Permeability of the corneal endothelium to nonelectrolytes

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The endothelial surface of the isolated rabbit cornea was perfused with modified KEI medium, according to the method reported previously. This in vitro cornea maintained its thickness within the normal range even after the epithelium was removed. The endothelial permeability of these in vitro corneas to tritiated water, \textsuperscript{14}C-labeled urea, sucrose, and inulin was determined. The reflection coefficients of the endothelium calculated according to the physical interpretation of Kedem and Katchalsky were in fair agreement with those determined previously from osmotic fluid flow across the endothelium. Perfusion of the corneal endothelium with calcium-free KEI medium resulted in a corneal swelling with an increased endothelial permeability. Perfusion with KEI medium containing ouabain (10\textsuperscript{-5} and 10\textsuperscript{-3} moles per liter) induced a corneal swelling with normal endothelial permeability.

In vitro perfusion of the endothelial surface of the isolated rabbit cornea with modified KEI medium was recently found to maintain thickness of the cornea within a normal range, even after removal of the epithelium from the cornea.\textsuperscript{1} It was concluded, therefore, that the endothelium is of major importance in maintaining normal corneal thickness. The method of this in vitro perfusion will therefore offer a very good system to study endothelial function in the maintenance of the corneal thickness. This endothelial function may be classified in two categories, namely: (1) active processes which require metabolic energy and (2) the function as a physical barrier. Some experimental evidence has been reported\textsuperscript{1-4} suggesting that aerobic metabolism of the endothelium is required for the maintenance of normal thickness. The second aspect, namely, the function as a physical barrier, has been the object of the present study.

The physical properties of a membrane involved in transport are characterized by three independent coefficients,\textsuperscript{5} i.e., the hydraulic conductivity, the reflection coefficients, and the permeability to solutes. The hydraulic conductivity of the endothelium and its reflection coefficients to various solutes were the subject of the

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This investigation was supported by the United States Public Health Service Research Grant NB 06309 and also by Grant NB 04968 (Cornea Center) from the National Institute of Neurological Diseases and Blindness. This work was also supported by a Grant-in-Aid from the National Society for the Prevention of Blindness, Inc., New York.

This paper was presented at the Eastern Section Meeting of the Association for Research in Ophthalmology, March 10-11, 1967, in Washington, D. C.

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mated the endothelial permeability to this by Maurice. Donn, Miller, and Mallett determined diffusional flux of tritiated water across the rabbit cornea and estimated the endothelial permeability to this substance. In the present investigation, the permeability of the endothelium perfused in vitro to nonelectrolytes has been determined, with the use of ¹⁴C-labeled urea, sucrose, and inulin, as well as tritiated water. The advantage of using these nonelectrolytes is that they are not consumed by the tissue and that permeation will not be modified by cellular activities.

A marked swelling of the cornea occurred following the perfusion of the endothelial surface with either the modified KEI medium free of calcium or with the medium containing ouabain. The endothelial permeability of these swelling corneas was compared with that of corneas having a maintained thickness, and two different swellings were demonstrated, namely, one with increased permeability and the other with normal permeability of the endothelium.

Materials and methods

The experiments were divided into two parts, namely: (1) determination of isotope efflux from the cornea and (2) determination of distribution ratio of the isotopes between the cornea and the medium.

Albino rabbits weighing 2 to 3 kilograms were used without regard to sex. The eyes were enucleated under anesthesia with pentobarbital. The cornea was isolated and perfused in vitro according to the method of Mishima and Kudo.

Efflux experiments.

Perfusion of the isolated cornea. The cornea with its scleral rim was isolated and clamped in a lucite perfusion chamber. This chamber was then placed in a larger moist chamber within a constant temperature water bath; the corneal surface was in contact with air saturated over 0.9 per cent saline. The endothelial surface was perfused with the modified KEI medium. The temperature of the cornea was 34° C. and the intraocular pressure was maintained between 10 and 20 mm. Hg by adjusting the height of the outlet tube of the perfusion system. The details of the preparation and perfusion were reported previously.

Perfusing medium. The basic perfusate was the modified KEI medium. This medium was made calcium free by substituting sodium for calcium. Details of the preparation and the composition of these media were reported elsewhere.

