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# PARACELLULAR IONIC AND TRANSCELLULAR WATER DIFFUSIONS ACROSS RABBIT CORNEAL ENDOTHELIUM

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SUMMARY

1. Corneal endothelial cell membrane electrical resistance is estimated at about 400 MΩ by using intracellular micro-electrodes.

2. If all ion diffusion across the endothelium were transcellular, electrical resistance of the endothelial monolayer would be about 1300 Ω cm², as there are about 3000 cells mm$^{-2}$.

3. Measured endothelial monolayer resistance is $12{\cdot}7 \pm 0{\cdot}8$ Ω cm² (mean ± S.E., $n = 6$), which indicates that about 99 % of ion diffusion does not cross the transcellular pathway, but must pass through the paracellular route. Arguments are presented which suggest that the proportion may be even higher.

4. Endothelial passive ion permeabilities are about the same as corneal stromal passive ion permeabilities.

5. Stromal water diffusional permeability is about the same as stromal ion permeability, after allowance is made for free diffusion coefficients. In contrast, endothelial water diffusional permeability is so high as to be unmeasurable.

6. It is concluded that ions diffuse across the corneal endothelium through the paracellular route, and that water diffuses across the endothelium mainly through the transcellular route.

## INTRODUCTION

The water relations of the cornea can be described as a dynamic equilibrium between the passive tendency of the stroma to take up water and swell, and the active transport of bicarbonate ions by the endothelium that effectively acts to oppose the inward movement of water. Included in this description is the nature of the membrane across which the active and passive movements take place (Mishima & Hedbys, 1967; Hodson, 1974; Hodson & Miller, 1976; Klyce & Russell, 1979).

In a previous work (Hodson & Wigham, 1983) we described the magnitudes of passive ion movement across the endothelium, using radiolabelled tracer techniques. We also used the formula derived by Hodgkin (1951) to compare rates of passive ion movement (the endothelial permeability to ions) and electrical conductivity. Endothelial permeability and electrical conductivity were shown to be equivalent, implying that passive ion movement followed the same pathway as ions moving down

an electrical gradient. Using stereology, we calculated that potentially all passive ion flow could traverse the paracellular route, with little, if any, transcellular movement.

Lim & Ussing (1982) have also measured passive ion diffusion across corneal endothelium using radiolabelled tracers, although they used the technique of non-steady-state flux analysis to determine flow routes. These authors estimate that 90 % of passive ion diffusion travels paracellularly and the remainder transcellularly. Although there is agreement that paracellular flow is greater, it is virtually impossible to resolve further the ratio of paracellular to transcellular flow by using tracer techniques alone.

The regular anatomy of the corneal endothelium presented us with the opportunity of an alternative approach. By using the technique of specular microscopy, corneal endothelial cells can be viewed directly (Maurice, 1968). Seen in this way, the single layer of corneal endothelial cells form a regular mosaic of homogenous cells, each roughly hexagonal in shape and with typical cell density of 3000 cells $mm^{-2}$ (Hodson & Wigham, 1983). The advantage gained from such a regular mosaic of cells is that a value for unit cell resistance (a unit cell consists of one cell and its proportion of the intercellular space volume) can be calculated from measured values of trans-endothelial resistance. If we then measure cell membrane resistance, using micro-electrode techniques, a comparison of unit cell and membrane resistance should help to resolve the proportions of passive ion fluxes travelling the paracellular and transcellular routes.

In addition, we were also interested in routes of passive water diffusion across corneal endothelium, because of the variety of techniques used and measurements obtained within the literature (Cogan & Kinsey, 1942; Donn, Miller & Mallett, 1963; Liebovitch, Fischbarg & Koatz, 1981; Fischbarg, 1981).

## METHODS

### *Flux measurements*

New Zealand white rabbits 3–4 months old were killed by intravenous injection of sodium pentabarbitone whilst they were calm and relaxed. Eyes, complete with conjunctiva and lids, were dissected (Dickstein & Maurice, 1972). Tissue preparation, incubation media and techniques used to measure electrical activity and passive ion fluxes were all as described previously (Hodson & Wigham, 1983).

Water and ion fluxes across the preparation, comprising first stroma plus endothelium and secondly stroma alone, were measured by perfusing the lens-side half-chamber with Ringer solution containing tritiated water (2·8 kBq $ml^{-1}$; 0·077 $\mu$Ci $ml^{-1}$) and either $^{22}NaCl$ (1·1 kBq $ml^{-1}$; 0·03 $\mu$Ci $ml^{-1}$) or $Na^{36}Cl$ (0·48 kBq $ml^{-1}$; 0·013 $\mu$Ci $ml^{-1}$).

