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Potentials in frog cornea and microelectrode artifact

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DAVIS, T. L., J. W. JACKSON, B. E. DAY, R. L. SHOEMAKER, AND W. S. REHM. *Potentials in frog cornea and microelectrode artifact.* Am. J. Physiol. 219(1): 178–183. 1970—Studies were performed on the frog's cornea in an intact globe preparation and also with an in vitro chamber technique. Intact corneas possessed a transcorneal potential difference (PD) of about 20 mv and a resistance of about 900 ohms cm². After removal of the epithelium, the PD of the remaining stroma plus endothelium was about zero, and the resistance was less than 100 ohms cm². Removal of only endothelium resulted in a PD and resistance which were essentially the same as the intact cornea. The corneal surface of the intact globe was about 20 mv negative to an electrode in the aqueous humor, and injury to the epithelium reduced this PD to about zero. With microelectrodes filled with 3.0 M KCl, the stroma was negative to an aqueous electrode by 23 mv, whereas with microelectrodes filled with Ringer the PD between the stroma and aqueous was zero. It is concluded that the PD across the endothelium is essentially zero and that microelectrodes filled with 3.0 M KCl give rise (for this tissue) to a substantial artifact.

cornea resistance; potentials of endothelium of cornea; resistance of epithelium of cornea; resistance of endothelium of cornea

ZADUNAISKY (10) has shown that the in vitro frog cornea actively transports Cl^- from the aqueous to the tear side and that the aqueous side is electrically positive to the tear side. These findings have been confirmed by Ploth and Hogben (7). Candia and Askew (1) have presented evidence suggesting that Na^+ may also be actively transported but at a substantially lower rate than Cl^-. Candia, Zadunaisky, and Bajandas (2) attempted to localize the site of the Cl^--transport mechanism by studying the potential profile across the cornea with microelectrodes. They reported that the stroma was negative to both outer surfaces and suggested that the Cl^--transport mechanism was located in the endothelium. They pointed out that their conclusions were predicated on the assumption that the microelectrodes were not the source of a substantial artifact. In our studies with macroelectrodes to be reported herein (and in preliminary form elsewhere (4)), we found that the potential difference (PD) across the endothelium was essentially zero. Since our findings differed from the microelectrode studies of Candia et al. (2), we performed experiments with microelectrodes in order to resolve the apparent conflict. The purpose of this paper is to show that the PD across the endothelium is about zero and that microelectrodes filled with 3 M KCl give rise for this tissue to a substantial artifact.

METHODS

Corneas of the bullfrog (*Rana catesbeiana*) were studied by several methods. In one group of experiments corneas were mounted between two Lucite chambers which were essentially the same as those described previously for the studies on the frog's gastric mucosa (8), except that the surfaces of the two opposing chambers were not flat but rounded to fit the natural curvature of the cornea. The area of the exposed cornea was 0.36 cm². These chambers were quite similar to those used by Zadunaisky (10). Two pairs of electrodes were used, one pair for measurement of the PD (calomel) and one pair for current sending (Pb-Pb acetate agar). For resistance measurements, current was sent across the cornea, and the resistance was determined as the ΔPD after 0.5 sec divided by the current (usually 5 μa/0.36 cm²). The system behaved as a linear bilateral system, with current densities up to several times the values used for resistance measurements.

In another series of experiments, chambers were used similar to the above, except that the end of the chamber facing the endothelial surface was open for the introduction of a microelectrode. This chamber is shown diagramatically in Fig. 1. In this modification, the current-sending electrodes consisted of coils of Ag-AgCl wires. The fluids in both chambers were connected to separate reservoirs (not shown in Fig. 1), and circulation was accomplished by means of gas lifts. The gas was 95% O_2–5% CO_2.

