International Journal of Pharmaceutics, 63 (1990) 1–16
Elsevier

IJP 02093

Research Papers

Electrophysiological and ultrastructural characterization of the cornea during in vitro perfusion

Yongyut Rojanasakul * and Joseph R. Robinson
School of Pharmacy, University of Wisconsin, Madison, WI 53706 (U.S.A.)

Received 4 May 1989
Modified version received 2 November 1989
Accepted 14 November 1989

Key words: Electron microscopy; Tissue viability; Tissue integrity; Membrane potential; Membrane resistance; Membrane capacitance; Membrane impedance; Charging kinetics; Phase angle

Summary

Functional and ultrastructural changes in rabbit cornea during in vitro perfusion were studied by means of electrical measurements and electron microscopy. Electrical measurements were used to provide information on membrane potential, resistance, and capacitance. Membrane potential reflects cellular metabolic activity and thus is used to monitor tissue viability. On the other hand, since electrical resistance and capacitance indicate charge flow and the ability of the membrane dielectric to store charges, they can be used to detect structural changes in the aqueous transport pathway, i.e., intercellular space, and membrane surface, respectively. Studies on the current-voltage relationship of the cornea indicate self-generated membrane potential and non-ohmic resistive behavior of the cornea. However, the electrical resistance remains constant and is invariant to the external applied voltage. Problems associated with the use of ohmic resistance as an indication for membrane resistance are discussed. During in vitro perfusion, the cornea undergoes a biphasic change in resistivity with an initial rise and subsequent fall. The initial rise of the resistance appears to be induced by a change in membrane potential while the declining portion, at longer time periods, correlates well with the change in tissue morphology, i.e., expansion of the aqueous intercellular spaces. With the exception of this structural change, the overall integrity of the cornea is preserved and its viability is maintained for at least 6 h in vitro. The effect of polarization current on the d.c. electrical resistance was investigated by means of discharging kinetic measurements. Results indicate that under direct current, the cornea, due to its capacitive property, undergoes rapid, exponential-like, charging and discharging with an average time constant of approx. 50 μs. The polarization current, however, was found to have no significant effect on determination of membrane resistance. In addition to the kinetic study, the capacitive property of the cornea was further investigated by phase difference and impedance measurements. The use of a range of frequencies of measuring current results in a model of the cornea as electrically equivalent to a parallel RC circuit. Unlike resistance, the capacitance of the cornea remains remarkably constant during in vitro perfusion. This finding is in agreement with electron microscopic studies of the tissue. The use of electrical methods to study membrane transport properties as well as to assess tissue viability and integrity is discussed.

Introduction

In vitro perfusion techniques have become a standard method in membrane transport and drug

* *Correspondence (present address)*: Y. Rojanasakul, School of Pharmacy, West Virginia University, Morgantown, WV 26506, U.S.A.

delivery studies, due mainly to their simplicity and the ease of experimental control. In many circumstances, specific information that is difficult to obtain in an in vivo system becomes accessible by this technique. However, due to instability of the biological membranes using in vitro methods, i.e., as a result of functional and structural changes, the use of such procedures to obtain reliable data that are truly representative of the conditions in vivo has been limited. Of most concern, especially in studies conducted for long time periods, are problems associated with tissue viability and integrity. The issue is of significance, but has not yet been systematically investigated. The intent of the present work is to address this matter and to develop methods for evaluating tissue condition as well as to study transport properties of the cornea. This investigation also serves as a foundation for ongoing research on the mechanisms of some penetration enhancers in the cornea.

The study of membrane transport in epithelia has been accomplished using basically two approaches. The first and commonly used approach is to measure fluxes of compounds of interest using specific analytical methods, i.e. radioactivity or HPLC assays. The second involves measuring electrical properties of the tissue, most notably membrane potential, resistance, and capacitance. The two methods are complementary on combined use, depending on the specific purposes of each study. In the present work, the electrical method was used to assess membrane transport properties of the cornea. This procedure also provides a rapid, non-invasive, and quantitative assessment of membrane viability and integrity.

The basis of the method lies in the fact that, under normal physiological conditions, a living cornea is capable of generating a transmembrane potential while a functionally dead cornea cannot. This potential is a result of active ion-transport mechanisms operating through membrane carriers and pumps. In rabbit cornea, this active potential was found to be initiated by an inward sodium transport from tear to aqueous humor (Donn et al., 1959; Green, 1965) and an outward chloride transport in the reverse direction (Zadunaisky, 1966; Klyce et al., 1973). The presence of an active potential indicates the active metabolic state of the cornea and thus can be used as an indication for tissue viability. On the other hand, electrical resistance and capacitance reflect ionic membrane permeability and the ability of the membrane to store charges, and therefore can be used to indicate tissue integrity. In addition to electrical assessment, simultaneous microscopic examination of the cornea during in vitro perfusion can relate morphological changes to membrane integrity and permeability.

Materials and Methods

Animals

Male albino New Zealand rabbits (New Franken, WI) weighing between 2.5 and 3.0 kg were used. Lighting was maintained on a 24 h basis in the caging facility, and the animals were fed regular diet with no restriction on the amount of food or water consumed.

In vitro perfusion studies

All chemicals were analytical grade and were used as received. All solutions were made using deionized, distilled water. Perfusion studies were carried out in glutathione-bicarbonate Ringer's solution (GBR), pH 7.4, according to O'Brien and Edelhauser (1977). Rabbits were killed by an intravenous injection of an overdose of sodium pentobarbital administered via the marginal ear vein. Corneas were removed by cutting the sclera 2 mm laterally to the limbus with subsequent removal of the lens and iris. Care was taken to avoid contamination and physical trauma to the tissue. The corneas were immediately mounted on corneal rings and clamped in the perfusion chamber (Machine Shop, University of Wisconsin-Madison). In contrast to the standard corneal mounting procedure utilizing a suction device, previously described (Schoenwald and Haung, 1983; Grass and Robinson, 1988), the present method provides better preservation of tissue integrity and viability as indicated by higher and longer membrane resistance and potential respectively. Aeration and circulation in the tissue bath were provided by means of bubbling with a mixture of 95% O_2 and 5% CO_2. All experiments were

carried out at 37 °C using a constant temperature bath with an external circulator connected through a water jacket of the perfusion chambers. A 0.67 cm^2 area of the cornea was exposed to the donor and receptor compartments, each having a volume of 7 ml. In some experiments, where degree of hydration was studied, corneas were removed from the perfusion chamber at appropriate time points and blotted dry. The water content was then determined by weighing the cornea before and after drying overnight in an oven at 60 °C.

Electrical measurements

Electrode preparation. The electrodes were prepared from silver wires (99.9% purity, Aldrich) of 5 cm × 1 mm size. They were cleaned by surface abrasion with sanding paper, placed in concentrated HCl solution for 1 h, and thoroughly washed in distilled water. The electrodes were then chlorided with direct current of 0.2 mA for 6 h in 0.5 M KCl solution. The finished electrode pairs were kept short-circuited in isotonic NaCl solution and stored in the dark for at least 24 h for intraelectrode potential stabilization prior to use. Asymmetry between electrodes was tested prior to each potential measurement. Only pairs of electrodes differing in potential by less than 0.1 mV were used.