Perfusates collected from both half-chambers were counted in a liquid scintillation counter; counts were corrected for background and cross-over. Labelled $Na^+$ and $Cl^-$ were added to identify any changes in passive ion permeability during the collection period but none were observed and all data were pooled.

### *Membrane resistance*

*Tissue preparation.* Rabbit eyes were dissected as described above. The corneal epithelium was removed using a rotating bristle brush and the central cornea stuck onto an 8 mm Perspex stub with cyanoacrylic glue. A second Perspex stub with a central hole 8·5 mm in diameter was slipped over the first stub and the sclera glued to this second stub with cyanoacylate. Both stubs were contoured to maintain corneal geometry (Fig. 1). Following removal of the posterior part of the eye, a 10 mm trephine cut was made around the central stub, allowing the central stub with the

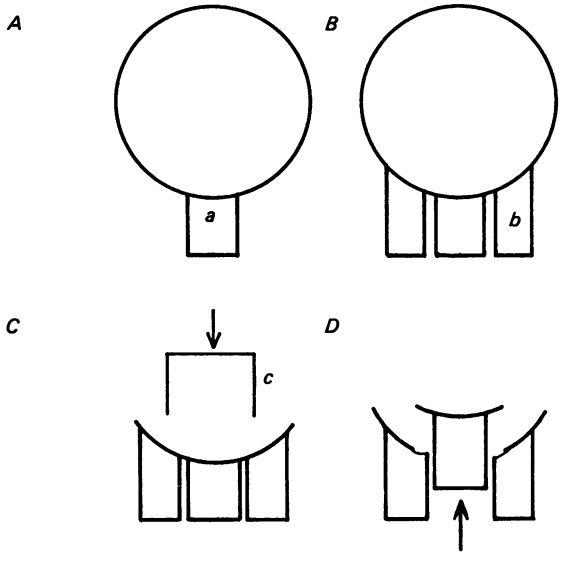


Fig. 1. Procedure for preparing a corneal button suitable for micro-electrode investigation of corneal endothelium. *A*, Perspex stub (*a*) glued to de-epithelialized surface of the cornea. *B*, outer mounting ring (*b*), glued to sclera. *C*, posterior eye removed and a cut made into endothelial surface with a 10 mm trephine (*c*). *D*, inner stub pushed up to isolate corneal preparation, with its normal geometry maintained.

corneal preparation attached to be pushed up away from the remaining cornea and sclera. The preparation on its stub was then mounted in a Perspex chamber containing Ringer solution. The chamber was then placed inside a temperature-controlled block, maintained at 35 °C. Inlet and outlet ports allowed perfusion of the tissue, whilst an intrachamber paddle ensured efficient mixing. The apparatus was placed inside a Faraday cage which stood on a vibration-damped table. Micro-electrodes were prepared from 1 mm (o.d.) filamented borosilicate glass which was pulled into micro-electrodes using a Narishige PN-3 micro-electrode puller. Micro-electrodes were backfilled with 3 M-KCl and held in a micro-electrode holder containing 3 M-KCl in contact with a sintered Ag–AgCl bead.

*Resistance measurement.* Micro-electrode tip resistance ($R_t$) and, following cell impalement, membrane resistance $R_m$ were measured by using the apparatus shown in Fig. 2*A*. The reference electrode was an agar-covered sintered Ag–AgCl bead. A potential difference (p.d.) of $\pm 100$ mV was generated across the tip by using a Time Electronics Model 404S millivolt source. The resultant current was measured by using a Levell Model TM9BP d.c. multimeter, which was connected to a pen recorder. $R_t$ was measured before and after cell impalement. $R_t$ and $R_m$ could also be monitored using an electrode resistance test facility built into the high impedance amplifier, a W.P.I. Model FD223, used to monitor transmembrane p.d. The principle employed by the test facility is to pass a known current and measure the p.d. generated. Although measurements of resistance, both tip and membrane, made using either technique were comparable, we found that measurement of p.d.-induced current gave more accurate data over a much wider resistance range.

*Cell impalement.* The micro-electrode holder was fixed to a Prior micromanipulator modified for remote control by attaching a stepper motor to the fine-movement screw. Single or multiple, up or down backlash-free movement steps less than 1 $\mu$m were possible. Following measurement of $R_t$, the measuring circuit was changed from that shown in Fig. 2*A* to that shown in *B*, that allowed measurement of transmembrane p.d. The output of the amplifier was connected to a pen recorder and an acoustic coupling device, W.P.I. Acoustic Baseline Monitor.

