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THE REGULATION OF CORNEAL HYDRATION BY A SALT PUMP REQUIRING THE PRESENCE OF SODIUM AND BICARBONATE IONS

BY STUART HODSON

From the Unit of Electron Microscopy, Welsh National School of Medicine, Heath Park, Cardiff, CF4 4XW

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SUMMARY

1. The use of polyacrylamide gel salt bridges enables trans-membrane potentials to be measured to an accuracy of 20 μV over long periods.

2. The technique is applied to measure electrical potentials across corneal endothelia of rabbits.

3. In de-epithelialized corneas which translocate water, a spontaneous potential of 550 μV is found across the endothelium (tissue resistance 20 Ω cm^2).

4. This electrical potential (and water translocation) is reduced to zero when sodium is absent from the Ringer, and by about 80% when bicarbonate ions are absent. Removal of chloride has no such effect.

5. Under a variety of conditions, the potential correlates with the observed translocation of fluid across corneal endothelium. The translocated fluid is shown to be isotonic with sodium in the Ringer and therefore the potential correlates with 'active' sodium transport.

6. The potential and water translocation are abolished in the presence of ouabain at concentrations greater than 10^{-5} M.

7. The potential (lens-side negative) is of the wrong polarity to explain the net sodium transport (into the lens-side) by a sodium ion 'pump'.

8. The current does not equal the net sodium flux under short circuit conditions. They differ in magnitude and polarity.

9. A model is proposed where the endothelium 'pumps' salt out of the corneal stroma into the aqueous humour.

10. Flux equations are derived for a condition where the membrane (corneal endothelium) separates an ion exchanger (corneal stroma) from free solution (aqueous humour), where the usual relationship for free-free solutions $\Delta\pi = c_s\Delta\mu_s$ does not apply.

11. The model is of use only when the stroma is well stirred. It may be used in whole corneas retaining their epithelium but it may not be used in de-epithelialized corneas.

12. The model predicts that the presence of an 'active' salt flux out across the endothelium would create passive water and salt fluxes. The passive water flux would also travel out of the stroma across the endothelium; the passive salt flux would travel, in the opposite direction, into the stroma across the endothelium.

13. The kinetics of the passive water efflux, as a swollen cornea reverts to physiological hydration (the temperature reversal phenomenon) are predicted extremely well if the 'active' salt flux is chosen at $3{\cdot}3 \times 10^{-7}$ m-mole.cm^{-2} sec^{-1}.

14. The value of the active salt flux which cannot be measured directly is extrapolated to be somewhat greater than $2{\cdot}8 \times 10^{-7}$ m-moles.cm^{-2} sec^{-1}; in good agreement with that required by the model to explain the temperature reversal phenomenon.

15. The model is further used to calculate the salt concentration difference across the endothelium (which drives salt passively into the stroma) at various stromal hydrations.

16. When an appropriate salt concentration is applied across the endothelium of de-epithelialized cornea, it generates a potential of the same polarity and similar magnitude to that found across the endothelium of equilibrated whole cornea. The endothelium acts like a cation exchange membrane.

17. Additionally the calculated salt concentration difference across the endothelium correlates well with the measured transendothelial potentials in whole cornea as the corneal hydration varies.

18. It is concluded that the model of an endothelial neutral salt 'pump' regulating corneal hydration is self consistent. The spontaneous potential found across the endothelium could be caused by the consequential passive flux of salt in the opposite direction.

INTRODUCTION

The transparency of the cornea depends critically upon its hydration (Maurice, 1957). The hydration of the cornea results from an equilibrium between two opposing forces: its passive tendency to swell (see Hodson, 1971*a*) is countered by an energy requiring process which 'dehydrates' the cornea. The equilibrium is stable. If a cornea is made thinner by a perturbation force (such as surface evaporation) it reverts to physiological thickness when the perturbation is removed. If a cornea is swollen (for example, by refrigeration) it also reverts to physiological thickness on re-warming to blood temperatures (the well known 'temperature reversal phenomenon'). By experiments on the 'temperature reversal phenomenon', it has been shown that the endothelium is responsible for regulating the stromal

hydration and whilst the epithelium acts primarily as a high resistance to ion and water fluxes it pays no active role (Mishima & Kudo, 1967; Riley, 1971; Dikstein & Maurice, 1972). The endothelial 'pump' requires the presence of sodium and bicarbonate ions in the bathing medium (Hodson, 1971*b*). If the epithelium is removed from the cornea, the stroma swells to a greater thickness and the endothelial 'pump' causes a net translocation of fluid across itself from the stromal to the free side (Maurice, 1972).

By measuring the electrical properties across the endothelium, it is hoped in this paper to identify what ion, salt, water is actively pumped across the endothelium.

METHODS

Over 300 corneas from 2 to 12 months old Dutch and Albino rabbits were studied *in vitro*. Animals were killed by air embolism, I.V. injections of sodium pentobarbitone or neck breaking. The method of killing had no effect on the results.

Incubation media. Normally, the salt solution contained: NaCl, 110 mM; $NaHCO_3$, 39 mM; KCl, 4 mM; K_2HPO_4, 1 mM; $MgSO_4$, 0·8 mM; $CaCl_2$, 0·6 mM; glucose, 5 mM; reduced glutathione, 0·5 mM. Phosphate, sulphate, calcium or magnesium free solutions were made by omitting the appropriate salts. Chloride free (nitrate) or chloride free (sulphate) were made by substituting the sodium, potassium and calcium salts. Otherwise, the chloride could be substituted by bicarbonate and vice versa, in which case the solution was buffered by 12 mM Tris maleate. Sodium-free solutions were achieved by substitution with choline salts. For radioactive tracer work, solutions included up to 2 $\mu c.ml.^{-1}$ $^{24}Na^+$. All solutions were bubbled through with a gas mixture of 88% N_2, 7% O_2 and 5% CO_2.

Electrical measurements

The technique of measuring transendothelial potentials is more comprehensible if the final results are briefly quoted. On de-epithelialized corneal preparations incubated at 35° C, transendothelial potential is about 540 μV and electrical resistance about 20 $\Omega.cm^2$. These results become more apparent as the best chamber design was evolving. Other difficulties arose. A hydrostatic pressure should be maintained across the cornea whilst it is mounted or the endothelial potential and resistance may irreversibly drop. Additionally, the aqueous solution near the endothelial membrane must be stirred. Only the final technique which accommodated these experimental difficulties is described here.

The incubation chamber. Fig. 1 shows the chamber tissue assembly in which most electrical measurements were taken.

A, B, C and D include salt bridges made of polyacrylamide gel connecting to external reservoirs of 3 M-KCl aqueous solution in which four calomel electrodes (not shown in Fig. 1) were immersed. Normally, reservoirs B and C contain the calomel electrodes which connect across a Vibron electrometer Model 33B-2 whose output is monitored on a pen recorder. Calomel electrodes in A and D are connected through a switch to a variable resistor, a microammeter and a battery. The electrometer and ammeter were calibrated against a Mallory battery and standard resistors to an accuracy of 0·2%.

Fluid inlets X and Y (Fig. 1) were connected to two independently variable fluid

injectors and they provided the stirring at the tissue surfaces. M, N, O and P (Fig. 1) are fluid outlets for filling and emptying chambers. Normally, the tubing on M and O were clamped and the outlet heights of N and P were adjusted to maintain a hydrostatic pressure of about 2×10^4 dyn.cm^{-2} across the tissue, endothelial side positive.

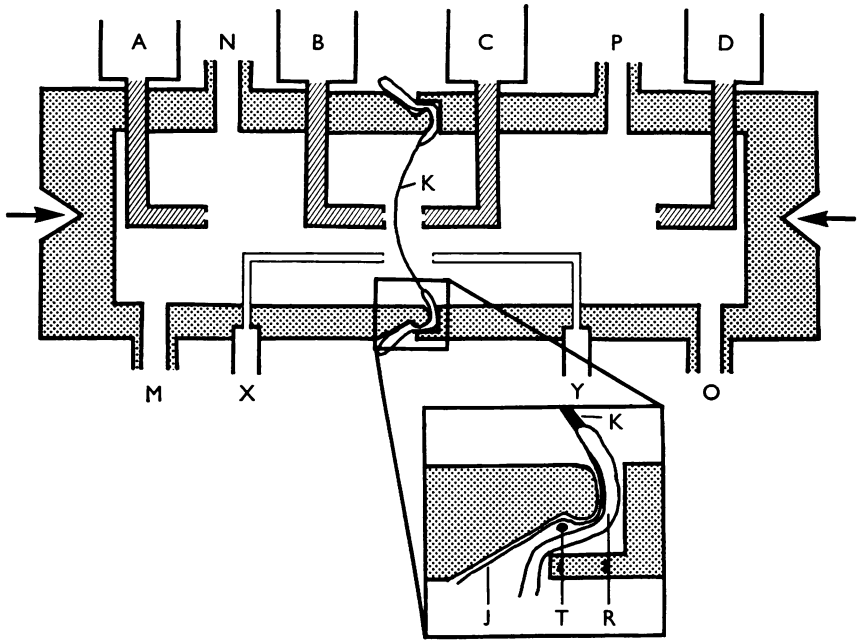

Fig. 1. The chamber in which most electrical measurements of corneal endothelium were taken. A, B, C and D are identical salt bridges filled with polyacrylamide gel. Calomel electrodes (not shown) sit in the top reservoirs in 3 M-KCl. Normally electrodes B and C connect across an electrometer, and electrodes A and D can be switched into a current regulated circuit. N and M (as P and O) are inlet tubes for filling or changing the aqueous medium. Normally, O and M are clamped and the height difference of the open ends of P and N determine the hydrostatic pressure across the cornea (K). X and Y are narrow inlets which, when connected to variable injector sources, allow the solution to be stirred near the tissue. The arrows (each side) show where the chambers are clamped together. The inset shows detail of how the chambers clamp the tissue. K is the cornea, R is the sclera, J is the conjunctiva and T shows the suture which holds the tissue during the mounting procedure.