Rate of perfusion. Since the exact rate of perfusion was necessary for the calculation of endothelial permeability, the rates were individually calibrated. The outlet tube of the perfusion system was connected to a graduated capillary pipette (0.01 ml. per division) and the time was measured during which the front meniscus of the perfusing medium traveled through 0.01 ml. The rate used for perfusion of ¹⁴C-labeled compounds was approximately 20 μL per minute and the rate for tritiated water was approximately 40 μL per minute.

Removal of the epithelium. After normal corneal thickness was attained and maintained for about one-half to one hour of perfusion, the epithelium was removed. It was crushed with a fine grain sand paper driven by a small electric motor. The crushed epithelium was then removed with a small scalpel. This method of removal caused very little stress to the cornea.

The measurement of corneal thickness. Corneal thickness was measured through the transparent door of the moist chamber with a Maurice-Giardini pachometer attached to a Haag-Streit slit lamp (Model 360). Ten readings for the central region of the cornea were averaged in each determination.

Isotope solutions and application to the cornea. ¹⁴C-labeled urea (sp. act. 2 to 10 mc. per millimole), sucrose (sp. act. 1 to 5 mc. per millimole) and inulin (sp. act. 1 to 3 mc. per gram) were obtained in crystalline form (New England Nuclear Corporation, Boston, Mass.). The isotopes were dissolved in distilled water to a concentration of 0.1 μc per microliter. Tritiated water (sp. act. 250 μc per gram) was obtained from the same source. Fluorescein was added to these isotope solutions to the concentration of 0.1%.

Approximately 1 μL of the isotope solution was aspirated into a fine polyethylene tube (Intramedic PE 10, Clay-Adams, New York) with a micrometer syringe. The tube was calibrated to contain 0.81 ± 0.01 μL per 10 mm. The solution was then applied on the bare stromal surface of the isolated cornea as evenly as possible. The distribution of fluorescein in the corneal stroma indicated the evenness of application.

Sampling of the perfusing medium. The perfusing medium was sampled at the outlet of the perfusion system in vials (Fig. 1) after the application of the isotopes to the corneal stroma. Sampling was done every 3 minutes for tritiated water, every 10 minutes for ¹⁴C urea and su-
Fig. 1. Application of isotope on the corneal stroma and sampling of the perfusing medium at the outlet.

Fig. 2. Reservoir-type chamber for the incubation of isolated rabbit cornea. For gas mixture, see text.

crose, and every 15 minutes for $^{14}$C inulin. Twelve to fifteen samples were collected for each experiment.

Assay of the isotope activity. Activities of the radiotracers were assayed with a liquid scintillation counter (Packard, Tri-Carb Liquid Scintillation Spectrometer, Model 3003, Packard Instrument Co., Downers Grove, Ill.).

Determination of distribution ratio. The distribution ratio of the isotopes between the corneal stroma and the medium was necessary to calculate the permeability of the endothelium. The ratios for urea and sucrose were therefore determined experimentally. Experimental determinations were not done for tritiated water and inulin.

Bathing of the endothelial surface. The cornea, with its scleral rim, was isolated as described previously and clamped in a lucite chamber as shown in Fig. 2. The whole specimen was placed in a small moist chamber described previously. In this reservoir-type chamber the modified KEI medium bathing the endothelial surface was re-circulated by bubbling a warm wet gas mixture (7 per cent oxygen, 5 per cent carbon dioxide, 88 per cent nitrogen). The rate of circulation was approximately 3 ml. per minute, ensuring good mixing of the medium. The total amount of medium bathing the endothelial surface was approximately 4 ml. The gas mixture maintained the pH of the medium at 7.3 throughout the experiment. The temperature of the main water bath was controlled to approximately 38° C, so that the temperature of the medium behind the cornea was maintained at 34° C.

Application of the isotope and sampling. When normal corneal thickness had been maintained during incubation for one-half to one hour, 20 μL of isotope solution were added to the medium. Rapid mixing was revealed by the distribution of fluorescein mixed into the isotope solution. At the end of the incubation, 10, 20, and 100 μL of the medium were sampled with Lang-Levy micropipettes and were subjected to the assay of the isotope activity.