The microelectrodes were held in a Brinkman micromanipulator, and the microelectrode was observed by means of a Zeiss operating microscope. In preliminary experiments long-taper microelectrodes were used with resistances (with 3 M KCl) around 15 megohms, but the tips of these electrodes broke almost as soon as a puncture was attempted. Consequently, microelectrodes with short tapers and with a resistance of about 2 megohms were used (with 3 M KCl). The microelectrodes were filled with either 3.0 M KCl, 1.0 M KCl, or a modified Ringer solution. (See below for composition.) They were first filled under vacuum with methyl alcohol and then allowed to equilibrate with the appropriate fluid. In all of the experiments the tips either broke during a puncture or were deliberately broken, and the tip potential was defined as the difference in potential before and after tip breakage with the micro-

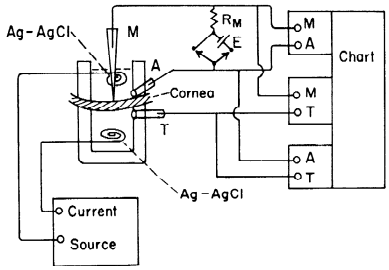

FIG. 1. Diagrammatic representation of method used in microelectrode studies. Cornea is mounted with aqueous side facing microelectrode; *A* and *T* indicate macroelectrodes in contact with Ringer on 2 sides of cornea. For resistance measurements of cornea, a current source was connected to Ag — AgCl electrodes. R_M represents a current source for measurement of resistance of microelectrodes. Three PDs are recorded simultaneously on a Grass polygraph, PD_{MA}, PD_{MT} and PD_{AT}. Amplifiers in addition to Grass amplifiers were used for measurements of PD_{MA} and PD_{MT}. Not shown on diagram are reservoirs, one for aqueous fluid and one for tear fluid, with gas lifts for circulation. A Zeiss surgical magnifier was used for observation of microelectrode.

electrode and reference electrode immersed in Ringer solution. In all but a few experiments, tip potentials were less than 4 mv. The resistance of the microelectrodes was determined at frequent intervals by sending pulses of direct current through the microelectrode as described previously (3). In other words, two types of resistances were measured, i.e., the resistance of the microelectrode itself, and the resistance of the cornea between the microelectrode and each of the macroelectrodes making contact with the bathing fluid.

In another series of experiments, an intact globe preparation was used. The frog was pithed or decapitated, small macroelectrodes made contact with the outer surface of the cornea, and reference electrodes were placed in either the aqueous humor or the vitreous humor. Calomel electrodes were used in both types of electrodes. The aqueous and vitreous electrodes consisted of glass tubes filled with Ringer and tapering to about 1 mm in diameter. The tapered end was not heat annealed, leaving sharp edges on the tip so that penetration of the globe was accomplished without marked deformation. In some experiments, electrodes were placed in both the aqueous humor and the vitreous humor, and the PD between them was zero. The small electrodes making contact with the tear surface had an internal diameter of about 1.5 mm and were filled with Ringer-agar solution. Agar protruded from the ends for about 0.5 mm, and these electrodes were placed in micromanipulators and brought close to the surface of the cornea without touching it, and electrical contact was made via a drop of Ringer solution. Control experiments revealed that moving an electrode so that the agar made contact with the surface did not result in a change in PD. Movement of the electrode so as to deform the cornea resulted in a reversible change in PD.

The Ringer solution had the following composition (in mM): 102 Na^+, 4 K^+, 1.0 Ca^{++}, 0.8 Mg^{++}, 81 Cl^-, 25 HCO_3^-, 1.0 phosphate, and 0.8 $SO_4^=$.

For purposes of clarity of presentation, the changes in PD arising from the measurements of the microelectrode resistance and the corneal resistances are not shown in the PD graphs.

RESULTS

Origin of the transcorneal PD. Table 1 shows a summary of the determination of the PD and resistance of corneas studied with the technique described in the first part of METHODS. In six corneas the epithelium was scraped in situ before the corneas were removed from the globe. Scraping was accomplished by moving the sharp side of the scapel blade across the cornea with the blade perpendicular to the corneal surface. In nine corneas endothelium was scraped after the cornea was removed by wiping the surface with gauze. Some corneas, after being prepared in the above manner, were fixed in neutral formalin and stained with hematoxylin and eosin. Examination of the corneas with the epithelium scraped revealed only small islands of epithelium and an intact endothelium; the reverse was true for the corneas in which the endothelium was removed. Even if some viable epithelium or endothelium were left, any activity would be readily nullified via the parallel denuded regions.

It can be seen from Table 1 that after the removal of the epithelium the PD is essentially zero, and the resistance is only a small fraction of that of the intact cornea. On the other hand, after the removal of the endothelium the characteristics are essentially the same as the intact cornea. On the basis of these observations, one would conclude that the PD across the endothelium is essentially zero and that the electromotive forces (emf) giving rise to the transcorneal PD arise in the epithelium. However, it is possible that in the process of mounting the corneas between the chambers we may have damaged the endothelium. As pointed out above, Candia et al. (2) reported with the microelectrode technique the presence of a large PD across the endothelium and, furthermore, in their studies, the PD across the epithelium had an orientation opposite to the transcorneal PD (i.e., the tear side appeared to be positive to the stroma). On the basis of these considerations, we performed the next group of experiments on the intact globe.

