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Regulation of Fluid Flow Through Corneal Stroma in the Bullfrog

Shingo Kubota,* Kazutaka Kani* and Hiroshi Kitasato†

*Departments of *Ophthalmology and †Physiology, Shiga University of Medical Science, Ohtsu, Japan*

Abstract: Regulation of fluid flow through corneal stroma was investigated in the bullfrog. Corneal specimens were mounted by clamping their limbal sclera between the two chambers of a Ussing-type chamber. The epithelial surface was covered with Ringer's solution, while the endothelial surface was superfused with Ringer's solution at various pressures ranging from 0–60 mm Hg. At 0 mm Hg, the cornea swelled, while at 10 mm Hg the corneal thickness remained unchanged. Further elevation of the hydrostatic pressure of the endothelial superfusion solution caused a decrease in corneal thickness, suggesting that the hydrostatic pressure in the in vivo frog corneal stroma is about 10 mm Hg. At 10 mm Hg of endothelial superfusion pressure, piercing the epithelial cell layer with a 30-gauge needle caused only slight corneal swelling. Removing glucose from the epithelial perfusion solution induced a slowly progressing increase in corneal thickness. Iodoacetate did not interfere with the swelling of the pierced cornea after the removal of glucose from the epithelial perfusion solution. To examine the possibility that the interstitial fluid flows across the stroma-scleral boundary, corneal specimens having unclamped sclera were incubated in Ringer's solution containing 3 mmol/L dextran of various molecular weights ranging from 8800–162 000, and the volume of the preparation was monitored by sequential measurement of the weight. In the presence of dextran with a molecular weight higher than 70 000, the corneal volume decreased at the beginning of incubation, and after reaching the minimal volume it slowly increased, indicating that the stroma-scleral boundary is permeable to dextran of even a molecular weight of 162 000, although dextran molecules diffuse much more slowly than water, and the concentration of unfilterable solutes in the stroma is lower than 3 mmol/L. In experiments using the Ussing-type chamber at 10 mm Hg of endothelial superfusion pressure, a decrease of NaCl in the superfusing solution to 1/2 caused rapid corneal swelling followed by slow recovery. Adding NaCl to the 1/2 NaCl Ringer's solution caused a further corneal thinning in a concentration-dependent manner. The same extent of decrease in corneal thickness as induced by adding NaCl was achieved by the same concentration of glucose as of NaCl, implying that the value of the reflection coefficient of the endothelial cell layer to either Na^+ or Cl^- is about half that of glucose. Our results show that even a small difference in the concentration of low molecular weight solutes (e.g., Na^+ and Cl^-) exerts a force that draws water from the cornea. **Jpn J Ophthalmol 1998;42:12–21**

Key Words: Bullfrog, corneal edema, corneal thickness, glucose, intraocular pressure, reflection coefficient of endothelium.

Introduction

Clinically observed corneal edema is frequently accompanied by an acute or persistent elevation of intraocular pressure (IOP). The phenomenon has been explained so far as a consequence of IOP elevation that may force fluid from the aqueous humor into the cornea. Under physiological conditions, the fluid content of the cornea is maintained at a constant level, while proteoglycans in the stroma of the cornea continuously exert a force to absorb fluids from the surrounding tissues.[1–4] The endothelial cell layer of the mammalian cornea has been postulated

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Address correspondence and reprint requests to: Shingo KUBOTA, MD, Department of Ophthalmology, Shiga University of Medical Science, Tsukinowa-cho, Seta, Ohtsu, Shiga 520-2192, Japan

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to be the site of the pump and barrier functions that maintain the constant hydration of the corneal stroma and the integrity of the cornea. It has been thought that the failure of these functions leads to corneal swelling.[4,5]

Fluid extrusion from the cornea depends on two factors in addition to the difference in hydrostatic pressure: one is the activity of the solute pump that maintains the concentration gradient of the solute across boundary membranes, and the other is the relative magnitude of the permeability of boundary membrane to the permeability of the solute. The latter factor is represented by the reciprocal of the reflection coefficient for the solute.

The best characterized solute pump is the Na^+/K^+-pump, which requires ATP supply to operate. Although there are some reports[6–10] indicating the importance of HCO_3^- in the corneal endothelial cell, the function of the HCO_3^- pump is still disputable.[11,12]

The driving force causing fluid flow across the endothelium was extensively investigated by Mishima and others, using cornea mounted on a Ussing-type chamber.[5,13–15] However, the fluid flow across the boundary between the cornea and the sclera has not been sufficiently studied. The goal of the present study is to characterize the forces affecting both the fluid flow across the corneo-scleral boundary and that across the endothelial cell layer.

Results obtained from the present study suggest that the interstitial fluid in the sclera is drawn into the corneal stroma by the unfilterable solutes in the stroma. In turn, the fluid in the corneal stroma is pulled into the endothelial superfusing solution, according to the concentration gradient of small solutes across the endothelium, formed by ionic pumps, which produces a force strong enough to overcome the force generated by the unfilterable solutes in the corneal stroma. The fluid flow through the stroma of the frog cornea may be more important to epithelial and stromal cells than to endothelial cells, from the viewpoint of nutrient supply. On the basis of our observations, the possible mechanism of the corneal edema that accompanies IOP elevation will be discussed.