To impale the cell, the micromanipulator was advanced until a change in pitch was heard,

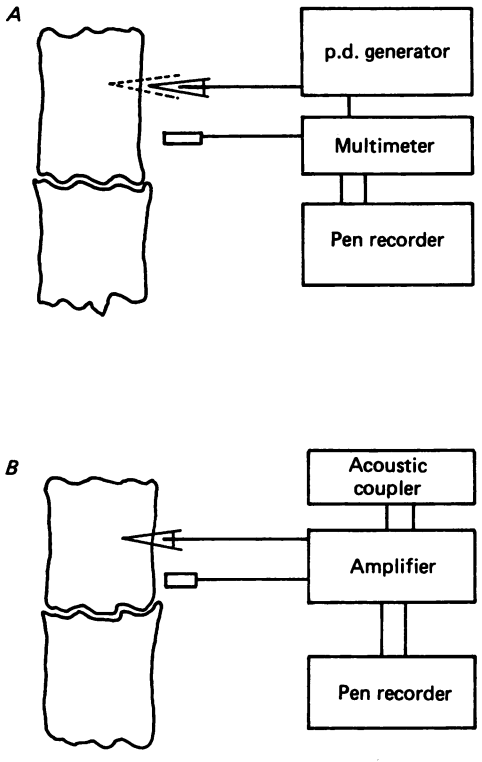


Fig. 2. *A*, apparatus for measurement of micro-electrode tip resistance ($R_t$) and corneal endothelial cell membrane resistance ($R_m$). *B*, apparatus for measurement of corneal endothelial cell transmembrane potential difference (p.d.).

indicating the change in measured p.d. as the cell membrane was crossed. Successful impalement was confirmed only when p.d. remained unchanged for a minimum period of 1 min. Following impalement, $R_m$ was measured by reverting to the apparatus of Fig. 2*A*. Measurement of $R_m$ was considered successful if $R_m$ remained stable for longer than 3 min. Transmembrane p.d. was monitored again as the micro-electrode was withdrawn from the cell. $R_t$ was measured after withdrawal. Any change between pre- and post-impalement $R_t$ of greater than 10% also rendered the experiment invalid. Our criteria were, therefore, (a) that transmembrane potential did not decrease during impalement, (b) that transmembrane resistance was stable for at least 3 min, and (c) that $R_t$ did not vary by greater than 10% during the collection of data.

Proportionality between applied p.d. and $R_m$ measurement was checked by generating a series of p.d.s across the cell membrane from +100 to −100 mV, and observing the induced current.

## RESULTS

### *Fluxes*

Following the initial settling-in period, transendothelial electrical parameters p.d. and resistance ($R_e$), remained stable for over 6 h. Measured p.d. compared well with previously published values of 500–700 $\mu$V (Hodson & Miller, 1976; Hodson & Wigham, 1983), at $564 \pm 43$ $\mu$V (mean ± S.E., $n = 6$). $R_e$ in this study was slightly lower than in previous studies; $12{\cdot}7 \pm 0{\cdot}8$ $\Omega$ cm$^2$ ($n = 6$) compared to 16·0 $\Omega$ cm$^2$

TABLE 1. Passive ion and water fluxes and diffusional permeabilities across de-epithelialized rabbit corneas

| | Flux ($\mu$equiv cm$^{-2}$ h$^{-1}$) | | Permeability (cm h$^{-1}$) | | |
|---|---|---|---|---|---|
| | E+S | S | E+S | S | E* |
| $Na^+$ | 19·1±1·0 | 37·3±0·1 | 0·124±0·005 | 0·242±0·002 | 0·230±0·014 |
| $Cl^-$ | 16·1±0·8 | 36·7±0·4 | 0·145±0·005 | 0·325±0·004 | 0·259±0·020 |
| $H_2O$ | 23274±412 | 22642±274 | 0·420±0·008 | 0·408±0·004 | Not calculable |

E+S = corneal stroma plus endothelium.
S = corneal stroma alone.
E* = endothelium alone calculated from previous two columns, as described by Hodson & Wigham (1983).
Values are mean ±S.E., $n = 6$.

(Hodson & Wigham, 1983). This was reflected in the slightly increased measurements of $Na^+$ and $Cl^-$ unidirectional flux and permeability (Table 1), compared with those of Hodson & Wigham (1983).

