

# EFFECT OF THIOL-OXIDATION OF GLUTATHIONE WITH DIAMIDE ON CORNEAL ENDOTHELIAL FUNCTION, JUNCTIONAL COMPLEXES, AND MICROFILAMENTS

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## ABSTRACT

Intracellular-reduced glutathione (GSH) was removed by thiol-oxidation with diamide during *in vitro* perfusion of the corneal endothelium. By 15 min the normal mosaic-like pattern of the endothelial cells was disrupted by serpentine-like lines of cell separation at the cell junctions. After 45 min of perfusion, individual clusters of cells formed cup-shaped islands. The resultant exposure of Descemet's membrane to the perfusion solution resulted in corneal swelling. Transmission electron microscopy revealed that the endothelial cells separated at the apical junctions and that the microfilaments in the apical cytoplasm of the cells formed dense bands, whereas the other subcellular organelles were normal in appearance. The change in cellular shape may be due to loss of cellular adhesion which results in the condensation of the microfilaments or contraction of the microfilaments. The addition of glucose to the perfusate prevented the diamide effect, and the diamide effect could be reversed upon removal and perfusion of a glutathione bicarbonate Ringer's solution. These results suggest that the ratio of reduced to oxidized glutathione in the endothelial cells plays a role in the maintenance of the endothelial cell barrier function.

Although the tripeptide glutathione is present in most living cells, its physiological and biochemical roles have not been clearly defined because it serves as a source of free sulfhydryls and has many interactions with cellular functions. For example, a role for glutathione has been implicated in the prevention of peroxide formation after cell irradiation, the oxidation of drugs by liver enzyme systems, the function of membrane ATPase and enzymes in the glycolytic cycle, and the maintenance of membrane structure. During *in vitro* perfusion of the corneal endothelium, the addition

of reduced glutathione (GSH) to a bicarbonate Ringer's solution has been shown to maintain endothelial ultrastructure (10), to increase the efficiency of the endothelial pump (4), and to prevent depletion of cellular ATP (1).

Diamide, a thiol-oxidizing agent, which stoichiometrically oxidizes intracellular glutathione to the disulfide (GSSG) (9), has been used in other cell systems to study the temporary effects of removal of GSH. For example, in lens (which normally contains high concentrations of GSH), diamide produces a decrease in the activity of the

cation pump and an increase in the permeability of the membranes of the lens fibers (5).

The endothelial cells of the cornea form a monolayer on the posterior surface of the cornea and function both as a barrier to the movement of water from the aqueous humor into the stroma and as a "pump" which actively maintains corneal water content by the active transport of ions across the endothelium from the cornea to the aqueous humor. Increases in corneal thickness due to fluid imbibition by the stroma can be measured during *in vitro* perfusion and is directly related to loss of endothelial function (10).

The purpose of the present study was to remove GSH in corneal endothelial cells (by oxidation with diamide during *in vitro* perfusion) in order to determine the effects on endothelial function and ultrastructure.

## MATERIALS AND METHODS

Paired eyes from albino rabbits (2–3 kg) were excised and the corneas were mounted in a dual-chambered specular microscope which permits constant perfusion of the endothelium, sequential measurements of corneal thick-

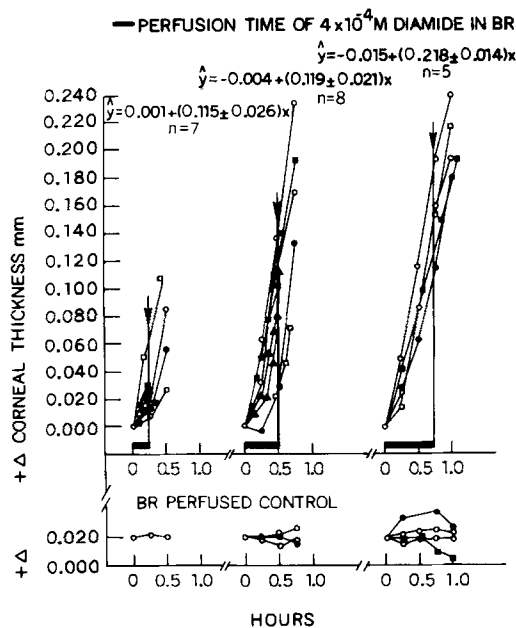


FIGURE 1 Changes in corneal thickness of rabbit corneas perfused with  $4 \times 10^{-4}$  M diamide in bicarbonate Ringer's (BR) or BR alone for 0.25, 0.5, and 0.75 h. After diamide perfusion, the corneas were perfused with BR (arrows). Equations are calculated regression lines for the rate of swelling during exposure to diamide for each time period.

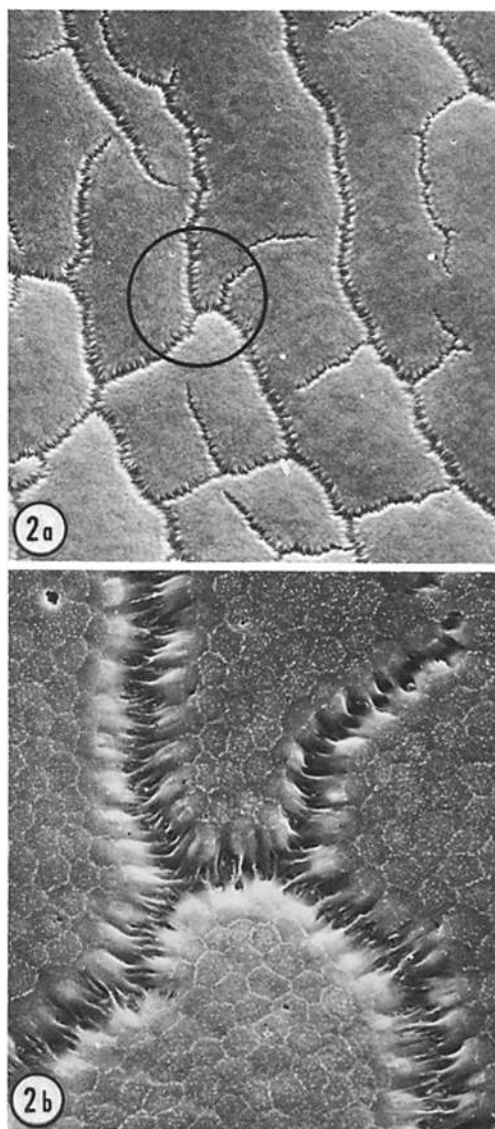


FIGURE 2 SEM of rabbit corneal endothelium after perfusion with  $4 \times 10^{-4}$  M diamide for 15 min. (a) The mosaic pattern of the endothelial monolayer is disrupted by serpentine-like lines of cell separation exposing Descemet's membrane.  $\times 90$ . (b) Higher magnification of the circled area. Many cellular processes can be seen spanning the areas of bare Descemet's membrane.  $\times 400$ .