Materials and Methods

The handling of animals followed the Helsinki declaration on the use of animals in research. Experiments were performed on bullfrogs (*Rana catesbeiana*), which were discarded after inhalation of ether. Both eyes with the conjunctiva were immediately enucleated and soaked in Ringer's solution until used. Corneas with scleral rim were prepared by cutting the sclera 2 mm posterior from the limbus. The lens and iris were removed from the posterior surface of the cornea very carefully to avoid damaging the corneal endothelium. All experiments were performed in an air-conditioned room. The temperature of solutions was kept at 22–26°C without using an incubator.

Perfusion Chamber, Optics, and Recording System

Figure 1 is a diagram of the semi-open Ussing-type chamber used for endothelial superfusion experiments in this study. The chamber was composed of lower and upper chambers. The lower chamber contained a cylindrical pool. The corneal specimen with scleral rim was placed on the top of the lower chamber, endothelial surface downward. The scleral rim was clamped between the lower and upper chambers. The endothelial surface of the specimen was superfused with either Ringer's solution or a variety of test solutions. The pressure applied to the superfusion solution was monitored by measuring the

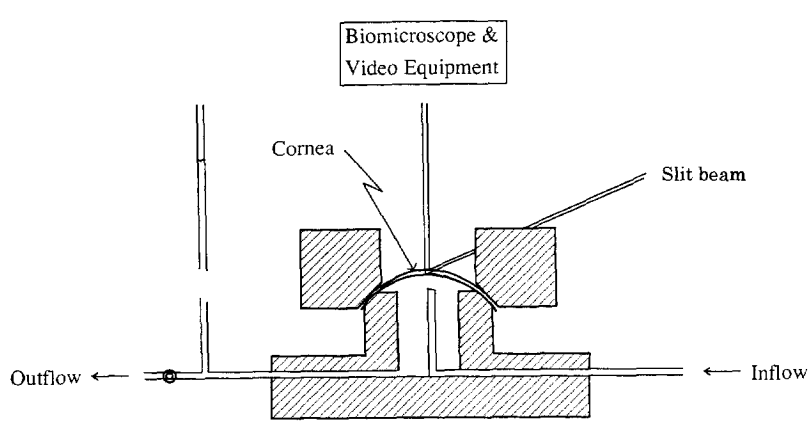

Figure 1. Diagram of apparatus used to measure corneal thickness in response to superfusion solutions. Corneal specimens were mounted in a Ussing-type chamber by fastening limbal sclera between the lower and upper chambers.

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fluid height in the glass tube connected to the lower chamber. Perfusion pressure was usually kept at 13.8 cm (10 mm Hg) by adjusting the screw set in the outflow tube or by changing the height of the inflow solution reservoir, unless stated otherwise. Perfusion flow rate was about 50 mL/hour. The upper chamber was filled with Ringer's solution.

The light beam of a slit-lamp microscope was set obliquely to the anterior surface of the cornea. The scattered light beam was recorded by a video camera attached to a microscope set perpendicular to the chamber, and the change in corneal thickness was recorded by measuring the beam width on a 37-inch TV monitor with a ruler. A 100-μm-thick cover glass was used for calibration. The reading error was ±1.4% (SEM; n = 10).

Weight Measurement

Corneas were carefully dissected and transferred to a petri dish filled with Ringer's solution. The fluid on the corneal surface was blotted with filter paper. This procedure was performed carefully to avoid damaging the endothelium. After measurement of the initial weight, the cornea was dipped in a 5-mL bathing solution and was sequentially measured in the same way described above, following the determined time schedule. The accuracy of the weight measurement was $\pm 1.0 \times 10^{-3}$ g.

Bathing Media and Chemicals

The compositions of the normal Ringer's solution, low-Na^+ Ringer's (1/2 NaCl, 3/4 NaCl), and glucose-free Ringer's solutions are shown in Table 1. All these solutions contain oxidized glutathione.[6–18] Solutions containing various concentrations of NaCl were prepared by adding test solutes to the 1/2 NaCl solution.

Table 1. Composition of Ringer's Solution

	Control	1/2 NaCl Ringer's	3/4 NaCl Ringer's	Glucose-free Ringer's
Na^+	102.6	63.8	83.2	102.6
K^-	1.01	1.01	1.01	1.01
Cl^-	82.4	43.6	63.0	82.4
Ca^{2+}	0.91	0.91	0.91	0.91
Mg^{2+}	1.0	1.0	1.0	1.0
HCO_3^-	25.0	25.0	25.0	25.0
Glucose	5.6	5.6	5.6	
Sucrose				5.6
Oxidized glutathione	0.3	0.3	0.3	0.3
Hepes	1.0	1.0	1.0	1.0

All values are in mmol/L. pH was adjusted to 7.40 with HCl.

