate diffusion in the mammalian retina with particular attention to the time factors involved and to possible diffusion barriers. In addition, diffusion in the normal retina is compared with the behavior of tracer material in the photocoagulated retina to gain a better understanding of the mechanism of action of this therapeutic modality.

Materials and methods

Fourteen mature Dutch pigmented rabbits, weighing approximately 2 kilograms each, were used in this study. In the right eye of each animal, photocoagulation was carried out with the Zeiss xenon arc coagulator at a setting of standard power 1, iris diaphragm 3.5, and field diameter 6 degrees. Sixteen confluent lesions were applied to the inferior retina approximately 5 mm. posterior to the ora serrata; the exposure time was regulated so that the retina became gray-white at the time of treatment and a pigmented chorioretinal scar developed subsequently at the treatment site.

Three weeks after photocoagulation, the animals were anesthetized with Nembutal and, in both eyes of 13 of the rabbits, horseradish peroxidase* (40 mg. dissolved in 0.2 ml. of 0.9 per cent saline) was injected through the pars plana into the inferior part of the vitreous close to the internal surface of the equatorial retina. To prevent an increase in intraocular pressure during the injection, an equal volume of aqueous humor was simultaneously aspirated from the anterior chamber. The fourteenth rabbit received no peroxidase injection, but it served as a control and was processed in an otherwise identical manner.

At intervals of 15 minutes (one rabbit), 30 minutes (two rabbits), 1 hour (three rabbits), 3 hours (three rabbits), 7 hours (three rabbits), and 12 hours (one rabbit), the rabbits were re-anesthetized and killed by transcardiac perfusion under constant pressure of 60 mm. Hg, with 1,500 ml. of fixative consisting of 0.8 per cent glutaraldehyde in 0.5M phosphate buffer containing 1.5 per cent glucose at a pH of 7.6. The total perfusion time was approximately 30 minutes. Three weeks after photocoagulation, the single control rabbit was killed by transcardiac perfusion under the same conditions.

The eyes were enucleated and, after removal of the anterior segment and lens, fixation was continued in the same solution for 12 hours. Following dissection into narrow strips, the inferior equatorial retina and choroid were separated from the sclera, and areas of scarred inferior retina in the right eye, as well as normal inferior retina in the left eye, were cut into pieces 3 x 6 mm. in size. The specimens were washed in phosphate buffer for two hours, sliced into 40 μ thick pieces with a Sorval TC2 Smith-Farquhar tissue sectioner, and incubated for one hour at room temperature in a solution of 3.3' diaminobenzidine tetrahydrochloride (5 mg.) and 1 per cent H₂O₂ (0.1 ml.) in tris-HCl buffer (9.9 ml.) at pH 7.6. The specimens were washed again in phosphate buffer, postfixed for one hour in a 1 per cent OsO₄-phosphate buffer solution, dehydrated in alcohol and propylene oxide, and embedded in Araldite. Thin sections were cut on the LKB ultratome, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop I.

Results

Pertinent findings were noted in the normal retinal tissue of the untreated left eyes and in the scarred retinal tissue of the photocoagulated right eyes.

*Type II from horseradish, Sigma Chemical Co., St. Louis, Mo.
Fig. 1. Sensory retina with peroxidase occupying all intercellular spaces (arrows). ILM = inner limiting membrane, NFL = nerve fiber layer, GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, OPL = outer plexiform layer, ONL = outer nuclear layer, OLM = outer limiting "membrane," IS = photoreceptor inner segments, OS = photoreceptor outer segments. (×450.)

Normal retina. Following intravitreal injection, peroxidase penetrated the inner limiting membrane and entered the intercellular spaces of the sensory retina (Fig. 1). Documenting this diffusion, peroxidase was readily identified in the intercellular spaces in the nerve fiber layer (Fig. 2, A and B), inner plexiform layer (Fig. 2, C), inner nuclear layer (Fig. 2, D), outer plexiform layer (Fig. 3, A), outer nuclear layer (Fig. 3, B), and photoreceptor layer (Fig. 3, C). In the photoreceptor layer, peroxidase was clearly evident between the inner segments (Fig. 3, D) and between the outer segments (Fig. 3, E and Fig. 4).