Positive injury potentials. The PD was measured between small macroelectrodes placed on the tear surface in the intact globe preparations and the change in PD was determined before and after removal of the epithelium in small localized regions. We were careful to prevent the

TABLE 1. *Potential difference and resistance of corneas mounted between Lucite chambers*

	PD, mv	Resistance, ohm cm²
Intact corneas (n = 33)	17.1 ± 5.3	820 ± 170
Endo + stroma, epi scraped (n = 6)	0.43 ± 0.34	92 ± 22
Epi + stroma, endo scraped (n = 9)	18.0 ± 7.6	1,100 ± 360

Values are means ± SEM. n = No. of corneas. Endo plus stroma means epithelium scraped off; epi plus stroma means endothelium removed.

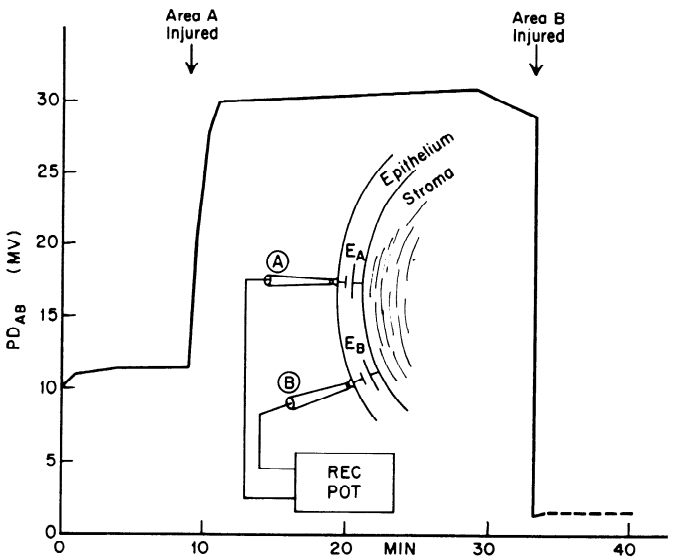

FIG. 2. PD_{AB} versus time, E_A and E_B are equivalent-circuit emf's for *regions A* and *B* in epithelium between surface and stroma. When PD_{AB} is positive it means *A* is positive to *B* in an external circuit.

TABLE 2. *PD between small macroelectrodes in contact with surface of cornea in intactglobe preparation**

PD_{AB}, mv		ΔPD_{AB}, (E_A), mv
Before injury	After injury to *A*, (E_B)	
+40	+44	+4
+27	+33	+6
+11	+27	+16
+6	+50	+44
+5	+24	+19
+4	+27	+23
+1	+27	+26
+1	+24	+23
0	+39	+39
0	+42	+42
−3	+14	+17
−7	+33	+41
−10	+23	+33
−41	+6	+47

PD_{AB}, when positive, means *A* is positive to *B* and vice-versa. Injury means the removal of a small area of epithelium by scraping.
* See diagram, Fig. 2.

corneal surface from drying; it was washed frequently with Ringer solution. A typical experiment is shown in Fig. 2. The PD between *electrodes A* and *B* (PD_{AB}) is plotted versus time. The convention used is that when PD_{AB} is positive it means that *A* is positive to *B* in an external circuit and vice versa. It can be seen that before injury *A* is positive to *B*. In general, a PD was usually present between two surface electrodes, and the magnitude of the PD ranged up to about 40 mv (see first column of Table 2). In one group of corneas we systematically explored the PD as a function of position of the electrodes on the surface, and it was found that in general an electrode near the center of the cornea was positive in an external circuit to a more peripheral one. However, there were exceptions to this, and no simple statement of the distribution of corneal potentials is possible.

