Transport of Na, Cl, and water by the rabbit corneal epithelium at resting potential

S. D. KLYCE
Division of Ophthalmology, Department of Surgery, Stanford University School of Medicine, Stanford, California 94305

KLYCE, S. D. Transport of Na, Cl, and water by the rabbit corneal epithelium at resting potential. Am. J. Physiol. 228(5): 1446-1452, 1975.—Theophylline (1 mM) produced a net transport of Na and Cl from aqueous humor to tears (0.02–0.04 μmol/cm²h) in the isolated rabbit cornea denuded of endothelium and in the presence of normal resting potential (25–35 mV). The active transport of Na (tears to aqueous) and of Cl (aqueous to tears), estimated with the Goldman constant-field equations, was confirmed. A 10°C rise in temperature produced changes close to those predicted for passive processes in both unidirectional fluxes of Na and in the tears-to-aqueous flux of Cl, but not for the aqueous-to-tears flux of Cl. Theophylline treatment doubled Cl permeability but did not significantly affect Na or urea permeability, suggesting specificity of effect. In separate experiments it was shown that stromal thinning occurred in previously swollen corneas when the endothelium was blocked by silicone oil and the epithelium was treated with theophylline. These findings provide further support for the argument that the mammalian epithelium could have an active role in the regulation of corneal thickness in situ.

The rabbit corneal epithelium generates a potential of 25–35 mV (9, 15) in situ. Oriented tear side negative, this resting potential must increase the passive backflux of Na and of Cl, thereby reducing the net ion transport from the levels measured with short-circuited preparations. Since the ocular conjunctiva provides only a small potential gradient for the radial spread of ionic current (9), the net charge transfer must be close to zero, and either the Na or the Cl transport will dominate solute transfer. While the possibility exists that the chloride transport in the mammalian cornea might lead to solute and water movement out of the stroma in situ, it is not possible to make this conclusion without first measuring ion and fluid movements across the open-circuit tissue. Such measurements have not been reported previously.

In this paper the movements of Na, Cl, and fluid were measured in the in vitro rabbit cornea denuded of endothelium and in the presence of normal corneal resting potential to further assess the role of epithelial ion transport in the regulation of corneal hydration in situ. Active and passive fluxes were differentiated by means of the Goldman-Hodgkin-Katz description (5, 8) of passive ion movements.

Methods

Flux measurements. The variations in corneal resistance between eyes of the same animal were systematically less than those encountered between eyes of different-aged animals. This allowed pairing of data, which was to have reduced error in the calculation of net fluxes and in the estimation of active fluxes in the presence of resting potential. For these experiments two identical Lucite chambers (11) were constructed. The chambers exposed 1 cm² of cornea to two well-stirred and aerated solutions of identical composition at 34°C with a hydrostatic pressure gradient of 1 cmH₂O to prevent corneal buckling.

Corneas were dissected from 8- to 12-pound albino rabbits shortly after death and were mounted in the double-sided Lucite chambers. In all experiments reported here, the endothelium was eliminated with Descemet's membrane to prevent the possibility that it contributes to the fluxes reported. This was accomplished after removal of the iris and lens by gently grasping Descemet's membrane at the limbus with fine toothed forceps and peeling it off as a sheet. Any remaining endothelium was abraded with a dry cotton swab. This left epithelium and stroma intact and resulted in a slow swelling of the latter throughout the experiment. However, despite the unsteady-state hydration profile produced by removing the endothelium, the restriction to the diffusion of Na and Cl offered by the stroma is only twice that of an aqueous solution. Hence, it was assumed that ion activities in the subepithelial fluid ap-
proximated those in the bulk solution during steady-state transport.

The normal Ringer fluid used in these experiments was similar to that previously described (11) with the substitution of 10 mM HCO₃ for the osmotic equivalent of sucrose. The final composition of the solution was 103.4 mM NaCl, 15.3 mM Na₂SO₄, 10 mM NaHCO₃, 2.2 mM K₂HPO₄, 0.5 mM KH₂PO₄, 5.24 mM H₂PO₄, 0.61 mM MgSO₄, 0.7 mM calcium gluconate, 26 mM glucose, and 20 mM tris(hydroxymethyl)aminomethane (Tris). The osmolarity and pH of the above solution were 305 mosM and 7.4, respectively.