ness, and continuous observation of the mosaic-like pattern of the endothelial monolayer (10). The isolated corneas were perfused at  $35\text{--}36^\circ\text{C}$  and 15 mm Hg pressure with either bicarbonate Ringer's solution (BR); BR plus glucose (GLU) 0.90 g/liter; BR plus  $4 \times 10^{-4}$  or  $10^{-5}$  M diamide (Calbiochem, La Jolla, Calif.); or BR

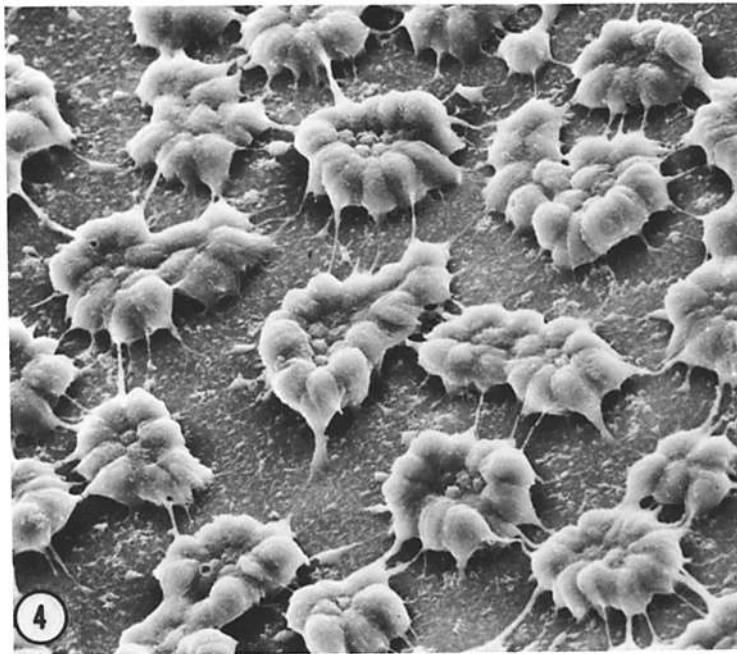
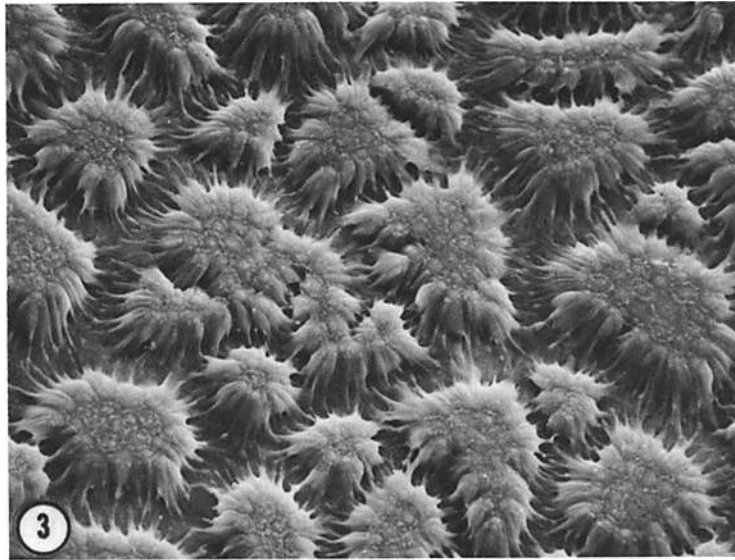


FIGURE 3 SEM of rabbit corneal endothelium after perfusion with  $4 \times 10^{-4}$  M diamide for 30 min. Clusters of cells form flat islands connected by fewer processes resulting in exposure of larger areas of Descemet's membrane.  $\times 400$ .

FIGURE 4 SEM of rabbit corneal endothelium after perfusion with  $4 \times 10^{-4}$  M diamide for 45 min. The endothelial cells form cup-shaped islands in which the peripheral cells are lifted off of Descemet's membrane. Only a few thin cellular processes extend between the islands.  $\times 500$ .

plus GLU plus  $4 \times 10^{-4}$  or  $10^{-5}$  M diamide. The composition of the bicarbonate Ringer's solution was NaCl (6.521 g/liter), KCl (0.358 g/liter),  $\text{CaCl}_2$  (0.115 g/liter),  $\text{MgCl}_2$  (0.159 g/liter),  $\text{NaH}_2\text{PO}_4$  (0.103 g/liter), and  $\text{NaHCO}_3$  (2.454 g/liter).

