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THE PERMEABILITY OF RABBIT AND HUMAN CORNEAL ENDOTHELIUM

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SUMMARY

1. The fluxes of sodium, chloride and bicarbonate across endothelium plus stroma and then stroma alone were measured in the direction from lens-side to tear-side in rabbit and human corneas *in vitro*, in order to measure passive permeabilities.

2. The results were used to calculate the permeability of the endothelium. Hodgkin's equation (1951) was then used to calculate the partial electrical conductivity of each ion crossing the endothelium.

3. The summated electrical conductivities of sodium, chloride and bicarbonate were equal to $89 \pm 8\%$ of the measured electrical conductivity, suggesting that the ions diffuse independently across the endothelium in the direction lens-side to tear-side.

4. Stereological analysis of the intercellular spaces supports the idea that the ions permeate through this route and that the physical shape of the spaces determines almost entirely the permeability of the endothelial layer.

5. Trans-endothelial sodium and chloride permeabilities are nearly equal, which may be explained by supposing the intercellular spaces include a cation exchanger of fixed negative charge capacity around 60 m-equiv $l.^{-1}$ intercellular fluid.

INTRODUCTION

When a cornea is at steady state, i.e. it is neither swelling nor shrinking, its hydration is governed by the relationship

$$J = \bar{\sigma} . S.P. \quad \text{(Mishima \& Hedbys, 1967)},$$

where J is the activity of the hydration-regulating pump in the corneal endothelium, ω and σ are, respectively, the permeability and reflexion coefficient of the endothelium to the ion pumped, and $S.P.$ is the swelling pressure of the corneal stroma. The permeability of corneal endothelium is therefore one of four parameters which interact in a 'pump-leak' mechanism, across the corneal endothelium (Hodson, 1974) to control almost completely (Klyce, 1975) corneal hydration and transparency (Maurice, 1957). Measurement of endothelial permeability is not easy, as the corneal endothelium is a 'leaky' cellular membrane whose permeability is approximately the same as the connective tissue on which it rests and from which it is surgically very

difficult to effect complete separation (Hodson, Miller & Riley, 1977). Measurement is complicated further by the recently emphasized anatomical feature (Mishima, 1982) of a morphologically distinct annular ring, 1–2 mm wide, of those cells adjacent to the limbus. As a consequence, data acquired from the whole cornea will mask the presence of two components.

We present here measurements of endothelial permeability in rabbits and post-mortem human corneas, taking these factors into account. Endothelial permeability to sodium, chloride and bicarbonate was measured across the central cornea in the direction lens-side to tear-side, fluxes which do not include active components coupled to the endothelial 'pump' (Hodson & Miller, 1976; Mayes & Hodson, 1978).

Further, we wished to investigate the relationship between ionic permeability and electrical conductance, because in the past we have used measured electrical resistance of the endothelium of post-mortem human corneas as an indication of their permeability, which is frequently too high to measure directly. The physiology of human cornea is sufficiently similar to that found in the rabbit cornea (Hodson, Wigham, Williams, Mayes & Graham, 1981; Wigham & Hodson, 1981) to justify studying the relationship between ionic permeability and electrical conductance using rabbit corneas and to include in the series those human corneas which became available during the course of this study.

METHODS

Theory and notation

It is not practical to isolate the corneal endothelium without the presence of the corneal stroma. A strategy was devised where ionic fluxes and electrical conductivity (g) or resistance (R) were measured across a preparation consisting of corneal stroma plus endothelium (including the associated unstirred layers on either side of the preparation with respect to the flux measurements and including the resistance of the Ringer solution between the voltage-sensing tips with respect to the electrical measurements), and then across the stroma alone after removing the endothelium. Our assumption is that the unstirred layers on either side of the preparation (Green & Otori, 1970) are the same in the presence and absence of the endothelium.