The two halves of the chamber were clamped together externally (arrows, Fig. 1) by a screw thread calibrated so that the tips of the salt bridges B and C were separated by 3 mm reproducible to 0·05 mm. The assembly was warmed by immersion almost to the tops of the reservoirs A, B, C and D, in a narrow paraffin oil bath which sat in an adjustable water-bath. Tissue temperature was recorded by a varnished thermocouple fixed in the chamber (not shown in Fig. 1).

Tissue mounting procedure. Eyes were dissected with their lids and conjunctival

after Dikstein & Maurice (1972). In most preparations, the epithelium was brushed off the cornea with rotating bristles. The tear-side chamber (A, B; Fig. 1) was clamped vertically and the eye was everted through the lids and seated with the cornea over the tear-side chamber. The lids were pulled downwards to take up the slack in the conjunctival membrane which was sutured near the mouth of the chamber (inset; Fig. 1). Excess lids, conjunctiva and extraocular muscle were trimmed off the eye. Incubation fluid filled the tear-side chamber, outlet N was clamped and outlet M lowered about 15 cm below the corneal surface to provide a suction pressure. It was then possible to open the eyeball and drop the intraocular pressure without wrinkling the cornea. The back of the eye was removed (Dikstein & Maurice, 1972; but see Hodson, 1971*b*) until the preparation consisted of only the cornea and a scleral flap. The flap was everted over the mouth of the tear-side chamber and the preparation rinsed in incubation medium. The aqueous-side chamber (C, D; Fig. 1) was clamped to the preparation crushing the scleral flap (inset; Fig. 1). Incubation fluid was introduced into the aqueous-side chamber, tube P was clamped and outlet O was held at a fixed height, usually 20 cm above outlet M. The whole assembly was immersed in the paraffin oil bath, described above, and outlets X and Y were connected to the injector sources. Finally, the calomel electrodes were inserted into the reservoirs A, B, C and D (Fig. 1) and the tissue temperature thermocouple had its reference junction immersed in melting ice. Its output fed into a galvanometer.

Potential difference. The greatest difficulty in measuring potential differences of about 0·5 mV is determining true zero. Instrument drift of about 200 μV.hr^{-1} precluded the use of a pre-set zero and the low specific resistance of the tissue precluded introducing a negligible resistance short.

The eventual solution was simple. To short the electrodes either electrode B was placed in salt bridge D or electrode C in salt bridge A (Fig. 1).

It can easily be shown that

$$V_{BC} - V_{BA} = V_t + ({}_AV_d - {}_CV_d) \quad (1)$$

and

$$V_{BC} - V_{DC} = V_t + ({}_DV_d - {}_BV_d), \quad (2)$$

where V_{BC} is the potential difference between the two electrodes when they are in salt bridges B and C, etc., V_t is the true potential difference across the tissue and ${}_AV_d$ is the summated liquid junction potential in salt bridge A, etc. The difference between the true potential across the tissue and the difference recorded as one electrode is switched between salt bridges is therefore the imbalance of diffusion potentials in salt bridge pairs A and C (eqn. (1)) or B and D (eqn. (2)). The remaining task was to match accurately the diffusion potentials in the salt bridges. All salt bridges were, therefore, machined to be as similar as possible. As a matrix for the salt bridges, agar and starch gels under a wide variety of conditions are unsuitable. Polyacrylamide under certain conditions is good. The composition eventually chosen is given here.

Solution A: KCl, 3 M; Cyanogum 41 (B.D.H. Chemicals, Ltd., Poole, Dorset), 30 g/100 ml.

Solution B: KCl, 0·75 M; NNN'N'-tetramethyl-1, 2-diaminoethane, 15% (v/v).

Solution C: ammonium persulphate, 13·5 mM.

20 ml. solution A was mixed with 20 ml. solution B. 40 ml. solution C was then added, rapidly mixed and poured into the Perspex chamber. The mixture gels within one minute but takes at least one hour to cure. For present purposes, there is no need to avoid atmospheric oxygen during curing. Extraneous gel was removed from the chamber and its surrounds and the salt bridges were stored for at least one day in 3 M-KCl top surface and 0·15 M-NaCl bottom surface. Salt bridges were tested

before and after each experiment by clamping dialysis membrane between the chambers and switching electrodes when from eq. (1),

$$V_{BC} - V_{BA} = {}_{A}V_{d} - {}_{C}V_{d}, \text{ etc.}$$

In no case did $({}_{A}V_{d} - {}_{C}V_{d})$ or $({}_{D}V_{d} - {}_{B}V_{d})$ exceed 20 μV in this model system. To get such accurate pairing the following precautions must be taken with electrodes. First, the temperatures in the 3 M-KCl reservoirs above the salt bridges must be identical. In practice, this means that the paraffin oil bath must be well stirred and cover at least 1 cm. of the reservoir. Secondly, the calomel electrodes should be shorted together in all non-experimental periods and preferably should be shorted together for several weeks before experimentation.

The polyacrylamide gel salt bridges were easy to make and trouble free in the first few weeks of experimentation. With this system it is possible to measure 0·5 mV to better than 4%.

Resistance measurements. Tissue resistance was so low that the resistance of the fluid between salt bridge tips B and C (Fig. 1) was not negligible. After each experiment, the chambers were broken open, the endothelial membrane was removed from the cornea by lightly abrading the surface with a squirrel hair brush and the tissue was reclamped to within 0·05 mm of its original position (see above). Series resistance was then measured and subtracted from the total resistance.

The preparation between the clamped chambers always included a conjunctival-scleral ring and the cornea. The chamber diameter was usually 1·3 cm. Other chambers were made in which the diameter was altered between 1·4 and 1·1 cm. Chamber diameter did not affect transendothelial potential or resistance. One chamber was made with a diameter of 3 mm in which it was possible to mount conjunctival-scleral flaps dissected adjacent to the cornea. These flaps developed no spontaneous potential and shunt paths through the conjuctiva-sclera were measured to be negligible in agreement with Oksala & Lehtinen (1960). The area of the curved corneal surface was measured as follows. At the end of each experiment the cornea was partially air-dried and the excess ring of sclera trimmed almost to the limbus. Four radial incisions were made into the cornea when it was compressed between the glass plates of a 35 mm projection slide. The image was projected on to a standard density paper and traced. The tracing was cut out and weighed and, by suitable calibrations, the corneal area could be calculated. Varying the hydration, depth or number of incisions on any one cornea altered the measured area by less than 3%. There was no significant image distortion by the projector.

Solution changing. Introducing a different aqueous medium into the chambers may alter the liquid junction potentials in the salt bridges (eqns. (1) and (2)). The magnitude of this effect was always checked before an experiment by testing across dialysis membrane. In all reported solution changes, the imbalance of liquid junction potentials was negligible except in two important cases. Choline for sodium substitution caused the imbalance to rise up to 70 μV. During any experiment the magnitude of the imbalance did not vary much and so the constant and small correction was applied to the results. Sulphate for chloride substitution caused a bigger imbalance up to 400 μV which was unacceptable. All experiments with sulphate solutions were made on corneas mounted between polyacrylamide salt bridges previously equilibrated in top end 3 M-KCl; bottom end chloride free (sulphate) solution.

The other difficulty in changing solutions is caused by the necessary proximity of salt bridge tip C to the endothelial membrane (Fig. 1). The chambers were refilled sequentially. The chamber to be refilled was held at atmospheric pressure and the transendothelial hydrostatic pressure was maintained by raising (or lowering) the free output of the other chamber. Chambers were always re-filled with fresh solution

at the same temperature (to within 0·5° C) as the original solution. One solution change was sufficient to give a fresh solution purity of better than 99% (tested by isotope dilution). Usually one change was sufficient, but when it was thought necessary, fresh solution was given up to three changes. It took about 3 min to change the solution in each half chamber.

Unidirectional sodium isotope fluxes. De-epithelialized corneas were mounted in the Perspex chamber (Fig. 1) and bathed in normal medium. After the trans-tissue potential had equilibrated (within half an hour of the start) the aqueous medium in one half chamber was replaced by a medium identical except for the inclusion of up to 2 $\mu c.ml.^{-1}$ $^{24}Na^{+}$. The flux through the tissue was determined by emptying and replacing the medium in the other half chamber at intervals of 15 min until the sodium flux was constant. The sodium flux in both directions could be measured sequentially on the same preparation as its electrical characteristics remained constant for the first 5 hr of its incubation. In other de-epithelialized corneas, the sodium fluxes in both directions were measured under short-circuit conditions (Ussing & Zerahn, 1951), correcting for fluid resistance by the method of Clarkson & Toole (1964).