Membrane potential and d.c. resistance. To measure the potential difference across the cornea, a high input-impedance, microvolt multimeter (Keithley-197) was used. Ag-AgCl electrodes were used as sensing devices. They were positioned 1 cm from each side of the corneal surfaces. Corneal resistance was measured using the four-electrode system; two electrodes were used to record potential difference, the other two (located 2 cm from each surface) being employed to inject current pulses. In all experiments, the anode was placed on the epithelial side and the cathode on the endothelial side. Pulses of variable duration (1–5 s) and intensity (up to ± 10 μA cm^{-2}) were generated from a constant-current source. Passive resistances were calculated from the slope of the *I-V* plots while apparent resistances were obtained from Ohm's law. To correct for the potential drop due to solution resistance between the sensing electrodes and the membrane, measurements were carried out before each membrane resistance determination using the same bathing solution but without the membrane in the perfusion chamber. The actual membrane resistance was then calculated by subtraction of the resistance obtained without the membrane from that determined with the membrane.

Discharging of membrane capacitor. To study discharging kinetics of the cornea, small steps of current having a magnitude of 10 μA cm^{-2} and frequency of 500 Hz (generated by a model PB-503 function generator, Global Specialties Corp.) were applied across the cornea through the current supplying electrodes. The potential response, detected through sensing electrodes, was then measured on an oscilloscope (Hitachi, model V-422).

Since the discharging of the cornea was found to follow exponential kinetics, a characteristic of an equivalent parallel *RC* circuit, analysis of the potential response was performed by using the following equation (Horowitz and Hill, 1980);

$$dV/dt = -I/C = -V/RC \quad (1)$$

The solution of this first-order differential equation has an exponentially decaying time course:

$$V = V_0 \exp(-t/RC) = V_0 \exp(-t/\tau) \quad (2)$$

where V, V_0, I, R, C, t and τ represent the voltage at time t, starting voltage, current, resistance, capacitance, time and time constant, respectively. The time constant is the period of time required for a capacitor to charge or discharge to 63.2% of the value of the original voltage. This time is the product of the resistance and capacitance of the membrane.

Impedance and phase relationship. The experimental setup for the impedance measurement was similar to that for d.c. resistance measurement except for the current source which, in this case, supplies alternating, sinusoidal current. The impedance of the excised cornea was obtained from the ratio of the voltage difference across the sensing electrodes and the current flowing through the tissue (each measured with an r.m.s. digital multimeter). Impedance measurements were made at a

current density of 1–10 μA cm^{-2} and at a number of frequencies encompassing a wide range (1 Hz–100 kHz). Since reversible Ag/AgCl electrodes and sinusoidal signals (average signal voltage of zero) were used, net polarization of the electrodes and the membrane was minimal. To obtain the true corneal impedance, the impedance between the two sensing electrodes, determined in the absence of the cornea, at each frequency was vectorially subtracted from that obtained in its presence.

The phase relationship between the applied and the membrane voltages was determined over the frequency range 1–100 kHz at a current density of 10 μA cm^{-2}. The sine wave output from the function generator was connected to the horizontal plates of the oscilloscope and the response signal from the membrane was detected through the vertical deflection plates. The phase angles between the two signals were determined from the Lissajous patterns reading from the oscilloscope (Plutchik and Hirsch, 1963).

Scanning electron microscopy

Rabbit corneas taken freshly or at 1, 6, and 12 h after in vitro perfusion were placed in a fixative solution containing 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.3, for 2 h. After washing in the same buffer, the tissues were post-fixed in 2% OsO_4/0.1 M phosphate buffer for an additional 1 h, and then dehydrated in 35, 50, 70, 90, 95 and 100% ethanol (each step for 10 min). The 100% ethanol bath was changed three times for a total of 30 min. The ethanol-dehydrated specimens were transferred to a critical-point dried chamber and the ethanol was replaced, under pressure, with liquid carbon dioxide. The liquid was subsequently converted into gas by raising the chamber temperature to 31° C. The specimens were then mounted on aluminum pin-type mounts and shadowed with a 100 Å layer of gold by means of Sputter coating (Balzers-SCD 030). Finally, the specimens were viewed in a scanning electron microscope (Jeol JSM-35C).

Transmission electron microscopy

Tissue preparation up to the dehydration process was identical to that used in scanning electron microscopy. The dehydrated specimens were embedded in Durcupan embedding medium, and sectioned using a glass knife on a microtome (Porter-Blum, MT 2-B Ultramicrotome). Sections were placed on copper grids and viewed with a transmission electron microscope (Jeol 100 CX).

Results and Discussion

Current-voltage relationship

Determination of membrane resistance can be accomplished by measuring voltage drop and current passing through the membrane. To calculate the resistance, Ohm's law ($V = IR$) is normally used. However, due to the complex nature of biological membranes, Ohm's law, which is only applicable to a passively resistive circuit, may not necessarily be valid. For example, the IV relationship may not be linear, indicating a voltage-dependent resistive property of the membrane (rectification) or the IV plot may not pass through the origin, indicating an intrinsic electrogenic property (selectivity) of the membrane. To determine accurately the membrane resistance, the IV relationship of the cornea was first investigated. A typical IV curve of the cornea, determined over a range of current density (0 ± 10 μA cm^{-2}), is demonstrated in Fig. 1. Linearity of the plot is evident, however, while the current goes to zero

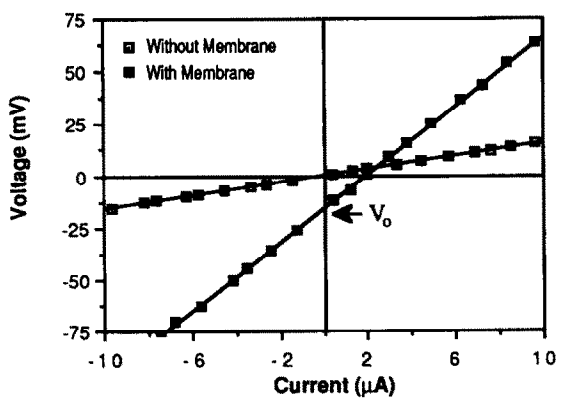

Fig. 1. A typical current-voltage relationship of the cornea. V_0 indicates negative zero-current potential (electromotive force). Current-voltage relation can be described according to modified Ohm's law; $V - V_0 = IR$. A relatively constant resistance over a wide range of voltage indicates non-rectification behavior of the cornea.

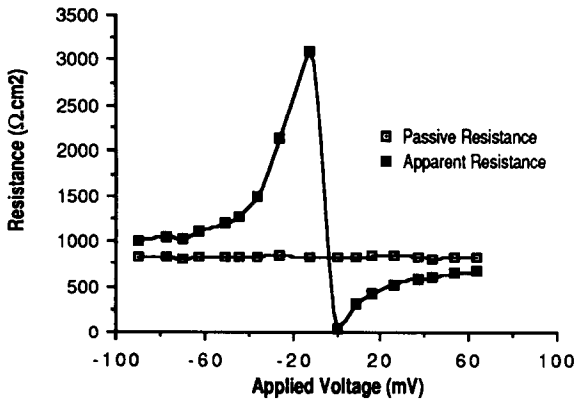

Fig. 2. Corneal resistance as a function of applied voltage. The apparent corneal resistances were calculated based on Ohm's law ($R = V/I$) and the passive resistances were evaluated according to the modified equation, $R = V - V_0/I$. The result indicates the influence of membrane potential on membrane resistance.

the voltage does not. The voltage at the intercept of the plot (zero current) is sometimes called the zero-current potential which, in this study, was found to be identical to the transmembrane potential determined separately in the absence of an external applied current. This result indicates stability of the corneal active potential during the *IV* study. The membrane potentials were consistently found to have negative polarity, i.e. epithelial side with respect to endothelial side, confirming the previous findings on an inward sodium and outward chloride active transport in the cornea. Membrane resistance, in this study, was determined from the slope of the *IV* plot according to the modified Ohm's law, $V = V_0 + IR$, where V_0 is the zero-current voltage. This resistance is referred to in the present study as passive resistance while that obtained directly from Ohm's law is designated as apparent resistance. Fig. 2 shows plots of the two as a function of external applied voltage. The result clearly demonstrates strong voltage dependence of the apparent resistance and thus its use as an indication of membrane resistance must be with caution.

