Table I. Calculated thickness of the epithelium (\(\mu m\)) from measurements of three subjects

<table>
<thead>
<tr>
<th>Subject</th>
<th>Observer</th>
<th>Session 1</th>
<th>Session 2</th>
<th>Subject's mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.E.M.</td>
<td>Mean</td>
<td>S.E.M.</td>
</tr>
<tr>
<td>D. H.</td>
<td>G.W.</td>
<td>54.5</td>
<td>0.7</td>
<td>52.5</td>
</tr>
<tr>
<td></td>
<td>D.O'L.</td>
<td>58.2</td>
<td>0.8</td>
<td>55.8</td>
</tr>
<tr>
<td>D. O'L.</td>
<td>D.H.</td>
<td>67.4</td>
<td>1.9</td>
<td>66.9</td>
</tr>
<tr>
<td>G.W.</td>
<td>G.W.</td>
<td>63.4</td>
<td>1.3</td>
<td>64.6</td>
</tr>
<tr>
<td></td>
<td>D.O'L.</td>
<td>64.7</td>
<td>1.7</td>
<td>66.7</td>
</tr>
<tr>
<td></td>
<td>D.H.</td>
<td>65.0</td>
<td>0.6</td>
<td>65.7</td>
</tr>
</tbody>
</table>

Each result is the mean of 10 measurements.

t_g = thickness of doubling plate (3.86 mm)
d = displacement after passing through the glass plate
M = magnification of slit by camera lens (0.144)
d' = displacement after passing through the camera lens
w = angle between slit and viewing system (60°)
v = angle between slit and fiduciary light (14°)
ne = refractive index of epithelium (1.49)
t_e = thickness of epithelium

For the sake of brevity, the above calculation does not include the radius of curvature of the cornea and, as a result, overestimates thickness by 0.3 \(\mu m\) for a 60 \(\mu m\) epithelium. This is an insignificant error compared with that due to uncertainty about the refractive index of the epithelium. A value of 1.49 was assumed in the calculation. If the true refractive index was as low as 1.38, a calculated epithelial thickness of 60 \(\mu m\) would be 5 \(\mu m\) too high.

Thickness of the epithelium was measured on three subjects. An assistant adjusted the doubling plate according to verbal instructions (+2°, −1°, +0.5°, etc.) given by the observer viewing into the biomicroscope and then noted the end point. By this method the observer had no knowledge of the magnitude of his settings until 10 end point determinations had been made. Later the 10 consecutive settings of the doubling plate were averaged, and the standard error was calculated. Later on, a further 10 measurements were made by a second observer. This procedure was followed for the three subjects to give a total of 60 measurements of epithelial thickness in the first session. The second session was run at least 48 hr after the first session and followed an identical procedure.

**Results and discussion.** The results for the two sessions are presented in Table 1. For any one subject, all the calculated thicknesses were similar even when measurements were made by separate observers. The mean value of all the 120 measurements was 62.1 \(\mu m\). Textbooks give a range of 50 \(\mu m\) to 100 \(\mu m\) for the thickness of the human corneal epithelium; however, we have been unable to find any published in vivo measurements of human epithelial thickness with which to compare our result.

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Key words: cornea, corneal epithelium, pachometry, specular microscopy

**Corneal endothelial autoradiography with the scanning electron microscope.** William H. Schutten, Diane L. Van Horn, Bonnie J. Bade, and Mary L. Faculjak.

A new technique for performing corneal endothelial cell autoradiography with the scanning electron microscope is presented. The scanning electron microscope is able to detect silver grains deposited over tritiated thymidine-labeled nuclei of regenerating corneal endothelium.