Electrical measurements on whole corneas. Corneas retaining their epithelium were similarly mounted in a chamber slightly modified from that shown in Fig. 1. The modifications were engineered to allow the cornea to be inspected through a long working distance binocular microscope. Hence, the end of the epithelium chamber was made from plain glass, the clamping device was shifted to the side and the epithelial side salt bridges were moved off axis. The binocular microscope was modified so that it could measure corneal thickness as described by Hale & Maurice (1969). To achieve greater accuracy, two alterations were made. (1) A slit aperture was fitted near the field stop of the Ramsden eyepiece. (2) the binocular movement was fitted with a fine thread adjustment. With these additional modifications, corneal thickness could be measured to an accuracy of 8 μm. As tissue resistances were not measured, for these experiments salt bridge tips B and C were separated by 1 cm. All experiments were conducted at 35° C with the cornea bathed in normal medium.

The reason for this series of experiments was as follows. Measurements on de-epithelialized corneal preparations are not equivalent to measurements across isolated endothelium as the preparation additionally consists of swollen stroma. I could not dissect off all the stroma without damaging the endothelium. Another approach to investigating transendothelial potentials is, therefore, to measure whole corneal potentials, and then remove the endothelium with a squirrel hair brush and re-measure trans-tissue potential. The difference in the two readings should indicate transendothelial potential.

In preliminary experiments, it soon became apparent that the transendothelial potential was a function of the state of the stroma. Only the leading experiment, done on eight pairs of eyes, will be described.

The rabbit was killed, its eyes dissected and stored at 4° C for 9 hr. The first cornea was then incubated in the chamber and its total thickness and trans-tissue potential recorded at intervals of about 5 min. The cornea was always swollen and after about a quarter of an hour it started to pump fluid out of the stroma (measured by its changing thickness). When it was established in pumping out fluid, usually after $1\frac{1}{4}$ hr, the chamber was broken open and the endothelium removed from the preparation. The de-endothelialized cornea was then re-mounted in the chamber and the trans-tissue potential recordings were continued for about 10 min. The preparation was then discarded and the second cornea, which had been refrigerated for about 11 hr, was then mounted in the chamber. Shortly it, too, started to pump fluid out from its swollen stroma. This cornea, however, was allowed to undergo to the full, the

temperature reversal phenomenon and the endothelium was brushed off the preparation only when the thickness of the cornea had stabilized to its physiological value.

Specular microscope measurements

Corneas were mounted under a specular microscope (Dikstein & Maurice, 1972). Some corneas were fresh, others were swollen by previous exposure to low temperatures for about one day. Initially they were all perfused with normal medium. Two corneas were exposed to medium in which all the chloride was substituted by bicarbonate and 12 mM Tris maleate. With four corneas, the hydrostatic pressure on the endothelial side of the cornea was varied by altering the height of the perfusion outlet tube between 8 and 50 cm above the cornea. Eight corneas were exposed to bathing solutions in which the bicarbonate:chloride ratios were altered to measure their relative reflexion coefficients after Mishima & Hedbys (1967). Six corneas were exposed to solutions supplemented by (non-penetrating) sucrose in order that the osmotically inactive volume fraction of the stroma could be measured after Mishima & Hedbys (1967).

Net water and sodium fluxes. De-epithelialized corneas were mounted under a specular microscope and the endothelial surface was perfused with normal medium. A standard volume (usually 150 or 200 μl.) of medium was pipetted on the top surface (de-epithelialized stroma) and covered with oil (Maurice, 1972). After about 1 hr the gap between the oil/normal medium interface and the endothelium narrows. Maurice (1972) has shown that this narrowing represents an active translocation of fluid across the endothelium by a process identical to that invoked in the 'temperature reversal phenomenon'. When the gap was decreasing at a linear rate the superficial oil and normal medium were aspirated with the aid of a fine catheter attached to a syringe. Recovery was greater than 98·7 %. A fresh, accurately known volume of normal medium was then titrated onto the de-epithelialized surface of the cornea and covered with oil. The operation was completed within two minutes. The preparation was left for up to 5 hr, during which time observations were made on the total fluid removed from the reservoir, between the oil and cornea, by calibrations described by Maurice (1972). The stromal thickness remained constant. At the end of the experimental period the fluid remaining in the reservoir was aspirated and, using only glassware pre-coated with silicone, its total sodium was measured by atomic absorption. The oil contained no contaminating sodium. By this method the rate of translocation of sodium and water could be measured simultaneously.

RESULTS

Electrical measurements

De-epithelialized corneas

At 35° C, de-epithelialized corneas spontaneously develop an electrical potential of 540 ± 20 μV (S.E. of mean) lens-side negative. The potential was present 20 min after the rabbit was killed, which was the earliest time that readings could be made, and persisted unchanged for about 5 hr when it decayed. In measurements taken from more than 150 de-epithelialized corneas the spontaneous potential was always found in the range of 480–640 μV. If the chambers (Fig. 1) were opened and the endothelium brushed off the preparation gently, with a squirrel hair brush, the transtissue potential immediately dropped to zero and remained there in-

definitely. The endothelial resistance was measured at $20 \pm 3\ \Omega.cm^2$ (S.E. of mean). The resistance remained constant throughout the first 5 hr of incubation and then dropped, slowly at first and then rapidly to immeasurably small values. Both potential and resistance were sensitive to temperature changes (S. Hodson, unpublished) so in all the further investigations reported below, great care was taken to ensure that the tissue temperature remained at $35{\cdot}0 \pm 0{\cdot}5°$ C.

Stirring. Surface stirring could be modified by changing the perfusion rates through inlets X and Y (Fig. 1). In that chamber, the spontaneous potential was at a maximum at perfusion rates greater than $4\ ml.hr^{-1}$. At lower perfusion rates, transendothelial potential decreased, but the resistance remained constant.

Streaming potentials. Because of hydrostatic pressure gradients across the tissue, experiments were made to detect any streaming potentials caused by forced fluid flows across charged membranes. Neither de-epithelialized cornea nor polyacrylamide gel salt bridges exhibited streaming potentials of greater than 20 μV when supporting pressure gradients up to 8×10^4, $dyn.cm^{-2}$. Friedman (1972) has argued that most of the pressure gradient across the cornea is likely to be dropped across the anterior fraction of the stroma (or the epithelium if it is present) but not across the endothelium.

Ouabain. Exposure to ouabain did not affect endothelial resistance, but at concentrations greater than 10^{-8} M it reduced the electrical potential (Fig. 2). The range of ouabain concentrations which reduce the potential is strikingly similar to that which causes the cornea to swell (Trenberth & Mishima, 1968). Any dose which decreases transendothelial potential cause whole corneas to swell.

Variable sodium solutions. When choline is completely substituted for sodium in the bathing medium, the transendothelial potential drops to zero over a period of about 10 min. If the sodium is maintained at a concentration of 40 mM, the transendothelial potential remains nearly constant except that it is apparently reduced instantaneously by about 30–40 μV. At intermediate concentrations, the potential is reduced (Fig. 3). The potential drop is completely reversible except when sodium is substituted below 10 mM, when it is only partially reversible. On re-substitution back from sodium-free solutions, the potential reverts to only about 60 % of its initial value. Endothelial resistance is reduced by about 10 % in sodium-free solutions.

Variable bicarbonate solutions. At concentrations higher than 12 mM bicarbonate, the transendothelial potential and resistance remained approximately constant. At lower concentrations, the resistance remained constant, but the potential progressively decreased until, in bicarbonate-

free solutions, it held at about 130 μV (Fig. 4). The potential changes were completely reversible.

Chloride-free solutions. When chloride was substituted completely by bicarbonate, the transendothelial potential and resistance remained con-

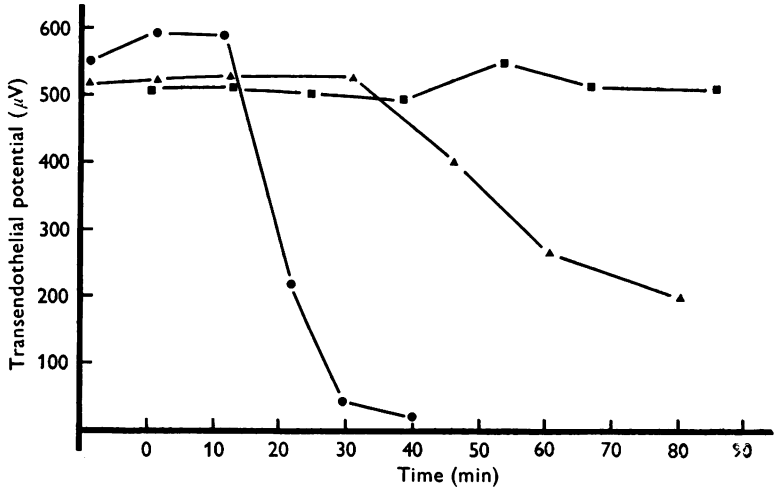

Fig. 2. The effect of ouabain in normal medium on endothelial potential at 35° C. Typical results of three corneas exposed to different concentrations are shown. Ouabain applied at time zero. ● 10^{-6} M ouabain; ▲ 10^{-7} M ouabain; ■ 10^{-8} M ouabain.