For the reversal studies, corneas were perfused with  $4 \times 10^{-4}$  M diamide in BR until serpentine-like lines of cell separation appeared in both corneas of a pair (15–30 min). At this time, one of the corneas was fixed for scanning electron microscopy (SEM) and transmission

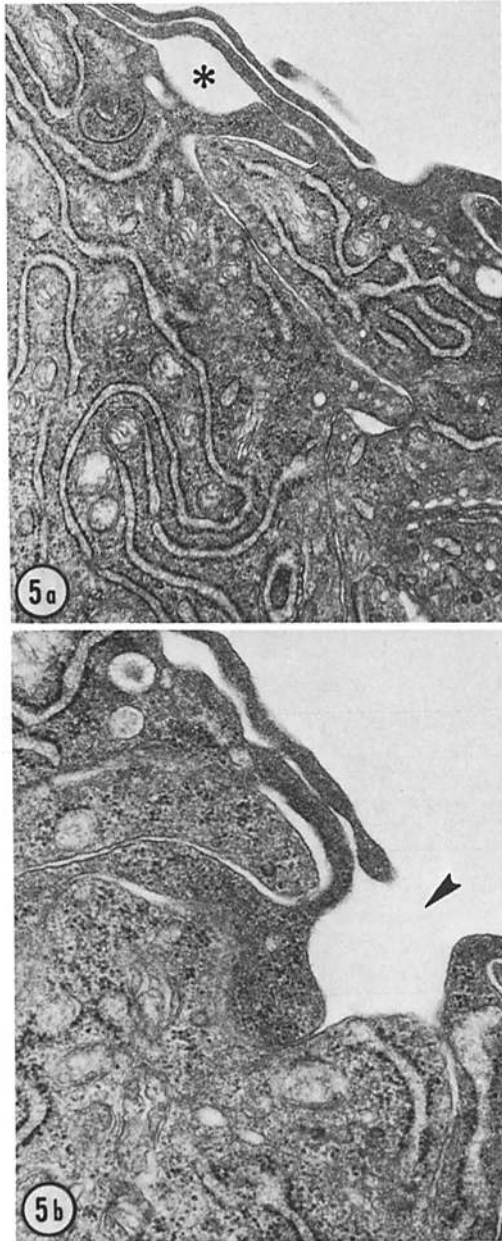


FIGURE 5 TEM of apical portion of junction between endothelial cells after exposure to  $4 \times 10^{-4}$  M diamide for 15 min. The intercellular space is focally dilated (\*) in the junction seen in (a). In (b), the cells are beginning to separate at the apical junction (arrow). (a)  $\times 19,000$ ; (b)  $\times 27,400$ .

electron microscopy (TEM). The diamide solution was removed from the other cornea and replaced with a glutathione bicarbonate Ringer's solution (GBR) which had the same chemical composition as BR with the

addition of 0.90 g/liter glucose, 0.133 g/liter adenosine, and 0.92 g/liter reduced glutathione. The corneal epithelium in all experiments was intact and covered with medical grade silicone oil (no. 20 CSKS Dow Corning Corp., Midland, Mich.).

At selected times during the perfusion, corneas were fixed for SEM and TEM in 3% glutaraldehyde (or 2% osmium tetroxide) in phosphate buffer (pH 7.2, 330 mOsm). For TEM, pieces from half of each cornea were flat-embedded in a low viscosity epoxy resin. Thin sections were cut with a diamond knife on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.) and viewed in an RCA-EMU-4C transmission electron microscope. For SEM, hot acetone was used to wash the resin from the endothelial surface of the other half of each cornea before polymerization (2). After polymerization, the specimens were coated rotationally with carbon and gold palladium metal, and viewed with an AMR-1000 scanning electron microscope.

## RESULTS

Perfusion of the corneal endothelium with  $4 \times 10^{-4}$  M diamide resulted in corneal swelling (Fig. 1) and loss of the normal mosaic pattern of the endothelial monolayer (Fig. 2). The corneas increased in thickness at a mean rate of  $115 \mu\text{m/h}$  during a 0.25- or 0.5-h perfusion; however, the mean rate of corneal swelling increased to  $218 \mu\text{m/h}$  when the endothelium was perfused with diamide for 0.75 h. Control corneas perfused with BR alone or BR plus GLU did not increase in thickness during similar time-periods (Fig. 1) and did not show alterations in the mosaic-like pattern of the endothelium or in the intracellular organelles.

SEM revealed that by 15 min the normal mosaic pattern of the endothelial monolayer was disrupted by serpentine-like lines of cell separation exposing Descemet's membrane (Fig. 2 a). At higher magnification, thin cellular processes could be seen spanning the bare areas of Descemet's membrane (Fig. 2 b). After 30 min of perfusion with  $4 \times 10^{-4}$  M diamide, clusters of cells formed flat islands (Fig. 3), and after 45 min the islands of cells were cup-shaped (Fig. 4).

TEM revealed that after 15 min of perfusion the endothelial cells were beginning to separate at the apical junctions (Fig. 5 a, b), and the microfilaments appeared to be condensed in the apical cytoplasm of the endothelial cells (Figs. 6 and 7). After 30 min, there was a change in nuclear and cellular shape in the peripheral cells of each island (Fig. 6). By 45 min the cells in the periphery of the islands could be seen to be lifted off of Descemet's