Subscripts:	e+s	endothelium plus stroma
	e	endothelium alone
	s	stroma alone
	i	one ion in the Ringer solution
Superscripts:	m	measured values or those calculated from Ohm's law
	c	calculated values.

$$R^{m}_{e+s} - R^{m}_{s} = R^{m}_{e} \tag{1}$$

$$\frac{1}{P^{m}_{e+s}} = \frac{1}{P^{c}_{e}} + \frac{1}{P^{m}_{s}} \quad \text{(Kedem \& Katchalsky, 1963)} \tag{2}$$

$$\frac{1}{R^{c}_{e}} = \sum_{i} g = \frac{F^{2}}{RT} \sum_{i} P_{i} . C_{i} \quad \text{(Hodgkin, 1951)} \tag{3}$$

Equation (3) is applied to the endothelium alone. P = permeability; C = the concentration of an ion in the Ringer solution; F = Faraday's constant and RT has its usual meaning. The applicability of Hodgkin's equation to the penetration of ions across the corneal endothelium is to be judged by comparing R^{m}_{e} (eqn. (1)) with R^{c}_{e} (eqn. (3)).

Tissue preparation

Dutch rabbits, 3–4 months old, were killed by intravenous injection of sodium pentobarbitone whilst they were calm and relaxed. The eyes together with lids and conjunctival membrane were dissected (Dikstein & Maurice, 1972). Human eyes were removed from cadavers following written consent given by immediate relatives of the deceased. Only fresh rabbit corneas were used; human corneas were used within 24 hr of death. The corneal epithelium was removed using a rotary bristle brush, and the remaining stroma plus endothelium preparation was mounted between the two halves of a modified Ussing chamber (Wigham, 1980; Wigham & Hodson, 1981). To prevent slipping of the tissue as the half-chambers closed together, a small ring of cyanoacrylic glue was applied to the sclera adjacent to the limbus in order to stick the stroma to the tear-side half-chamber. To minimize further cell damage and so prevent the formation of low-resistance pathways, the leading edges of the half-chambers were shaped to match the contours of the cornea, with the exception of the contact point on the lens-side half-chamber, which was flattened to produce an annulus of

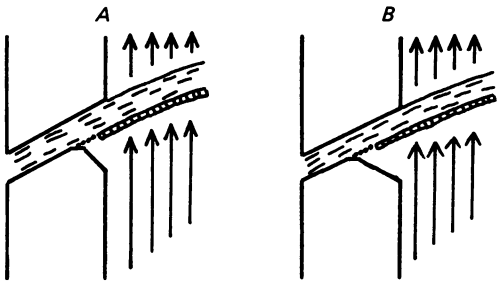

Fig. 1. Where the half-chambers meet, the endothelium is crushed. Damage spreads about 1 mm from the point of contact (indicated in *A* and *B* by the absence of endothelial cells). The contribution of the consequent low-resistance pathways is negligible if either: *A*, the damaged area is just outside the tube of flow (arrows) or *B*, well outside the tube of flow.

approximately 0·5 mm width. The diameter of the annulus was slightly larger than the main chamber to mask any cell damage from the current pulse used to determine endothelial resistance. The possible problems of stagnant pools and varying effective exposed tissue area were examined by building two chambers with different leading-edge geometries (Fig. 1). There was no significant difference between the results obtained with the two chambers, and all results were pooled. A small hydrostatic pressure of 2 cmH_2O (lens-side positive) was applied across the preparation during each experiment in order to prevent the tissue from 'flapping' in the chamber.

Incubation media

Rabbit corneas were incubated in rabbit Ringer solution containing (mM): NaCl, 106; $NaHCO_3$, 37; KCl, 6·7; $MgSO_4$, 0·6; Na_2HPO_4, 5·55; glucose, 4·45; reduced glutathione, 1; $CaCl_2$, 0·56 and bubbled to pH 7·32 with a 5% CO_2, 7% O_2, 88% N_2 gas mixture. Human corneas were incubated in human Ringer solution containing (mM): NaCl, 117·5; $NaHCO_3$, 24; KCl, 4; Na_2HPO_4, 1; $MgSO_4$, 1; glucose, 4·45; reduced glutathione, 1; $CaCl_2$, 2·54 and bubbled to pH 7·38 with a 5% CO_2, 7% O_2, 88% N_2 gas mixture. Rabbit Ringer solution would not sustain a human cornea, nor would human Ringer maintain a rabbit cornea.

