several times with similar results. We have reported only the results obtained from the study of four lenses from two rabbits to emphasize the ability of this technique to obtain a great deal of information from a minimum amount of tissue. Very recently, analogous 31P-NMR measurements on the intact lens have been reported, thus affording a complementary approach to the study of lenticular metabolism. Similar studies are also underway in this laboratory.

From the Department of Chemistry, University of California, Santa Cruz, Calif., and the Howe Laboratory of Ophthalmology, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary, Boston, Mass. (J. A., L. T. C.). This work was supported by grants from the UCSC Faculty Research Committee, the Division of Natural Sciences at UCSC, the American Diabetes Association, and by the Estate of the late Marjory L. Davidson (to T. S.). L. T. C. was supported by National Institutes of Health grants EY-01276 and EY-03247. Submitted for publication Oct. 26, 1981. Reprint requests: Dr. Thomas Schleight, Department of Chemistry, University of California, Santa Cruz, Calif. 95064.

Key words: lens, cataractogenesis, 13C-nuclear magnetic resonance, diabetic cataract, sugar cataract

REFERENCES
Fig. 1. Exploded view of corneoscleral button and mounting collars with metric dimensions. A, Impaling of button by needles of thin collar; B, corneoscleral button, endothelial surface up; C, holding collar with receiving holes for the four needles. Alignment notches positioned for ease of penetration of needles into receiving holes. Orientation notch allows repositioning of corneoscleral button and the holding collar in the well as shown in Fig. 2.

maintained in McCarey-Kaufman (MK) medium held at 4°C until our observation.

An inverted Nikon SM microscope with a 10x objective modified for Hoffman modulation contrast observation (Modulation Optics, Inc., Greenvale, N. Y.) plus an extra-long working distance condenser were used to observe the unstained corneal endothelium.

During the standardization experiments to define the morphologic changes seen before and after vital staining, a location device and relocation as described below were used. The holder for the excised corneoscleral button was manufactured out of acetal resin (Delrin) and machined according to the specifications on Figs. 1 and 2. As shown in Fig. 1, the donor cornea (B) was impaled at its scleral ring with four needles protruding from a thin collar (A) that fitted a thicker vertical collar (C), securing the button in a manner similar to that described by Krohn and Breitfeller. An orientation notch on both collars A and C allowed the needles to be aligned with the corresponding holes in collar C. The endothelial surface faced collar A, and at all times the endothelium was bathed in MK medium.

The corneoscleral button, in position between the collars, was then inverted with endothelial surface down into an acrylic (Plexiglas) well (Fig. 2) filled with MK medium. If necessary, the collar and cornea were tilted slightly to allow escape of air bubbles caught in the concavity of the endothelial surface. The collar with impaled button was then fitted snugly into a recess in the floor of the Plexiglas well, collar A down, with an orientation notch aligned with a corresponding notch in the well floor, with the entire cornea submerged in the MK medium. The base of the Plexiglas well, through which the endothelium was to be observed, was a thin coverslip glass only. The entire well was then placed firmly in the calipers of an XY micrometer stage on the inverted microscope.

By the technique described by Hoffman, maximal morphologic details of the endothelial surface were obtained. Focus on the endothelial surface was then obtained by centering the corneal button over the objective lens and racking the objective lens up toward the endothelial surface and focusing sequentially on the two surfaces of the coverslip, through the MK medium, and on to the endothelial surface. Because of the curvature of the endothelial surface, the entire field was in focus only if the central area of endothelium was viewed. When moving the corneal button to one side or the other, a continuous movement with the focusing knob allowed only a segment of the endothelial surface to be in focus at any one time.

Photographs of representative areas of the endothelium were obtained with a Nikon microscopic attachment and Tri-X film. After the position of the X and Y scales of the micrometer stage were recorded, the well was removed from the microscope and the collar with sandwiched cornea was inverted in a Petri dish with endothelial surface upward. With the staining technique of Spenz and Peyman the endothelial surface was vitally stained with trypan blue and alizarin red. An initial rinse of the endothelial surface with normal saline resulted in better vital staining.