³⁶Cl Ringer (3 μCi/ml) and ²²Na Ringer (2 μCi/ml) were prepared by substituting equimolar amounts of Na³⁶Cl or Na²²Cl for Ringer NaCl. For the measurement of urea fluxes [¹⁴C] urea was incorporated (4 μCi/ml) into Ringer with a final urea concentration of 2 mM. Samples of "hot" side solutions were taken with a 10-μl constricted micropipette 3 times during the experiment, while 200- to 250-μl samples were withdrawn from "cold" sides at 30-min intervals, this volume being replaced with Ringer, and counted in Bray’s solution. Unidirectional fluxes were calculated as previously described (11).

Potentials of the corneas were measured through agar bridges and calomel half-cells with a high-input impedance voltmeter (H. Fein, Yale University). Prior to each isotope sampling, corneal resistance was estimated by measuring the PD produced by a 5-μA, 3-μV hyperpolarizing current pulse applied via agar-Ringer bridges. Drugs used in these experiments were added following a 2-h control period. Theophylline or N₆, O₂'-dibutyryl adenosine 3', 5'-cyclic AMP (Sigma Chemical) was incorporated into both sides of the chambers following the control period.

Estimation of active fluxes and ion permeabilities. To complement the net fluxes of Na and Cl measured in the paper and to compare values to data at SCC (11), estimates of active transport and ion permeability were made. Active transport components were estimated assuming that the inward Na pump and the outward Cl pump were unidirectional processes and that the passive fluxes obeyed the flux ratio equation (20). Permeabilities were calculated from the unidirectional fluxes in the direction of presumed passive transport (e.g., tears to stroma for Cl permeability) with the Goldman-Hodgkin-Katz approach (5, 8) (cf. APPENDIX, Mandel and Curran (13)).

Thickness measurements. Correlative experiments were performed to measure the effect of theophylline on the thickness of the cornea at open circuit using the specular microscope apparatus designed by Maurice (3, 16) for endothelial studies. The corneal endothelium was dissected away as described above, and the stroma was allowed to imbibe Ringer freely from the bare surface for 5 min. Subsequently, that fluid was replaced with silicone oil (Dow Corning 200 fluid; viscosity: 20 cSt). In an initial experiment with the original chamber of the apparatus, the epithelium-stroma preparation was found to thin at the rate of 15 μm/h. This thinning was not reversed by transport inhibitors but was fully reversed by refreshing the epithelial side Ringer. Therefore, to prevent the effects of evaporation, the original chamber was replaced with a previously described chamber (9), allowing continuous perfusion of the epithelial surface and minimizing thermal gradients. The experimental arrangement is shown in Fig. 1.

Generally the epithelium was perfused with Ringer + 1 mM sucrose preheated to 34°C at 10 ml/h. Thickness measurements were made at intervals of 10 min or less by averaging several readings. After 60 min of linear thickness change, the perfusing solution was changed to Ringer + 1 mM theophylline, and thickness measurements were continued.

RESULTS

Effect of theophylline on Na and Cl fluxes. The effect of 10⁻³ M theophylline on the unidirectional fluxes of Cl, corneal potential, and resistance is shown in Fig. 2. Following theophylline treatment there was an increase in both unidirectional fluxes. The flux-ratio equation would predict that J_Na/Cl⁰ = 0.3 for a univalent anion with a membrane potential of 30 mV. It is obvious from the data in Fig. 1, which are typical, that the observed ratio is greater (J_Na/Cl ≈ 1) than that prediction, which confirms the fact (11) that the corneal epithelium transports Cl to the tears.

Figure 3 presents an experiment testing the effect of theophylline on the unidirectional fluxes of Na at open circuit. Unlike its effect on the chloride results, theophylline produces no marked effect on Na fluxes. It is again obvious from this paired experiment that the flux-ratio criterion is not met, since J_Na/Cl ≈ 1 rather than 3.8 as predicted for a passive process.