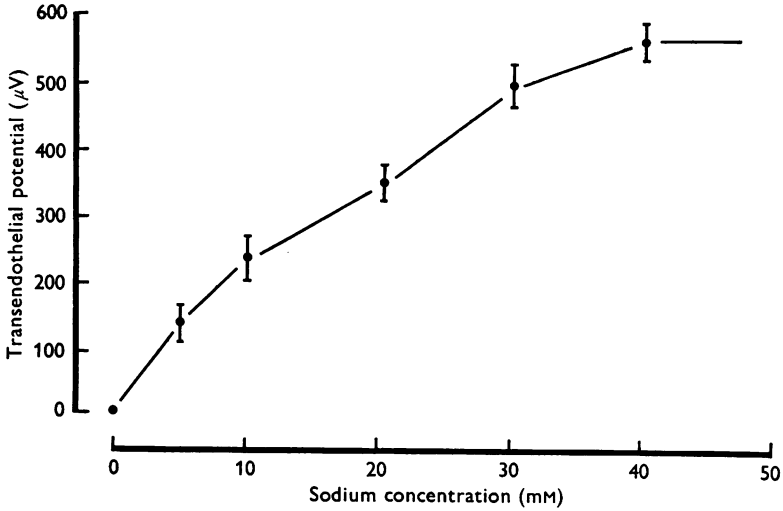

Fig. 3. The effect of substituting choline for sodium in the bathing medium on the transendothelial potential (stromal side positive). The endothelial resistance remains nearly constant.

stant at 550 μV and 20 $\Omega.cm^2$. When chloride was substituted by sulphate, the potential rose to about 700 μV and the resistance to about 30 $\Omega.cm^2$. When chloride was substituted by nitrate, the potential dropped to about 250 μV and the resistance to about 10 $\Omega.cm^{-2}$. It seems most likely that

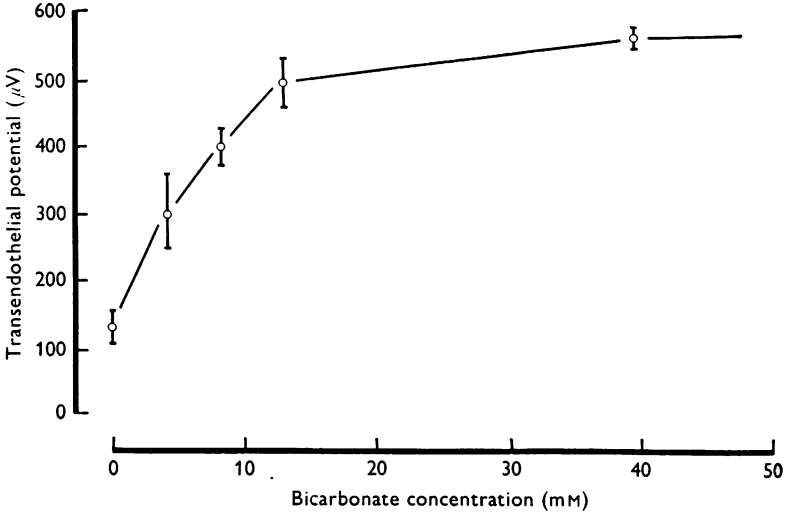

Fig. 4. The effect of substituting chloride for bicarbonate in the bathing medium on the transendothelial potential (stromal side positive). The endothelial resistance remains constant.

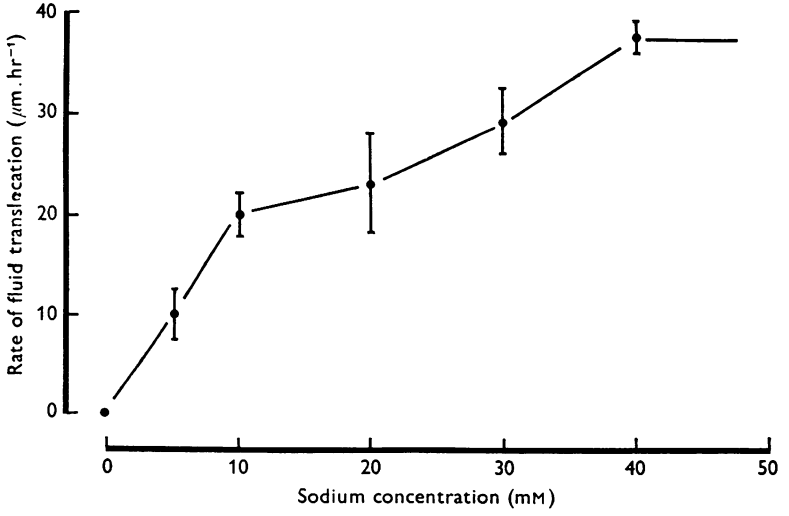

Fig. 5. De-epithelialized corneas translocate fluid into their lens-side. The rate of fluid translocation is a function of the sodium concentration in the bathing medium.

chloride, unlike sodium or bicarbonate, does not affect the electrical activity across the endothelium other than through a passive shunt.

Calcium-free solutions. When calcium was removed from the bathing solution, the endothelial resistance dropped to immeasurably low values within minutes. The decreased resistance is probably caused by the great increase in the size of the intercellular spaces, which occurs in calcium deprivation (Kaye, Mishima, Cole & Kaye, 1968).

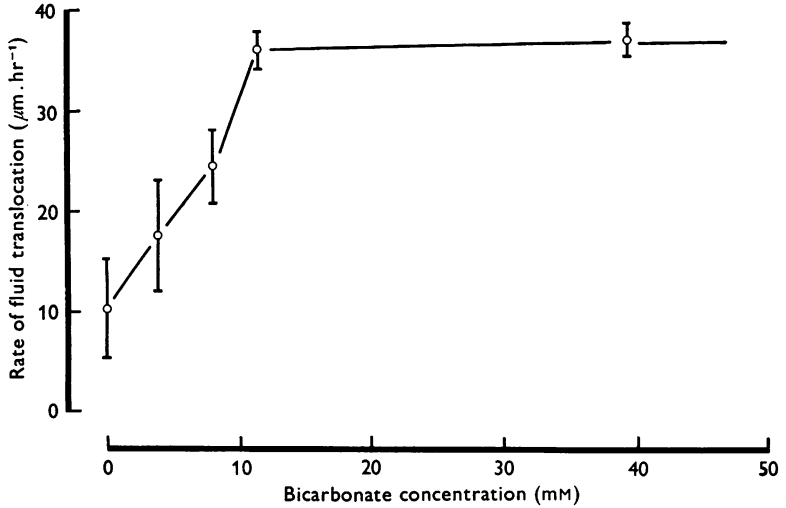

Fig. 6. The rate at which de-epithelialized corneas translocate fluid into their lens-side is a function of the bicarbonate concentration in the bathing medium. The pH is adjusted to be constant.

Magnesium, phosphate- and sulphate-free solutions. Removal of any of these three ions had no detectable effect on potential or resistance in the first few hours. Similarly, their absence has no effect on the 'temperature reversal phenomenon' (Hodson, 1971*b*).

Glutathione deprivation. Two de-epithelialized corneas were deprived of reduced glutathione. In both cases, the potential steadily decreased to about half its normal value after the first 2 hr, after which the resistance which had earlier remained constant, abruptly decreased. Dikstein & Maurice (1972) have shown that the 'temperature reversal phenomenon' of whole cornea is often not fully exhibited in simple ionic media unless reduced glutathione is present.