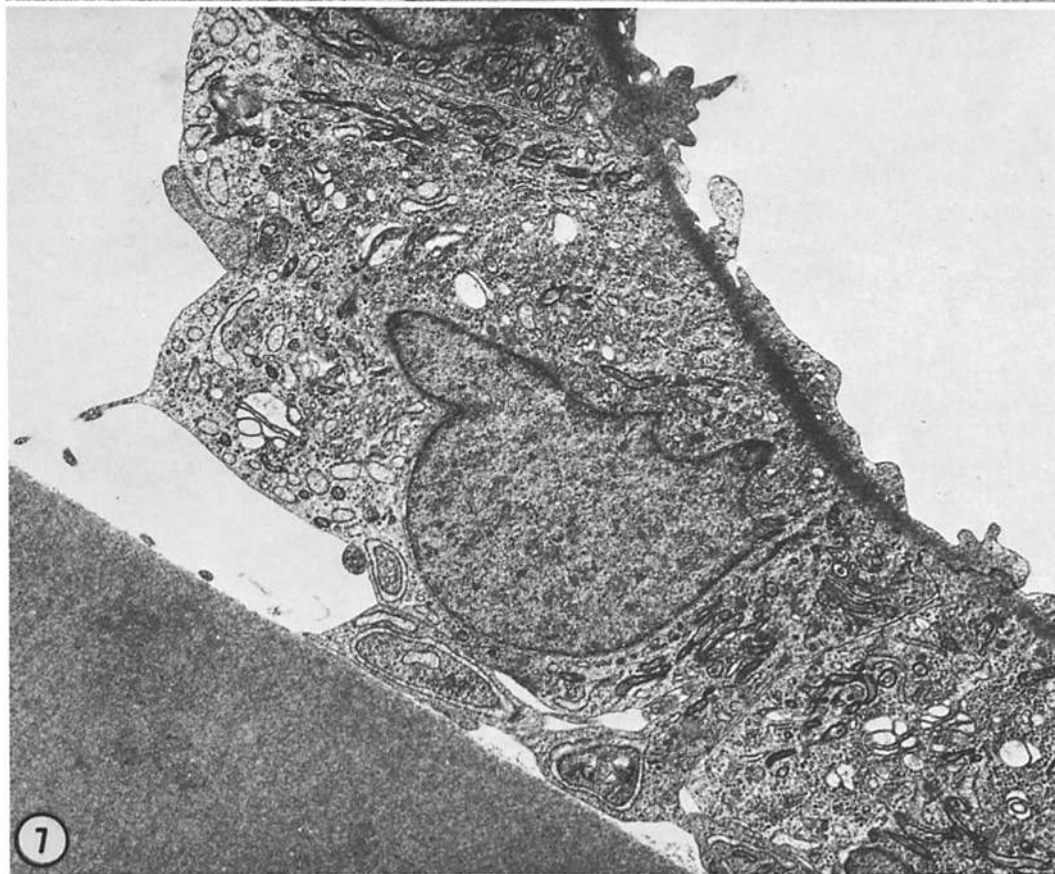
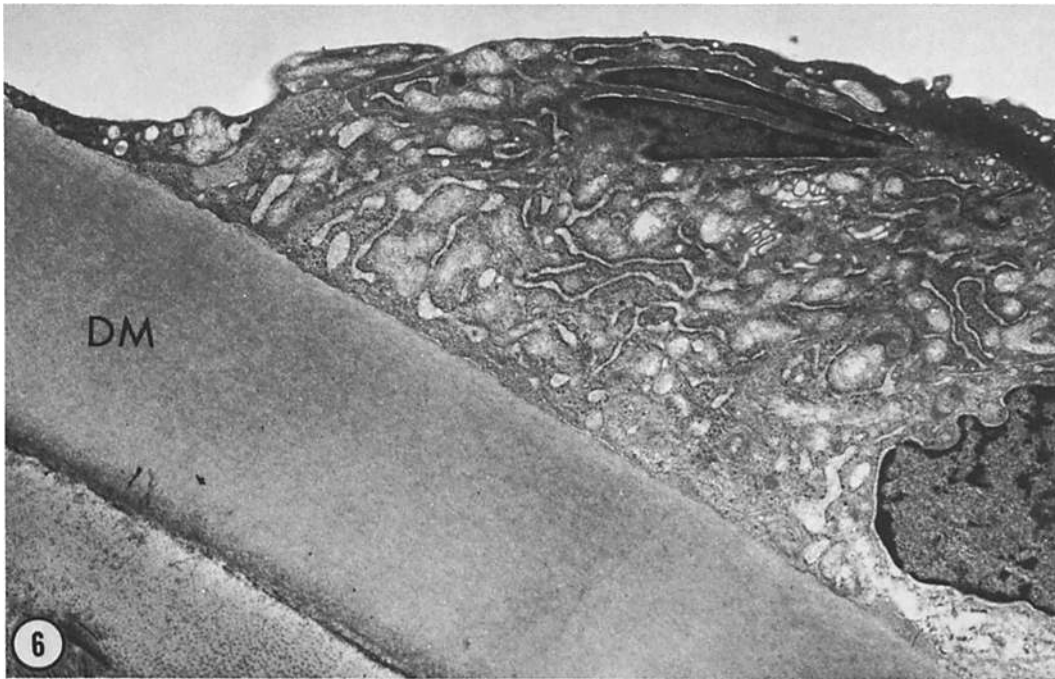


FIGURE 6 TEM illustrating distortion of cellular and nuclear shape in an endothelial cell in the periphery of an island after 30 min of exposure to  $4 \times 10^{-4}$  M diamide. The microfilaments in the apical cytoplasm of the cell are condensed. Other subcellular organelles are normal. A cell with a normal shaped nucleus is at right. A thin cell process covers Descemet's membrane (DM) on the left side.  $\times 13,000$ .

FIGURE 7 TEM of cells in the periphery of a cup-shaped island in a cornea exposed to  $4 \times 10^{-4}$  M diamide for 45 min. The microfilaments form a prominent and continuous band, and the apical cytoplasm is convoluted. The most peripheral cells are lifted off of Descemet's membrane. Some subcellular organelles are swollen.  $\text{OsO}_4$  fixation.  $\times 13,000$ .

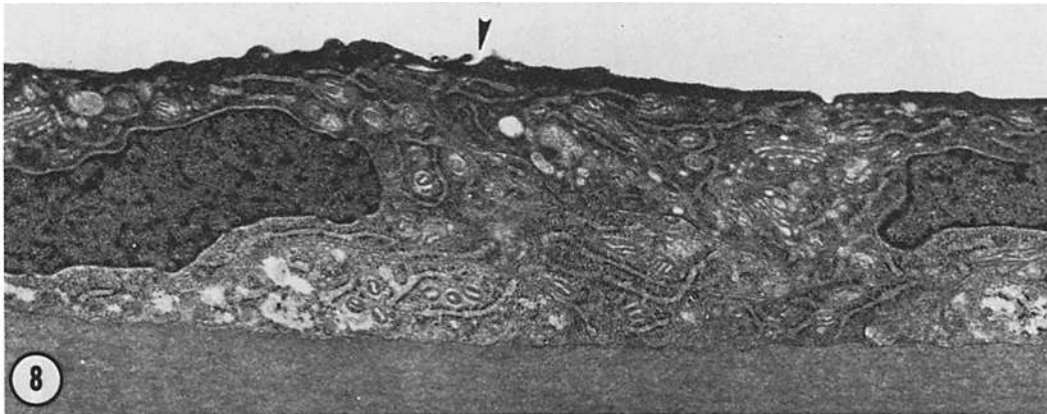


FIGURE 8 TEM of control cornea perfused 0.75 h with bicarbonate Ringer's. Apical end of intercellular junction is tight (arrow); the terminal web area of cytoplasm is free of other organelles.  $\times 12,700$ .