Assay of isotope activity in the corneal stroma. At the termination of the incubation, the cornea was removed from the incubation chamber, rinsed briefly with nonradioactive medium and blotted. The epithelium and endothelium were then removed with a razor blade and the stroma was sandwiched between two parafilms. The previous procedures were carried out in a transfer chamber saturated with moist air over 0.9 per cent saline. A stromal button of 9 mm. diameter was then punched out. It was weighed and dried in vacuo to a constant weight at 60° C. after phosphorus pentoxide. The dry weight was determined, and the amount of the stromal water calculated. The dry cornea was then extracted in 1 ml. of distilled water by vigorous shaking for at least 7 hours. This time was chosen since Otori11 reported that sodium, potassium, and chloride were almost completely extracted after 5 hours. After the extraction, 400 μL of the extract was sampled with the Lang-Levy micropipette and the activity was assayed.

Mathematical methods. The time course of the concentration changes of the isotopes in the perfusing medium at the outlet sampling site was the function of the rate of perfusion, permeability of the corneal endothelium, volume of the anterior chamber, volume of the cornea, and area of the corneal endothelium. A mathematical relation among these factors was formulated in order to calculate the permeability of the corneal endothelium.

Calculation of endothelial permeability. In the perfusion system, shown in Fig. 1, let us assume
that most of the resistance to diffusion of the substance in the cornea is in the endothelium and that the anterior-chamber and the corneal stroma were well stirred. The former assumption may be substantiated by the fact that the corneal stroma has very low resistance to diffusion compared with that of the endothelium (less than 1/200, Maurice7-s). The latter assumption of well-stirred corneal stroma was not correct in these experiments; the error caused by this assumption will be treated in the next section.

The basic equation for the solute flux across the unit area of the endothelium under the previous assumption and the steady state conditions were obtained as follows, according to the theory of membrane permeability (Kedem and Katchalsky5).

\[
J_s = \frac{\omega RT (C_a - r C_c) + (1 - \sigma) \bar{c}}{J_s} \tag{1}
\]

where symbols are defined in the following:

\(J_s\) = Rate of net solute flow per unit area (moles per second per square centimeter), \(J_v\) = Rate of volume flow per unit area (cubic centimeter per second per square centimeter), \(\omega RT\) = Permeability coefficient of the endothelium (centimeter per second), \(C_a\) = Concentration of the solute in the anterior chamber (moles per cubic centimeter), \(C_c\) = Concentration of the solute in the cornea (moles per cubic centimeter), \(r\) = Distribution ratio of solute between the anterior chamber and the cornea, \(\sigma\) = Reflection coefficient, \(\bar{c}\) = Average concentration of the solute in the endothelium, i.e., \(\bar{c} = (r C_c + C_a)/2\).

By dividing Equation 1 by the volume of the cornea (Vc) and applying the equation for the entire area of the endothelium (A), one can obtain the following:

\[
\frac{dC_a}{dt} = \frac{A}{V_c} \left( \frac{K_a C_a - r K_c C_c}{J_s} \right) \tag{2}
\]

where \(K_a = \omega RT + (1 - \sigma) \frac{J_s}{2}\) and \(K_c = \omega RT - (1 - \sigma) \frac{J_s}{2}\).

For the concentration of the anterior chamber (\(C_a\)), one can write:

\[
\frac{dC_a}{dt} = -\frac{(AJ_v + v_i C_a)}{V_a} \tag{3}
\]

where \(V_a\) is anterior chamber volume, and \(v_i\) is rate of anterior chamber perfusion. This equation can be transformed to more practical form by using Equation 1:

\[
\frac{dC_a}{dt} = \frac{(AK_a C_c - (AK_a + v_i) C_a)}{V_a} \tag{4}
\]

Equations 2 and 4 may be solved, if one can assume that the rate of volume flow, namely, of corneal swelling (\(J_v\), is constant* and that the change of the corneal volume during the experiment is not large so that the steady state of the system can be approximated. The solution of these equations is the following:

\[
C_a = M \left(e^{-\alpha t} - e^{-\beta t}\right) \tag{5}
\]

where \(M\) is a constant given by the amount of isotope applied, and \(x\) and \(y\) are given by the following equations:

\[
x \text{ and } y = \frac{\beta \sqrt{b^2 - 4d}}{2}
\]

\[
b = \left(\frac{AK_a}{V_a} + \frac{v_i}{V_a} \frac{AK_c}{V_a}\right)
\]

\[
d = \frac{\alpha K_v}{V_a V_c}
\]

Since \(y\) is larger than \(x\), the second term of Equation 5 will be negligible after a certain time interval, and therefore the value of \(x\) will be obtained graphically from semilogarithmic plotting of the \(C_a\)-time relations.