The effect of injury was determined by scraping the epithelium in a small area; the electrode was removed and then replaced on the scraped area. It can be seen that, following injury to *site A*, the PD_{AB} increased from about 11 to 30 mv, i.e., the injured area became more positive to the uninjured area (a positive injury potential). Removal of the epithelium at *site A* should enable electrical contact to be made with the stroma, and the resulting PD should yield a measure of the magnitude and orientation of the PD at *site B*, the uninjured site. Assuming electrical contact was made with the stroma, then the PD across the epithelium at *B* is 30 mv. In the diagram in Fig. 2, E_A and E_B represent the equivalent circuit emf's for the *regions A* and *B*; hence

$$PD_{AB} = E_B - E_A \qquad (1)$$

After injury to *site A*, E_A goes to zero (see next section), and PD_{AB} then equals E_B. Therefore

$$(PD_{AB})_{inj\ A} - PD_{AB} = -PD_{AB} = E_A \qquad (2)$$

where $(PD_{AB})_{inj\ A}$ indicates the PD between *A* and *B* after injury to *A*. Hence, in the experiment in Fig. 2, $E_A = 30 - 11 = 19$ mv. If direct electrical contact with the stroma is made after scraping the epithelium at *site A*, then scraping the epithelium at *site B* should result in a reduction of the PD to about zero. It can be seen that following injury to *B* the PD decreases from about 30 to 1.5 mv. Following scraping of the epithelium at both *A* and *B*, the average PD_{AB} was 0.32 mv (SD ± 0.8) (14 experiments).

In 14 experiments on 14 corneas, there was no exception to the finding that injury resulted in a positive injury potential, i.e., ΔPD_{AB} was always positive as can be seen in column 3 of Table 2. On the basis of *equations 1* and *2*, the value of PD_{AB} after injury equals E_B and ΔPD_{AB} equals E_A. In later experiments (see next section) results similar to those shown in Table 2 were obtained but for simplicity are not included in Table 2. In these latter experiments, there was no exception to the rule that injury always results in a positive injury potential.

In some experiments an attempt was made to study the effect of varying grades of injury. Minimal mechanical movement of the scapel blade over a small area usually produced a small reversible increase in the PD with respect to another electrode on the surface. With more intensive scraping the change in PD was always positive and soon became irreversible. We never saw a temporary decrease in the PD of an injured region with respect to an uninjured one. On the basis of these experiments it is clear that in the intact globe preparation the stroma is positive to the tear side. It is also clear that removal of the epithelium enables direct electrical contact to be made with the stroma. The problem next arises as to the magnitude of the PD across the stroma and endothelium in the intact globe preparation, and this is dealt with in the next section.

PD from stroma to aqueous humor or to vitreous humor. In this section experiments were performed on the intact globe in which the PD was measured between an electrode on the surface of the cornea and one in the aqueous or vitreous humor. A typical experiment is shown in Fig. 3. The PD_{VA}

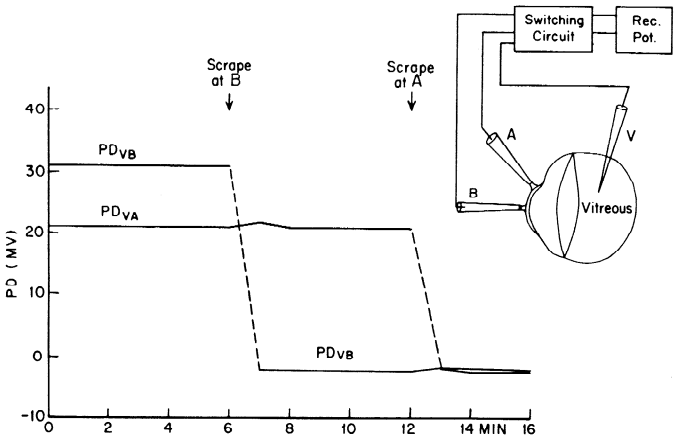

FIG. 3. PD_{VA} and PD_{VB} vs. time where V refers to vitreous electrode and A and B to two small macroelectrodes on surface of cornea.

is the PD between *electrode V* in the vitreous and *electrode A* on the surface. Scraping the epithelium at the site of *electrode A* results in a fall in the PD to about −2 mv. In all of these experiments, the vitreous or aqueous humor were positive to an electrode on the uninjured surface of the cornea, confirming results of Zadunaisky (10) and also our own observations in Table 1. After scraping the epithelium under one of the surface electrodes, the PD between the vitreous and surface electrode decreased to an average value of 0.9 mv (SD ± 0.7) with a range of from −2 to +4 mv.