The corneal endothelium is a single layer of cells on the posterior aspect of the cornea which functions as a pump to regulate corneal stromal hydration in order to maintain corneal transparency. Although human corneal endothelium has little, if any, regenerative capacity, animals such as rabbits and cats are capable of varying degrees of corneal endothelial cell regeneration. In order to
Fig. 1. Scanning electron micrograph (upper) and light microscopic autoradiograph (lower) of the same group of $^3$H-thymidine–labeled kitten corneal endothelial cells 3 weeks after freeze injury. (Both ×500.)
has occurred. It is necessary to be able to determine accurately the extent to which cell division has occurred.

Autoradiography of incorporation of \(^3\)H-thymidine into nuclear DNA is a useful procedure for studying the regenerative capacity of the corneal endothelium. Because of established techniques for flat-mount preparations and the ease of analysis, light microscopy has been the preferred method for corneal endothelial autoradiography.

The use of light microscopy for autoradiography is somewhat limited by the magnification of the particular optical system being used. In order to increase the magnification of autoradiographic specimens, it has been suggested that scanning electron microscopy (SEM) be used to detect silver grains on the surface of a specimen.

In this paper, we present a method for detecting the uptake of \(^3\)H-thymidine in regenerating corneal endothelium by using autoradiography with the scanning electron microscope.

**Methods and materials**

**Transcorneal freezing.** Eight- to 10-week-old kittens were used to demonstrate this technique. Brass probes, machined to the curvature of the corneal surface were selected, which would destroy 25% of the corneal endothelium during transcorneal freezing. The brass probes were cooled in liquid nitrogen (\(-196^\circ\) C) and applied to the central region of the cornea for 15 sec. Prior to freezing, the eyes were anesthetized with topical application of 0.4% benoxinate HCl.

**Injection of \(^3\)H-thymidine.** \(^3\)H-thymidine was diluted in balanced salt solution to give a concentration of 1 \(\mu\)Ci of \(^3\)H-thymidine/0.01 ml of solution (20.0 mCi/mmol). Seventy-two hours after the transcorneal freeze, the corneas were again anesthetized with 0.4% benoxinate HCl. A 30-gauge needle was inserted through the limbus, and 2 \(\mu\)Ci of \(^3\)H-thymidine were injected into the anterior chamber.

**Flat-mount technique.** Three weeks after the freeze, all animals were sacrificed. The eyes were enucleated and placed in 10% neutral buffered formalin. After 2 weeks, the eyes were removed from the formalin and washed in tap water for 4 hr. The cornea was removed from the globe and placed in a container of warm (34° to 37° C) tap water for 5 min to promote swelling of the stroma. The cornea was then grasped with forceps while the stroma was grasped with another forceps, and Descemet’s membrane was stripped from the stroma by gently pulling the two forceps in opposite directions with one continuous motion. The stripped layer with Descemet’s membrane was placed endothelial side up on a microscope slide coated with gelatin and chrome alum. Four radial peripheral slits were made through the mount to allow it to lie flat. The slide was drained of excess water and placed on a warming plate (37° C) for 48 hr. Excess gelatin was removed from the slide by a series of ascending ethanol washes. The slide was then “defatted” in xylene, rehydrated back to distilled water, and air-dried for 24 hr.

**Autoradiography.** In a darkroom, the thin-mount slides were dipped in a 1:1 mixture of Kodak NT-B2 emulsion and distilled water. After the emulsion dried, the slides were placed in a light-free plastic slide box containing Dry-Air Humicaps. The slides were exposed for two weeks at 4° C.

After exposure was complete, the emulsion was developed in Kodak D-19 for 3 min, rinsed in distilled water, fixed with Kodak Rapid-Fix without hardener for 5 min, and washed in distilled water for 45 min. The slides were then stained with routine hematoxylin and eosin stain.

**SEM.** After staining, slides were air-dried for 24 hr in a dust-free container. They were subsequently sputter-coated with 60% gold–40% palladium and studied by means of SEM. After SEM study, each slide was cover-slipped and observed by light microscopy.