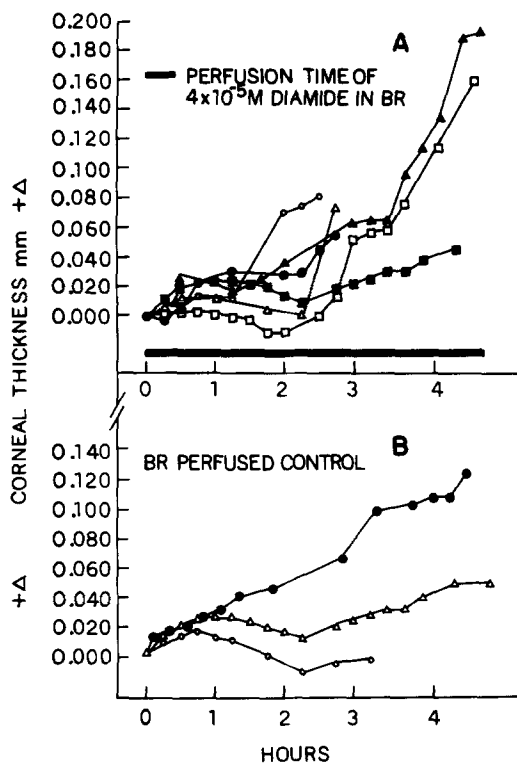


FIGURE 9 Changes in corneal thickness of rabbit corneas perfused with  $4 \times 10^{-5}$  M diamide in bicarbonate Ringer's (BR) or BR alone.

membrane forming the lip of the cup-shaped islands. The microfilaments within these cells formed a prominent band, and the apical cytoplasm was convoluted (Fig. 7). Other subcellular organelles were similar in appearance to those in

control tissue (Fig. 8) in both the glutaraldehyde- and osmium tetroxide-fixed tissues. In contrast to these junctional changes that occurred with diamide perfusion, control BR-perfused corneas had intact junctions and a flat continuous plasma membrane.

Continuous perfusion of  $4 \times 10^{-5}$  M diamide in BR produced a slower increase in corneal thickness (Fig. 9) than did  $4 \times 10^{-4}$  M diamide; however, the rate of swelling increased rapidly after 3 h, and the endothelial cells at the end of that time were clustered in flat islands (Fig. 10 a). Condensation of the microfilaments was again observed by TEM. Although the control corneas perfused with BR alone gradually increased in thickness (Fig. 9 b), they did not have the rapid increase in thickness after 3 h and the normal mosaic pattern (Fig. 10 b) and ultrastructural appearance of the endothelial cells was maintained.

The addition of glucose to BR containing  $4 \times 10^{-4}$  M diamide reduced the rate of corneal swelling during 3 h of continuous perfusion (Fig. 11), and endothelial integrity was maintained for about 2 h (Fig. 12 a). After that time, some cellular swelling was observed by SEM (Fig. 12 b). The addition of glucose to BR containing  $4 \times 10^{-5}$  M diamide completely prevented corneal swelling during 5 h of continuous perfusion (Fig. 11), and the integrity of the endothelial monolayer was maintained during that time period (Fig. 12 c). TEM of these corneas showed that normal endothelial ultrastructure was maintained except for cytoplasmic swelling in some cells (Fig. 13).