Values for water flux across the stroma and endothelium were rather unexpected. As can be seen from Table 1, water flux was not increased when the endothelium was removed. In fact there was a small, but not significant, decrease. The endothelium appears to present very little barrier to diffusional exchange of water compared to corneal stroma.

*Membrane resistance*

Micro-electrodes used in this study had a mean $R_t$ of 43·6±5·8 MΩ, ($n = 46$), measured in the bathing Ringer solution. Following measurement of $R_t$ the micro-electrode was advanced into a cell and $R_m + R_t$ measured. To determine if the cell membrane was acting as an ohmic resistor, applied p.d. was varied and induced current measured. There was an almost linear response with a slight deflexion in electrical resistance about 20 mV depolarized from resting potential, which averaged −32 mV in this series. It is possible that the deflexion is a consequence of voltage-dependent gating activity in the membrane but we would not, at this stage, wish to comment further on this observation. The linearity was sufficient to give confidence to the measurements of $R_m$ of better than ±10%.

Mean $R_m$ obtained by subtraction of $R_t$ from $R_m + R_t$ was 969 MΩ, $n = 46$. No standard error of mean is given on this data, since it can be seen from Fig. 3 that there is considerable variation in the data.

## DISCUSSION

The spread of values for measured $R_m$ (Fig. 3) raises the question as to what is the true value of $R_m$, because the distribution is much more dispersed than the spread in endothelial cell size. It is our suggestion that values for $R_m$ at the lower end of the spread, approximately 400 MΩ, represent true $R_m$. The remainder of the values, 1000 MΩ and upwards, we believe could be from impalements where the micro-electrode tip lies in the cell nucleus or mitochondrion. A stereological analysis of

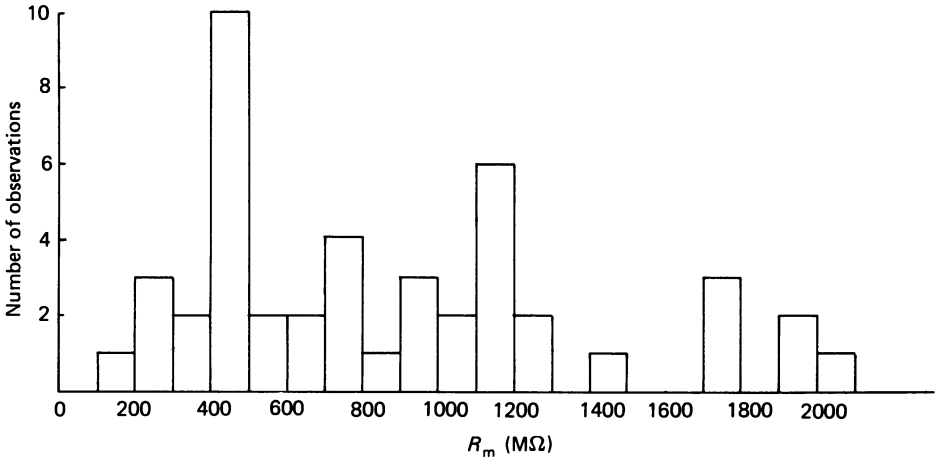


Fig. 3. A frequency distribution of rabbit corneal endothelial cell membrane resistance ($R_m$), measured using micro-electrodes.

endothelial cells shows the cytoplasm to occupy 65%, the nucleus 18% and mitochondria 17% of cell volume.

The data of Fig. 3 can be divided into three resistance groups, 0–1000, 1000–1400 and 1400–2000 mΩ, where the proportion of impalement in each group resembles quite closely the proportion of cell volume occupied by the cytoplasm, nucleus and mitochondria, respectively. This suggestion of course implies that these organelle membranes present a significant barrier to ion movement. Alternatively it could be that the endothelial cell membrane population is as heterogenous as the distribution shown in Fig. 3, but there is no equivalent heterogeneity morphologically.

Typical cell density for rabbit corneal endothelium is 3014 cells $mm^{-2}$ (Hodson & Wigham, 1983). By using this value for cell density and our value for $R_e$ of 12·7 Ω $cm^2$, we calculate unit resistance ($R_u$), the resistance of one cell and its corresponding proportion of the paracellular space (Fig. 4), to be 3·8 MΩ.

The resistance of the transcellular route (Fig. 3) is about 100 times greater than $R_u$, which includes both the transcellular and the paracellular routes measured in parallel. This difference suggests that the diffusion of ions across the corneal endothelium meets least resistance from the paracellular route and takes place almost exclusively between the cells. This conclusion becomes even firmer if we consider the approximations we have made in arriving at it. They include the following.