Each half-chamber was perfused with Ringer solution at 10 ml. hr^{-1} supplied by a digital syringe pump (Vickers Ltd), and stirring was effected by intrachamber paddles rotated at 40 rev/min in all experiments to standardize unstirred layers adjacent to the preparation. The intrachamber stirring and constant perfusion maintained all corneas without deterioration for the duration of the experiment and allowed efficient and trauma-free solution changes. Tissue temperature was regulated at 35 °C by immersing the assembled chamber in a thermostatically controlled oil-bath.

Electrical measurements

Electrical parameters of corneal endothelium were measured as described by Wigham & Hodson (1981); trans-edothelial potential difference (p.d.) was measured continuously and resistance (*R*) measured at frequent intervals.

Measurement of passive ion fluxes

Each preparation was incubated for a 1 hr period before experiments were started, which allowed human corneas to recover from the cold post-mortem storage conditions and rabbit corneas to stabilize from transients often seen within the first 10–15 min. The main experiment was carried out in two parts as follows.

(a) Appropriate Ringer solutions including first ^{22}NaCl, 0·03 μCi ml.$^{-1}$ and NaH^{14}CO$_3$, 0·16 μCi ml.$^{-1}$ and secondly Na^{36}Cl, 0·013 μCi ml.$^{-1}$ were perfused at 10 ml. hr^{-1} through the lens-side half-chamber. Samples were collected from the outlet tube of each half-chamber at 12 min intervals directly into glass vials containing 20 ml. scintillation fluid (5 g 2,5-diphenyloxazole plus 0·2 g 1,4-di-[2-(5-phenyloxazolyl)]benzene in 1 l. toluene plus 500 ml. Triton X – 100). This was to prevent loss of ^{14}C activity due to loss of CO_2 during collection and similar losses seen when Polythene vials were used. Samples were counted in a liquid scintillation counter.

The electrical potential of the preparation was short-circuited by a current supplied by Ag/AgCl disk electrodes positioned on the back face of each half-chamber. The series resistance component of the stroma and Ringer solution between the sensing tips must be compensated for when short-circuiting the endothelium. In this preparation $R_e \doteq R_s + R_{Ringer}$. If no compensation is made then under normal 'short circuit', with p.d. across the sensor tips set to zero, there will be a non-zero p.d. across the endothelium of

$$E_e = V\frac{R_s + R_{Ringer}}{R_e + R_s + R_{Ringer}},$$

where V is the open-circuit p.d. Ion fluxes will be driven partly by an electrical p.d. and estimates of ion permeability coefficients will be inaccurate. We are able to make the necessary compensations because variations in series resistance ($R_s + R_{Ringer}$) between preparations are negligible. After we measure R_{total} we can calculate by difference R_e, and then pass a short-circuiting current equal to V/R_e.

(b) The endothelium was removed *in situ* by wiping with a cotton-wool bud. (Histological evidence showed the removal to be complete.) Access to the endothelium was by a small port situated at the end of the lens-side half-chamber. Removal of the endothelium *in situ* allowed the blank electrical resistance between the tips of the voltage-sensing probes to be measured more accurately than by opening the chamber, removing the endothelium and remounting.

Measurement of ion fluxes across the stroma alone was repeated. No short-circuit current was necessary, since removal of the endothelium abolished the potential difference across the preparation.

RESULTS

De-epithelialized corneas bathed in Ringer solution at 35 °C spontaneously develop an electrical potential, polarity lens-side negative, of typically 500–700 μV in the rabbit (Fischbarg & Lim, 1974; Hodson, 1974; Hodson & Miller, 1976) and 200–500 μV in the human (Wigham & Hodson, 1981; Hodson *et al.* 1981). In this series endothelial p.d. was 672 ± 66 μV (S.D., $n = 9$) in the rabbit and 280 μV and 150 μV in the human.