Partial ion substitution. In these experiments, a small fraction of the ionic medium on one side of the preparation was substituted by approximately equi-osmotic solutions of small neutral molecules. Such substitutions never significantly altered the potential across either whole corneas

or dialysis membranes. They did not, therefore, significantly upset the balanced diffusion potentials in the salt bridges (equations 1 and 2). Such substitutions always cause potential changes across de-epithelialized corneas (Fig. 7). Controls, in which small neutral molecules were added to (and not substituted for) the salt solution, proved that the induced potential difference was not generated by an osmotic pressure gradient (Fig. 7) but by the salt chemical potential difference across the endothelium. The

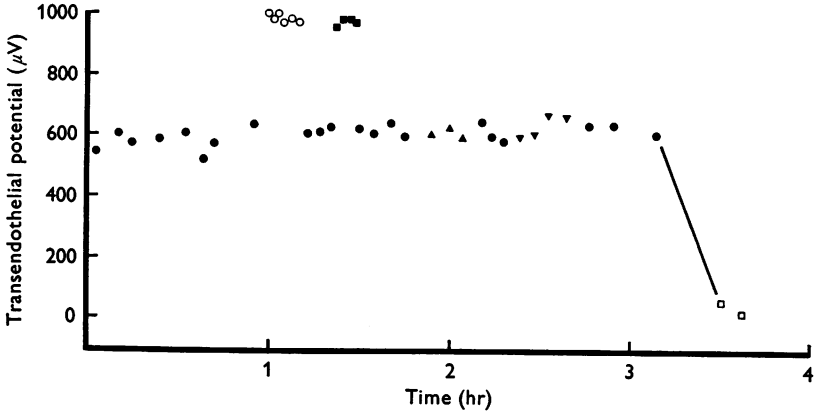

Fig. 7. The transendothelial potential found in de-epithelialized corneas at 35° C (stromal side positive). The endothelial surface was bathed in normal medium; the de-epithelialized stromal surface was variously bathed in: ● Normal medium. ○ Normal medium plus 300 mM sucrose 20:1 v/v. ■ Normal medium plus 300 mM urea 20:1 v/v. ▲ Normal medium in which is dissolved 15 mM sucrose. ▼ Normal medium in which is dissolved 15 mm urea. The open squares show the trans-tissue potential after the endothelium has been removed. Such experiments show corneal endothelium to act as a cation-exchange membrane.

partially substituted medium could be exchanged with the lens-side fluid when an equal magnitude opposite polarity potential difference was observed across the preparation. Because solution changes on this side were technically more difficult without damaging the tissue (see Methods), substitution experiments were mainly done in the tear-side chamber. The change in electrical potential was a linear function of the salt chemical potential difference across the membrane. The polarity is such that the endothelium acts like a cation exchange membrane. When there is a salt concentration difference of 10 mM across the stroma plus endothelium, that part of the gradient which drops across the endothelium generates an electrical potential of 450 μV; positive on the side of depleted salt. The potential is essentially the same whether the salt gradient is in NaCl or $NaCHO_3$. Similar magnitude salt gradients across the stroma alone gene-

rates no detectable electrical potential, even though corneal stroma is a cation exchange resin (Hodson, 1971*a*). Clearly the endothelium is a much 'stronger' cation exchange membrane than the stroma.

TABLE 1. The absence of any relationship between short-circuit current and net sodium flux across corneal endothelium

	Na^+ flux into lens-side*	Na^+ flux into tear-side*	Net Na^+ flux*	Short-circuit current*
Open circuit	$+1{\cdot}0(5) \pm 0{\cdot}2$	$-0{\cdot}4(5) \pm 0{\cdot}1$	$+0{\cdot}6 \pm 0{\cdot}2$	0
Short circuit	$+1{\cdot}2 \pm 0{\cdot}2$	$-0{\cdot}8 \pm 0{\cdot}2$	$+0{\cdot}4 \pm 0{\cdot}2$	$-0{\cdot}97$

* Units: $mC.cm^{-2}.hr^{-1}$.

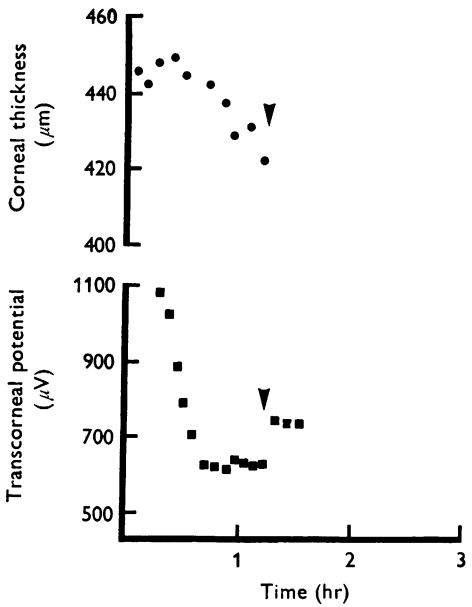

Fig. 8. The potential across whole cornea (previously refrigerated at 4° C for 9 hr) during the temperature reversal phenomenon. In contrast to the other figures in this paper (except Fig. 9) a positive potential indicates lens side positive. Whilst the endothelium is actively assisting in removing water from the stroma (arrow), its potential is small (measured by difference. The arrow indicates where the endothelium was removed from the preparation). Compare with Fig. 9.

Isotopic sodium fluxes. Measurements were taken on eight de-epithelialized corneas. Four were measured in open circuit; four were measured in closed circuit conditions (Ussing & Zerahn, 1951). The results are summarized in Table 1. The net sodium flux in conditions of open circuit is into

the lens-side. When it is recalled that the potential is lower on the lens-side of the endothelium, it would appear, superficially, that the sodium flows downhill. That there is not an electrogenic sodium pump (as defined by the equivalent circuit of Ussing & Zerahn (1951)) operating in the endothelium is more clearly shown in closed circuit conditions. There is no quantitative relationship between the net sodium flux and short-circuit current either in magnitude or polarity.

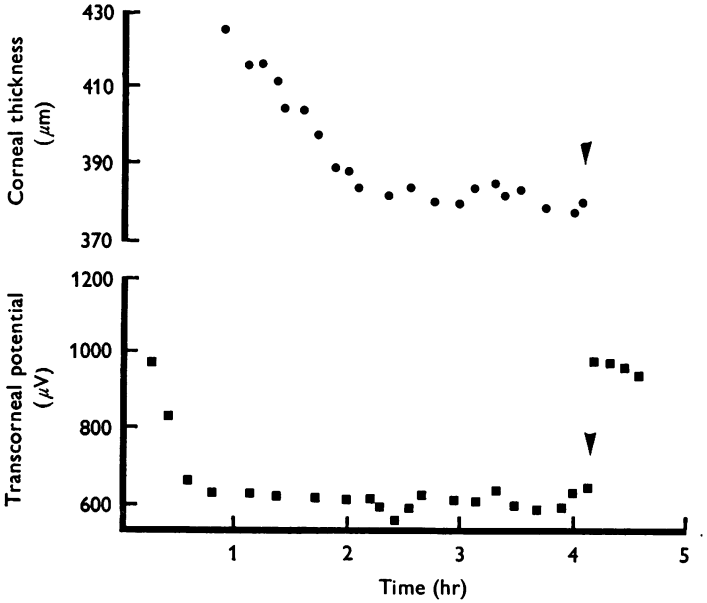

Fig. 9. The potential across whole cornea (lens side positive) (previously refrigerated at 4° C for about 11 hr) during the temperature reversal phenomenon. When the cornea is re-equilibrated and there is no net removal of water from the stroma (arrow) the endothelial potential is large (measured by difference. The arrow indicates where the endothelium was removed from the preparation). Compare with Fig. 8.

Whole corneas

A typical pair of results is shown in Figs. 8 and 9, which show the measurements of whole corneal thickness and trans-tissue potential in corneas previously swollen by refrigeration at 4° C for about 10 hr. The electrical potential is the algebraic sum of that across the epithelium and that across the endothelium. The trans-epithelial potential in fresh corneas is about 25 mV, lens-side positive (Donn, Maurice & Mills, 1959), which is considerably reduced in these refrigerated corneas, but nevertheless remains sufficiently large to dominate the opposite polarity (lens-side negative) transendothelial potential. The cornea whose properties are

shown in Fig. 8 was actively pumping fluid out of corneal stroma when the endothelium was removed (arrow). The transendothelial potential was small (actual readings – 140, 120, 120, 100, 20, 0 and 0 μV; mean $\pm$ S.E. = 70 ± 30 μV). In corneas which have re-equilibrated to physiological thickness (Fig. 9) the transendothelial potential is larger (actual readings – 440, 440, 380, 380, 380, 360, 360, 300 μV; mean $\pm$ S.E. = 380 ± 20 μV).

The transendothelial potential is not invariant. It is a function of stromal hydration.

Optical measurements

De-epithelialized corneas

The rate of fluid translocation across de-epithelialized corneas could be accurately measured with the aid of the specular microscope (Maurice, 1972) in the space of a few minutes. In contrast, the accurate measurement of net sodium fluxes by chemical methods required the pumping characteristics of the endothelium to remain constant for several hours.

Reducing the sodium below 40 mM (Fig. 5) or the bicarbonate below 12 mM (Fig. 6) caused a reduction in the rate of fluid translocation. The dose–response curves for transendothelial potential (Figs. 3 and 4) and fluid translocation (Figs. 5 and 6) were strikingly similar for each ion. When they were plotted, as a function of ionic concentration, against each other the relationship showed a one-to-one linearity with a correlation coefficient of 0·95 (variable bicarbonate) or 0·97 (variable sodium). Each sodium dose–response curve (Figs. 3 and 5) was, however, significantly different from each bicarbonate dose–response curve (Figs. 4 and 6).

The sodium concentration in the translocated fluid was measured on a variety of preparations providing that their rate of fluid translocation remained constant for a few hours. Measurements were taken on de-epithelialized corneas exposed to 10^{-5} M ouabain, lowered bicarbonate concentrations (but not lowered sodium concentrations) and normal medium. The results are plotted in Fig. 10.

The continuous line through the points is that calculated from regression analysis (correlation coefficient, 0·83). The interrupted line is that which would prevail if the translocated fluid were isotonic in sodium. The slight difference which suggests that the translocated fluid is marginally hypertonic in sodium is not statistically significant.