The collar with sandwiched cornea was again introduced, in a fashion similar to that previously described, into the well with the orientation marks on the collar and the well base again aligned. The entire well was then repositioned in the same orientation in the calipers, and the X and Y coordinates noted previously during photography were reset. A similar focusing method was used, and photographs were obtained of the resultant areas within 5 min of the vital staining.

In addition, endothelial trauma was inflicted on some relatively normal corneoscleral buttons by
Fig. 2. Exploded view of Plexiglas well with metric dimensions (D and E) for receiving corneoscleral button (B) sandwiched between collars (A and C). The inverted button in its collar is placed into the recessed receiving hole in the well. The orientation notches are aligned for exact repositioning before and after staining. E, A cover glass is glued to the bottom of the well for optimal microscopic viewing.

scraping the surface with a 1 mm plastic pipette tip. These corneas were examined in the same manner as the other donor buttons. The photographed areas obtained prior to staining and after staining were then compared.

**Results.** Examination of the unstained corneal endothelium in these donor buttons revealed that the endothelial surface was generally a rather featureless plane, with underlying stromal or Descemet's striae apparent. Individual endothelial cell boundaries or intracellular details were generally not visible. However, in some sections of the cornea, indistinct cell boundaries, apparent cell nuclei, and areas of granularity could be observed. The endothelial surface could effectively be viewed to within 1 to 2 mm of the scleral rim. In corneas that had been stored for longer periods of time, it was generally possible to see more of the morphologic details of the individual endothelial cells.

The use of our relocation technique permitted examination of exactly the same field of endothelial surface before and after the vital staining. This was confirmed by the unique characteristics of stromal or Descemet's structure visible through the normal endothelial layer, as well as by small unique areas of abnormality (Fig. 3). Once the general endothelial area was found by the relocating technique, areas of changes noted in the unstained endothelial layer could be compared “cell for cell” with the stained cells by visually relocating these cells by their characteristic morphologic appearance in the general field of view.

It was evident from vital staining that most corneal endothelial layers that appeared as featureless and on occasion slightly granular in the unstained examination were seen after staining to consist of apparently normal and vital endothelial cells. Areas of apparent “nuclear” shapes in the unstained preparation correlated well with areas of later nuclear staining with trypan blue. This would indicate damage to the cell membrane3 (Fig. 4).

Areas of endothelium were purposefully debrided of cells by mechanical means. These areas also appeared devoid of detail and similar to those endothelial areas containing normal cells. However, small areas of such total endothelial loss were generally even smoother and less granular than areas of normal endothelium. In addition, the mechanical trauma involved in removing these endothelial cells, e.g., swab debridement and surgical instrument removal, generally caused a roughened but distinct interface of damaged cells between the areas totally devoid of endothelial cells and areas of relatively normal endothelium. After a 1 mm plastic pipette tip was gently scraped on the endothelial surface of a corneoscleral button, the relocation technique was used to photograph the entire area before and after staining. The resultant montages are illustrated in Fig. 5. The unstained defect is demarcated from normal surrounding endothelium by a ridge of damaged
Fig. 3. A, HMCS photomicrograph of unstained corneal endothelial surface with stromal striae (A and B). B, HMCS photograph of exactly the same endothelial surface area after staining with trypan blue and alizarin red. Points A and B reveal the same underlying stromal identifying points as those in A. (×174.)
Fig. 4. A, HMCS photomicrograph of unstained corneal endothelial surface with "pock" marks (arrow) of nuclei of damaged endothelial cells. B, HMCS photomicrograph of exactly the same area as that in A after staining with trypan blue and alizarin red. Trypan blue uptake in nuclei of damaged cells (arrow) reveals identical configuration with "pock" marks of A. Note surrounding endothelial cells, although well outlined with alizarin red, are viable as indicated by absence of trypan blue uptake. (Trypan blue and alizarin red; ×177.)
Fig. 5. For legend see facing page.
endothelium (asterisks). Similarly, a small area of residual normal endothelium within the entire defect is demarcated from the surrounding denuded Descemet's membrane by another ridge interface (solid black arrows). Within the denuded area small islands of residual damaged endothelial cells can be seen (numeral 1) and cell fragments can be noted (numeral 2). Striae of damaged cells radiating from this denuded area, implying cell damage, can also be detected (brackets).