The peroxidase was concentrated adjacent to the inner surface of the retinal pigment epithelium and extended for a short distance into the apical portion of the intercellular spaces of the pigment epithelium. Diffusion was then abruptly halted and there was no peroxidase in the more external portions of the pigment epithelium intercellular spaces of adjacent to Bruch's membrane.

The full extent of this diffusion was present in retinal tissue fixed 15 minutes after intravitreal injection of peroxidase and was unchanged in specimens fixed 30 minutes and one, three, seven, and 12 hours after intravitreal injection.

In effect, after intravitreal injection, peroxidase diffuse rapidly from the vitreous through the entire sensory retina. Diffusion was then halted abruptly near the apical portion of the intercellular spaces of the pigment epithelium. The full extent of this diffusion was present 15 minutes after intravitreal injection and remained unchanged throughout a relatively long period of subsequent observation.

Photocoagulated retina. Three weeks after photoagulation, the sensory retina and pigment epithelium were replaced with a scar. On the vitreal side, this scar was composed of cells that were derived probably from the sensory retina and often contained a relatively large number of ribosomes (Fig. 5, A). Blended with these, and also located externally, were several layers of cells that were derived probably from the pigment epithelium. Some of these cells contained numerous filaments (Fig. 5, B), while others were filled with clusters of melanin granules (Fig. 5, C).

Following intravitreal injection, peroxi-
Peroxidase diffused readily through the inner portion of the scar (Fig. 5, A) and continued without obstruction through the outer portion of the scar (Fig. 5, B and C) to come in contact with Bruch’s membrane. As in the noncoagulated tissue, the full extent of this diffusion was evident 15 minutes after intravitreal injection.

In essence, following intravitreal injection, peroxidase diffused rapidly from the vitreous, passed through all layers of the photoagulation scar derived from the
Fig. 3. Peroxidase-filled intercellular spaces (arrows) in the outer layers of the sensory retina. (A) Photoreceptor synapse in outer plexiform layer. Note the peroxidase in the synaptic cleft (arrow). (×14,000.) (B) Outer nuclear layer, outer limiting "membrane," and photoreceptor inner segments. N = visual cell nucleus, IS = photoreceptor inner segments. (×14,000.) (Inset) High power view of the outer limiting "membrane." (Note: Peroxidase in intercellular space; the densities in the lining cytoplasm indicate a typical zonula adhaerens.) (×48,000.) (C) The villous processes (fiber baskets) of Müller's cells beyond the outer limiting membrane are covered with peroxidase (arrows). (×17,500.) (D) Peroxidase between the two inner segments. (×60,000.) (E) Peroxidase around photoreceptor outer segment. (Note: No tracer particles appear between the discs.) (×26,000.)
sensory retina and pigment epithelium, and came in contact with Bruch's membrane. This diffusion process was rapid, and there was no evidence of a diffusion barrier at the level of the pigment epithelium.

**Discussion**

In discussion of these results, consideration will be given to (1) the anatomical features of epithelial cell junctions, (2) the anatomical basis for diffusion through the sensory retina, (3) the anatomical basis for a diffusion barrier in the pigment epithelium, (4) the physiologic implications of this diffusion system, (5) the effect of photocoagulation on the pigment epithelium diffusion barrier, and (6) the mechanism of the action of photocoagulation in certain retinal diseases.

In 1963, Farquhar and Palade described a complex of intercellular junctions that, with certain variations, were present between adjacent cells of almost all epithelia. The elements of these junctions succeed each other in an apical-basal direction and are described as the zonula occludens, zonula adhaerens (Fig. 6), and macula adhaerens. (The latter is also called the desmosome.) The zonulæ occludentes, which are closest to the apices of the cells, form continuous horizontal belts around the cells and are characterized by fusion
of the cell membranes of adjacent cells. This fusion obliterates the intercellular space. Next in the basal direction are zonulae adhaerentes. They also form horizontal belts around the epithelial cells, but these are characterized by a widening of the intercellular space to approximately 200 Å and by lines of dense fibrillar material within the cell, but adjacent to and parallel with the cell membrane. Even more basal are the maculae adhaerentes or desmosomes. These are discontinuous button-like structures that are not limited to the junctional complexes.