Whole corneas

Measurements made on whole corneas situated under the specular microscope (Dikstein & Maurice, 1972) showed the following features. Substituting all the chloride by bicarbonate did not change the thickness of an equilibrated cornea, nor did it distort the 'temperature reversal phenomenon' (Fig. 11). Altering the hydrostatic pressure on the cornea

between 8 and 50 cm water caused the whole cornea to move but did not affect corneal thickness (Fig. 12). Substituting equimolar sodium chloride for sodium bicarbonate in the bathing medium caused no osmotic shrinking or swelling of the corneal stroma. The extreme case is illustrated in Fig. 11. The reflexion coefficient of sodium bicarbonate is therefore equal to that of

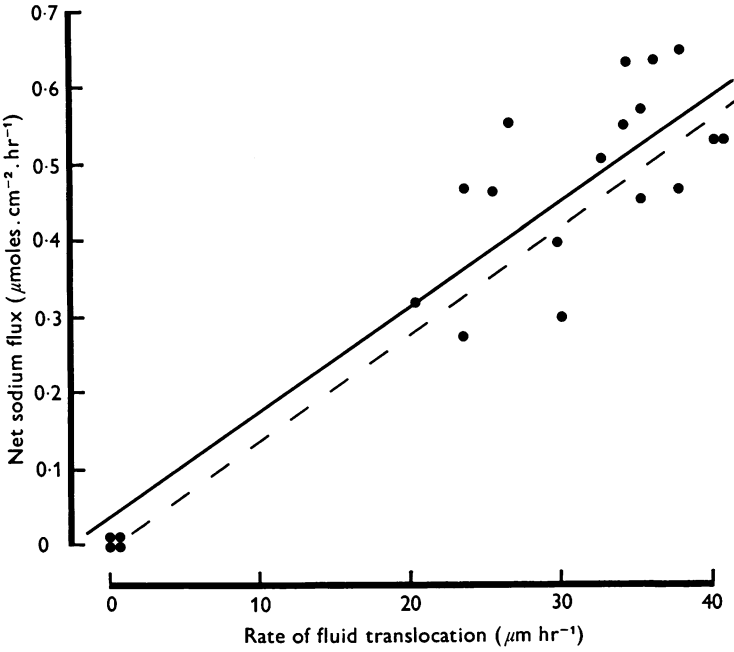

Fig. 10. Simultaneous measurements on the rate of water and sodium translocations into the lens-side of de-epithelialized corneas. The data points at the origin were taken from de-epithelialized corneas exposed to 10^{-5} M ouabain. The continuous line is derived from regression analysis; the interrupted line would pertain if the translocated fluid were isotonic in sodium.

sodium chloride in corneal endothelium, which has been shown to be 0·6 (Mishima & Hedbys, 1967). Changing the osmotic pressure of the bathing medium by adding a non-penetrating solvent caused the cornea to shrink (Fig. 13) by amounts already accurately measured by Mishima & Hedbys (1967). Fig. 13 gives the additional information of what fractional shrinkage is contributed by the stroma alone.

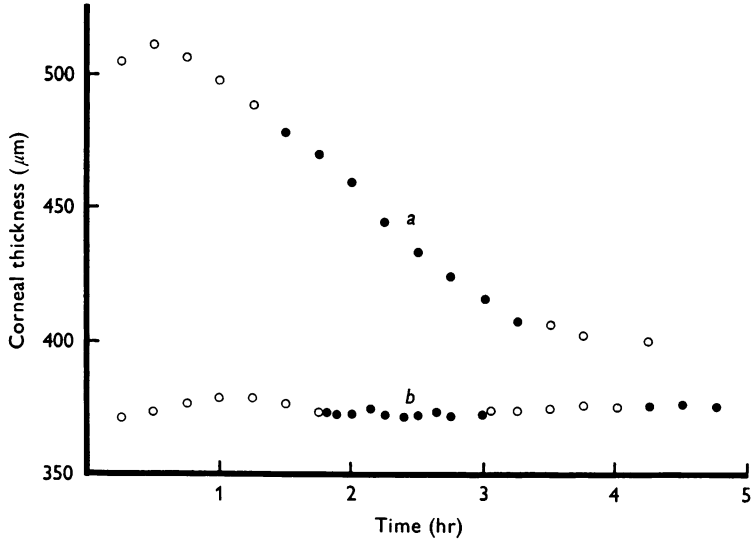

Fig. 11. Substituting bicarbonate and a little Tris for all the chloride in the bathing medium does not effect (*a*) the kinetics of the temperature reversal phenomenon or (*b*) the thickness of an equilibrated cornea. ○ Normal medium; ● Chloride-free medium.

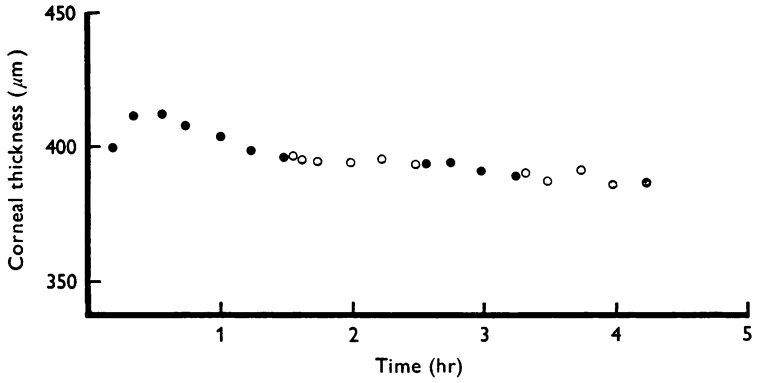

Fig. 12. Showing the absence of effect on corneal thickness of altering the hydrostatic pressure across the whole cornea. ○ Hydrostatic pressure 8 cm water. ● Hydrostatic pressure 50 cm water.

DISCUSSION

If de-epithelialized corneas are incubated in a suitable aqueous medium, a small electrical potential difference exists across the endothelium, lens-side negative. In its dependence on the presence of sodium (Fig. 3 and 5), bicarbonate (Figs. 4 and 6), reduced glutathione and its ouabain sensitivity (Fig. 2), the electrical potential correlates with the endothelial 'pumping'

mechanism which regulates the hydration of the cornea (Mishima & Kudo, 1967; Trenberth & Mishima, 1968; Hodson, 1971*b*; Riley, 1971; Dikstein & Maurice, 1972; Maurice, 1972). The overall direction of water and sodium flow is into the lens-side (Table 1, Figs. 4, 6 and 10; also Maurice, 1972) and as Davson (1949) and Maurice (1951) have pointed out, the lens-side must be the direction into which the active mechanism 'pumps'.

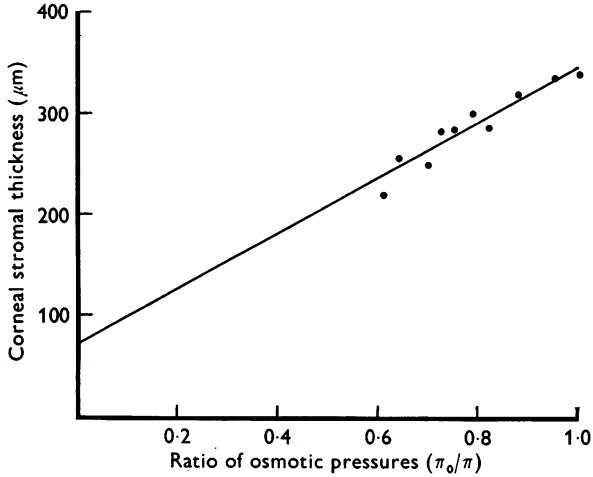

Fig. 13. Corneal stromal thickness as a function of osmotic pressure of the bathing medium. The osmotic pressure of the normal medium (π_0) was increased to π by adding sucrose. Regression analysis shows that as $\pi \rightarrow \infty$, stromal thickness $\rightarrow$ 70 μm.

The intention of this work was to find what species is actively 'pumped', but a simple and direct interpretation of the results is not possible because the spontaneous potential exhibits many surprising features. For instance, in its ouabain sensitivity (Fig. 2) and its sodium dependence (Fig. 3), the mechanism acts like a sodium ion pump. Such it cannot be, because the net sodium flux is into the region of lower electrical potential (and see Table 1). The potential might be explained by an electrogenic anion pump, but the sodium requirement (Figs. 2 and 4) seems to contradict this hypothesis. A third possibility is that the endothelium 'pumps' salt into the lens-side (compare Figs. 3 and 4, and 5 and 6). Initially, it might be thought that the presence of the electrical potential would exclude the interpretation of a neutral salt pump. In the treatment that follows the opposite conclusion is reached: an electrically neutral salt 'pump' across the endothelium would inevitably (but indirectly) generate an electrical potential of the same polarity and approximate magnitude to that measured. The treatment also explains why the transendothelial potential

is a function of stromal hydration (Figs. 8 and 9). Additionally, the treatment predicts the kinetics of the 'temperature reversal phenomenon' extremely well.