The components of the same areas of endothelial surface after vital staining confirm the significance of the features in the montage of the unstained defect.

**Discussion.** The observation of the unstained endothelium in excised human corneas is restricted at present to the use of the specular microscope. This permits the viewing of individual endothelial cell boundaries and also allows correlation between cellular morphologic appearance and the endothelial function in the area as measured by serial pachometry. To date, the only attempt to view individual cells in excised corneal tissue by specular microscopy with comparison of the same cells by other histologic techniques has been the work of Sherrard.

We have found that by using the Hoffman modulation contrast system (HMCS), which is designed to maximize the surface morphologic relief of unstained corneal layers, we could observe features of corneal endothelium in the excised human cornea.

By the use of the relocation technique described above and photography before and after vital staining, a comparison could be made of the structure of the unstained corneal endothelium in relation to exactly the same areas after vital staining.

The staining and relocation procedures revealed that areas of unstained normal endothelium appeared as a nearly featureless surface when viewed by the HMCS, whereas areas of minimal endothelial disturbance (as confirmed by trypan blue uptake) were readily observed in the contrast system as nuclear-like shapes in the featureless plane of endothelium. Appreciation of areas of Descemet's totally devoid of endothelial cells is difficult with this technique. Relatively small areas of mechanically debrided endothelium will generally appear featureless but will be separated from the normal endothelial layer by a roughened interface consisting of damaged cells that surround the area of debrided endothelium.

The use of this new relocation technique allows correlation of the morphologic characteristics of specific endothelial cells with the results of vital staining of these same cells. Thus a glossary of endothelial changes observed in the unstained cornea was developed in relation to endothelial cell viability. This glossary could then be applied to other corneas, thus permitting the interpretation of endothelial viability in a noninvasive fashion, requiring no manipulation or staining procedures.

The described relocation technique used in laboratory experiments may permit investigation of endothelium in different media, pH, etc., by the correlation of stained and unstained morphologic appearance.

From the Wilmer Institute, Johns Hopkins Hospital, Baltimore, Md., and Adelphi University, Garden City, N. Y. (R. H.). This study was supported in part by NEI grant EY 02476-01 (L. W. H.), NEI grant EY 01765-05, and by a research grant from the Medical Eye Bank, Inc., of Baltimore, Md. Presented at the Annual Meeting of ARVO, Sarasota, Fla., 1981. Submitted for publication July 21, 1980. Reprint requests: Lawrence W. Hirst, M.D. The Wilmer Institute, The Johns Hopkins Hospital, 600 N. Wolfe St., Baltimore, Md. 21205.

**Key words:** corneal endothelium, MK medium, vital staining, Hoffman modulation contrast system, relocation technique

**REFERENCES**


---

**Fig. 5.** A, HMCS photomicrograph of unstained corneal endothelial surface montaged from 12 adjoining fields of the corneal endothelial surface after damage. Features include an interface between areas marked by asterisks enclosing area of Descemet's denuded of cells in whose center is a small residual island of normal cells marked by arrows. Numeral 1 illustrates area of residual damaged cells, while numeral 2 indicates cellular remnants (enclosed areas of damaged cells in the otherwise normal endothelial layer outside the induced defect). B, HMCS photomicrograph of exactly the same area montaged from 12 adjoining fields after trypan blue and alizarin red staining. Features noted in A are confirmed by vital staining with trypan blue throughout the entire central defect apart from the area of residual cells marked by the arrow. Numeral 1 and the areas enclosed by brackets take up trypan blue to indicate cellular damage, as do the areas of roughened interface marked by the asterisks. (×62.)
Kinetics of ocular herpes simplex virus shedding induced by epinephrine iontophoresis. BYOUNG S. KWON, LOUIS P. GANCAROSA, SR., KEITH GREEN, AND JAMES M. HILL.