Changes in membrane resistance and structure

Fig. 3 shows passive membrane resistance of the cornea in vitro as a function of time. Unexpectedly, the resistance profile of the cornea shows an initial rise during the first hour and a subsequent drop in the later hours. Attempts were made to determine the cause of this resistive behavior. Two possible contributing factors, namely swelling and deswelling of membrane and change in membrane potential, were investigated. The former was studied by determining the degree of hydration while the latter was determined by measuring transmembrane potential. The results of the two studies are shown in Fig. 4. The results from the study of hydration show that corneal swelling occurs rather uniformly and continuously and thus does not appear to be the major contribution, at least during the initial period, for the observed resistance change. On the other hand, the change in membrane potential correlates well with the change in membrane resistance. In fact, it was observed that, at any time, the resistance always followed the potential. To confirm this finding, a separate study conducted at a number of fixed membrane potentials was carried out and the resistances at various time intervals determined. Maintenance of membrane potential was achieved by clamping the voltage across the membrane using an external, constant voltage, power supply. It was shown (Fig. 5) that membrane resistance was actually controlled, especially in the early period, by membrane potential. The insensitivity

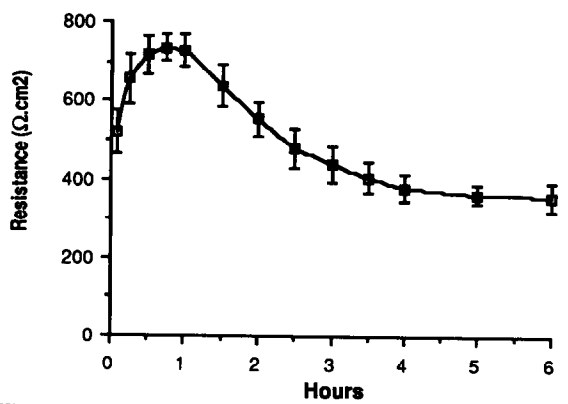

Fig. 3. A typical resistance-time profile of the cornea. The resistance was measured by applying d.c. pulses of variable duration (1–5 s) and intensity (up to ± 10 μA cm^{-2}) and recording the voltage drop across the cornea. Bars indicate 1 SE; $n = 4$.

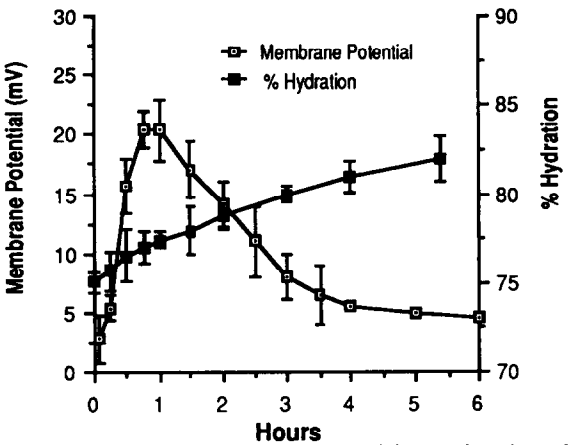

Fig. 4. Hydration and membrane potential as a function of time. Continuous swelling and biphasic potential change of the cornea are presented. Transmembrane potential, developed when two identical solutions are placed on both sides of the membrane, indicates active ion transport and thus the viable state of the tissue. Bars indicate 1 SE; $n = 4$.

of the resistance to membrane potential in later hours indicated another contributory factor which was subsequently found to be the expansion or swelling of the corneal intercellular pathway. In the voltage-clamp experiment, when bubbling of the bathing solutions was switched from oxygen to nitrogen, membrane potentials dropped to zero (nitrogen inhibits active transport that gives rise to membrane potential) and the resistances were consistently higher than those in the control (average peak potential ≈ -20 mV). With increasing negative potentials (-25 and -50 mV), the resistances decreased accordingly. It should be noted that the actual membrane potential observed in vivo is approx. -25 mV (Potts and Modrell, 1957; Maurice, 1967). Based on this study, it appears that the change in membrane resistance occurs in the direction that promotes preservation of normal membrane potential. The mechanism for this change is not yet clear but is believed to be due to changes in tight junction permeability, which is the subject of an ongoing investigation. Results from this study also suggest that, in order to mimic the actual in vivo conditions more closely, transport studies conducted in vitro should be performed under conditions of physiological potential. The contribution of the membrane potential is even more significant in permeation studies of charged species due to the influence of an additional electrostatic barrier caused by this potential.

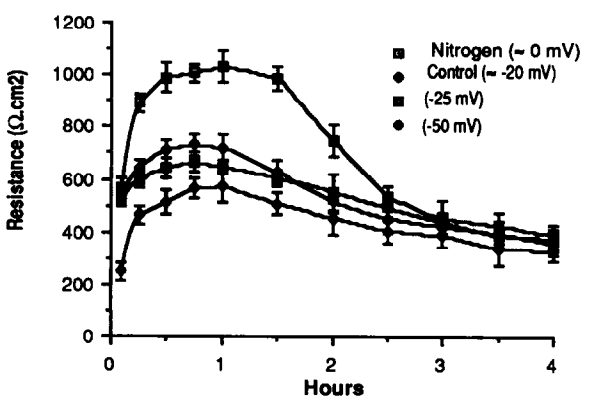

Fig. 5. Effect of membrane potential on electrical resistance as a function of time. Membrane potentials were kept constant by means of voltage clamp (-25 and -50 mV) or use of nitrogen (≈ 0 mV). The membrane potential in the control has an average peak potential of approx. 20 mV (see fig. 4). Bars indicate 1 SE; $n = 4$.

Ultrastructural changes of the cornea during in vitro perfusion were also investigated using electron microscopy. Tissues obtained freshly (time zero) and after 1, 6, and 12 h were processed and subjected to microscopic examination. Electron micrographs of these tissues are shown in Figs 6–9. No detectable change was observed between the tissues at 1 and 0 h and thus only results from the latter are presented. Fig. 6(a–c) shows transmission electron micrographs of the corneal epithelium at 0, 6, and 12 h respectively. Results indicate that, in all tissues, the epithelial cell layers remain intact, however, progressive cell separation is evident, i.e. widening of the intercellular spaces occurs. Expansion of the superficial intercellular spaces and tight junctions can be clearly observed in the 12 h tissue. These results substantiate previous findings on the decrease in membrane resistance observed during later hours, since electrical resistance is a measure of charge flow across the membrane and this occurs primarily via an aqueous intercellular pathway. The finding on intercellular expansion also explains, in part, why membrane potential could not be maintained. According to the current concept on epithelial regulation of ionic conductance (Binder, 1980), the mem-