Results

Corneal Thickness and Perfusion Pressure

Figure 2 shows the effect of endothelial perfusion pressure on the thickness of cornea mounted on the Ussing-type chamber. The endothelial perfusion pressure was varied in a range from 0–60 mm Hg. At 0 mm Hg, the corneal thickness gradually increased. After the endothelial perfusion pressure was raised to 10 mm Hg, the corneal thickness reached a steady value (Figure 2A). The ratio of thickness change to the control thickness at 0 mm Hg of endothelial perfusion pressure was determined. Corneal thickness decreased rapidly and maintained a constant value during the period of pressure elevation from 10–20 mm Hg. Raising the endothelial pressure from 20–40 mm Hg caused a further decrease in the corneal thickness. The thickness at 40 mm Hg was about 0.6 of the control thickness. A further pressure elevation from 40–60 mm Hg did not induce any noticeable change in the thickness. At 60 mm Hg, the cornea became opaque, and the solution in the upper chamber increased gradually. When the pressure was reduced to 10 mm Hg, the corneal thickness recovered its initial level at 10 mm Hg. The number of experiments was three. The thickness ratios to the initial thickness at 0 mm Hg in a steady state were plotted against the endothelial perfusion pressure (Figure 2B). A decrease in the corneal thickness by pressure elevation indicates that the applied pressure forces fluid through the corneal stroma into the upper chamber solution, implying that the outermost layer of cornea (i.e., the epithelial cell layer) acts as a barrier against the applied pressure.

Effect of Adding Glycogenolysis Inhibitor on Corneal Thickness

It is reasonable to consider that metabolic substrate is required for the cornea to maintain its normal thickness. When the limbal portion is clamped, it is difficult for a supply of glucose to reach the cells in the corneal stroma, because little exchange of fluid takes place across the endothelium in a steady state. To supply metabolic substrate to the cells in the cornea, a small hole was made by piercing the epithelial cell layer with a 30-gauge needle. The endothelial surface was superfused with the normal Ringer's solution at 10 mm Hg throughout the experiments. Figure 3 shows the time course of the change in thickness of corneal specimens after epithelial cell layers were pierced. In the presence of 5.6 mmol/L glucose,

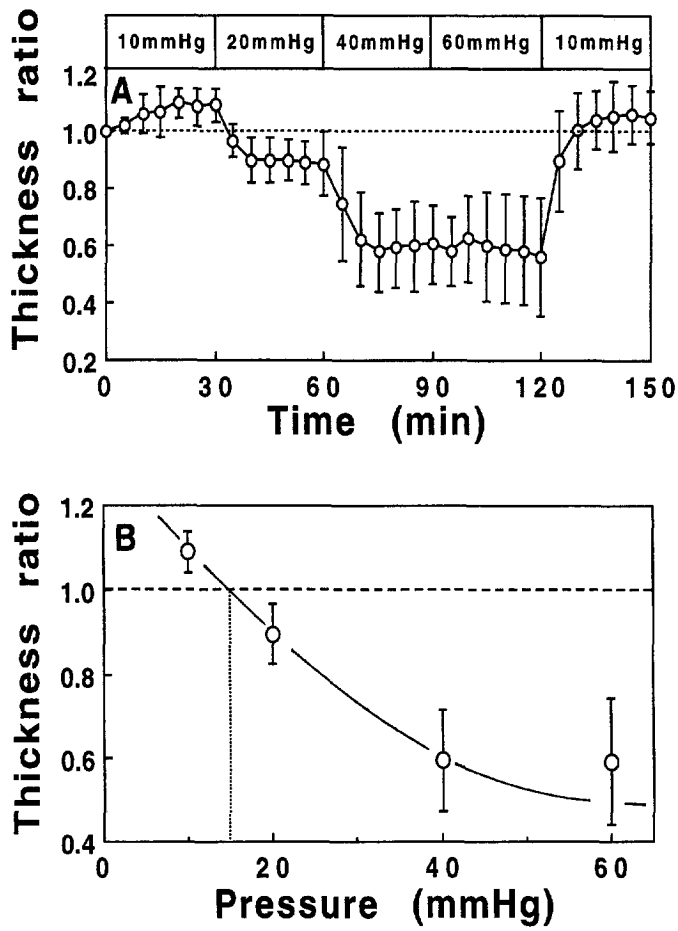

Figure 2. Effects of superfusion pressure elevation on corneal thickness. Pressure of the solution-perfusing endothelial surface was adjusted by changing the height of the inflow solution reservoir. (**A**) Effect of pressure elevation on corneal thickness. The endothelial surface was superfused with normal Ringer's solution at various denoted pressures. (**B**) Relationship between thickness ratio and applied pressure. Data are taken from the experiments shown in Figure 2A. The curve is drawn arbitrarily (bar = standard deviation; n = 3).

no noticeable change was observed in the corneal thickness. Removal of glucose from the Ringer's solution in the upper chamber caused progressive swelling, suggesting that the influx of glucose into the cornea had been utilized to generate the energy to pump out fluid from the cornea. Adding iodoacetate, an inhibitor of glycogenolysis, to the glucose-free solution in the upper chamber did not interfere with the swelling of the cornea. This finding suggests that stored carbohydrates do not play an important role in maintaining the activity of the fluid pump in the frog eye under aerobic conditions.