Endothelial resistance was calculated as the difference between measured electrical resistance in the two runs (eqn. (1)). In order to express resistance in standard units it was necessary to determine the exposed tissue area. A formula for calculating the surface area of a sphere sector was used, with the posterior radius of curvature of the cornea as the sphere radius and main chamber diameter as the sector diameter. The resulting value of 0·76 cm^2 correlated very well with measurements of corneal area determined by staining methods (Hodson, 1974). This was reassuring with regard to chamber geometry and the small pools described in Fig. 1, since the pools present a sufficiently large diffusional pathway to be effectively independent of the main chamber. Corrected values for measured endothelial cell resistance in the rabbit and

TABLE 1. The diffusional permeability of various ions across de-epithelialized rabbit cornea. Values are the means ±S.E. of means, $n = 9$

Ion	P_{e+s}* (cm hr^{-1})	P_s* (cm hr^{-1})	P_e† (cm hr^{-1})
HCO_3^-‡	0·101±0·004	0·197±0·007	0·214±0·013
Na^+	0·100±0·004	0·236±0·007	0·181±0·014
Cl^-	0·117±0·003	0·320±0·010	0·186±0·009

P = Permeability. Subscripts: e+s = corneal stroma plus endothelium; s = corneal stroma; e = endothelial cell layer.

* These stroma were swollen to about 2½ times their physiological hydration.

† Calculated from the previous two columns.

‡ Bicarbonate data derived from total HCO_3^- plus CO_2 flux measurements; as a result, they over-estimate true ionic permeablity and conductance.

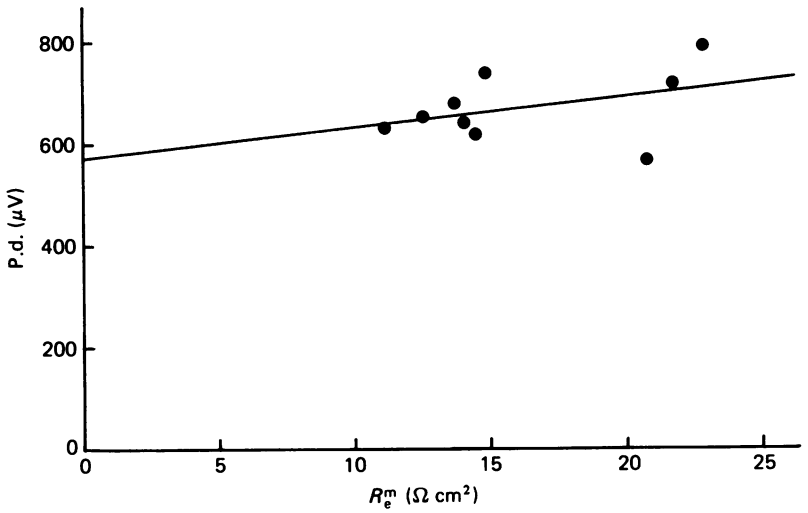

Fig. 2. The trans-endothelial potential difference, V (μV, polarity lens-side negative) is nearly independent of trans-endothelial resistance, R (Ω cm^2). Best fit gives $V = 574 + 6R$.

human cornea were 16·20±4·08 Ω cm^2 (S.D., $n = 9$) and 10·6 and 10·26 Ω cm^2 respectively.

Counts from samples collected from both half-chambers were corrected for background radiation and cross-over between channels when two isotopes were counted simultaneously. Flux rates for the three labelled ions were calculated from activities in the collecting, tear-side, half-chamber. Ion permeabilities were calculated from the flux data, which enabled endothelial permeabilities to be calculated (eqn. (2)). Results are shown in Table 1.

Hodgkin's equation (1951) was used to calculate partial endothelial conductivity for each ion. Summing individual conductivities gave a value for total conductivity, from which R_e^c was obtained. Calculated endothelial resistance was 17·91±2·88 Ω cm^2 (S.D., $n = 9$) in the rabbit and 9·14 and 13·3 Ω cm^2 in the human.