It occurred to us that we should explore the unlikely possibility that injury to a small section of the epithelium resulted in the transmission of some influence that reduced the PDs or the emf's in the various layers of the cornea. This unlikely possibility was eliminated from further consideration by performing experiments like that shown in Fig. 3. It can be seen that the PD between the vitreous electrode and an electrode on the surface was not changed by injury to another region of the epithelium, the injury being of sufficient magnitude to result in a decrease in PD between it and the vitreous humor to about zero. On the basis of the experiments performed so far, it is difficult to escape the conclusion that the PD across the endothelium is zero or very close to zero. These results are in conflict with the microelectrode studies of Candia et al. (2). In an attempt to resolve this conflict, experiments with microelectrodes were performed and are presented in the following section.

Experiments with microelectrodes filled with 3.0 M KCl. In these experiments the PD between the microelectrode (represented by M) and each macroelectrode (A for aqueous and T for the tear side) and also the PD between A and T were simultaneously recorded on the polygraph, i.e., PD_{MA}, PD_{MT}, and PD_{AT}. The resistance of the microelectrode, the resistance between the microelectrode and the *electrodes A* and T, were determined at frequent intervals. The data for the PD and the calculated values for the resistance are plotted in Fig. 4 for a typical experiment with a microelectrode filled with 3.0 M KCl. The abscissa represents time, but for the sake of convenience of presentation no scale is placed on the axis. The whole experiment took about 30 min. The discontinuities in PD_{MT} and PD_{MA} are associated with small inward advancements of the microelectrodes. The cornea showed dimpling, so the amount of movement of the microelectrode did not correspond to the depth of penetration into the corea. Before the tip broke (indicated by the marked fall in microelectrode resistance; see scale labeled tip resistance) the PD_{MA} decreased from its original value of zero to −25 mv, and PD_{MT} decreased from its original value of 15 to −10 mv. In other words, right before the tip broke the stroma was negative to the tear side by 10 mv and negative to the aqueous side by 25 mv. Without other data one would be tempted to conclude that the PD across the endothelium was 25 mv and across the epithelium, 10 mv. During the penetration of the microelectrode, the PD across the cornea between A and T did not change; in fact, it can be seen that this PD was constant throughout the entire experiment.

In the foregoing studies (Table 1) it was shown that practically all the transcorneal resistance originated in the epithelium. We see this confirmed in Fig. 4. Before the tip broke the value of the resistance R_{MT} (between the microelectrode and the tear side) was much greater than that of R_{MA} (between the microelectrode and the aqueous side).