A series of 10 paired experiments were done with ³⁶Cl and ²²Na to determine whether a significant net flux of either ion could be demonstrated at open circuit (Table 1). During the control period the net fluxes of Na and Cl were not significantly different from zero, and corneal resistance and potential were similar to previous reports (9, 11). The calculated active fluxes confirmed the presence of the stroma-to-tears active transport of Cl and the active transport of Na in the opposite direction. The mean Na permeability of 3.1 × 10⁻⁴ cm/h was at the low end of the range of 3-9 × 10⁻⁴ cm/h measured in vivo by Maurice (14). Cl permeability (5.1 ± 0.2 × 10⁻⁴ cm/h)
FIG. 2. Response to theophylline of unidirectional \(^{36}\text{Cl}\) fluxes, potential, and resistance of an isolated rabbit corneal pair at resting potential.

was slightly greater than the value of \(4.3 \times 10^{-4}\) cm/h measured with short-circuited preparations (11). Whether this was due to a slight difference in technique or to polarizing effects of sustained current application is uncertain.

Following the theophylline treatment there was a net flux of both Na and Cl in the direction of aqueous to tears of 20-40 nmol/cm\(^2\) h (Table 1). Corneal resistance was reduced by half. Theophylline increased active Cl transport 2.5 times, while apparently decreasing the tears-to-aqueous Na transport by one-half. After theophylline treatment, apparent Na permeability increased by 42%, but the major effect was on the Cl permeability, which showed a 115% increase. If the action of theophylline were confined primarily to an increase in a unidirectional chloride transport mechanism, the latter result would be unexpected.

Hence the increase in Cl permeability, which is calculated from the tears-to-aqueous flux, suggests that the action of theophylline is on a permeability barrier in series with the pump and/or that the pump is bidirectional.

Since the Cl transport produces a net outward Cl flux at open circuit, an equivalent amount of Na must also move in the outward direction to preserve electroneutrality. This is apparently accomplished through both inhibition of Na inward transport as well as an increase in the outward unidirectional flux (Table 1). The total increase in the latter flux can be attributed to the maintenance of electroneutrality independent of that part of the flux driven by the electrochemical potential gradient across the permeability barrier. Hence, the true epithelial Na permeability is probably not affected by theophylline.
To further demonstrate the specificity of cyclic AMP action in this epithelium, unidirectional fluxes of the non-electrolyte urea were measured in five corneas. During the first 30–120 min, urea permeability averaged 1.93 ± 0.12 × 10⁻³ cm/h, and this was unchanged during the next 150–300 min either by subsequent treatment with 1 mM theophylline (P<sub>A</sub> = 1.84 ± 0.11 × 10⁻⁵ cm/h) or by prolonged incubation (P<sub>A</sub> = 1.70 ± 0.07 × 10⁻³ cm/h). These controls suggest that the cyclic AMP effect on corneal epithelial transport is anion specific.

**Effect of dibutyryl cyclic AMP on Cl⁻ fluxes.** Experiments similar to those reported above were done with dibutyryl cyclic AMP to further demonstrate net CI transport at open circuit. The mode of action of theophylline is presumably through the inhibition of a cyclic nucleotide phosphodiesterase, which in turn would allow the accumulation of tissue cyclic AMP. The response to topical catecholamines, while these have been found effective in stimulating the diesterase, which in turn would allow the accumulation of exogenous cyclic AMP nor dibutyryl cyclic GMP was found to produce this response in pilot studies.

Dibutyryl cyclic AMP was found to produce a net flux of Cl from aqueous to tears (Table 2) similar to the action of theophylline. Furthermore, dibutyryl cyclic AMP increased both active CI transport and Cl permeability to the same extent as theophylline. This provides support to the argument that the action of theophylline in this system is confined to the inhibition of phosphodiesterase as opposed to direct effects on membrane permeability.

**Effect of temperature on Na⁺ and Cl⁻ fluxes following theophylline treatment.** Corneas were incubated at 24°C in the presence of 10⁻³ M theophylline and either ⁴Cl⁻ or ²²Na⁺ until a fairly stable potential and resistance were recorded. The response of the unidirectional fluxes was predicted with the Goldman approach, assuming each was a passive diffusion process with a Q₁₀ of 1.9. Following an increase in temperature to 34°C, both unidirectional fluxes of Na as well as the tears-to-aqueous flux of Cl were close to prediction (Figs. 4 and 5). The aqueous-to-tears flux of Cl alone showed a large deviation from prediction. These results provide good additional support for the argument that the tears-to-aqueous CI flux is a passive phenomenon, whereas the opposite flux contains an active component. Pairing the two Cl fluxes, the Q₁₀ for the active component of J<sub>Cl</sub> was calculated to be 2.6, whereas that for the active component of the Na experiments was not significantly different from the Q₁₀ expected for free diffusion. The significance of the latter result is not yet fully understood, yet it does imply that some unaccountable external passive force or experimental error may be associated with the "active" tears-to-aqueous transport of Na by this epithelium and under these conditions. The effects of temperature do, however, favor the use of the constant-field equations to predict the behavior of the passive fluxes in estimating the magnitude of the active flux of Cl.