By solving Equation 6 in terms of the permeability coefficient of the endothelium (\(\omega RT\)), one obtains:

\[
\omega RT = \frac{x}{V_a V_c V_a} + A \left(\frac{v_i}{V_a V_c} - \frac{x}{V_a} - \frac{r}{V_a} \frac{(1 - \sigma) J_s}{2}\right)
\]

\[
A \left(\frac{v_i}{V_a V_c} - \frac{x}{V_a} - \frac{r}{V_a}\right)
\]

When the cornea does not swell, the second term of the numerator becomes zero, and the calculation becomes simplified.

Calculations of dimensions. At the end of each experiment, the distance between the corneal apex and the base of the anterior chamber was measured by the use of a caliper to the accuracy of 0.05 mm. The depth of the anterior chamber (d) is obtained by subtracting the corneal thickness. Since the diameter of the cornea clamped to the perfusion chamber was 11 mm., the radius of the corneal curvature (r) in millimeters was calculated, assuming that the cornea had a single curvature throughout.

\[
r = \frac{(5.5^2 + d^2)}{2d} \tag{8}
\]

Volume of the anterior chamber (\(V_a\)) and the area of the cornea (\(A\)) are, respectively,

*In corneas perfused with calcium-free KRB medium (Fig. 4) and also treated with ouabain (see reference 17), the swelling was found to be practically linear in the first 2 to 3 hours.
$V_c = \pi d^2 \left( r - \frac{d}{3} \right)$

$A = 2\pi rd$

Volume of the cornea, with its solid mass excluded, is obtained by

$V_c = A \left( q - 0.08 \right)$

where $q$ is the corneal thickness in millimeters and 0.08 mm. corresponds to the thickness of the solid mass.\(^6\)

The calculations of these dimensions lead to a solution of Equation 7 to yield $\omega_{RT}$ values for the corneal endothelium.

**Correction for unstirred layer.** Since the assumption used in the preceding section that the corneal stroma is well stirred does not hold, one must correct for the errors resulting from this assumption. Correction for the apparent permeability coefficient for unstirred layer was studied by Ginzberg and Katchalsky.\(^{12}\) Their equation was modified to give the following equation:

$$\frac{1}{\omega_{RT}} = \frac{1}{\omega^{RT}} - \frac{Q}{D'}$$  \hspace{1cm} (9)

where $\omega_{RT}$ is the permeability of the endothelium corrected (centimeters per second), $\omega^{RT}$ is the apparent permeability (centimeters per second) calculated from equation 7, $Q$ is the thickness of the stroma (centimeters), and $D'$ is the diffusion coefficient of the solute for unit thickness of the

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**Fig. 3.** Corneal thickness during the efflux experiment: the perfusion with the modified KEI medium. $E$, Epithelium was removed. $I$, Isotope solution ($^{14}$C sucrose) applied on the stromal surface.

**Fig. 4.** Corneal thickness during the efflux experiment: perfusion with Ca free medium. $E$, Epithelium was removed. $I$, Isotope solution ($^{14}$C inulin) applied on the stromal surface.
Perfusion of corneal endothelium

Results

Corneal thickness during the experiments.

Perfusion with the modified KEI medium. When the isolated cornea with its epithelium removed was perfused with the modified KEI medium, the cornea could maintain constant thickness for several hours. This observation, made in more than 10 perfusions, confirmed the previous finding of Mishima and Kudo. When an isotope solution of sucrose, inulin, or tritiated water was applied to the stromal surface, the cornea swelled to less than 5 per cent of its thickness (Fig. 3). When urea was applied, however, swelling to about 10 per cent of the thickness was measured. During the subsequent perfusion, the thickness initially tended to reduce toward the original value, and then to maintain constant thickness during the remainder of the experimental period. Similarly, when the cornea was incubated in the reservoir-type chamber, the corneal thickness remained constant for more than 6 hours of incubation.

Perfusion with Ca free medium. The isolated cornea was first perfused with the modified KEI medium and then with the Ca free medium. Slight corneal swelling was noticed after about one hour, followed by remarkable swelling, again confirming the previous results of Mishima and Kudo. The rate of swelling was 0.08 ± 0.03 mm. per hour (5 corneas) when the epithelium was present and 0.07 ± 0.02 mm. per hour (11 corneas) without the epithelium. The presence of the epithelium did not alter the rate of swelling.