(a) When we measure $R_m$ we are not measuring the resistance of one membrane, but the resistance of two membranes, apical and basolateral, in parallel. This is because the paracellular pathway has clearly such a low resistance relative to the plasma membrane, that it acts as a short circuit for current flowing across the basolateral membrane even when the reference electrode is in the solution bathing the apical surface. Any ion passively diffusing across the cell will have to cross the resistances of both apical and basolateral membranes in series, effectively more than doubling resistance of the transcellular pathway recorded with the micro-electrodes.

(b) If these cells are electrically coupled then again current flow will be divided

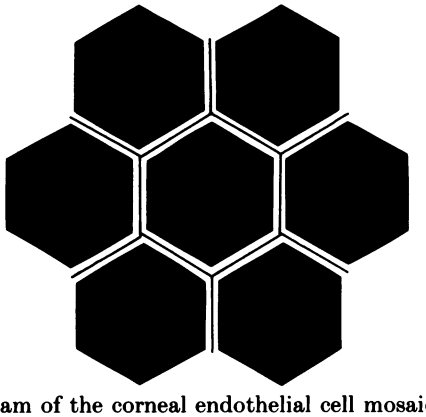

Fig. 4. A schematic diagram of the corneal endothelial cell mosaic. The shaded portion indicates the cell whilst the area surrounding each cell within the confines of the continuous line indicates the paracellular component of that one cell. The cell and its paracellular space we describe as a unit cell. Typical cell density is 3000 cells $mm^{-2}$, each side of the cell being 11·3 $\mu$m in length.

between the coupled cells and we would have underestimated $R_m$ by a factor dependent on the average number of cells in electrical contact.

The assumption made by Hodson & Wigham (1983), that the conductivity to ions of the paracellular route is many times greater than the transcellular route, is confirmed by this study. Although the corneal endothelium falls into the category of a 'leaky' epithelium, transendothelial resistance is 12–16 $\Omega$ $cm^2$; the cells that comprise the epithelium are extremely impermeant to the passive movements of ions. The maximum passive ion flux across the cell is less than 1 % of the total passive flux; the remainder passes paracellularly.

We suggest that the 'tightness' of these cell membranes is such that for any ion to enter the cell at physiologically significant rates, there must be a membrane–ion interaction, be it a gate, channel or transport protein. This could be advantageous to a transporting epithelium since it eliminates one pathway for the dissipation of any electrochemical gradients generated by the tissue.

In contrast to ions, water diffusion across corneal endothelium is so fast that it is not possible to resolve a difference in passive water fluxes with or without the endothelium present on the corneal stroma. The endothelium is effectively 'transparent' to water. Measurements of endothelial diffusional permeability to water by others have produced similar results, but we believe that earlier workers were modest in stating the obvious conclusion (Cogan & Kinsey, 1942; Donn *et al.* 1963). It is interesting to note that more recent published values of endothelial water permeability tend to have increased as techniques are refined (Mishima & Hedbys, 1967; Fischbarg, 1979; Liebovitz *et al.* 1981; Fischbarg, 1981). We propose that they may be so high as to be effectively unmeasurable. From our data, it seems that they could be of the same order of magnitude as the diffusional permeability of the same thickness (about 5 $\mu$m) of unstirred fluid.

We considered whether our estimates of endothelial electrical resistance could be

caused by trauma and the occasional deletion of cells from the mosaic. We assumed that $R_u$, i.e. the resistance of one cell and its associated share of the paracellular space, (Fig. 4) was that indicated by the intracellular micro-electrodes, i.e. about 400 MΩ, and that the measured $R_u$ of 3·6 MΩ was an error resulting from cell loss. The average resistance of a deletion in the mosaic caused by the loss of a cell would be 10600 Ω (specific resistance of Ringer solution, 70 Ω cm; cell dimensions, $3{\cdot}3 \times 10^{-6}$ cm² area, $5 \times 10^{-4}$ cm width) so that one deletion in seventy-three cells of the endothelial mosaic would reduce transendothelial resistance from that required for the transcellular hypothesis to pertain i.e about 1300 Ω cm² to that measured, i.e. about 13 Ω cm². We scanned about 18000 cells of the endothelial mosaic which had undergone the appropriate dissection and mounting procedures. We saw no deletions in the endothelial mosaic and concluded that our data did not result from the occasional loss of cells from the endothelial mosaic.

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