**Effect of theophylline on corneal thickness.** Corneas swollen and mounted in the specular microscope apparatus with the endothelial surface blocked by oil were generally found to thin at the rate of a few micrometers per hour. This thinning was enhanced when 1 mM theophylline was substituted for 1 mM sucrose in the Ringer bathing the epithelial surface, but swelling was easily produced by further treatment with known modifiers of corneal epithelial.

**Table 1. Effect of 10⁻³ M theophylline on Cl⁻ and Na⁺ fluxes at resting potential**

<table>
<thead>
<tr>
<th></th>
<th>Single Experiments</th>
<th>Paired Data</th>
<th>Chloride</th>
<th>Sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potential, mV</td>
<td>Resistance, kΩ cm⁻¹</td>
<td>Flux, μeq/cm² h</td>
<td>Flux, μeq/cm² h</td>
</tr>
<tr>
<td><strong>Control period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tears-aqueous</td>
<td>34.1 ± 1.7</td>
<td>5.60 ± 0.23</td>
<td>-0.094 ± 0.005</td>
<td>-0.008 ± 0.006</td>
</tr>
<tr>
<td>Aqueous-tears</td>
<td>34.3 ± 1.3</td>
<td>6.04 ± 0.35</td>
<td>+0.085 ± 0.008</td>
<td></td>
</tr>
<tr>
<td><strong>Treated period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tears-aqueous</td>
<td>28.1 ± 1.0</td>
<td>2.69 ± 0.07*</td>
<td>-0.104 ± 0.006*</td>
<td>+0.019 ± 0.009†</td>
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<tr>
<td>Aqueous-tears</td>
<td>32.7 ± 0.8</td>
<td>2.58 ± 0.07*</td>
<td>+0.023 ± 0.011</td>
<td></td>
</tr>
<tr>
<td><strong>Control period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tears-aqueous</td>
<td>29.1 ± 1.9</td>
<td>1.06 ± 0.24</td>
<td>-0.069 ± 0.006</td>
<td>-0.001 ± 0.008</td>
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<tr>
<td>Aqueous-tears</td>
<td>26.2 ± 1.8</td>
<td>6.26 ± 0.37</td>
<td>+0.068 ± 0.008</td>
<td></td>
</tr>
<tr>
<td><strong>Treated period</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tears-aqueous</td>
<td>30.4 ± 0.9</td>
<td>3.27 ± 0.13*</td>
<td>-0.057 ± 0.004</td>
<td>-0.036 ± 0.006†</td>
</tr>
<tr>
<td>Aqueous-tears</td>
<td>23.6 ± 1.0</td>
<td>2.9 ± 0.06*</td>
<td>+0.093 ± 0.010*</td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SE from 10 paired ⁴Cl experiments and 8 paired ²²Na experiments. Observations were pooled as follows: control period—3 fluxes (30–120 min); treated period—5 fluxes (150–300 min). Representative experiments are displayed in Figs. 1 and 2. * The difference between control and treated periods was significant (P < 0.05) for these parameters. † The difference between these values and zero was statistically significant (P < 0.05). By convention, a positively signed flux was in the aqueous-to-tears direction, whereas a negatively signed flux was oppositely directed.
transport. A sample experiment is illustrated in Fig. 6. Following the theophylline response, the epithelium was bathed with Ringer containing $10^{-5} \text{ M Ag}^+$, which markedly increases inward Na transport in this tissue and which is partially reversible by reduced glutathione (unpublished data). Steady corneal thickness changes before and after theophylline are listed in Table 3. The thickness changes reported were confined to the stroma, since epithelial thickness was unaltered (45.3 $\pm$ 0.6 $\mu m$ before treatment and 45.5 $\pm$ 0.7 $\mu m$ afterward) in these experiments.