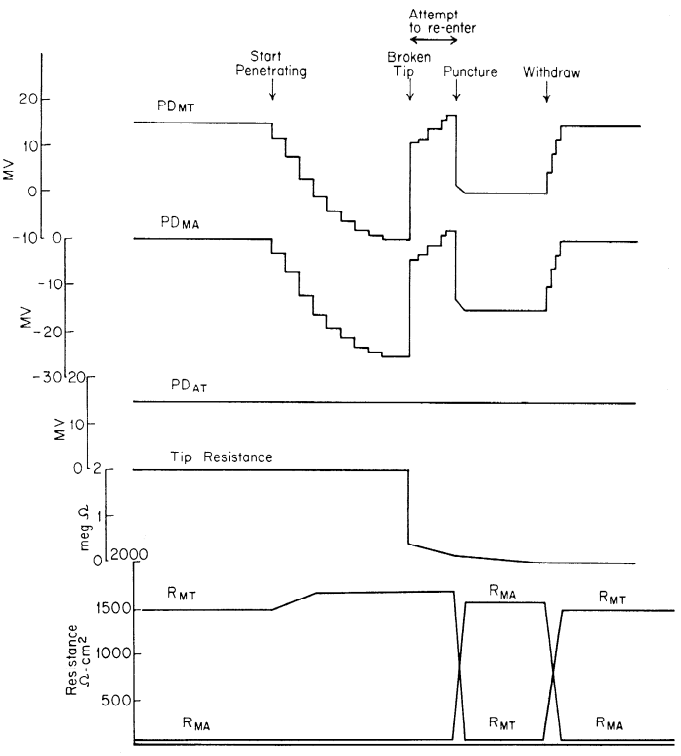

FIG. 4. Experiments with 3.0 M KCl filled microelectrodes. PD_{MT}, PD_{MA}, PD_{AT} refer to PD between microelectrode (M) and tear electrode (T), between M and aqueous electrode (A) and between A and T, respectively. *Abscissa* represents time, but no scale is used for convenience of presentation, since exact timing of various procedures is not germane to results. Tip resistance refers to resistance of microelectrode, and R_{MT} and R_{MA} refer to resistance between microelectrode and tear electrode and microelectrode and aqueous electrode. Microelectrode was moved into cornea from endothelial side at time indicated. Time of contact of microelectrode with cornea was easily determined because of dimpling. At time indicated, tip broke (marked decrease in tip resistance). Penetration with microelectrodes after tip had broken resulted in a crossover of R_{MA} and R_{MT}. Withdrawal of electrode back into fluid bathing aqueous side was at time indicated.

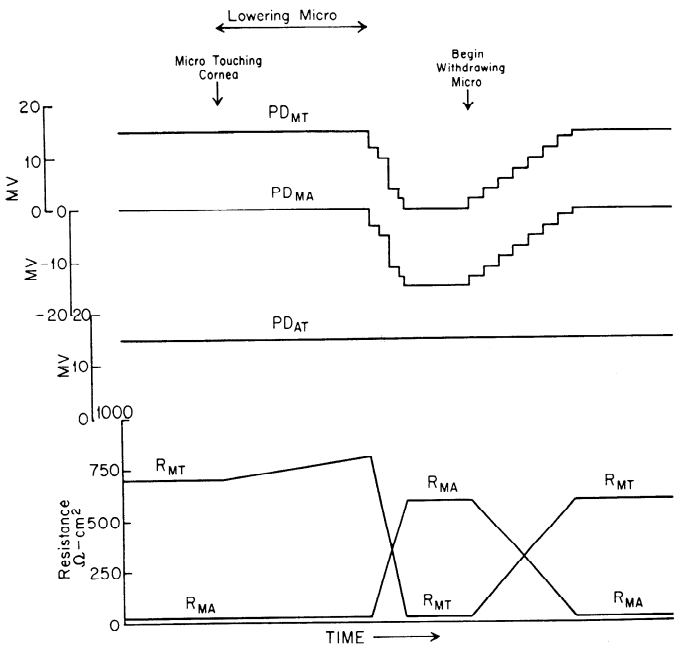

FIG. 5. Microelectrodes filled with Ringer. Symbols have same meaning as described in legend for Fig. 4. Tip did not break during this experiment, so tip-resistance measurements are omitted.

A puncture was made after the tip broke, and it can be seen that the change in PD was associated with the crossover of the resistances R_{MA} and R_{MT}, indicating that the tip penetrated through the entire cornea and made contact with the solution on the tear side. This was typical of punctures made with broken tips; it was not feasible to place a broken tip in the stroma. During the crossover period, the PD between the microelectrode and the tear macroelectrode (PD_{MT}) was zero and that between the microelectrode and the aqueous electrode (PD_{MA}) was -15 mv. This latter value was exactly that predicted from the PD between the aqueous and tear electrodes ($PD_{AT} = -PD_{TA}$). Upon withdrawal of the microelectrode, we see the PD between the microelectrode and the aqueous electrode returned to zero and that between the microelectrode and the tear electrode to the same value as that between the two macroelectrodes. We also see that the resistances R_{MT} and R_{MA} return to approximately their original levels.

In 28 punctures on 7 corneas with 3.0 M KCl microelectrodes, the average maximum PD between the microelectrode and the aqueous electrode was -22.6 mv (SD = ± 6.0) and the range was from -13 to -31 mv.

Studies with microelectrodes filled with 1.0 M KCl and with Ringer. Studies similar to those described above were performed with microelectrodes filled 1.0 M KCl, and with 25 punctures on 7 corneas the average PD_{MA} equaled -14.6 mv (SD $\pm$ 4.5) with a range of from -8 to -22 mv.

In Fig. 5 an experiment is shown with a microelectrode filled with Ringer solution. The protocol for this experiment is essentially the same as that shown in Fig. 4. During the period before the crossover of R_{MT} and R_{MA}, the microelectrode was advanced repeatedly, and no change was seen in the PD. It can be seen in both Figs. 4 and 5 that during the period of penetration the magnitude of R_{MT} increased. An increase of R_{MT} occurred during penetration of the cornea by the microelectrode and was associated with dimpling of the cornea.