Effects of Osmolality of Unfilterable Solutes in Stroma Through the Stroma-Scleral Boundary

Thickness measurement experiments using the Ussing-type chamber are not able to give information about the fluid flow across the corneo-scleral boundary (the limbal portion). To evaluate the fluid flow across the limbal portion, cornea specimens with unclamped rim were incubated in a variety of test solutions. Under these conditions, the intracorneal pressure is considered to be almost equal to that of the incubation solution (i.e., 0 mm Hg). The volume of cornea with unclamped scleral rim was monitored by sequential weight measurement. The cornea with unclamped scleral rim swelled spontaneously in the normal Ringer's solution (data not shown), indicating that the osmolality of the interstitial fluid in the stroma is higher than that of normal Ringer's solution. Figure 4 shows the time courses of volume change in the corneas with unclamped scleral rim. The changes in volume were expressed by a ratio of the weight at a given time to that at the beginning of the incubation. High osmolality incubation solutions were prepared by adding dextran of various molecular weights (MWs), ranging from 8800 to 162 000, to the normal Ringer's solution. The concentration of dextran was 3 mmol/L in this series of experiments. Dextran of MW 8800 did not prevent corneal swelling. Dextran of higher MW transiently prevented the

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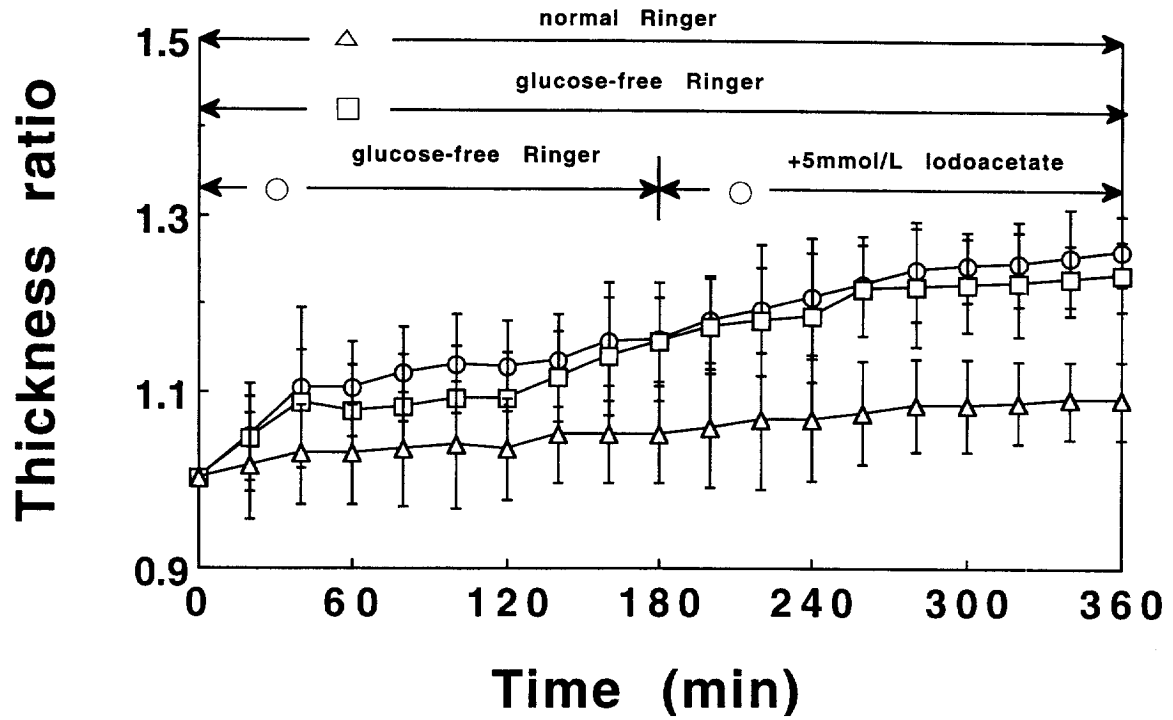

Figure 3. Effects of removal of glucose from the solution in the upper chamber on the corneal thickness of the pierced epithelial specimens. The endothelial surface was superfused with normal Ringer's solution at 10 mm Hg. The solution in the upper chamber was normal Ringer's solution (△), glucose-free Ringer's solution (□ or ○), or glucose-free Ringer's solution containing 5 mmol/L iodoacetate (○). Iodoacetate was added to the glucose-free Ringer's solution for a period indicated by the bar (bar = standard deviation; n = 3).

swelling. The higher the MW, the more prominent the prevention of swelling was at the beginning of the incubation. Dextran of MW 162 000 caused a prolonged decrease in the corneal volume (Figure 4A). The finding that even dextran of MW as high as 162 000 could not sustain a volume loss but induced only a transient decrease indicates that all dextrans examined can pass across the corneo-scleral boundary from the incubation solution into the corneal stroma, although the diffusion rate of dextran molecules is much lower than that of water. From the fact that 3 mmol/L dextran of MWs higher than 70 000 induced a transient volume decrease, the osmolality of unfilterable solutes within the stroma is estimated to be lower than 3 mmol/L. This value is lower than that reported in rabbit corneas.[8]

Figure 4B shows the time course of the volume change induced by dextran (MW 39 100) of various concentrations. At concentrations lower than 3 mmol/L, dextran was not able to prevent spontaneous swelling. At a concentration of 3 mmol/L, the initial slope was very close to zero, while at concentrations higher than 3 mmol/L the weight transiently decreased. With a concentration causing the same transient volume decrease as 3 mmol/L dextran with higher MWs, the diffusing rate of dextran of MW 70 000 is about half that of MW 39 100 dextran.