In the final set of experiments, paired corneas



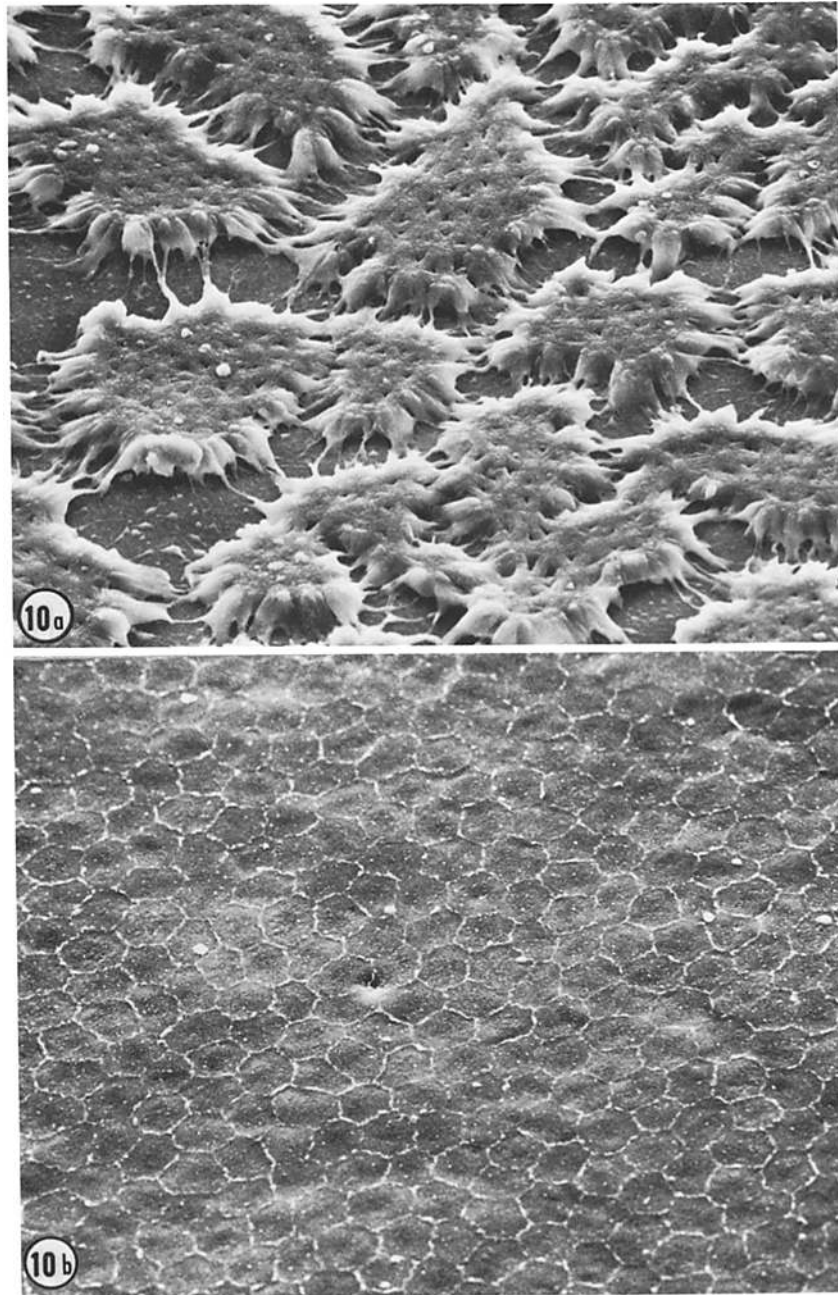


FIGURE 10 (a) SEM of rabbit corneal endothelium exposed to  $4 \times 10^{-5}$  M diamide for 4 h. The endothelial cells are clustered in flat islands connected by a few cellular processes. Large areas of Descemet's membrane are exposed.  $\times 500$ . (b) SEM of control rabbit corneal endothelium exposed to bicarbonate Ringer's solution for 4 h. Endothelial cells have maintained the normal mosaic pattern.  $\times 500$ .

were perfused with diamide until the endothelial cells separated from each other and the corneas began to increase in thickness (Fig. 14, lower curve; Fig. 15, upper). The diamide effect was reversed in the paired corneas by continuous perfusion of GBR. Corneal thickness stabilized after 1 h and began to decrease after 2 h of perfusion (Fig. 14, upper curve). At the same time,

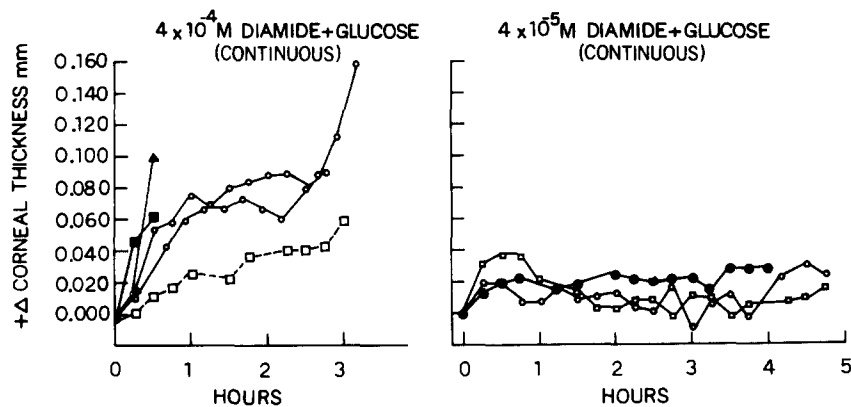


FIGURE 11 Changes in corneal thickness of rabbit corneas during continuous perfusion with  $4 \times 10^{-4}$  M or  $4 \times 10^{-5}$  M diamide plus glucose.

the normal mosaic-like pattern of the endothelial monolayer was restored (Fig. 15, lower). The junctional complexes reformed and the microfilaments were no longer condensed (Fig. 16).

#### DISCUSSION

These results demonstrate that during *in vitro* perfusion of the corneal endothelium with diamide (a thiol-oxidizing agent which is supposed to be specific for intracellular glutathione), loss of cellular adhesion in a serpentine-like pattern occurs, the microfilaments in the endothelial cells become condensed, and there is a change in cell shape. After 30 and 45 min, the cells form islands and cup-shaped clusters. Because of the breakdown of the endothelial barrier and the exposure of Descemet's membrane to the perfusion solution, the corneas swell at a rapid rate. All these effects could be prevented by the addition of glucose to the perfusion solution and reversed by the removal of diamide.

The change in cell shape may be due to loss of cellular adhesion or contraction of the microfilaments in the apical endothelial cytoplasm. This condensation of the microfilaments may be secondary to the loss of cellular adhesion, which results in the corneal swelling, and the microfilaments become condensed due to the cells balling up from the elasticity of the microfilaments.

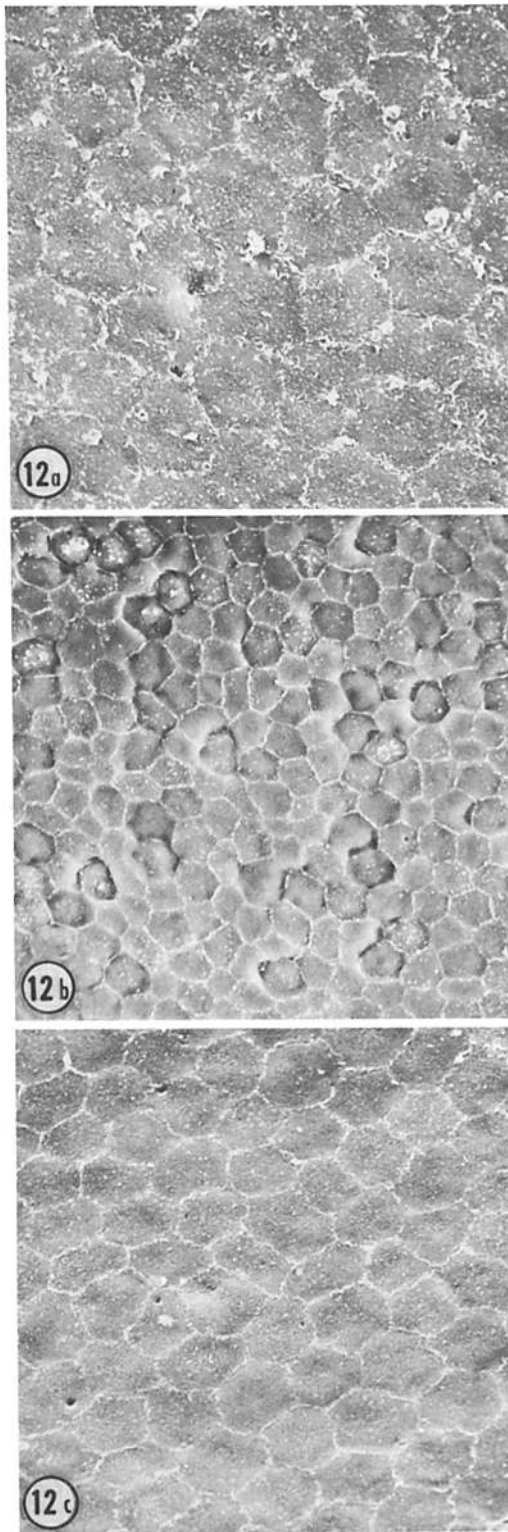
Alternatively, the microfilaments may be induced by diamide treatment to contract with such force that intercellular adhesions are disrupted. It was not possible to distinguish between these two possibilities, because both junctional disruption and microfilament condensation were present as early as 15 min after diamide perfusion.