Since the reflection coefficient of the epithelium for NaCl is close to 1 (0.9997 can be calculated from the information given by Mishima and Hedbys (18)), the transport of 20–40 nmol/cm² h of salt from the stroma should be accompanied by isotonic water movement of about 0.2 $\mu l$/cm² h. This would yield a thinning rate of 2 $\mu m$/h, which is similar to the mean difference in thickness change of 2.27 $\mu m$/h (Table 3). The agreement between solute transported and fluid moved is good and strengthens the argument that the epithelium might play a role in the regulation of corneal hydration in situ by means of its Cl transport system.

**DISCUSSION**

Net outward transfer of Na and Cl in the presence of corneal potential could be demonstrated only after treatment with theophylline or dibutyryl cyclic AMP. This may attest to the dependence of Cl transport on adequate levels of intracellular cyclic AMP. Incubation of corneas has been observed to result in a decrease in cyclic AMP levels to one-quarter of their in vivo level, and while treatment with both epinephrine and theophylline increased the cyclic AMP content, the latter was still only one-half the in vivo value (11). The reason for the fall in cyclic AMP content on isolation of the tissue is unknown. However, dissection of the cornea eliminates two possible sources of adenylate cyclase stimulants: corneal adrenergic fibers (19) and the circulation. Both sources of catecholamines may sustain the high epithelial cyclic AMP measured in fresh tissue over the levels for the isolated preparation.

Apart from the stimulation of active Cl transport, the major effect of theophylline and dibutyryl cyclic AMP was an increase in Cl permeability, which on the average was double the control value. At the same time, treatment with theophylline had no great effect on urea permeability or on Na permeability when allowance for ion drag was made. These results imply that the mechanism for cyclic AMP-induced Cl permeation is anion specific. Theophylline also increases both unidirectional fluxes of thiocyanate across the epithelium (unpublished data). Therefore, one cannot exclude similar effects of cyclic AMP on HCO₃⁻ permeability.

The increase in theophylline or dibutyryl cyclic AMP-induced Cl permeability and active Cl transport was accompanied by a 50% reduction in epithelial resistance. Such a large change in epithelial resistance could occur only in the outer regions of the epithelium, since the measured transverse resistance of the inner membrane of the basal cells was only 0.3 kΩcm² (9), a value 10 times less than the resistance change observed in the present study. Further, the major effect of these cyclic AMP-inducing agents must be on transcellular ion pathways, since an increase in Cl permeability through an extracellular route (the tight junctions of the apical epithelium, for example) of the magnitude demonstrated ought to strongly depolarize the preparation. Instead, corneal potential is relatively unchanged following the resistance decrease. This has been ascribed to the fact that the outer membrane potential of the epithelium is normally close to the 40 mV predicted by the Nernst equation for Cl equilibrium across that membrane. Therefore, a further increase in the Cl permeability of the outer membrane should not significantly affect its potential or that of the cornea as long as other serosal membrane potentials remain constant (10).

The existence of Cl transport in the frog cornea has been well documented (1, 2, 19, 22, 23) since the initial observation by Zadunaisky (21). Less has been reported on the direct relationship between the transport and epithelial or stromal hydration. Zadunaisky and Lande (23) have shown a positive correlation between corneal transparency and Cl transport in the frog, but the latter methods utilized did not discriminate between possible effects on epithelial transparency and stromal transparency with the measurement of light transmission. In the present paper the stromal thickness has been measured directly, and the influence of theophylline has been demonstrated in the presence of corneal resting potential.

Using the short-circuit current technique (20), it has been presumed the active transport of Cl from the subepithelial stroma to the tear side solution may proceed without a net transfer of solute, since Cl would be removed at the tear...
side current bridge and liberated at the aqueous side-current bridge. For this reason an osmotic gradient external to the epithelium is difficult to postulate, and the mechanism of stromal thinning is obscure. In the present study it has been shown that Na accompanied the Cl transport at resting potential. In the experiments in which the endothelial side was blocked by silicone, stromal thinning can be postulated on osmotic grounds alone, since the solute was transported from a closed compartment.