Effects of Lower Osmolality in Perfusion Solutions on Corneal Thickness

To examine the relative permeability of the endothelial cell layer for NaCl compared to water, the corneal thickness was monitored using a low-NaCl concentration of superfusion solution (Figure 5). Immediately after replacing the normal Ringer's solution with 1/2 NaCl Ringer's solution, the corneal thickness rapidly increased and then slowly decreased (Figure 5A). Forty minutes after replacement with 1/2 NaCl Ringer's, the thickness reached a new steady level. The increase in thickness observed immediately after replacement with 1/2 NaCl solution suggests that the endothelial reflection coefficient for NaCl is considerably high. When ouabain was added to the endothelial superfusion solution (Figure 5B), the corneal thickness did not return to a

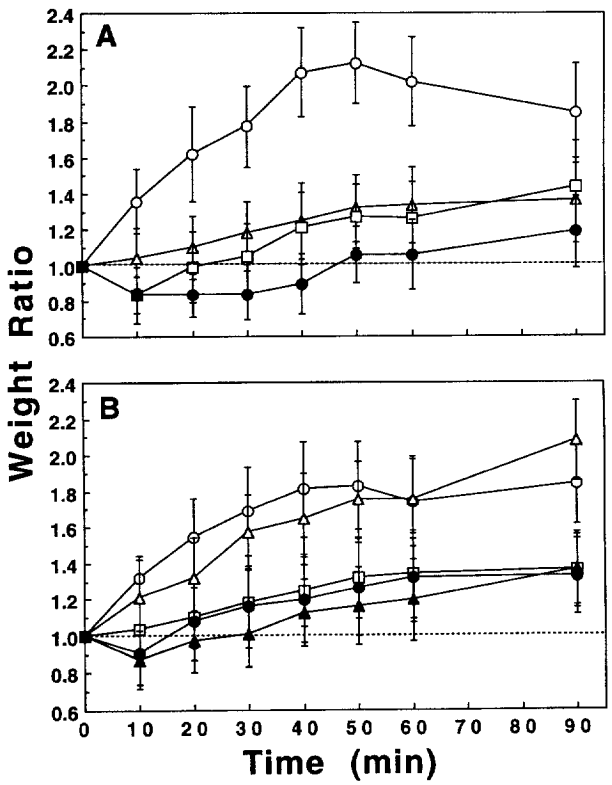

Figure 4. Effects of dextran added to the Ringer's solution on weight of the cornea with unclamped scleral rim. (**A**) Time course of weight ratio changes in the Ringer's solutions containing 3 mmol/L dextran of various molecular weights (MWs) (○ = MW 8800; △ = MW 39 100; □ = MW 70 000; ● = MW 162 000). (**B**) Time course of weight ratio changes in the corneas incubated in Ringer's solutions containing dextran (MW 39 100) at various concentrations (○ = 1 mmol/L; △ = mmol/L; □ = 3 mmol/L; ● = 4 mmol/L; ▲ = 6 mmol/L) (bar = standard deviation; n = 3).

less hydrated level but continued to increase slowly. During the period of superfusion with 1/2 NaCl Ringer's solution containing ouabain, adding 3 mmol/L dextran (MW 39 100) induced a thickness decrease to a level slightly higher than the control level. After reaching the lowest value, the thickness slowly increased, indicating a continuous elevation in the osmolality of the stromal interstitial fluid during the period of exposure to ouabain, probably due to the suppression of Na^+ extrusion resulting from the inhibition of the Na^+/K^+ pump.

Evaluation of Endothelial Reflection Coefficient for Glucose and NaCl

Figure 6 shows the thickness decrease induced by increasing the osmolality of solute concentrations. In advance of exposure to a high-glucose solution, the cornea had swelled after replacing the normal Ringer's solution with 1/2 NaCl Ringer's solution (63 mmol/L Na^+). During the period of perfusion with 1/2 NaCl Ringer's solution, the cornea reached a less hydrated state after an initial increase in thickness, as mentioned above. The effect on the corneal thickness of adding glucose to the 1/2 NaCl Ringer's solution was examined at various concentrations from +3 mmol/L to +60 mmol/L. By adding glucose to 1/2 NaCl Ringer's solution, a decrease in thickness was observed in a concentration-dependent manner (Figure 6A). This finding indicates that the endothelial reflection coefficient for glucose also is considerably high. Figure 6B illustrates the thickness decrease evoked by increasing NaCl concentration in the endothelial perfusion solution. The thickness decrease induced by adding 30 mmol/L NaCl was almost equal to that after adding 30 mmol/L glucose. Considering the fact that NaCl completely dissociates in aqueous solution, these results seem to indicate that the value of the endothelial reflection coefficient for either Na^+ or Cl^- is about half that for glucose.