A plot of p.d. against R_e^m (Fig. 2) shows that p.d. is almost independent of R_e^m, suggesting that natural variations in R_e^m and short-circuit current (i) mutually

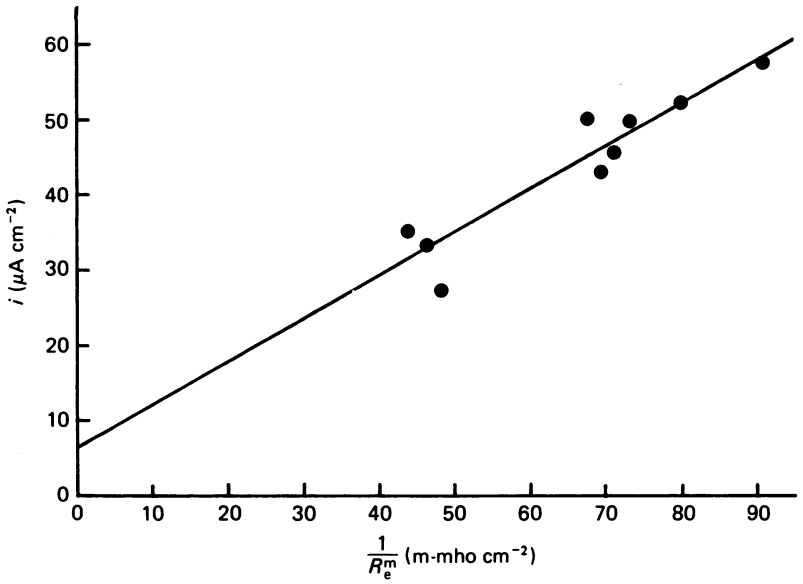

Fig. 3. Trans-endothelial short-circuit current, $i(\mu A\ cm^{-2})$ is roughly proportional to trans-endothelial electrical conductance, $1/R_e^m$ (m-mho cm^{-2}). Best fit gives

$$i = 5{\cdot}6 + 0{\cdot}58 \frac{1}{R_e^m}.$$

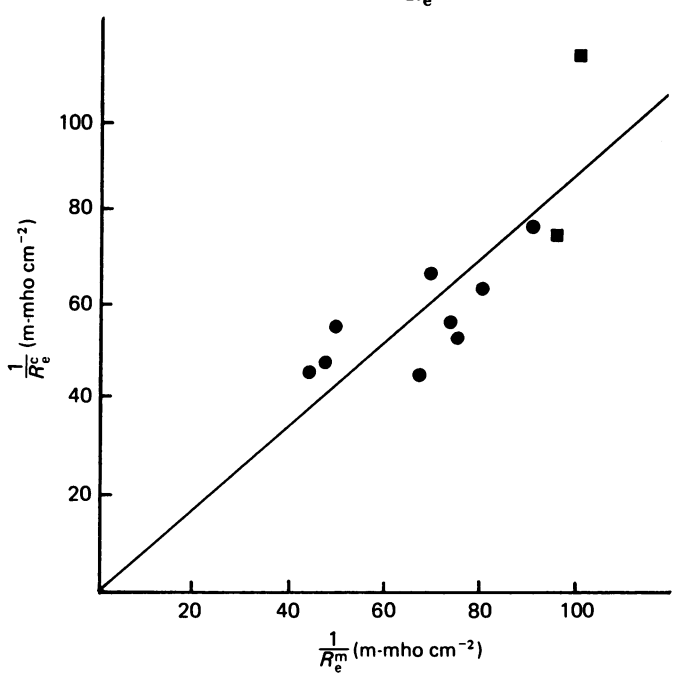

Fig. 4. There is a linear relationship between trans-endothelial electrical conductance, $1/R_e^m$ and that calculated from measured ionic fluxes using eqn. (3) of the text $1/R_e^c$. Circles are data taken from rabbit; squares are data taken from human. Best fit gives

