A whole-mount cholinesterase technique for demonstrating corneal nerves: Observations in the albino rabbit

Dennis M. Robertson and R. K. Winkelmann

A histochemical staining technique for cholinesterase is reported for the study of corneal nerves in whole-thickness rabbit cornea. The technique is relatively simple, yields reproducible results, allows good definition of the nerve fiber pattern, and provides the investigator, using a binocular microscope, with the advantage of three-dimensional study. When tissue is stained for specific cholinesterase, definition is sufficient to allow visualization of individual axons and their terminations, as well as large nerve fiber bundles. The stain for nonspecific cholinesterase appears to demonstrate the large nerve fiber bundles but not individual axons. The anatomy and cholinesterase content of corneal innervation is discussed in relation to the enzyme demonstrated.

Key words: cholinesterase, cornea, nerve, rabbit, whole mount.

Although several hundred publications have dealt with corneal innervation, in many the data have been based on observations dependent upon techniques which have involved not only special tissue preparations, such as lamellar sectioning, but complex staining procedures as well. The present report discusses a whole-mount cholinesterase technique for staining corneal nerves; it is relatively simple, yields consistently reproducible results, provides an elegant demonstration of the patterns of nerve fiber distribution within the various layers of the cornea, and, when visualized with the binocular microscope, allows three-dimensional observation.

Methods

The Gomori technique for cholinesterase as modified by Winkelmann and Schmitz was used. All tissue preparations were made from adult albino rabbit corneas. Immediately after the animals were put to death, the corneas were carefully removed with corneal scissors. To conserve tissue, facilitate handling, and minimize wrinkling when the tissue was later mounted, the corneal buttons were cut into quarter sectors. Each rabbit thus provided eight corneal pieces.

We learned that incubation with either substrate gave optimal staining when the pH was between 5.5 and 6.0. With acetylthiocholine...
iodide as the substrate, staining was optimal when the incubation period was 4 hours. With butyrophthiocline iodide, staining was most definite at 5 hours. Accordingly, the pH was routinely maintained at 5.58 while the incubation periods used were either 4 or 5 hours, depending on the substrate.

After incubation the tissue was rinsed over a 10 to 15 minute interval in three changes of saturated sodium sulfate. It was then immersed in dilute ammonium sulfide (2 drops of NH₃S in 10 ml. of H₂O) for 8 minutes, washed thoroughly (two 5 minute rinses with distilled water), secured between two glass slides, and dehydrated overnight in 80 per cent alcohol. Dehydration was continued in absolute alcohol changed daily for 5 days, twice the following week, and once during each of the 2 successive weeks. Two changes of xylene were used over the next 4 days. The tissue was mounted in Permount between two slides which were then weighted.

In an effort to clarify the enzymatic activity, several enzyme inhibitors were used to pretreat the tissue at 37° C. for 1/2 hour before adding the substrate. These included di-isopropylfluorophosphate (DFP) at 10⁻²M concentration, physostigmine (eserine) at 10⁻⁵M concentration, Ro-2-0683* at 10⁻⁹M concentration, B.W. 284-C-511 at 10⁻⁸M concentration, 30 per cent dimethyl sulfoxide (DMSO), and ouabain at 4.0 × 10⁻⁶M concentration.

Results

Excellent histologic demonstration of the corneal nerves was achieved. The definition allows visualization of the arrangement of the nerve bundles, recognition of the behavior of certain individual axons in large nerve bundles, as well as in plexiform nerve masses, and even visualization of the terminations of axons. In the tissue treated for acetylcholinesterase (that is, "true" or "specific" cholinesterase), beading of the terminal branches was easily visible both in the stroma and in the epithelium. Plexiform arrangement, branching of the nerve bundles, and beading of the terminal axons can be seen in Figs. 1 and 2. Unfortunately, the photomicrographs can show only a portion of the nerve network in focus since we are dealing with thick-tissue whole mounts. Figs. 3 and 4 are taken of the same areas but with the camera focused at different depths in an attempt to demonstrate the usefulness of full-thickness preparations. With the binocular microscope, one can easily trace the nerves throughout the cornea as they travel from one layer to another.

Full-thickness cornea stained for butyro-

---

*Kindly supplied by Dr. W. E. Scott, Hoffmann-La Roche, Inc., Nutley, N. J.
**Kindly supplied by Dr. Richard Baltzly, Burroughs, Wellcome & Company, Inc., Scarsdale Road, Tuckahoe, N. Y.
***Kindly supplied by G. C. Chiu, M.D., the Lilly Research Laboratories, Eli Lilly & Company, Indianapolis, Ind.
cholinesterase (nonspecific or pseudocho-
linesterase) showed a pattern distinctly
different from that demonstrated by spe-
cific cholinesterase. In Fig. 5 one can see
large nerve bundles which branch or di-
vide and then eventually terminate rather
abruptly without any evident beading. The
fine, beaded axons, which are shown by
the specific cholinesterase technique, could
not be seen with stains for nonspecific
cholinesterase even with prolonged incu-
bation up to 24 hours.