Discussion

The present study revealed that the cornea having unclamped scleral rim swelled after a transient volume decrease when incubated in Ringer's solution containing 3 mmol/L dextran of MWs higher than 70 000 (Figure 4A), while specimens clamped at the limbal sclera using the Ussing-type chamber did not swell when superfused by Ringer's solution at a pressure of 10 mm Hg (Figure 2A). This finding indicates that the stroma-scleral boundary is highly permeable to most solutes in the interstitial fluid and even to dextran of MW as large as 162 000. High permeability of the stroma-scleral boundary to these solutes suggests that there is almost no difference in osmolality among the low MW solutes in the stromal and scleral interstitial spaces. Furthermore, no rigid boundary structure strong enough to sustain the pressure difference between stromal and scleral interstitial fluids was observed histologically except for the distribution of collagen fibrils. Under these conditions, the force responsible for the absorption of the Ringer's solution from the scleral interstitial space would be derived solely from the osmolality of unfilterable solutes in the stroma. The main solutes exerting such absorption force may be proteoglycans. The absorption force corresponds to the imbibition pressure, which has been defined by Hedbys et al.[19] There are some reports[20–22] on the hydrostatic

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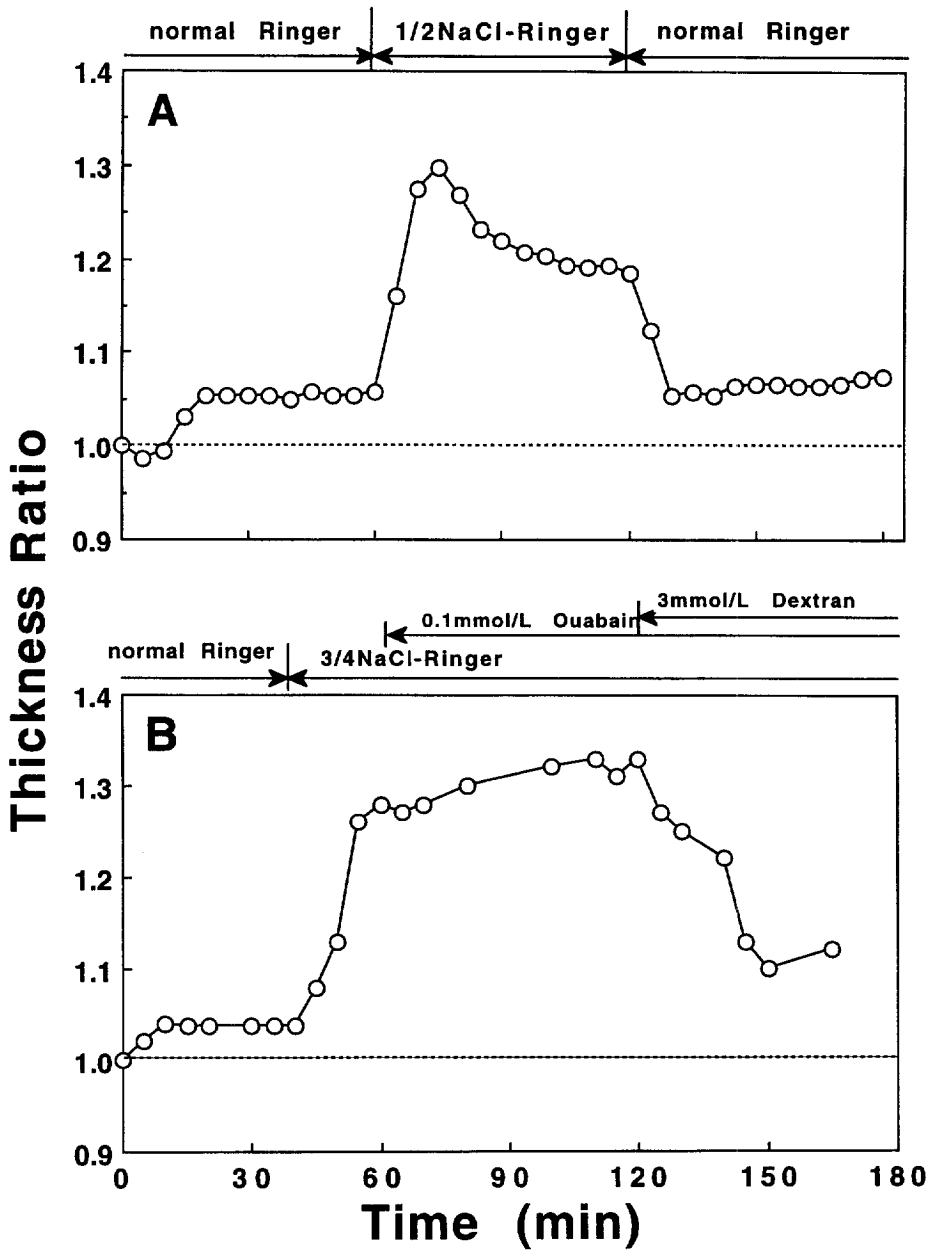