$$\frac{1}{R_e^c} = 0{\cdot}22 + 0{\cdot}89 \cdot \frac{1}{R_e^m}.$$

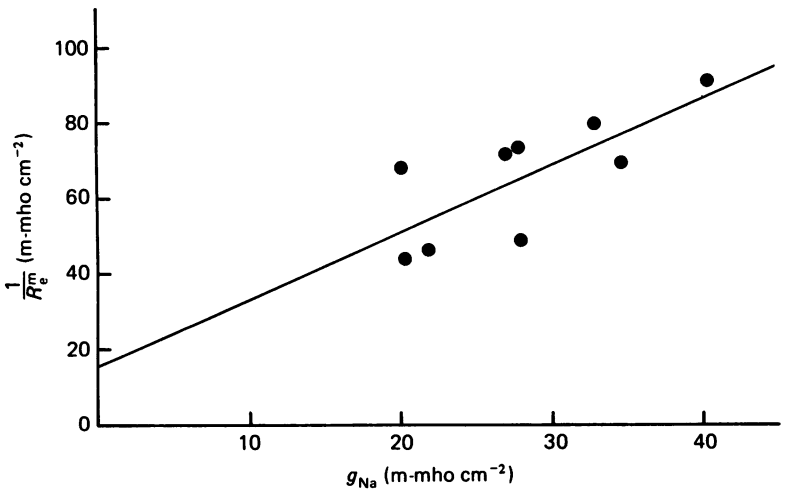

Fig. 5. Partial sodium conductance varies with measured conductance in rabbit corneal endothelium.

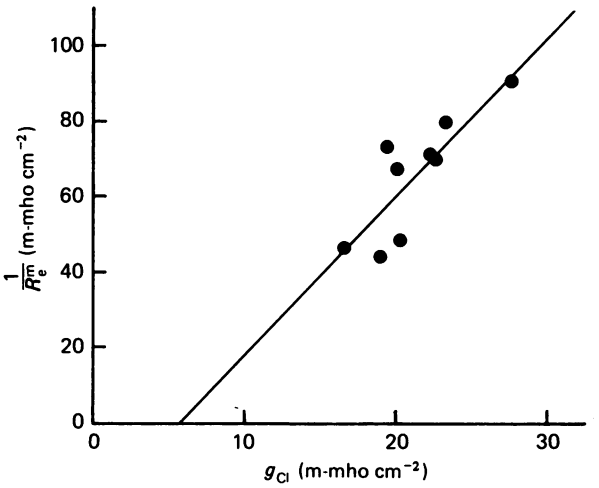

Fig. 6. Partial chloride conductance varies with measured conductance in rabbit corneal endothelium.

compensate. A plot of i against $1/R_e^m$ ($\Sigma_i g_i^m$) (Fig. 3) supports this assumption. As endothelial conductance increases (indicating a more leaky preparation), the short-circuit current (which reflects the activity of the electrogenic bicarbonate ion pump) also increases.

If ions move freely and independently across the endothelium then total conductance calculated from Hodgkin's law $1/R_e^c$, would be expected to resemble the measured electrical conductance, $1/R_e^m$, closely, since there is a common pool of freely diffusing ions, passively diffusing across the cells and carrying the externally applied current used to measure resistance electrically. A plot of $1/R_e^m$ against $1/R_e^c$ ($\sum_i g_i^c \propto \sum_i g_i^m$)(Fig. 4) shows a very close relationship between the two. We observed that $1/R_e^c$ was slightly smaller than $1/R_e^m$.

DISCUSSION

Hodgkin's equation (1951; eqn. (3) in this paper), which relates ionic fluxes across epithelia to partial electrical conductivity, predicts our data well. The permeation of sodium, chloride and bicarbonate across corneal endothelia accounts for $89 \pm 8\%$ of the measured electrical conductance of the tissue. Including the fluxes of the minor ions across the endothelium, by assuming their permeability to be proportional to their ionic mobility (see later), generates an even closer fit between Hodgkin's equation and the phenomena. It seems reasonable to use measurements of endothelial electrical conductance as an indication of the ionic permeability of the cellular layer.