**Results.** The surface architecture of the central corneal endothelial wound area resulting from the transcorneal freeze could be well visualized with SEM. In addition, SEM revealed the granular nature of the silver particles overlying \(^3\)H-thymidine labeled cell nuclei (Fig. 1, upper). Light microscopy could be subsequently performed on the same region to again demonstrate the silver grains overlying cell nuclei (Fig. 1, lower).

**Discussion.** We have demonstrated that SEM can be used for detecting uptake of \(^3\)H-thymidine as well as for visualizing the surface architecture of regenerating corneal endothelium.

From the Departments of Ophthalmology and Physiology, The Medical College of Wisconsin, Milwaukee, and Research Service, Veterans Administration Center, Wood (Milwaukee), Wisc. This investigation was supported by NEI Research grant EY-01436, Ophthalmic Research Center grant EY-01931, the Veterans Administration, and Research to Prevent Blindness, Inc. Submitted for publication June 6, 1979. Reprint requests:
Acute effect of epinephrine on aqueous humor formation in the timolol-treated normal eye as measured by fluorophotometry. ROBERT G. HIGGINS AND RICHARD F. BRUBAKER.

A double-blind, randomized, placebo-control study of the effect of epinephrine on the rate of aqueous formation in eyes pretreated with a betaadrenergic blocking drug was carried out in 25 normal subjects with the use of fluorophotometry. All eyes were pretreated with timolol maleate before epinephrine or placebo was given. The effect of timolol maleate alone was to lower intraocular pressure and to reduce the rate of formation of aqueous humor. In the presence of timolol, aqueous formation was decreased further, approximately 7% more, in the epinephrine treated-eye as compared to the placebo-treated eye. This additional decrease in aqueous formation was statistically significant. No statistically significant difference was found in intraocular pressure or in tonographic C value between the epinephrine-treated and the placebo-treated eye. However, intracocular pressure was so low in both eyes that differences in intraocular pressure were not expected and tonographic tracings were difficult to interpret.

Epinephrine is an adrenergic agonist which is an effective ocular hypotensive agent in some subjects and is used as a topical treatment for glaucoma. Recently, it has been shown that the initial effect of epinephrine in the normal eye is to increase aqueous formation and increase aqueous humor outflow. The resultant effect, since the outflow effect is greater, is to decrease intraocular pressure. Timolol maleate is a beta-adrenergic blocker which is now used for treatment of chronic open-angle glaucoma. Topical application of this drug produces a rapid and prolonged lowering of intraocular pressure by decreasing production of aqueous humor.

The complex relationship between the adrenergic system of the eye and intraocular pressure in the human eye is not fully understood. Because epinephrine, a combined alpha and beta agonist, acts to increase aqueous formation and timolol maleate, a beta blocker, acts to decrease aqueous formation, we hypothesize that the initial action of beta agonists must be to stimulate the eye in some way to produce more aqueous humor. The purpose of this study was to see whether timolol was capable of blocking the early stimulatory effect of epinephrine on aqueous formation.

Methods. Twenty-five normal subjects were studied. An eye examination of each subject was carried out, consisting of best corrected visual acuity, external examination, slit-lamp examination, fundus examination (without dilation), and tonometry. The admission criteria were that both eyes were normal (except for ametropia) and that intraocular pressure of the two eyes differed by 3 mm Hg or less.

A commercially prepared solution of 0.5% timolol maleate (Timoptic; Merck, Sharp & Dohme, West Point, Pa.) and 1% epinephrine hydrochloride (Epiprin; Allergan Pharmaceuticals, Irvine, Calif.) were used along with an identical appearing placebo vehicle solution (Liquifilm; Allergan). The epinephrine/placebo bottle pairs were randomized by a hospital pharmacy to the right/left eye pairs of the subjects. The containers were identical and labelled as follows: "Subject 1, OD; Subject 1, OS; Subject 2, OD; Subject 2, OS; etc." The code was not known to the examiner until the data of all the subjects had been calculated. In determination of the effect of the drug, the treated eye of the subject was compared to the fellow placebo eye.

On the morning of the study, both eyes of the