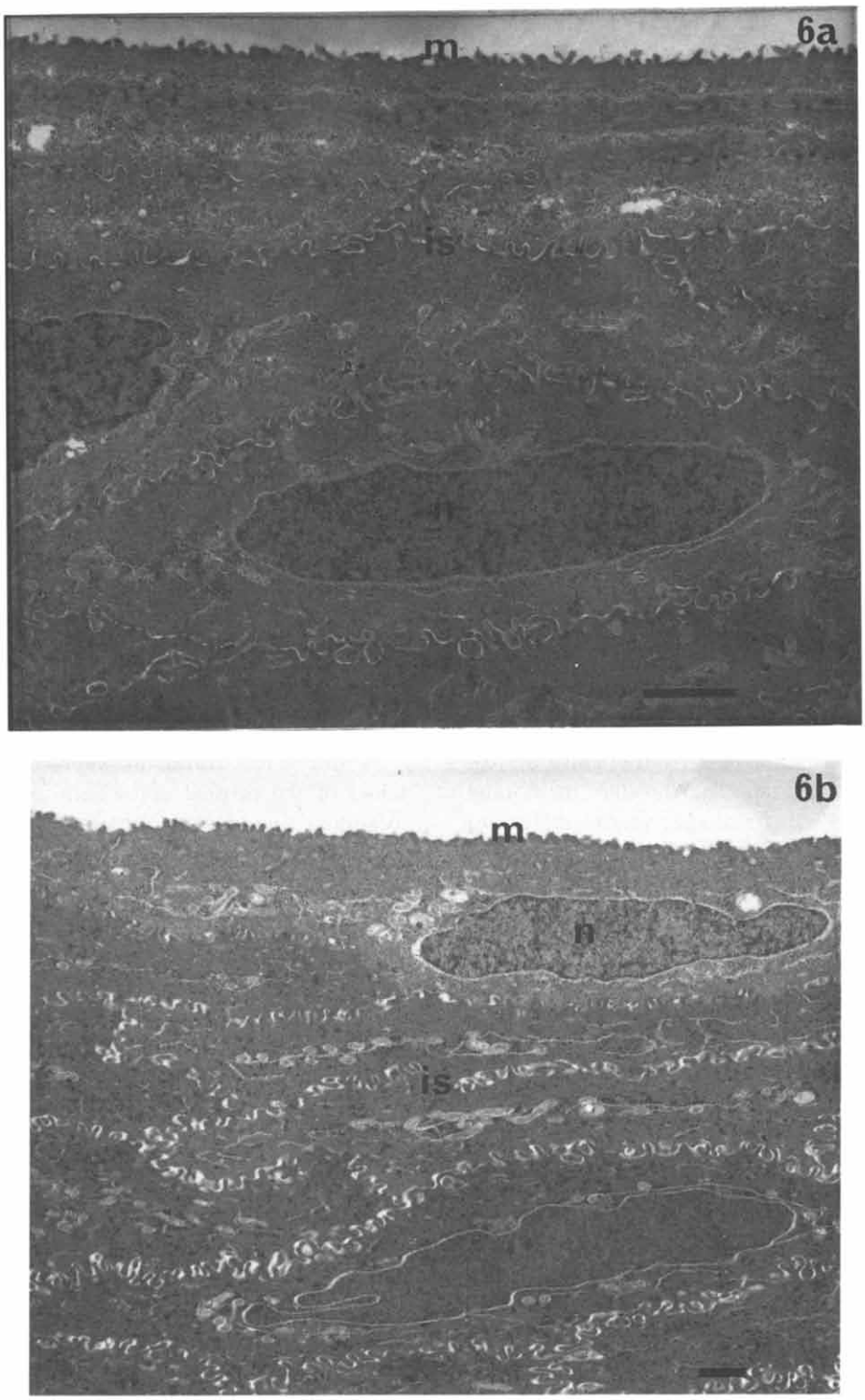

Fig. 6. Transmission electron micrographs of the rabbit corneal epithelium after (a) 0 h, (b) 6 h, and (c) 12 h in vitro perfusion in GBR solution at 37° C. Progressive cell separation as a result of expansion of the intercellular spaces (is) is evident. Note the location of nucleus (n), and microvilli (m). Bar: 2 μm.

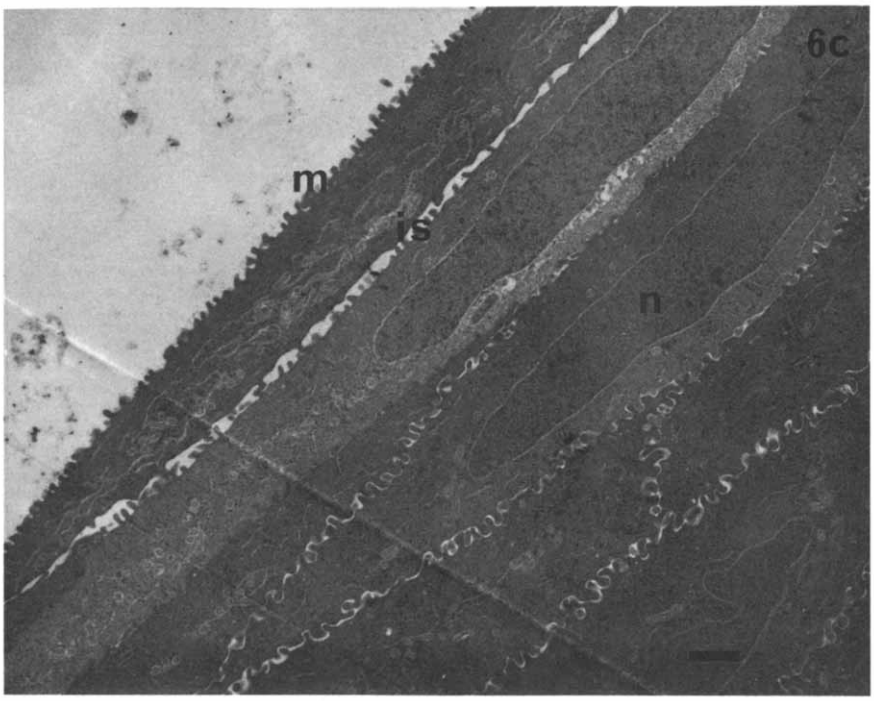

Fig. 6 (c).

brane potential is controlled by the active uptake of specific charged species through membrane pumps and passive leakage (back diffusion) through junctional pathways. In vivo, membrane potential is maintained at a fixed value as a result of the net effect of these two transport systems. In this study, the drop in membrane potential can be attributed to a decrease in pump activity as well as an increase in junctional permeability. The presence of the corneal potential observed in later hours, however, indicates that the cornea remains viable, although weakened, for at least 6 h under our experimental conditions.

Transmission electron micrographs of the corneal endothelium after 0, 6, and 12 h perfusion are shown in Fig. 7(a–c). At 6 h, the endothelial cells have minimal structural damage and the intercellular junction appears normal. A few vacuoles are present in the cytoplasm. At 12 h, loss of cellular organization has occurred. The nucleus and the intercellular junction are swollen and expanded and the posterior plasma membrane and cytoplasm are disrupted.

Scanning electron micrographs, showing surface views of the corneal epithelium and endothelium at similar time points, are shown in panels a–c of Figs 8 and 9, respectively. At 6 h, the superficial cells of the epithelium appear normal except for swelling of some of the dark cells. No apparent expansion of the tight junction can be seen. At 12 h, widening of some of the intercellular junctions is evident. Disruption of the intercalating protein network at the cell junctions is clearly seen. In the endothelium, progressive intercellular expansion, similar to that observed in the epithelium, can also be seen.