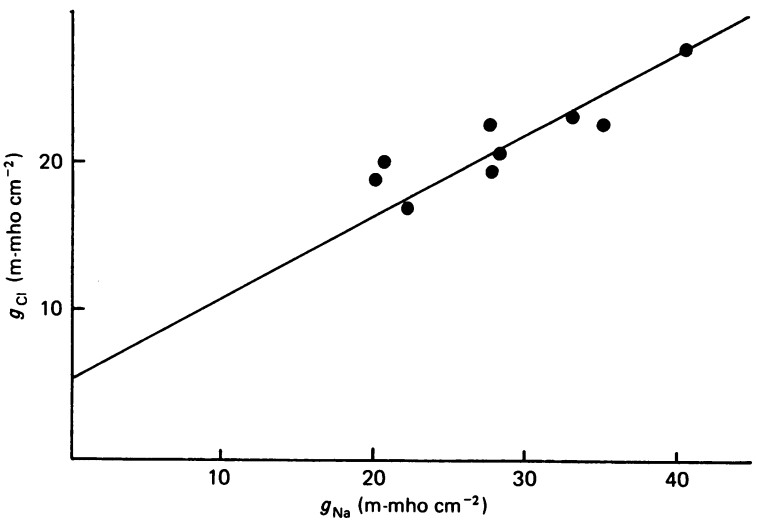

Fig. 7. The best linear relationship between sodium (g_{Na}) and chloride (g_{Cl}) conductances in rabbit corneal endothelium is

$$g_{Cl} = 6{\cdot}4 + 0{\cdot}53\, g_{Na}.$$

Figs. 5 and 6 may be used for this purpose to estimate sodium and chloride permeabilities in different preparations. Although the electrical resistance of individual preparations varies considerably, a point to which we shall return at the end of this Discussion, Fig. 7 indicates that there is no corresponding change in the ratio of the permeability of sodium to chloride over the range. They remain approximately equal and proportional to the over-all electrical conductance. This equality in their permeability across the corneal endothelium contrasts with their diffusion coefficients in free solutions.

The free diffusion coefficients of sodium and chloride at 35 °C in 0·15 M aqueous solution are taken as $1{\cdot}21 \times 10^{-5}$ cm^2 sec^{-1} and $1{\cdot}85 \times 10^{-5}$ cm^2 sec^{-1} respectively (mutual diffusion coefficient and equivalent conductivities taken from Robinson & Stokes, 1959, and substituted in the Nernst-Hartley equation).

According to Table 1, corneal stroma is less permeable to sodium ($6{\cdot}7 \times 10^{-5}$ cm sec^{-1}) than chloride ($8{\cdot}9 \times 10^{-5}$ cm sec^{-1}), but when due allowance is made for the even greater difference in their free diffusion coefficients, the mobility

of sodium in corneal stroma is relatively enhanced over the mobility of chloride by a factor of $1{\cdot}15 \pm 0{\cdot}05$. A similar phenomenon is observed in their mobilities across corneal endothelium (Table 1) and here the phenomenon is more pronounced. The increased mobility of sodium relative to chloride in their passage across the endothelium is $1{\cdot}49 \pm 0{\cdot}08$.

The flux of ions, J, across simple tissues as measured in the present experiments is mostly given by

$$J = P.C. \tag{4}$$

Although, by convention, C is taken to be the concentration of ion in the bathing medium, properly C should be taken as the concentration of ion in the membrane and is usually unknown. In the case of corneal stroma the concentration of ion in the membrane can be calculated reliably, since corneal stroma behaves like a non-selective, simple cation exchange resin (Hodson, 1971). At the hydrations recorded in the present series of experiments, where the stroma was swollen to approximately two and a half times its physiological hydration, the cation exchange capacity is about 18 m-equiv $l.^{-1}$ stromal fluid. Under these conditions, stromal sodium concentration would be about 1·06 times greater than its concentration in the bathing medium and stromal chloride concentration would be about 1·06 times lower than in the bathing medium. The sodium:chloride concentration ratio would be 12% higher in the stroma than in the bathing medium, which very well accounts for the measured 15 ± 5% enhanced mobility of sodium over chloride in the stroma. There is consequently no need to invoke differential diffusional mobility in corneal stroma to explain the relative enhanced mobility of sodium over chloride. The phenomenon is explained adequately by their differential concentrations in the corneal stroma as a result of the Donnan potential generated by the acidic groups of the glycosaminoglycans.