In other studies of experimentally treated corneas, it has been shown that microfilaments may or may not be associated with disruption of cell adhesion. During perfusion with a calcium-free medium the corneal endothelial cells take on a cobblestone appearance due to disruption of the apical microfilament network resulting in breakdown of the intercellular junctional complexes (6, 7), whereas during perfusion with cytochalasin B there is also a focal disruption in the apical microfilament network resulting in cytoplasmic bulging. However, the endothelial junctions retained their morphologic integrity despite the attenuated cytoplasm (8). In neither of these cases of microfilament disruption were cup-shaped islands formed: the corneas increased in thickness, but at a slower rate than the diamide-perfused corneas.

The level of intracellular-reduced glutathione is normally maintained by the NADPH generated by the direct oxidation of glucose through the pentose shunt (3). The reduction of the diamide effect by the presence of glucose in the perfusion solution may be due to the increased production of NADPH in this pathway. In the lens, the level of intracellular GSH has been reported to be maintained as long as glucose is present in the medium, and the effects of diamide on membrane permeability and the cation pump are also minimized (5).

Although the addition of glucose to the diamide medium prevents the breakdown of the endothelial barrier and condensation of the microfilament network in the corneas perfused with  $4 \times 10^{-4}$  M diamide, the corneas increase in thickness at a rate of approximately  $40 \mu\text{m}/\text{h}$ . This rate of swelling is equal to the passive fluid movement which occurs





after inhibition of the corneal Na/K ATPase by ouabain (12) or after substrate deprivation (1) during perfusion of the corneal endothelium. Therefore, the low rate of corneal swelling that occurs in the presence of diamide plus glucose may be the result of thiol-oxidation of the active membrane sulfhydryl (-SH) groups associated with the corneal endothelial ionic pump, since it has been shown that an -SH group at the active site of the Na-K-activated ATPase must remain reduced for normal pumping activity (11). Although the precise interrelationship between membrane sulfhydryls and intracellular GSH levels is not clear, it is possible to conclude that thiol-oxidation of GSH leads to disruption of the endothelial barrier.

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FIGURE 12 SEM of rabbit corneal endothelium exposed to: (a)  $4 \times 10^{-4}$  M diamide plus GLU for 0.75 h; (b)  $4 \times 10^{-4}$  M diamide plus GLU for 2.75 h; and (c)  $4 \times 10^{-5}$  M diamide + GLU for 4 h. The normal mosaic pattern is altered only in b due to cytoplasmic swelling in some cells. (a)  $\times 1,000$ ; (b)  $\times 500$ ; (c)  $\times 900$ .

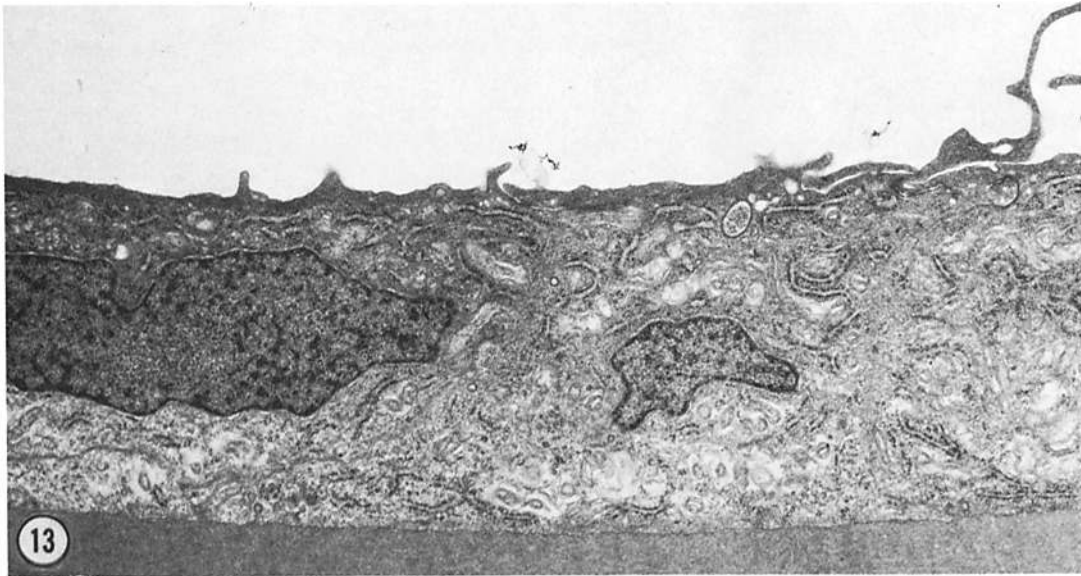


FIGURE 13 TEM of rabbit corneal endothelium exposed to  $4 \times 10^{-6}$  M diamide plus glucose for 4 h. Normal endothelial ultrastructure is maintained except for slight swelling in basal cytoplasm. The microfilaments are not condensed and the junctional complexes are tight.  $\times 13,000$ .