Table I summarizes the effects of the
various inhibitors on the staining character-
istic of each substrate. None of the agents
seemed to exhibit differential inhibition
within the same stained whole mount. For
example, in the specimens stained for
specific cholinesterase, if the large nerve
bundles were partially inhibited, so were
the fine, beaded axons.

In all experiments with inhibitors, con-
trols were found to be stained in the usual
manner.

Discussion

In a review of several histochemical
methods of staining neural tissue, Weddell
and Zander demonstrated patterns and
details of the corneal nerves most precisely
with methylene blue. The methylene blue-
staining technique, as Weddell and Zander
have emphasized, is somewhat difficult and

Table I. Effects of inhibitors on substrate
staining

| Inhibitor agents | Acetylthio-
|                 | choline | Butyrothio-
<table>
<thead>
<tr>
<th></th>
<th></th>
<th>choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>4*</td>
<td>4</td>
</tr>
<tr>
<td>Phospholine iodide</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Eserine</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ouabain</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>DMSO</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Ro-2-0683</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>B.W. 284-C-51</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ro-2-0683</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>B.W. 284-C-51†</td>
<td>4</td>
<td>3+</td>
</tr>
</tbody>
</table>

*Inhibition is graded from 0 (none) to 4 (complete
inhibition of staining).
†All inhibitors were used to pretreat tissues for 15 hour.
In addition, a 3 hour pretreatment incubation time was
employed with Ro-2-0683 and B.W. 284-C-51.
demonstrates strict attention to technical procedures. For example, the methylene blue must be injected carefully into the anterior chamber of the living animal 30 minutes before removing the tissue for later study. Keratocytes, cytoplasmic processes of Schwann cells, and even Schwann cell nuclei may be stained with the methylene blue and can be a source of confusion. In contrast, the cholinesterase method is relatively simple and is easily reproducible; it does not appear to stain non-neural tissue and, though there is some loss of specificity, it does provide an elegant demonstration of the corneal nerves that compares favorably with the methylene blue preparations. Unlike methylene blue, however, cholinesterase cannot be used for in vivo staining.

Silver stains require thin-tissue sections, may stain non-neural tissue as well, and only rarely demonstrate the final ramifications of the nerve fibers such as are present in the epithelium. These nerve fibers seem to be easily demonstrated with cholinesterase.

Gold chloride methods demonstrate the nerve pattern nicely in whole mounts but the results do not compare with the detail provided by the cholinesterase stain. The relative permanence of the cholinesterase stain is another advantage over gold chloride. In whole mounts, the potential value of the cholinesterase technique in the study of innervational characteristics, certain aspects of corneal physiology, and patterns of nerve degeneration and regeneration is apparent.

In an effort to confirm the validity of the histochemical results, a variety of inhibitor agents were employed choosing agents with proven anticholinesterase activity. Inhibition occurred with all of the agents used, tending to confirm the idea that cholinesterases at the level of the nerve were indeed responsible for the stain. The fact that some agents did not behave in a totally typical manner is probably explained by the thickness of the whole mount, which causes variation in penetration of the inhibitor agent.

Since cholinesterase enzymes are widely distributed in neural tissue, and have been demonstrated in highly sensitive organized sensory end organs of the skin, it is not surprising that the corneal nerve network could be demonstrated with stains for these enzymes. Pseudo-cholinesterase, for example, is invariably present in organized sensory end organs of the skin. Specific cholinesterase, though not a significant constituent of end organs, has been found occasionally in nerve fibers in the dermis and epidermis, and about the hair follicles, as well as in its more usual association with autonomic nerve fibers about eccrine glands and blood vessels.

Cholinesterases have also been recognized in the cornea, especially in the epithelial layer. However, the fact that the corneal nerves themselves appear to be rich in cholinesterase has not been fully appreciated. Petersen and co-workers found significant amounts of cholinesterase in the corneal epithelium but only small amounts in the stroma. They believed the small amount of cholinesterase found in the stroma was due to residual epithelium, which had been incompletely removed, or to cholinesterase within the nerve fibers in the stroma. Our studies, showing the abundant cholinesterase present in the stromal nerves, support the latter explanation.