Ions permeate passively across the corneal endothelium, apparently almost entirely through the paracellular route (Maurice, 1969; Lim & Fischbarg, 1981). If the Donnan theory is used to explain the differential mobilities of sodium and chloride across the endothelium, it can be calculated that the extracellular spaces of the endothelium are filled with a cation exchanger of capacity about 60 m-equiv of free acidic groups per l. intercellular fluid, which is comparable with the fixed negative charge density in the corneal stroma at physiological hydration (48 m-equiv $l.^{-1}$ stromal fluid; Hodson, 1971).

Electron microscopical observations of intercellular spaces show first, a 'terminal complex' at the apical end of the cell where the intercellular channel narrows to less than 2 nm, and secondly, that intercellular space volume is a function of fixative osmolarity (Hodson & Mayes, 1979). High osmolarity fixatives (> 330 m-osM) show a uniform intercellular space width of 20 nm; below 320 m-osM and down to at least 280 m-osM intercellular space volume increases roughly linearly with decreasing osmolarity. Reflexion coefficients are not known in the complex situation of chemical fixation; as a result it is not possible to know which fixative strength neither introduces nor removes fluid from the intracellular spaces.

Stereological analysis (Hodson & Mayes, 1979; S. Hodson & G. Thomas, unpublished) shows the endothelial matrix to be composed of a monolayer of

hexagonal cells of side 11·3 μm and depth 3·6 μm. Tortuosity of the intercellular space increases the mean intercellular channel length to 10·3 μm.

Ignoring the terminal complex and assuming that the true intercellular spaces are 'closed' at a width of 20 nm, we can calculate the permeability of the cell layer to be 0·11 cm hr^{-1} (measured 0·185 cm hr^{-1}) and the electrical resistance to be 34·5 Ω cm^2 (measured 16·2 Ω cm^2). This discrepancy between calculated and observed values is minimal if we assume an average intercellular space width of 38 nm. Then, permeability is calculated at 0·20 cm hr^{-1} (measured 0·185 cm hr^{-1}) and electrical resistance calculated at 18 Ω cm^2 (measured 16·2 Ω cm^2). This assumption leaves no significant contributory role for the 'terminal complex' in permeability. In reality only if average intercellular space width is greater than 40 nm can the 'terminal complex' contribute towards endothelial permeability.

It seems likely that the 'terminal complex' is the site of partial solute filtration that corresponds to the measured reflexion coefficient of salt, which is between 0·6 and 0·4 (Mishima & Hedbys, 1967; Klyce & Russell, 1979). If this is the case then endothelial reflexion coefficient and endothelial permeability result from physically distinct structures, the first from the 'terminal complex' and the second from the volume of fluid in the intercellular spaces and its geometry.

A tentative model of passive salt permeability across the endothelium is that ions diffuse across the paracellular route and most of the resistance to the diffusion results from geometric narrowness and tortuosity of the intercellular space. The intercellular spaces are slightly 'open' (possibly because of the presence of lakes intermittently observed there) and intercellular fluid contains a cation exchange capacity of about 60 m-equiv $l.^{-1}$.

The variation in the rabbit population is of great interest. Endothelial electrical resistance shows a much greater variability than the relatively constant potential difference (Fig. 2). This may be a consequence of the nearly linear relationship between short-circuit current and electrical conductance (Fig. 3). We know from the present study that electrical conductivity reflects, fairly accurately, endothelial permeability, and Hodson & Miller (1976) showed the short-circuit current to reflect the activity of an electrogenic bicarbonate ion pump in the endothelium which is the 'pump' in the 'pump-leak' balance of corneal water relations.

We observe for the first time a relationship between the 'pump' and one component of the 'leak'. When one is large then so is the other and vice versa. We cannot imagine a trivial reason (such as trauma or chamber design) for this observation, and conclude that the balance between the 'pump' and the 'leak' which maintains corneal transparency is not the unregulated expression of genetic characteristics, but that the hydration and transparency of the cornea must be monitored and regulated by mechanisms as yet undetermined.

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