Polarization and discharging kinetics of the cornea

In addition to containing many conducting pathways, the lipid matrix of the epithelia, due to its dielectric property, is capable of storing charges on its surface and generating its own electrical field. In a way, the lipid matrix acts as a capacitor. For this reason, if a direct current is applied across the tissue, i.e. during resistance measurement, polarization and subsequent current flow

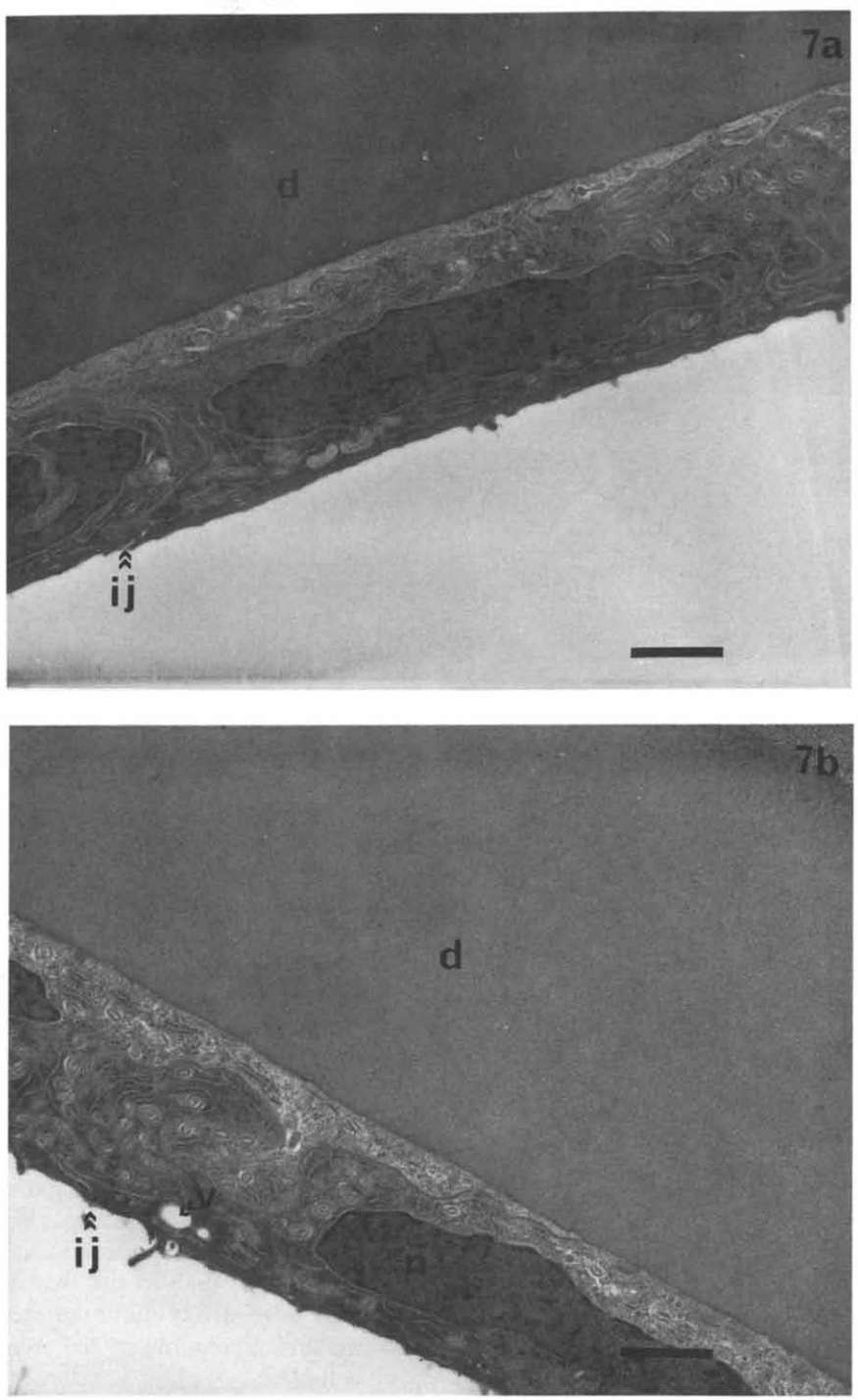

Fig. 7. Transmission electron micrographs of the corneal endothelium after (a) 0 h, (b) 6 h and (c) 12 h in vitro perfusion in GBR solution at 37 °C. Vacuoles (V) can be seen after 6 h and complete loss of cellular organization is evident after 12 h. Note the location of Descemet's membrane (d), nucleus (n), and intercellular junction (ij). Bar: 2 μm.

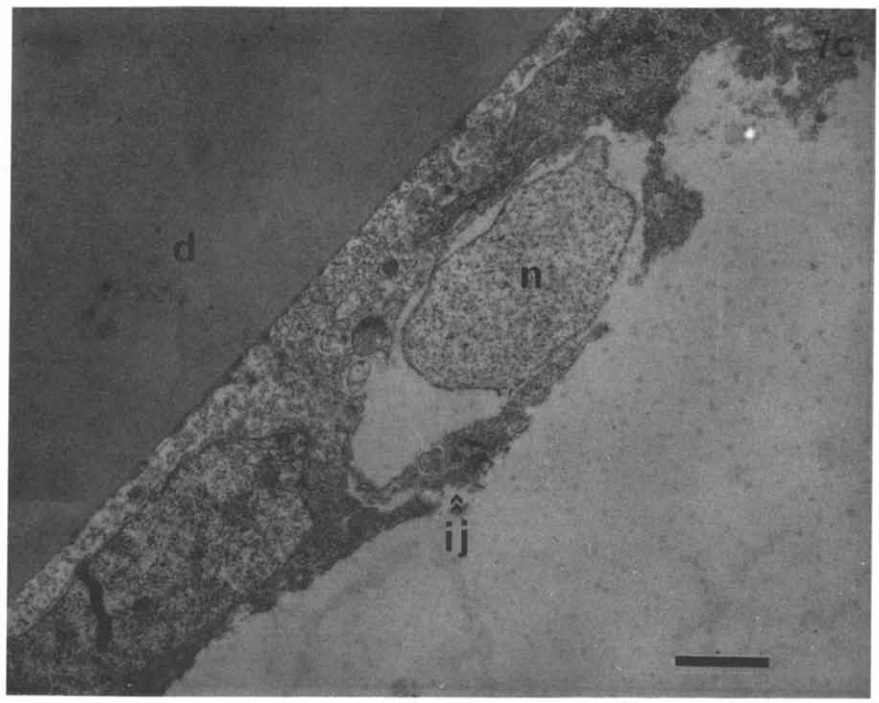

Fig. 7 (c).

through these conducting pathways can occur. The resulting polarization current, which is transient in nature, has been shown to cause an interfering effect, depending on its magnitude and duration, on determination of membrane resistance (Lawler et al., 1960; Plutchik and Hirsch, 1963). In order to determine the polarization effect on d.c. resistance, a study of the discharging kinetics of the tissue was conducted. In this study, a step current generated from a function generator with a current density of 10 μA cm^{-2} was initiated and the voltage response of the cornea was detected on an oscilloscope. The resulting oscillograph is shown in Fig. 10. The voltage decay which corresponds to discharging of membrane surface charges shows exponential-like behavior, a characteristic of a parallel *RC* circuit. The time constant, i.e. time required for the membrane to discharge 63.2% of its initial voltage, is approx. 50 μs. Assuming that a complete discharge ($\approx$ 99%) occurs after 4–5 time constants, the presence of this capacitance-induced polarization current should diminish in approx. 200–250 μs after initiation or cessation of the external applied current. All the currents passing through the membrane after this period thus reflect charge movement in only the conducting pathways. Note that the time required for a complete reading during membrane resistance measurement is approx. 1–2 s. Thus, the resistance determined in this study should contain no interfering effect resulting from tissue polarization and should also represent the true electrical resistance of the membrane. This finding is further confirmed by an a.c. impedance measurement, subsequently reported. The polarization effect is expected to be of importance if the membrane has high capacitance and resistance. In this case, the time required for complete charging or discharging may be long and determination of membrane resistance may be difficult. An example of this is the in vivo experiment conducted with tissue of high resistance such as the skin. In this situation, due to a relatively large surface area (infinite capacitance), charging of the membrane may not be complete.