The importance of the stroma

All experiments in this paper were made on corneal endothelium (thickness $\simeq 5\ \mu$) plus corneal stroma at various states of hydration (thickness between 320 and 650 μm). Corneal stroma acts as a non-cross linked cation exchange resin (Hodson, 1971*a*). Although the electrical potential is generated entirely across the endothelium, it is well to remember that the presence of a cation-exchange resin on one side of the membrane will significantly alter the passive fluxes across the endothelium. In my hands, it was not possible to separate the endothelium and the stroma without causing effectively irreversible damage to the endothelium (detected by a decrease of the endothelial resistance).

When a membrane separates two (ideal) salt solutions, the osmotic pressure difference ($\Delta\pi$) and the concentration dependent chemical potential difference ($\Delta\mu_s$) across the membrane are related by

$$\Delta\mu_s = \frac{\Delta\pi}{c_s}, \tag{3}$$

where c_s is the average salt concentration in the membrane.

It is shown in the appendix that when the membrane has an ion-exchanger, and not free solution, on one side the relationship is

$$\Delta\mu_s = \frac{\Delta\pi}{c_s} - \frac{RTQ^2}{4c_s^2}, \tag{4}$$

where Q is the ion-exchange capacity of the resin.

The difference between eqns. (3) and (4) is crucial. For instance, eqn. (3) allows for diffusional equilibrium between the two solutions ($\Delta\mu_s = 0$ and $\Delta\pi \neq 0$), but there can never be diffusional equilibrium of both solute and solvent in eqn. (4) without one of two additional conditions.

(*a*) the ion-exchanger swells ($Q \to 0$),

(*b*) the ion-exchanger cross-links and maintains a hydrostatic pressure within the resin – but this is not possible in corneal stroma (Hodson, 1971*a*).

Dynamic equilibrium could be achieved if the membrane 'pumps' an ion, salt or water into the region of lower osmotic pressure (and out of the ion-exchange resin).

The difference between eqns. (3) and (4) is also significant for the technical reason that the flux equations derived by Kedem & Katchalsky (1958) are all derived using eqn. (3). Using eqn. (4), and following the notation of Katchalsky & Curran (1965), it is shown in the appendix that the flux equations are slightly different, and

$$J_v = L_p\left(\Delta P - \frac{RTQ^2}{4c_s}\right) - \sigma Lp\left(\Delta\pi - \frac{RTQ^2}{4c_s}\right), \tag{5}$$

$$J_s = c_s(1-\sigma)J_v + \frac{w}{\sigma}\left(\Delta P - \frac{RTQ^2}{4c_s}\right). \tag{6}$$

The corneal model

The model is partially justified in the appendix and is illustrated in Fig. 14. It can only be applied when the stroma is well stirred, i.e. in whole cornea (Mishima & Hedbys, 1967), but not in de-epithelialized corneas

whose bare stromal surface is exposed to aqueous solutions. Trans-epithelial and translimbal fluxes are ignored. The tissue is bathed in a mono-monovalent salt (see Fig. 11). All fluxes pass across the endothelium and these number three: (i) an active flux of salt into the lens-side; (ii) a passive flux of salt (for purposes of convention shown into the lens-side but in practice always into the stroma); (iii) a passive flow of water.

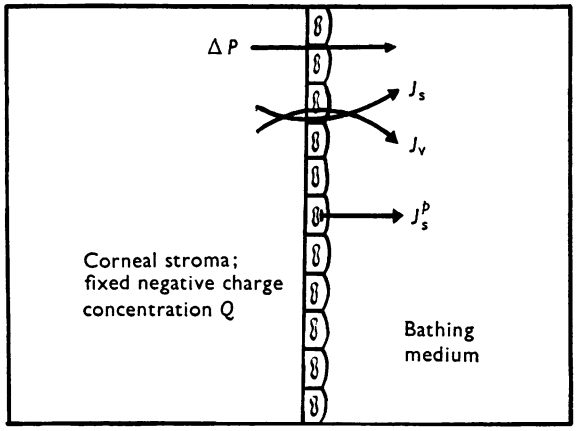

Fig. 14. The model used to predict the properties of the cornea. The endothelium separates two compartments; the bathing medium and the stroma. The stromal compartment may expand or contract as water flows in or out across the endothelium. The stroma contains an invariant quantity of uniformly distributed fixed negative charge whose concentration varies as the stromal volume alters. The endothelium 'pumps' outwards a neutral salt flux J_s^p which remains constant. Associated with this active flux are coupled passive fluxes of salt J_s and water J_v. The passive salt flux J_s is always negative, i.e. passive salt flows only into the stroma. ΔP is the hydrostatic pressure across the endothelium which is shown to be negligible.

The model predicts that if a cornea is swollen (by refrigeration, for example), it will return to its physiological thickness x_p. The change of its thickness x is related to time, t by:

$$-(x-x_s)+(x_p-x_s)\coth^{-1}\frac{(x-x_s)}{(x_p-x_s)}=\frac{J_s^p}{c_s\sigma}t+\alpha \qquad (7)$$

where x_s is the thickness occupied by the solid substances of the stroma (mainly collagen fibres), J_s^p is the flux of salt through the pump, and as before c_s is the salt concentration in the membrane (equals the salt concentration in the bathing medium) and σ is the reflexion coefficient of the salt in the membrane. The boundary conditions at the start of the experiment give the constant α.

Only one constant is unknown; J_s^p the rate at which the salt pump operates. When eqn. (7) is compared to the data of Riley (1971), the fit is seen to be extremely good if J_s^p is chosen at $3{\cdot}4.10^{-7}$ m-mole.cm^{-2}.sec^{-1} (Fig. 15). Fitting eqn. (7) to the data on the 'temperature reversal phenomenon' published by Hodson (1971*b*), Riley (1971) and Dikstein & Maurice (1972) gives $J_s^p = 3{\cdot}3 \pm 0{\cdot}2 \times 10^{-7}$ m-mole.cm^{-2}.sec^{-1} (mean and S.D.).

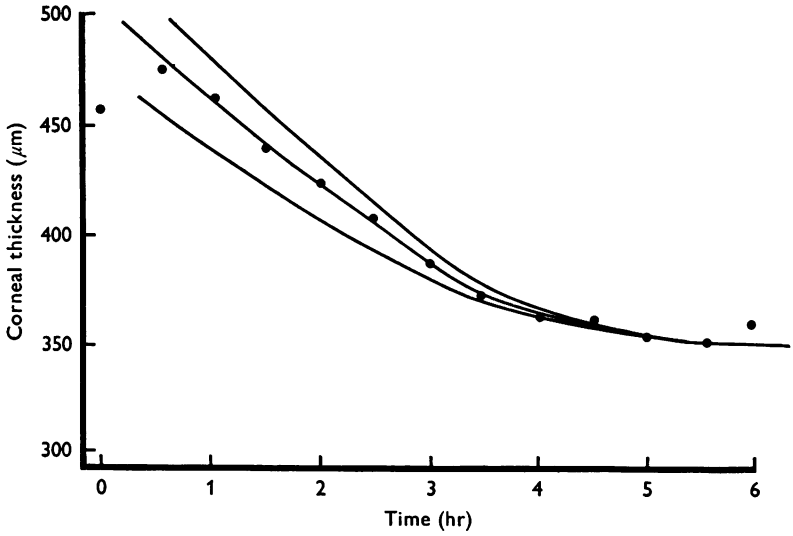

Fig. 15. Fitting the theory presented in this paper to the data of Riley (1971). All three curves are fitted to an equilibrated corneal thickness of 360 μm. The uppermost curve is calculated for an outward directed neutral salt pump in the endothelium, J_s^p, rated at $4{\cdot}0 \times 10^{-7}$ m-mole.cm^{-2} sec^{-1}; for the fitted curve, J_s^p is rated at $3{\cdot}4 \times 10^{-7}$ m-mole.cm^{-2} sec^{-1}; for the lowest curve, J_s^p is rated at $2{\cdot}5.10^{-7}$ m-mole.cm^{-2} sec^{-1}. The first two data points given by Riley represent an initial reversible swelling of the cornea. The magnitude and duration of this early swelling are variable and the cause probably trivial. The final datum point indicates where the equilibrated cornea swells irreversibly. It represents the end of the useful lifetime of the *in vitro* corneal preparation (data points reproduced by permission of Academic Press).

The direct measurement of J_s^p, according to the model, is not easy. Any measured flux would be that of the active flux out of the stroma minus the back leak of the ubiquitous passive flux into the stroma. J_s^p may be estimated from two related observations. First, the fluid actively translocated across de-epithelialized corneas is approximately isotonic in sodium (Fig. 10). Secondly, if the stroma is mostly removed from the preparation the rate of fluid (but not sodium) translocation is measured as high as

65 μm.hr^{-1} (Maurice, 1972). Extrapolating the sodium concentration to these higher rates of fluid translocation shows J_s^p to be somewhat greater than $2{\cdot}8.10^{-7}$ m-mole.cm^{-2}.sec^{-1} – in good agreement with the fitted value of $3{\cdot}3.10^{-7}$ m-mole.cm^{-2}.sec^{-1}.