The epithelial transport of Na by the isolated rabbit cornea was originally discovered by Donn, Maurice, and Mills (4). Since the direction of this transport was from tears to stroma, no obvious relationship of this transport and corneal dehydration was anticipated. Green (6, 7) confirmed the presence of Na transport and showed that the SCC and corneal thickness were dependent on tear-side Na. However, his conclusion that stromal thickness was determined by inward-Na transport has been proven unwarranted, since it has been shown that when adequate cell levels of cyclic AMP are maintained, the inward Na transport is more than balanced by the outward transport of Cl. Hence with Cl transport thought now to be the dominant system, the function of the Na transport remains unknown.

The present studies confirm the existence of the active Cl transport system in the rabbit corneal epithelium and establish the fact that there is net transfer of Na and Cl.

**Fig. 4.** Response of $^{36}$Cl fluxes, corneal potential ($\alpha$), and corneal resistance (○) to 10°C temperature rise. This corneal pair was incubated with 1 mM theophylline from time 0. Dashed lines indicate predicted response of each flux for a passive process.

**Fig. 5.** Response of $^{22}$Na fluxes, corneal potential ($\bullet$), and corneal resistance (○) to 10°C temperature increase. These corneas were incubated in presence of 1 mM theophylline from time 0. Dashed lines are predicted levels of fluxes for passive processes.
from the stroma to the tears in the presence of the cornea. Further, it is shown that under similar conditions, the cornea thins in response to theophylline which opposes and reduces the extent of

**REFERENCES**

Prior to the knowledge of epithelial Cl transport, the role of the epithelium has been described as a permeability barrier against the accumulation of fluid from the tears. In view of the recent findings, it is proposed that in addition the epithelium—the site of an outwardly directed pump—may produce effective impermeability in situ by balancing the slow flow of solute into the stroma that would otherwise occur. It would appear that the epithelium as well as the endothelium may synergistically control mammalian corneal hydration through active processes.

Although the maximal rate of stromal thinning achieved by the endothelium is 30 times the rate reported here for the epithelium thinning a swollen cornea, the contribution to normal corneal hydration may be similar in situ. The possibility also exists that stimulation of epithelial Cl transport in a cornea with a dystrophic endothelium may dcter stromal swelling and the eventual requirement for corneal transplantation.

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**TABLE 3. Effect of 1 mM theophylline on corneal thickness change**

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Control, μm/h</th>
<th>Theophylline, μm/h</th>
<th>Difference, μm/h</th>
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<tbody>
<tr>
<td>021573</td>
<td>-1.30±0.33</td>
<td>2.86±0.45</td>
<td>-0.92</td>
</tr>
<tr>
<td>021673</td>
<td>-1.75±0.27</td>
<td>-1.75±0.23</td>
<td>-3.50</td>
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<tr>
<td>051873</td>
<td>-1.72±0.23</td>
<td>0.15±0.21</td>
<td>3.27</td>
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<tr>
<td>052173</td>
<td>-2.17±0.37</td>
<td>-3.05±0.48</td>
<td>0.88</td>
</tr>
<tr>
<td>052273</td>
<td>+0.97±0.08</td>
<td>-0.35±0.19</td>
<td>-0.66</td>
</tr>
<tr>
<td>052974</td>
<td>+0.89±0.06</td>
<td>+1.75±0.09</td>
<td>4.07</td>
</tr>
</tbody>
</table>

Grand means ±SE

Values are means ± SE calculated with linear least-squares method during steady changes in corneal thickness (see Fig. 6) for approximately 60 min preceding and after treatment. Corneas were initially swollen 75–125 μm above their in vivo thickness. The mean of the differences (–2.27) is statistically significant \((P < 0.01)\) for paired observations. Thinning is indicated by a negative sign.

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**FIG. 6. Effect of various agents on corneal thickness in specular microscope apparatus with endothelial surface blocked by silicone oil and epithelial surface perfused at 10 μl/h. Steady thinning rates are indicated by lines calculated with least-squares method for comparison to steady-state ion fluxes. In order of occurrence, slopes were: \(2.17 ± 0.37\), \(-3.05 ± 0.48\), \(21.3 ± 3.2\), and \(6.10 ± 1.27 μm/h\).**