Corneal impedance and phase relationship

The difficulty associated with polarization cur-

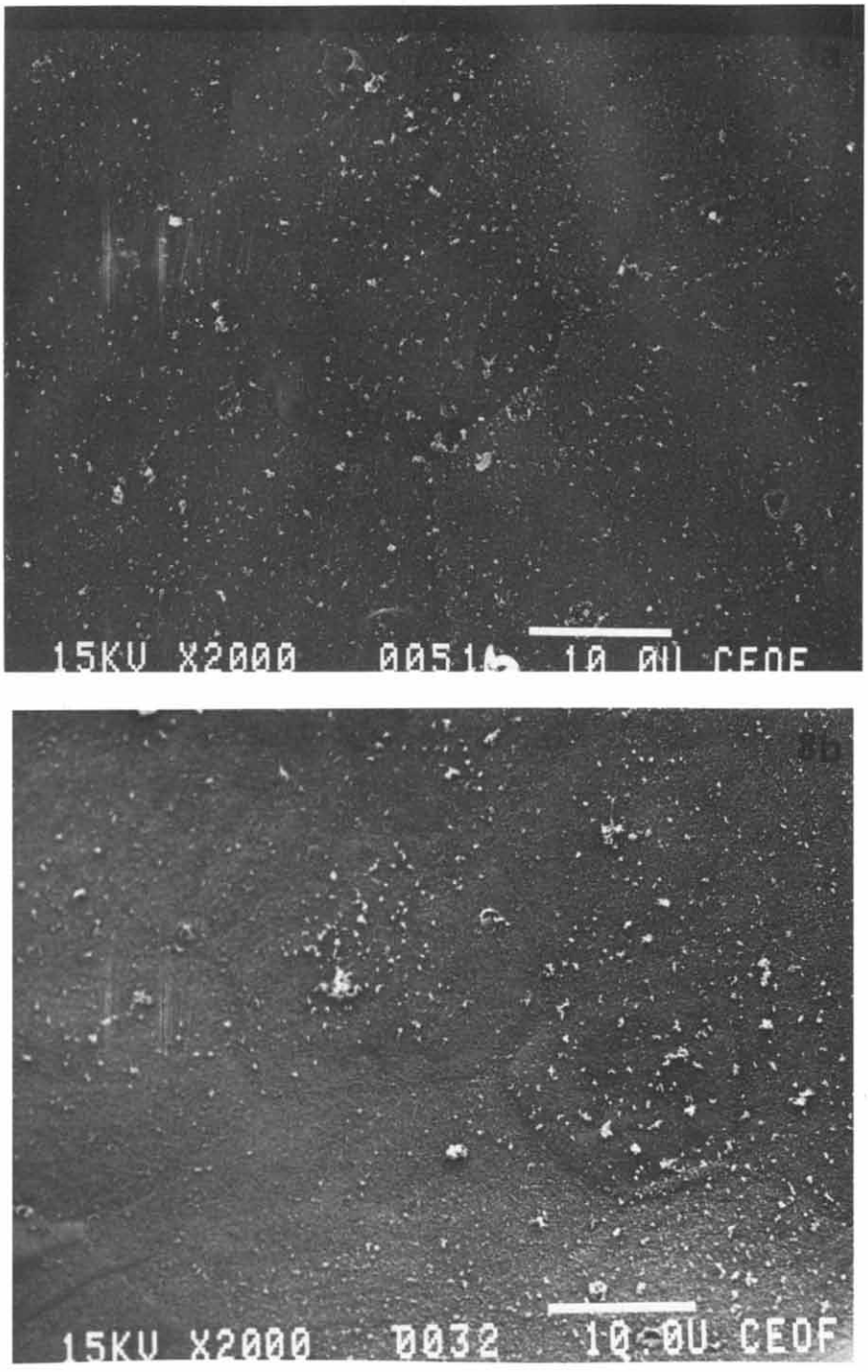

Fig. 8. Scanning electron micrographs of the corneal epithelium after (a) 0 h, (b) 6 h and (c) 12 h in vitro perfusion in GBR solution at 37° C. The epithelial cells are normal in appearance except for expansion of the intercellular junctions in some of the epithelial cells at 12 h. Bar: 10 μm.

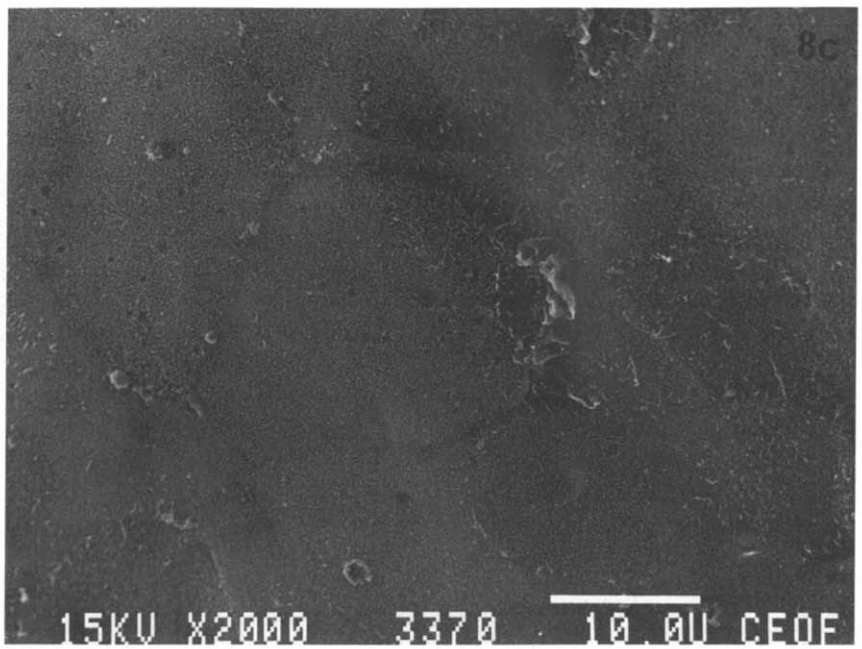

Fig. 8 (c).

rent can be overcome by using an alternating current and measuring membrane impedance or total electrical opposition to current flow. The impedance consists of a resistive component, related to movement of charged species, and a capacitive component, associated with the dielectric property and polarization of the tissue. Thus, by measuring impedance, information on both membrane resistance and capacitance can be obtained.

In an alternating current measurement, the membrane is constantly charged and discharged according to the change in applied voltage. Once the membrane is initially charged, the voltage across it acts as a voltage source. Its effect is to oppose changes in an external applied voltage. Since the membrane must charge or discharge to follow changes in the applied voltage, the resulting current flow is out of phase with changes in the applied voltage. The magnitude of the phase shift is determined by the amount of resistance and capacitance. Thus, by measuring phase difference, information on these two membrane parameters can be obtained.

Fig. 11 shows plots of impedance magnitude and phase angle of freshly excised corneas as a function of frequency ranging from 1 Hz to 100 kHz. Increasing the frequency of the alternating current results in a decrease in corneal impedance and a biphasic response in the phase angle. At first glance, it would appear that as the frequency increases the overall transport of charged species across the cornea increases. However, this may not necessarily be true. To understand this it is necessary to examine charge behavior that constitutes a current flow in an a.c. circuit. When a constantly charging voltage is applied to a membrane, the polarity of the applied voltage alternates, causing charges in the dielectric to shift from one direction to the other. Because of the lipid nature of the membrane, no charges in the dielectric can actually break through and flow into the external circuit. However, shifting of the charges, which is frequency dependent, constitutes a current flow and causes the capacitive impedance to change. In fact, the capacitive impedance is inversely proportional to frequency (Horowitz and Hill, 1980).