In a total of 16 punctures in 5 corneas, there was no change in PD before the crossover of the resistance R_{MT} and R_{MA}, except in one experiment in which the PD became as large as -4 mv, i.e., $PD_{MA} = -4$ mv.

In the experiment shown in Fig. 5 following the crossover of R_{MT} and R_{MA}, the $PD_{MA} = -15$ mv (or $PD_{AM} = +15$ mv) which is the magnitude found for PD_{AT} ($PD_{TA} = -PD_{AT} = PM_{MA} = -15$ mv). Upon the withdrawal of the electrode, the crossover of R_{MT} and R_{MA} was reversed (they returned to approximately their original level), and PD_{MT} and PD_{MA} returned to their levels.

DISCUSSION

The experiments performed after removal of the epithelium or the endothelium indicate that the PD across the endothelium is zero or very nearly zero and that the emf's giving rise to the transcorneal PD originate in the epithelium. They also indicate that the resistance across the endothelium and stroma is very low and that the high resistance of the cornea resides in the epithelium. The experiments on the intact globe show that the surface of the cornea is not an isopotential region; there are sizeable PDs between the various regions on the surface of the cornea. They also show that injury to the epithelium always results in a positive injury potential. (The injured region always became more positive with respect to the uninjured region.) These experiments show that the stroma is always positive to the tear side, although its magnitude varied from region to region.

The nonisopotentiality of the corneal surface is of interest with respect to the problem of the role of ion transport mechanisms in epithelial tissues. It is generally assumed that the sole function of these mechanisms is the net transport of ions from one side of a cell layer to the other. However, with marked differences in PD between adjacent regions, the flow of current may play a role in the movement and distribution of ions within the stroma. For example, in Fig. 2 where $E_B > E_A$, current flow coupling the two regions could result in movement of Cl^- from the stroma to the tear fluid at *B* and in the opposite direction at *A*. The flow of current in the stroma from *A* to *B* may be important in regulating ion gradients in this tissue.

The experiments in which the PD was measured between small macroelectrodes on the surface of the cornea and aqueous or vitreous electrodes showed that removal of the epithelium from a small region results in a drop of the PD to zero, indicating that the PD across the stroma and the endothelium is zero in the intact globe preparation. They further showed that localized injury to the epithelium does not result in a significant change in the PD in other regions.

On the basis of the above we conclude that the potential difference across the endothelium is essentially zero. Maurice (6), in comparable experiments on the corneas of rabbits, also concluded that the PD across the endothelium is essentially zero.

The experiments with the microelectrodes filled with Ringer further support the above conclusion that the PD

is zero across the endothelium. It is apparent that the KCl-filled microelectrodes when in contact with the stroma give rise to a junction potential. One is tempted to speculate on the possible origin of the junction potential. The results of studies on the electrolyte composition of stroma (9) indicate that there are fixed negative charges in the stromal connective tissue. With fixed negative charges in the stromal connective tissue, the mobility of the K^+ diffusing from the microelectrode into the stroma would be significantly greater than that of the Cl^-, which would give rise to a PD between the microelectrode and the electrode in the Ringer solution on the aqueous side oriented so as to make the microelectrode negative. The density of fixed negative charges would not be high enough to result in the stroma acting as a membrane exclusively permeable to cations, since if this were the case much larger potentials would be expected; with a 3.0 M KCl solution the cation concentration ratio would be about 30 to 1, i.e., 3 Eq/liter of K^+ to about 0.1 Eq/liter of cations in the Ringer on the aqueous side. A perfect cation-selective membrane with no discrimination between K^+ and Na^+ would yield a calculated PD of around 90 mv. With microelectrodes filled with the same solution as that of the aqueous side (Ringer), one would expect the PD to be zero.

The stroma is not a homogeneous tissue since Descemet's membrane, Bowman's layer, and the remaining stroma all have distinct histological characteristics (5). Appropriate future studies of the liquid-junction potentials in the various domains of the stroma should lead to a furthering of our understanding of the relationship between the structure and function of this tissue.

The situation in the cornea offers a unique opportunity to test the validity of measurements with microelectrodes. In this particular tissue we conclude that microelectrodes filled with 3.0 M KCl give rise to an artifactual potential of an average value of 23 mv in which the microelectrode is negative in an external circuit. Obviously, the question remains unanswered as to the adequacy of these electrodes for PD measurements in other tissues where there is no easily available independent way for determining whether the measurements are valid or not.

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