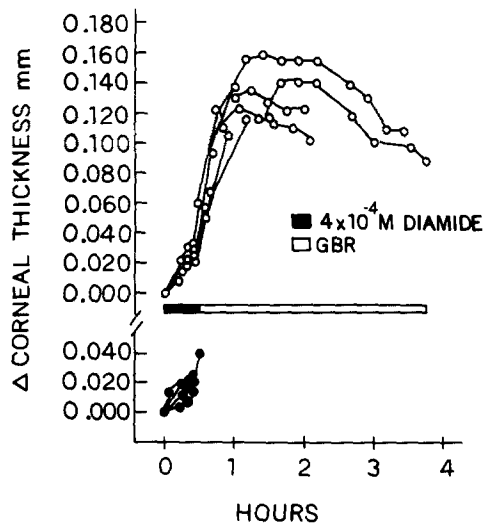


FIGURE 14 Changes in corneal thickness of paired rabbit corneas perfused with  $4 \times 10^{-4}$  M diamide for 15-30 min (closed circles) and of paired corneas in which the diamide was removed and perfusion with GBR was carried out for an additional 2-4 h (open circles). Corneal thickness stabilized after 1 h and began to decrease after 2 h, illustrating reversal of the diamide effect.

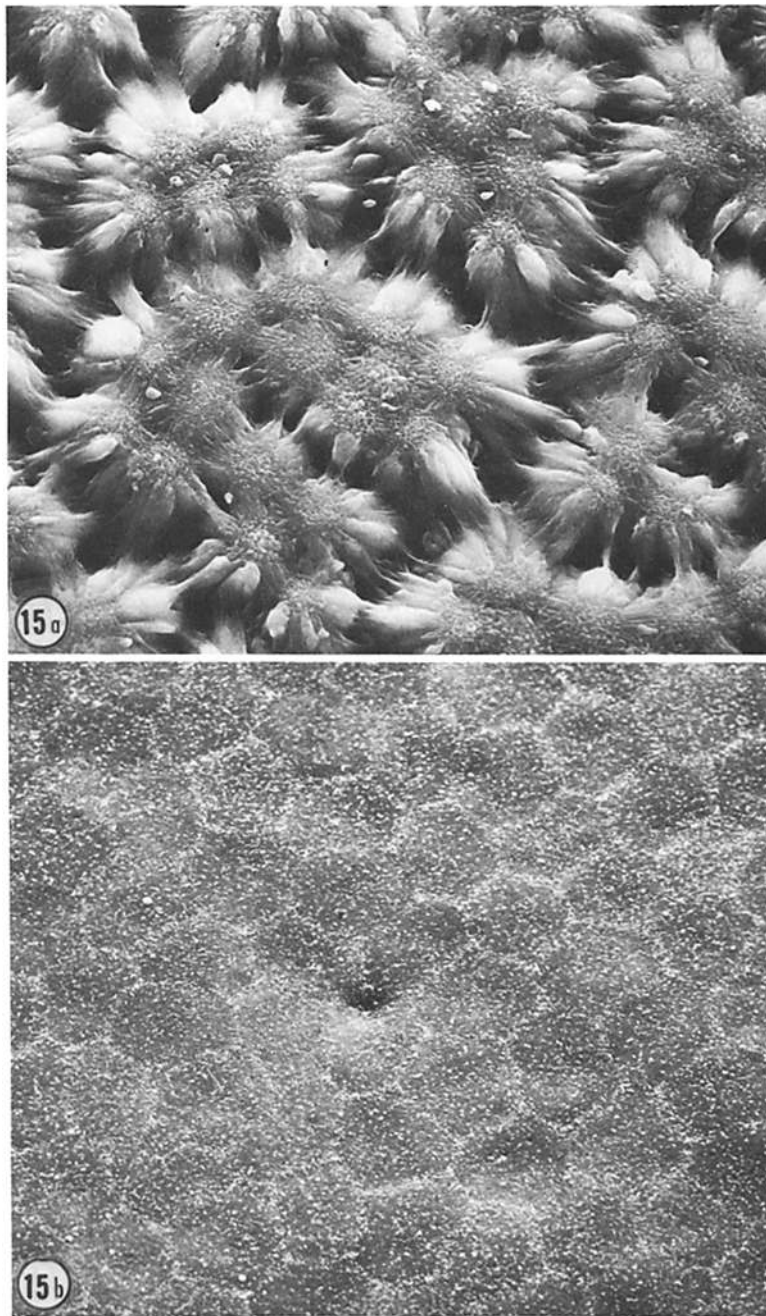


FIGURE 15 (a) SEM of the endothelium of a cornea after 15-30 min of perfusion with  $10^{-4}$  M diamide in BR. The cells are beginning to form isolated clusters.  $\times 1000$ . (b) SEM of the endothelium of the paired cornea after reversal of the diamide effect with GBR. The normal mosaic-like pattern of the endothelium is restored.  $\times 1000$ .

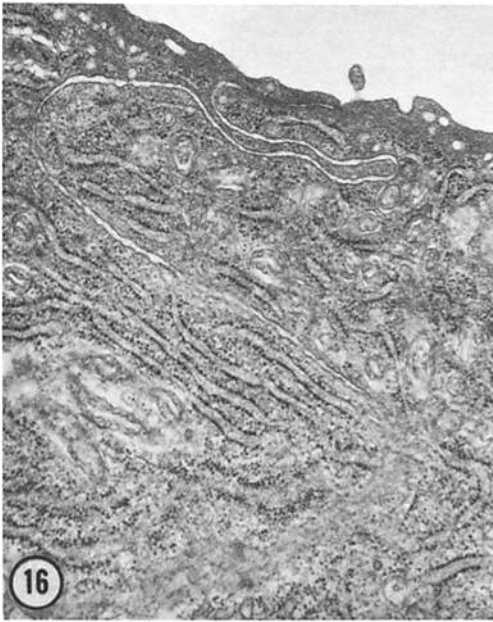


FIGURE 16 TEM of endothelial cells in same cornea as Fig. 15 *b*. The junctional complex has been restored and the microfilaments are normal.  $\times 19,100$ .

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