Results from phase angle measurement further

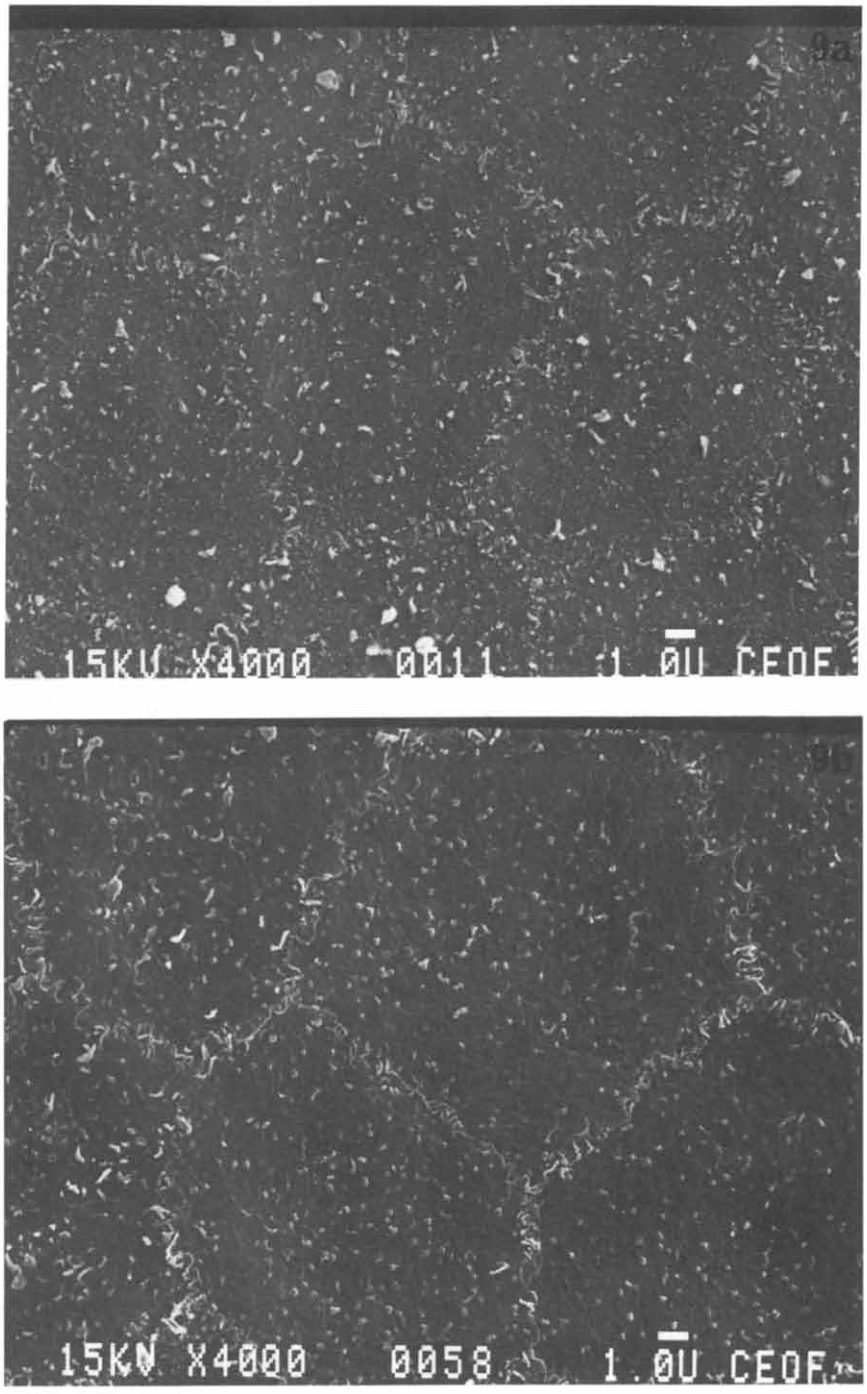

Fig. 9. Scanning electron micrographs of the corneal endothelium after (a) 0 h, (b) 6 h and (c) 12 h in vitro perfusion in GBR solution at 37 °C. Note the progressive expansion of the intercellular junctions. Bar: 1 μm.

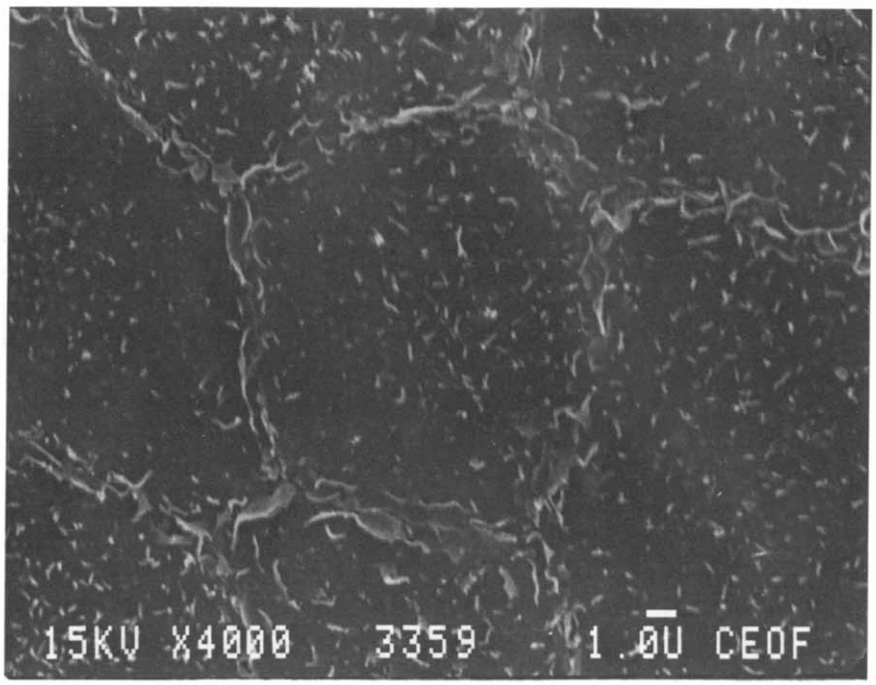

Fig. 9 (c).

demonstrate that the cornea does not behave simply as a passive resistor but also exhibits a certain capacitive behavior. This is because in a purely resistive circuit (no capacitance), no phase difference should be present while in a strictly capacitive circuit (no resistance), the phase angle would

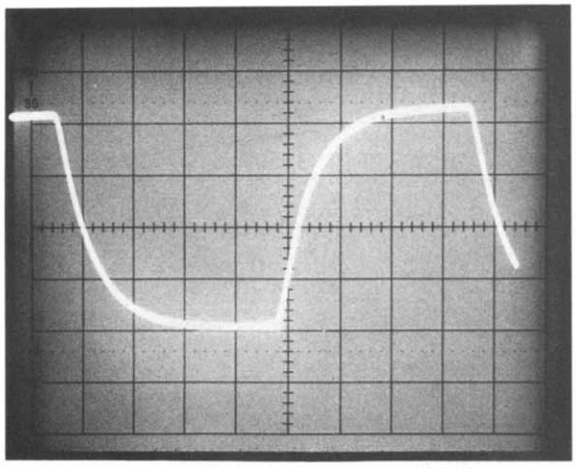

Fig. 10. Discharge of the electrical capacitance of the cornea. A step current (current density, 10 μA cm^{-2}; frequency, 500 Hz) was applied across the cornea from a function generator and the voltage response of the cornea was detected on an oscilloscope. The time required for the trailing edge to come back to the baseline was the discharging time of the membrane which was 200 μs (99% discharged).