Ocular herpes simplex virus type 1 (HSV-1) shedding induced by epinephrine iontophoresis was characterized by determining the titers and kinetics of viral appearance in the tear film. HSV-1 in the tear film was recovered by washing the ocular surface with Dulbecco's phosphate-buffered saline containing 2% albumin. Epinephrine (0.01%) iontophoresis (0.8 mAmps for 8 min) was performed once a day for 3 consecutive days. The frequency of induced shedding was 90% of eyes and 100% of rabbits (0.01%) iontophoresis (0.8 mAmps for 8 min) was performed once a day for 3 consecutive days. The frequency of induced shedding was 90% of eyes and 100% of rabbits at 90 days after inoculation. Seventy-eight percent of virus were as follows: 70% on day 2, 90% on day 3, 80% on day 6, 70% on day 4, 70% on day 5, 40% on day 6, and 10% on day 7 after the first epinephrine iontophoresis. The mean titer of wash of HSV in the tear film increased gradually from 6.4 × 10^6 plaque-forming units (PFU)/ml) to 2.7 × 10^7 PFU (day 4) and then decreased to 3 × 10^5 PFU, remaining relatively constant until virus was not detected. The lowest titer was obtained on the first day of shedding (day 2 after the initiation of epinephrine iontophoresis), and the highest titer was obtained on the third day of shedding (day 4 after the initiation of epinephrine iontophoresis).

The duration of induced shedding averaged 4 days. The results indicate a highly reliable animal model for the induced appearance of HSV-1 into the tear film. This model can be used for the elucidation of basic mechanisms of the HSV latency-reactivation-recurrence processes. (INVEST OPHTHALMOL VIS SCI 22:818-821, 1982.)

We have recently developed a model for herpes simplex virus (HSV) shedding and reactivation in rabbit eyes. The induction of ocular HSV type 1 (HSV-1) shedding from latently infected rabbits was achieved by administering epinephrine into rabbit eyes via iontophoresis. We have also shown that during the induced shedding there was a reactivation of HSV in the rabbit neural tissues. The characterization of the model is necessary for further studies on the elucidation of basic mechanisms of the HSV latency-reactivation-recurrence processes. The present study was designed to quantitate kinetically the HSV appearance in the tear film after epinephrine iontophoresis.

Materials and methods.

Animals. New Zealand white rabbits were used. Viruses and cells. HSV-1 McKrae strain (1 × 10^6 plaque-forming units [PFU]/ml) was used throughout the experiment. Viruses were propagated and titrated on primary rabbit kidney cell (PRK) monolayers. Inoculation of HSV into rabbit eyes, ocular disease severity, induction of HSV shedding, assay of virus in tissues, and identification of viral isolates have been described previously. 1-3

Detection and titration of HSV shedding

Eye wash. Rabbit eyes were opened by spreading the upper and lower eyelids. An aliquot (0.1 ml) of wash medium consisting of Dulbecco's phosphate-buffered saline plus 2% bovine serum albumin was instilled into eyes and the eyelids were closed and massaged. The wash medium (0.5 ml) was used to rinse the cornea while the upper and lower eyelids were held open. The medium was collected with a 1 ml disposable syringe. The procedure (rinse and collection) was repeated three times with a total of 1.5 ml of wash medium.

Viral detection. An aliquot (0.2 ml) of the collected wash medium was inoculated onto a PRK monolayer. The remaining eye wash was stored at -75° C for later use in viral titration. When specimens were positive for HSV, titration was performed by means of a plaque assay.

Experimental design. Latently infected rabbits that shed HSV spontaneously, at least once, from both eyes between 40 and 60 days after inoculation were selected. The spontaneous sheddings were determined by an eye swab procedure as previously described. 1 These rabbits were eye-washed between 60 and 78 days after inoculation to detect spontaneous shedding by the procedure described below. The eyes of five rabbits, randomly selected from all rabbits positive in both eyes by eye swab and eye wash, were then treated with epinephrine iontophoresis (0.01% epinephrine, 0.8 mAmps for 8 min) daily for 3 consecutive days, beginning at 90 days after inoculation. Eye washes were taken...