Figure 5. Effect of lowering NaCl content in endothelial-superfusing solution on corneal thickness. The endothelial surface was superfused with Ringer's solution at 10 mm Hg. (**A**) Normal Ringer's solution was replaced with 1/2 NaCl Ringer's solution during the period indicated by the bar. (**B**) Effect of ouabain on thickness recovery of corneas preswollen by exposing to 3/4 NaCl Ringer's solution. Ouabain (0.1 mmol/L) was added to 3/4 NaCl Ringer's for the period indicated by the bar. Partial recovery was achieved by adding dextran (molecular weight = 39 100) of 3 mmol/L during the period of exposure to ouabain (bar = standard deviation; n = 3).

pressure in the stroma. However, it is not clear whether the hydrostatic pressure is higher in the stroma than in the sclera when the physiological structure is maintained; the cornea is so thin that a small change in volume can cause serious deviation in measured values.

From the histological viewpoint, it may be reasonable to assume that the corneal epithelium composed of many epithelial cell layers is less stretchable than the endothelium and is mechanically strong enough to withstand IOP. In any case, the fluid flow across the corneal epithelial cell layer is negligibly small.[14,23] These observations suggest that the stromal hydrostatic pressure is nearly equal to both IOP and scleral interstitial pressure under physiological conditions.

The other important finding in the present study is that the cornea mounted on the Ussing-type chamber decreased in thickness after increasing the hydrostatic pressure of the solution superfusing the endothelial surface (Figure 2). This finding indicates that the epithelial cell layer is the main barrier for