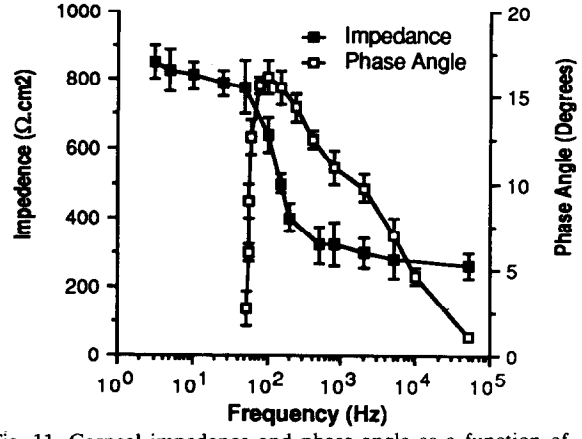

Fig. 11. Corneal impedance and phase angle as a function of frequency. A constant a.c. current (current density = 10 μA cm^{-2}) at several discrete sinusoidal frequencies ranging from 1 to 100 kHz was applied across the cornea and the corresponding voltages and phase differences were recorded. Phase angles were determined by the Lissajous method (see text). Bar: 1 SE; $n = 4$.

be 90° (Horowitz and Hill, 1980). The phase difference between 0 and 90°, characteristic of a combined *RC* circuit, confirms the validity of the parallel *RC* model of the cornea. Measurement of the phase angle may provide an alternative approach to assessment of membrane transport properties and tissue condition.

Alternating current resistance and capacitance

The a.c. resistance and capacitance of the cornea were calculated from impedance data according to a frequency-dependent, parallel *RC* circuit model. The expression for the impedance of such a circuit, according to Burnette and Bagniefski (1988), is

$$1/Z^2 = 1/R^2 + (2\pi C)^2 f^2 \qquad (3)$$

where Z is the impedance, f is frequency, and R and C have the usual meanings. Note that as the frequency approaches zero, impedance becomes the resistance. Physically, at low frequency, the membrane dielectric represents essentially an open circuit or infinite resistance and only the resistive component of the membrane conducts. Thus, the low-frequency impedance is a measure of resistance. To determine the capacitance, a number of impedance values within a known frequency range are determined and C can be obtained from the slope of Eqn 3. In this calculation, it is assumed that over a narrow enough frequency range the resistance and capacitance are approximately constant. Fig. 12 shows time profiles of a.c. resistance (1 Hz) and capacitance (500 Hz) of the cornea during the in vitro perfusion. Biphasic resistive behavior of the cornea, similar to that observed in the d.c. experiment, is evident. The resistance values of both methods are almost identical, indicating consistency between the two and detection of the true electrical resistance of the cornea, i.e. no polarization interference. The result also indicates that the observed change in corneal resistance is not affected by the current-induced tissue polarization. In contrast to resistance, the capacitance of the cornea appears to be remarkably constant during in vitro perfusion with an average value of approx. 0.4 μF cm^{-2}. Since capacitance reflects the ability of the membrane to hold charges on its

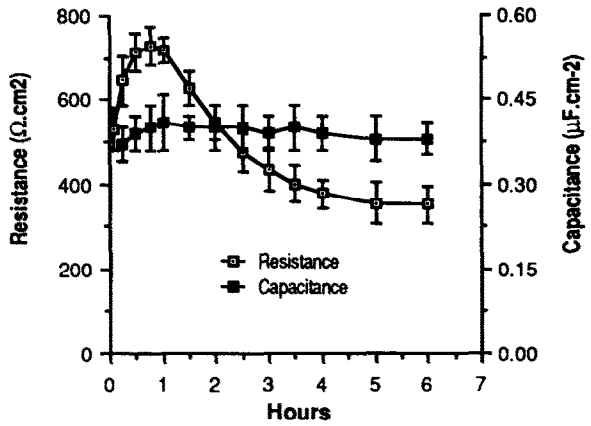

Fig. 12. Plot of a.c. resistance and capacitance as a function of time. A sine wave (current density = 10 μA cm^{-2}) was applied across the cornea and the resistance (1 Hz) and capacitance (500 Hz) were determined. Note the similarity of the a.c. and d.c. resistive behavior (see Fig. 3). Corneal capacitance is relatively constant. Bar: 1 SE; $n = 4$.

surface, any change in membrane integrity, i.e. disruption of the membrane, would result in a decrease in capacitance. The fact that capacitance remains constant suggests the preservation of the membrane surface integrity. This is in good agreement with previous results obtained via electron microscopy. An additional experiment conducted with an excised, denuded, cornea shows a considerably low capacitance of approx. 0.01 μF cm^{-2}. Currently, the method has been successfully used to provide information on the promoting effect of various penetration enhancers in the cornea. The results of this study will be presented in a separate publication.

Conclusions

In addition to electrical and structural characterization of the cornea, this paper presents an overview of problems commonly encountered in in vitro perfusion studies. Particular attention is focused on those associated with tissue integrity and viability and methods of assessing these membrane parameters. The cornea exhibits unique electrogenic, resistive, and capacitive properties. Results from active potential measurement indicate that the viability of the cornea is maintained

for at least 6 h in vitro. Changes in membrane potential and resistance occur in a parallel and biphasic fashion. Corneal capacitance, however, remains relatively constant throughout the experiment. Results from electron microscopy show that the drop in resistance correlates well with changes in intercellular integrity while maintenance of the capacitance is consistent with preservation of the surface integrity. The electrical technique has been shown to provide a simple, non-invasive, and quantitative means of evaluating membrane conditions.

References

Binder, H.J., *Mechanisms of Intestinal Secretion*, A.R. Liss, New York, 1980, pp. 275–286.

Burnette, R.R. and Bagniefski, T.M., Influence of constant current iontophoresis on the impedance and passive Na^+ permeability of excised nude mouse skin. *J. Pharm. Sci.*, 77 (1988) 492–497.

Donn, A., Maurice, D.M. and Mills, N.L., The active transport of sodium across the epithelium. *Arch. Ophthalmol.*, 62 (1959) 748–757.

Grass, G.M. and Robinson, J.R., Mechanisms of corneal drug penetration. I: In vivo and in vitro kinetics. *J. Pharm. Sci.*, 77 (1988) 3–14.

Green, K., Ion transport on isolated cornea of the rabbit. *Am. J. Physiol.*, 209 (1965) 1311–1316.

Horowitz, P. and Hill, W., *The Art of Electronics*, Cambridge University Press, Cambridge, U.K., 1980.

Klyce, S.D., Neufeld, A.H. and Zadunaisky, J.A., The activation of chloride transport by epinephrine and DB c-AMP in the cornea of the rabbit. *Invest. Ophthalmol.*, 12 (1973) 127–139.

Lawler, J.C., Davis, M.J. and Griffith, E.C., Electrical characteristics of the skin. *J. Invest. Dermatol.*, 34 (1960) 301–308.

Maurice, D.M., Epithelial potential of the cornea. *Exp. Eye. Res.*, 6 (1967) 138–140.

O'Brien, W.J. and Edelhauser, H.F., The corneal penetration of trifluorothymidine, adenine arabinoside, and idoxuridine: A comparative study. *Invest. Ophthalmol. Vis. Sci.*, 16 (1977) 1093.

Plutchik, R and Hirsch, H.R., Skin impedance and phase angle as a function of frequency and current. *Science*, 141 (1963) 927–928.

Potts, A.M. and Modrell, R.W., The transcorneal potential. *Am. J. Ophthalmol.*, 6 (1957) 284–290.

Zadunaisky, J.A., Active transport of chloride in the frog cornea. *Am. J. Physiol.*, 21 (1966) 506–512.