When definite swelling of the cornea was measured, isotope solution was applied evenly on the bare corneal stroma. After the isotope application, swelling continued without change of rate (Fig. 4).

Distribution ratio of urea and sucrose between the corneal stroma and the medium. The isolated cornea was incubated for various lengths of time after the isotope was mixed in the medium. The activity in the corneal water was expressed as the ratio with that in the medium. When urea was used, the ratio was found to be steady between 2 and 6 hours of incubation. The average of ratios in 6 experiments incubated for this time period was regarded as the distribution ratio; it was 0.95 ± 0.07.

The ratios of the activity in the stromal water to that in the medium at various intervals after sucrose application were plotted in Fig. 5. Considerable scattering of the data was noticed, but the result was
compatible with the assumption that the distribution ratio was practically unity.

For tritiated water, the ratio of one was used without experimental determination. For inulin, the ratio was assumed to be unity without experiment. It was not possible to incubate the cornea long enough (more than 12 hours) without the risk of damage to the endothelium, to make the actual determination for inulin which is taken up very slowly by the cornea.

**Calculation of the permeability coefficients.**

**Permeability for the cornea with a maintained thickness.** When normal corneal thickness had been maintained for one-half to one hour of perfusion, the epithelium was removed. After a certain period to allow recovery of the cornea from evaporation during the procedure, the isotope solution was applied on the bare stromal surface and the perfusing medium was sampled at the outlet of the perfusion system at intervals (Fig. 1). The activity of the isotope in the samples was plotted semilogarithmically, as shown in Figs. 6 and 7. The activity changes can be expressed by double exponential equation, as expected from the mathematical analysis. The time required for maximum activity to be reached in the perfusing medium became longer and the subsequent decrease of activity slower as the size of the test molecules increased. The rate of the activity decrease after the maximum was reached was determined graphically and the permeability coefficients calculated. The results are given in Table I.

Since the assumption for this calculation, that the corneal stroma constitutes a well-stirred compartment, does not hold, corrections were made for this unstirred layer by the method described in the previous section. The error was approximately 40 per cent for tritiated water and about 10 per cent for urea. The corrected values are given in Table I. The error was negligible for sucrose and inulin.

Diffusion coefficients of these molecules in the aqueous medium at 34°C were calculated from previous sources and are given in Table I. The ratios of the permeability coefficients for the endothelium and the diffusion coefficients in aqueous medium were calculated on the assumption that the endothelial thickness was 5 μ. These results are also given in Table I.

**Permeability for the swelling cornea.** When corneal swelling was definitely noticed in the perfusion with Ca free medium, the epithelium was removed. After the corneal thickness had recovered from temporary thinning because of evaporation during the removal of the epithelium, the isotopes were applied on the stromal surface (Fig. 4). The perfusing medium was then sampled and the activity counted. The permeability coefficients were calculated for urea and inulin. The calculation with the obtained swelling rate of the cornea showed that the second term of Equation 7, which is due to the corneal swelling, was negligible (less than 5 per cent of the permeability value at the maximum). The subsequent calculation was therefore made
by neglecting this swelling rate and by using an average corneal volume during the experimental period. The permeability coefficients of the endothelium of the cornea swelling during perfusion with Ca free medium are listed in Table I. The permeability of the endothelium is found to be considerably increased over normal values.

In another investigation, it was found that the perfusion with ouabain-containing medium (10⁻³ and 10⁻⁵ moles per liter) resulted in a corneal swelling; the endothelial permeability of these swelling corneas was determined. The permeability coefficients for these corneas to urea and sucrose are given in Table I for comparison. The coefficients in these cases are not significantly different from those values for corneas with a maintained thickness.

Table I

<table>
<thead>
<tr>
<th>Test molecule</th>
<th>Tritiated water</th>
<th>Urea</th>
<th>Sucrose</th>
<th>Inulin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>wRT × 10⁶ cm./sec</td>
<td>Ds, c.</td>
<td>Ds, c. ±</td>
<td>σ calculated</td>
</tr>
<tr>
<td>Tritiated water</td>
<td>164 ± 52 (6)</td>
<td>3.0§</td>
<td>360</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>21 ± 2.2 (6)</td>
<td>1.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>6.5 ± 1.7 (5)</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>1.25 ± 0.45 (6)</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*All data are presented as average ± standard deviation (number of experiments).
†Ax: thickness of the endothelium was assumed to be 5 µ.
‡From Mishima and Hedbys (1967).
§Wang, Robinson, and Edelman (1953).
||Longworth (1954).
#Longworth (1953).
$Calculated from Einstein-Stokes equation assuming radius for sucrose 5.3 Å, inulin 12 Å (Durbin, 1960).
**For raffinose.