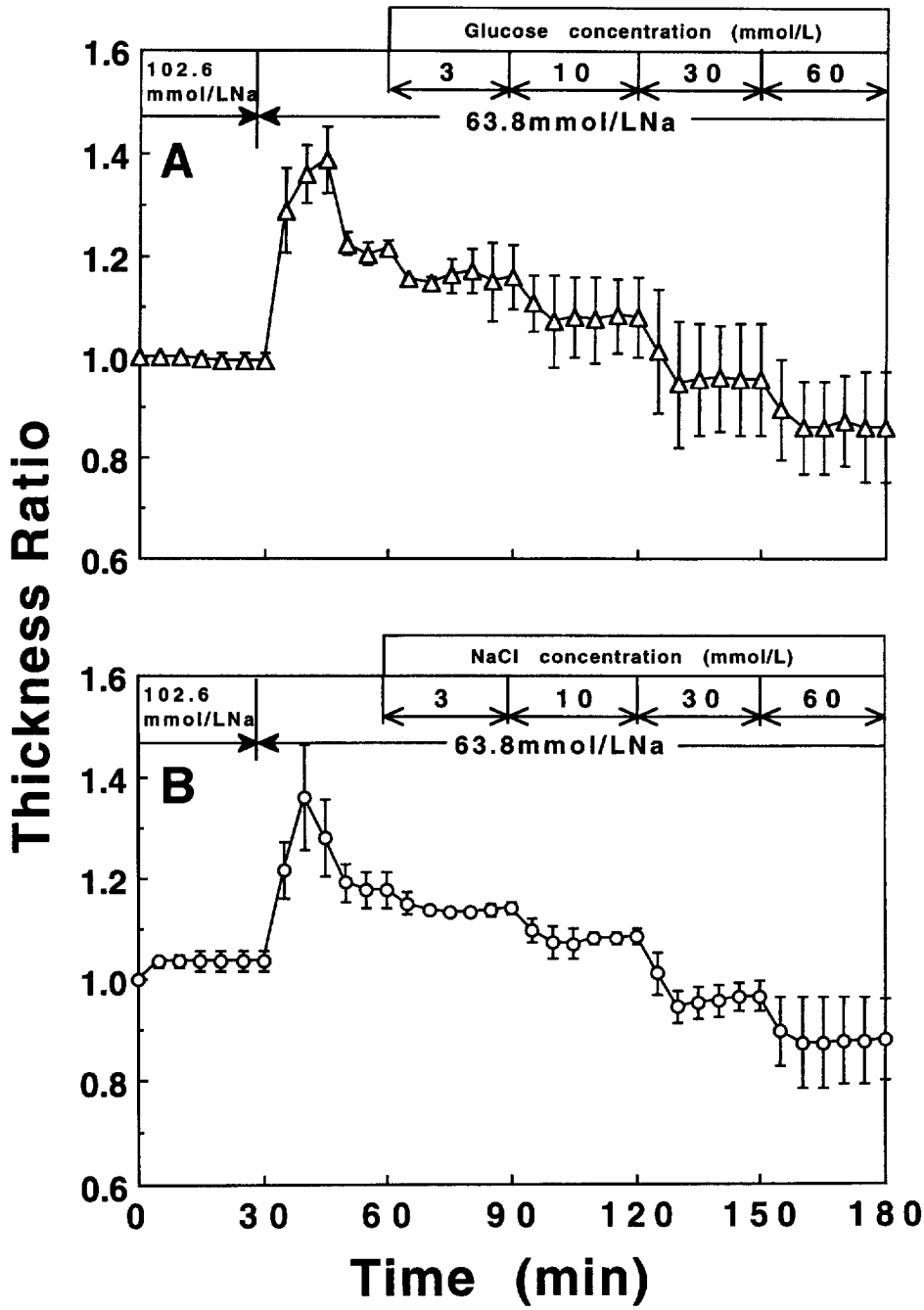

Figure 6. Effect of raising solute concentration in the endothelial-superfusing solution on thickness of preswollen corneas. The endothelial surface was superfused by a variety of test solutions at 10 mm Hg. Corneal specimens were preswollen by exposing to 1/2 Na Ringer's solution. During the period of exposure to low NaCl Ringer's solution, the solute concentration was sequentially elevated. (**A**) Time course of the thickness change induced by elevating the glucose concentration from 0–60 mmol/L. (**B**) Time course of the thickness change induced by adding NaCl. Pressure of the superfusing solution was 10 mm Hg (bar = standard deviation; n = 3).

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fluid flow from the endothelium to the epithelium and that increasing the hydrostatic pressure of the endothelial-superfusing solution decreases the thickness of the stroma, as we theorized above. When the hydrostatic pressure within the stroma is higher than the osmolality of unfilterable solutes in the stroma, the cornea becomes thinner. If there is no hydrostatic pressure gradient among intraocular spaces, the stroma, and the scleral interstitial spaces, the interstitial fluid should continue to flow from the surroundings into the stroma until the osmolality of unfilterable solutes in the stroma becomes low enough to be equal to that of existing unfilterable solutes in the surroundings.

Fluid flow across the limbal boundary has been seldom discussed. Using mammalian cornea, Maurice compared the distribution of fluorescein between the cornea and the aqueous humor with that between the cornea and limbal vessels, and found more fluorescein in limbal vessels than in the aqueous humor.[24] Recently, Walther and Martin found that fluid movement across the anterior and posterior surfaces of the cornea is about one-fourth of the fluid conductivity of the limbal portion.[25] This indicates that the limbal area is the main pathway of fluid flow into the stroma.

Our results suggest that the maximum osmolality of unfilterable solutes in the stroma of the frog is lower than 3 mmol/L. The endothelial reflection coefficient for NaCl in the rabbit has been reported as being close to 0.6.[14]

Our results also show that the value of the endothelial reflection coefficient for NaCl is about 0.5. The osmotical driving force for the fluid flow across the endothelium results from two opposing influences: one is the stromal unfilterable solutes and the other, small filterable ions, probably Na^+ and Cl^-.

Hydraulic conductivity of the epithelial cell layer is expected to be much lower than that of the stroma-scleral limbal boundary. Therefore, the interstitial fluid flows from the scleral interstitial space across the limbal boundary into the stroma, and then the fluid is driven out across the endothelial cell layer into the aqueous humor space, resulting in zero volume change in the cornea. This fluid flow may carry nutrients to the cells in the cornea, not only to the endothelial cells but also to stromal and epithelial cells. There are many reports indicating that corneal edema has been induced by severe damage to the corneal endothelium.[26] This phenomenon is accountable to the fall in the endothelial reflection coefficient for small filterable solutes. It is proposed that the Na^+/K^+ pump and anion exchange take part in corneal dehydration.[27–30] The existence of the Na^+/K^+ pump is reconfirmed by ouabain experiments in the present study.

An elevation of IOP is known to cause corneal edema. Since all the boundaries of the cornea have no rigid structure preventing swelling, it is difficult to suppose that the IOP alone can vary without corresponding changes in both stromal and scleral interstitial pressures. The elevation of IOP itself cannot exhibit the force to push fluid into the stroma from intraocular space. Possible causes of corneal edema are a decrease in the endothelial reflection coefficient for NaCl, a fall in the fluid pump activity of endothelial cells, and the elevation of the osmolality of solutes in the stroma. A rise in IOP causes distension of the eyeball. Since the sclera is less stretchable than the cornea,[3] an elevation in IOP may result in distension of the cornea. The mechanical distension of the corneal endothelium may induce a partial disruption of intercellular junctions, leading to a fall in the endothelial reflection coefficient for NaCl.

An increase in corneal volume results in a fall in the osmolality of unfilterable solutes in the stroma, which causes a decrease of fluid flow from the scleral interstitial space to intraocular space through the stroma. The decrease of fluid flow may bring about a decrease of nutrient supply. Endothelial cells face both to the stroma and the aqueous humor. So, endothelial cells may be supplied with glucose from the aqueous humor as well as from stromal interstitial solution. Taking the direction of the fluid flow through the stroma into account, the interstitial fluid flow through stroma is more important to stromal and epithelial cells than to endothelial cells from the viewpoint of nutrient supply. Although the present experiments were conducted using frog corneas, the discussion presented above may also apply to the mammalian eye.

This work was conducted in the Department of Physiology, Shiga, University of Medical Science.

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