Petersen and associates concluded that cholinesterase deposits were present intracellularly in the epithelium, in addition to being present in especially high concentrations in fine, hairlike structures between the cells. From their studies, they could not conclude that these structures were nerves, though this was suggested. Using cross sections of epithelium stained for specific cholinesterase, Laties and Jacobowitz demonstrated that these fibers were nerve structures. Cholinesterase-positive

---

*The presence of cholinesterase-positive neural tissue in the cornea is not to be interpreted as an indication of autonomic function. For example, the presence of acetylcholinesterase need not be related to either cholinergic neural activities or neurohumoral transmission.*
corneal nerves have also been demonstrated by Lassmann and Wawrzyunik. However, their work was based on the study of thin pieces of tissue, either cross sections or lamellar sections, rather than on whole mounts.

Our study on the whole-mount specimens stained for specific cholinesterase show the nerves within the epithelium to be composed of an extensive network of cholinesterase-rich axons. Though we did not study cross sections, several observations support our belief that we can identify and localize the fibers within the epithelial layer: (1) With the binocular microscope, axons can be traced from the deep nerve fiber bundles into the most superficial layers of the cornea. (2) The beaded pattern of axons present in this layer resembles the nerve pattern in the epithelial layer demonstrated by other techniques. (3) This rich network is absent in specimens in which the epithelium has been removed. (4) As the nerve fibers turn perpendicularly to penetrate apertures in Bowman's membrane, they sometimes divide into a leash of up to 15 fibrillas. This structural transition into fibrillas is seen frequently in the superficial cornea where the fibers presumably penetrate the region of Bowman's membrane (see arrow, Fig. 2). (5) With prolonged incubation, using the stain for specific cholinesterase, the epithelial cells eventually can be visualized as they become outlined by diffused stain (Fig. 6). These cells are in the same layer as the most superficial, beaded axons. By the time the epithelial cells are seen, however, the nerve fibers are generally less well defined since considerable diffusion has taken place. Indeed, it is possible that the high concentration of specific cholinesterase found in the epithelium by methods using differential hydrolysis of epithelial homogenates depends largely on the presence of neural tissue rich in specific cholinesterase, rather than on an intracellular location per se.

We could not demonstrate neural tissue in the epithelial layer when we used nonspecific cholinesterase stains. This is in keeping with the studies of Petersen and associates, who found little evidence for nonspecific cholinesterase activity in epithelial homogenates and absence of staining for nonspecific cholinesterase in cross sections of epithelium.

It is apparent, when comparing the appearance of the patterns of corneal nerves demonstrated with each of the two cholinesterase stains (specific vs. nonspecific), that there is a difference in enzyme distribution. Pseudocholinesterase appears to demonstrate the large fiber bundles in the stroma. Specific cholinesterase appears to demonstrate these same large fiber bundles more intensely and, in addition, those finer fibers which are known to be bare axons. Even with prolonged incubation up to 24 hours, these finer fibers could not be seen with the pseudocholinesterase stain.

Recognizing this difference in enzyme distribution, it is reasonable to speculate that it might be related to differences in structure. Tewari and Bourne suggested that pseudocholinesterase distribution was related to the presence of myelin. Although the nerves entering the cornea do contain a myelin sheath, these sheaths extend only for a distance of 0.3 to one mm. from the limbus. Since we did not observe a sig-
significant difference in the concentration of enzyme present in nerves near the limbus as contrasted with those situated more centrally, another explanation for the enzyme variation was needed. Koelle stated that nonspecific cholinesterase of the ciliary ganglion was confined to the glial cells which surround the neurons and the Schwann sheath cells of the preganglionic and postganglionic trunks. In the rabbit cornea the Schwann sheath cells are known to be associated with the nerve fibrils when they are aggregated into bundles. Sometimes the Schwann cells also accompany single axons for a short distance when they travel alone in the stroma. However, for a large part of their terminal course in the stroma, the axons are naked. When the fibers terminate in the epithelium rather than in the stroma, the fiber bundles shed their sheaths of Schwann cells and proceed between the basal epithelial cells, frequently dividing into a leash of fibers as previously mentioned. These terminations of the Schwann sheath cells appear to correlate with the apparently abruptly terminating nerves demonstrated with pseudocholinesterase.

It seems quite possible then, at least in the rabbit cornea, that the presence of pseudocholinesterase is related to the presence of Schwann cells and that specific cholinesterase alone is present at the level of the axon. Further studies will be necessary to validate this speculation.

We wish to acknowledge the assistance of Mrs. Alice Hagen, who performed the laboratory staining techniques for this study.

REFERENCES