Fig. 7. Time course of the activity change in the perfusing medium at the outlet: solid circles, urea; open circles, sucrose; crosses, inulin.
Discussion

Direct determination of corneal endothelial permeability to the nonelectrolytes used in this investigation is not found in the literature. The present data may be compared, however, with estimated values for tritiated water (Donn and co-workers\(^9\)) and with determined values for small ions (Maurice\(^7,8\)). Donn and others measured diffusional flux of tritiated water across the whole layer of rabbit corneas, cornea with epithelium removed, and the cornea with the endothelium removed. From their data, endothelial permeability of tritiated water may be calculated, assuming that the diffusion coefficients in swollen stroma remained virtually unchanged from those in normal stroma. A calculation from their data indicated that the obstruction of the endothelium to tritiated water (ratio between the endothelial permeability and free diffusion) was about the order of 1/250, which is in fair agreement with 1/360 of the present study.

The permeability coefficient found for urea in this study was almost the same as those found for sodium and other small ions in the living cornea (Maurice\(^8\)). In the previous investigation,\(^6\) it was found that both urea and sodium chloride had about the same reflection coefficients to the corneal endothelium. Since the reflection coefficients are the function of water and solute permeabilities across the membrane, these results indicate that the endothelium of the perfused cornea has been kept practically intact as in these previous investigations.

The reflection coefficients may be expressed by using the available area of the membrane for diffusion of solute and water, according to the physical interpretation of Kedem and Katchalsky\(^18\):

\[
\sigma = 1 - \frac{\omega_v}{L_p} - \frac{A_e}{A_v}
\]

where \(\omega_v\) is the partial molar volume of the solute, \(L_p\) is the hydraulic conductivity of the membrane, and \(A_e\) and \(A_v\) are, area of the membrane available for diffusion of the solute and water, respectively. It is, therefore, of interest to calculate the value of \(\sigma\) from the present permeability values and to compare this with the values of \(\sigma\) determined previously from osmotic fluid flow across the endothelium. The results are listed in Table I. The calculated and the determined values may be regarded to be in fair agreement, when it is realized that the determination of hydraulic conductivity and the permeability coefficient, especially of tritiated water, were subject to rather large standard deviation of the values.

A comparison of the permeability values for the swelling cornea with those for corneas with maintained thickness brings out an interesting point concerning mechanisms of the corneal swelling. The perfusion with Ca-free medium resulted in a swelling with markedly increased permeability of the endothelium. In the other investigation,\(^19\) it was found that the perfusion with Ca-free medium resulted in a dissolution of the terminal bar and separation of the endothelial cells. Wide intercellular separation obviously increased the permeability of this layer and fluid was imbibed through this impaired endothelium by the imbibition pressure of the corneal stroma.\(^20\)

The cornea also showed a marked swelling after the endothelial surface was bathed with the medium containing ouabain.\(^17\) The swelling occurred with ouabain concentrations between \(10^{-5}\) and \(10^{-3}\) moles per liter at the maximum rate of about 0.04 mm. per hour. A slower rate of swelling occurred with lower concentrations of ouabain and the half rate of maximum swelling was obtained with an ouabain concentration of \(3 \times 10^{-7}\) moles per liter. The average permeability of the endothelium to urea and sucrose is given in Table I for these corneas swelling in the presence of \(10^{-5}\) and \(10^{-5}\) moles per liter ouabain. It is found that endothelial permeability remained practically the same as normal corneas during the swelling.

It is thus clear that one can demonstrate two types of corneal swellings: (1) swell-
ing with increased permeability, as in the case of perfusion with Ca free medium, and (2) swelling with practically normal permeability, as in the case of ouabain application.

The authors are very grateful to Dr. D. M. Maurice and Dr. I. Flatt for their helpful criticism during this investigation. Acknowledgment is also due to Miss L. E. Valentin and Miss Susana Limaco for their skillful technical assistance.

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