Experimental observations indicate that the zonulae occludentes seal off the intercellular spaces from the luminal surface and form diffusion barriers. Functioning in a different manner, the zonulae adhaerentes and the maculae adhaerentes act as mechanical attachment devices and do not constitute barriers to diffusion.

Applying this concept of epithelial junctions to the retina and pigment epithelium, it is pertinent to note that, embryologically, these structures develop from the primary optic vesicle. As this vesicle is converted to the optic cup, the anterior portion gives
rise to the sensory retina and the posterior portion becomes the pigment epithelium, but the intercellular junctional elements in both layers retain their basic orientation toward the original lumen of the optic vesicle.

Therefore, there is no barrier to diffusion from the vitreous into the retina, and diffusion continues readily through the intercellular spaces of the nuclear and plexiform layers. The junctional area is represented by the outer limiting membrane but, as Sjöstrand\textsuperscript{11} and others have pointed out, this is not a real membrane. In fact, as Spitznas\textsuperscript{14} has demonstrated, the external limiting membrane is composed entirely of zonulae adhaerentes, which join adjacent cells mechanically and do not contain any zonulae occludentes. Consequently, there is no diffusion barrier in this area, and intravitreal peroxidase rapidly diffuses into the intercellular space between the sensory retina and the pigment epithelium.

Interestingly, no peroxidase was present between the discs of the retinal rods. This observation indicates that a cell membrane forms a diffusion barrier that completely surrounds the rods (Fig. 3, E).

Basal to the zonulae occludentes the intercellular space widens, and there are characteristic zonulae adhaerentes which do not obstruct diffusion. This is demonstrated by noting that—after introduction into the choroidal circulation—peroxidase diffuses from the choroid, across Bruch's membrane, and into the basal portions of the pigment epithelium intercellular spaces.\textsuperscript{5} This diffusion extends past the zonulae adhaerentes, but is halted abruptly at the basal ends of the zonulae occludentes. Transfer from the choroid across the pigment epithelium to the retina apparently occurs in one direction only (from choroid to retina), and transfer is accomplished by means of active transport through pinocytotic vesicles.\textsuperscript{1}

Physiologically, demonstration of rapid diffusion from the vitreous to the pigment epithelium indicates that nutritive materials and metabolic products in the vitreous have access to all layers of the sensory retina. Significantly, in terms of this interaction, the vitreous contains nutrient materials derived from the blood vessels of the ciliary body\textsuperscript{15} and, in all probability, from the optic nervehead.\textsuperscript{16-18} Moreover, unobstructed diffusion in the intercellular spaces of the retina permits metabolic materials transferred from the retinal capillaries by active transport through pinocytotic vesicles\textsuperscript{4} and across the pigment epithelium by similar pinocytotic vesicles\textsuperscript{5} to be distributed readily throughout all layers of the sensory retina.

It is likely, also, that the diffusion barrier at the level of the zonulae occludentes of the pigment epithelium plays an important role in physiology and pathologic processes. It limits the passage of materials.
from the retina to the choroid and, conversely, restricts the transport of materials from the choroid to the retina.

Significantly, photocoagulation disrupts the normal morphology of the retina and pigment epithelium and is followed by formation of a scar that does not possess an orderly arrangement of zonulae occludentes and, therefore, does not function as a diffusion barrier. Consequently, there is a rapid diffusion from the vitreous to Bruch’s membrane.

Destruction of the diffusion barrier by photocoagulation may contribute to the disappearance of sub- and intraretinal fluid associated with central serous retinopathy, retinoschisis, diabetic retinopathy, and other disease processes. In essence, and understanding of the effect of photocoagulation on the pigment epithelium diffusion barrier offers insight into the mechanism of action of photocoagulation.

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