Possible generation of the electrical potential by the induced passive salt flux. The passive salt flux would, according to the model, always flow into the stroma. Because the endothelium acts as a cation exchange membrane (Fig. 10), this passive salt flux would induce a potential across the endothelium of the same polarity as that measured experimentally (lens-side negative). Predicting the magnitude of the potential presents greater problems. It has been shown that a 10 mM concentration difference of salt across de-epithelialized cornea generates a potential difference of 450 μV (see Fig. 7) but one assumption and one fact must be made explicit. The assumption is that the potential generated by the (small) changes of salt concentrations across the cornea may be algebraically added to that generated indirectly by the salt pump. The fact is that the salt concentration difference across the corneal endothelium is not equal to that across the whole preparation. Part of the gradient is dropped across the swollen stroma. It can easily be shown that at equilibrium, the concentration difference across the endothelium ΔC_e is related to the concentration difference across the preparation ΔC by

$$\frac{\Delta C_e}{\Delta C} = \frac{D_s}{P_e x + D_s}, \tag{8}$$

where D_s is the apparent diffusion coefficient of salt in the stroma (cm^2.sec^{-1}), x_s is the thickness of the swollen stroma (cm), and P_e is the permeability of the corneal endothelium to the salt (cm.sec^{-1}). Equation 8 assumes that the diffusion coefficient of salt is constant throughout the width of the stroma, but this is not the case. In all de-epithelialized corneal preparations which are equilibrated to a constant thickness by active processes in the endothelium, the stroma itself must be considered to maintain electrochemical gradients of ions and chemical gradients of water between its two surfaces. In a non-cross linked ion exchange matrix such as the stroma the collagen fibre separation is a function of the local salt and water activity (Hodson, 1971*a*). As the collagen fibres separate, so the local cation exchange capacity decreases (Hodson & Meenan, 1969) and the ionic diffusion permeabilities alter. Therefore salt would diffuse more slowly in the stroma as it neared the endothelial surface, but the derivation of a more reliable equation than (8) introduces other more impenetrable problems.

Endothelial permeability to sodium bicarbonate, P_e, is $5{\cdot}4 \times 10^{-5}$ cm.sec^{-1} (Appendix). Stromal thickness, x_s, was measured on average at 600 μm. The diffusion coefficient of sodium bicarbonate in the stroma has to be guessed. Maurice (1961) has shown that in equilibrated corneal stroma at physiological thickness the apparent diffusion coefficients of a variety of salts is about half that found in free solution. In the absence of any other data then, this factor will be used in (8) to give

$$\Delta C_e \simeq 0{\cdot}5\ \Delta C. \tag{9}$$

Therefore an approximate salt concentration difference of 5 mM across the endothelium generates a transendothelial potential of 450 μV.

According to eqn. (6) (also see the appendix and Hodson, 1971*a*) the salt concentration difference across corneal endothelium in a normal intact cornea is 1·8 mM. The transendothelial p.d. is 380 μV (Fig. 9). The estimated value from the 'direct'

measurement (165 μV/1·8 mM) and the value calculated from the model (380 μV/1·8 mM) are of the same order of magnitude. That they are not equal is not significant because of the several sources of error pointed out above, but the calculations and experiments do show that an outward directed electrically neutral salt pump across the endothelium would generate an electrical potential of the same order of magnitude and polarity as that found experimentally.

Eqn. (6) also shows that during the 'temperature reversal phenomenon' the passive salt flux inwards across the endothelium would vary as J_v and Q varied. If the transendothelial p.d. is induced by the passive salt flux, then the transendothelial p.d. should also vary during the dehydration process. Figs. 8 and 9 show this clearly to be the case. Table 2 shows how the three fluxes, the calculated passive salt flux, the extrapolated active salt flux and the directly measured water flux alter with the hydration of the cornea. The transendothelial potential correlates positively with only the passive salt flux.

TABLE 2. Correlations between trans endothelial fluxes and electrical potential during the temperature reversal phenomen

	Normal stroma	Deturgescing stroma about 60 μm thicker than normal
Measured potential (V $\times 10^5$; stroma positive)	38	7
Calculated passive salt flux (inwards; m-mole.cm^{-2} sec^{-1} $\times 10^8$)	33	7
Extrapolated active salt flux (outwards; m-mole.cm^{-2} sec^{-1} $\times 10^8$)	33	33
Measured water flux (outwards μm.hr^{-1})	0	35

DISCUSSION

The arguments above strongly suggest that the endothelium 'pumps' salt into the aqueous humour on its lens-side. The question arises as to what salt is pumped, and the answer cannot be given. The apparent certainties of measuring fluxes under short circuit conditions do not apply because the equivalent circuit of Ussing & Zerahn (1951) is not applicable to a salt concentration cell with transference. More likely, the sodium fluxes measured under short-circuit conditions (Table 1) give us some information on the transport number of sodium. Nonetheless, it can be stated that the presence of chloride ions is not required for the operation of the corneal endothelial pump (Figs. 4, 6 and 11) but sodium and bicarbonate ions (Figs. 4, 5, 6 and 7) are essential. This takes us little farther than a confirmation of the studies of Hodson (1971*b*) which showed these two ions to be essential for the 'temperature reversal phenomenon'. The present study does show that the endothelium is unlikely to operate two 'pumps', a sodium 'pump' and a bicarbonate 'pump' which act indepen-

dently but at similar rates, because the single ion substitution experiments never caused an increase of potential (by unmasking one – an electrogenic pump). Any mechanism which would explain the physiological phenomena must be in terms of a salt 'pump' or a tightly coupled cation and anion 'pump pair'. Even the constituent ions of the salt 'pump' (or 'pump pair') cannot be unequivocally stated. It is almost certain that the cation is sodium and possibly that the anion is bicarbonate. However, the bicarbonate substitution data show that even in bicarbonate free solutions there is residual pumping activity (Fig. 6). It is possible that the endogenous supply of CO_2 from the essential aerobic metabolism of corneal endothelium (Riley, 1969; Freeman, 1972) could provide a substrate for a pumping activity (Carlisky & Lew, 1970). It should be added that the anion of the 'pump' could equally well be a carboxylated molecule, and that the (partial) removal of bicarbonate from the bathing medium could influence the 'pump' activity not directly but indirectly by altering the intracellular pH. Such speculation illustrates that a mechanistic interpretation of the data given here on the endothelial 'salt pump' is not at present possible.

Corneal endothelium is a smooth homogeneous membrane consisting of a single layer of non-dividing cells which are all of one type – the simplest cellular membrane that can be envisaged. Yet all the electrical measurements across the endothelium are misleading, for they would seem to reflect the properties of the induced passive flux of salt across the membrane and not the 'pump' proper. The greatest source of confusion, according to the model given in this paper, concerns the intimate relationship between the 'pump' and the induced passive salt flux. Thus, whenever the 'pump' is arrested, the passive salt flux stops also (quite abruptly in de-epithelialized corneas) and therefore the transendothelial potential difference vanishes. It could easily be imagined, because of this inevitable causal chain, that the electrical properties of an electrogenic ion pump were being examined. The main observation which prevented this interpretation was that the p.d. had the 'wrong' polarity, although most of the rest of this paper has been dedicated to providing overwhelming support against an electrogenic ion pump in corneal endothelium.

Consider a membrane made from two cell layers, each having one property of corneal endothelium. The first cell layer pumps salt into the intercellular spaces between the two layers. The second cell layer has the cation exchange properties of the endothelium. Then if the salt cannot diffuse back across the first cell layer it must diffuse across the cation exchange membrane of the second cell layer (provided the cells do not physically separate as in the model of Diamond & Bossert, 1967) and generate a potential whose polarity would be consistent with an electrogenic sodium pump. In such multicellular membranes it would be much easier to confuse a neutral salt pump with an electrogenic sodium pump. In particular, if the transport number

of the cation were large compared to the transport number of the anion, the short-circuit current could easily be similar to the net sodium flux under short-circuit conditions.

This work was initiated at the Institute of Ophthalmology, University of London. I thank Dr David Maurice for his suggesting experiments with polyacrylamide gel salt bridges. Miss Audrey Meenan gave outstanding technical assistance. Mr L. Williams built the specular microscope.

APPENDIX

Consider a cation-exchange resin capacity Q equiv/l. of resin fluid, separated by a membrane from a free solution of mono-monovalent salt CA. For electrical neutrality in the resin and free solution

$$[C]_i = [A]_i + Q, \tag{A 1}$$

$$[C]_o = [A]_o, \tag{A 2}$$

where subscripts i and o refer to the solution inside and outside the stroma respectively.

If $\Delta\pi$ is the osmotic pressure excess inside the stroma, then for ideal solutions according to van't Hoff's equation

$$\Delta\pi = RT([C]_i + [A]_i - [C]_o - [A]_o). \tag{A 3}$$

Let $\Delta\mu_s$ be the chemical potential excess of the salt in the resin over that in the free solution. Remembering that the electrical component of the potential cancels and ignoring the very small pressure dependent term.

$$\Delta\mu_s = RT \ln \frac{[C]_i[A]_i}{[C]_o[A]_o}. \tag{A 4}$$

Provided Q is numerically smaller than $[C]_o$ then to a very good approximation, (A 1), (A 2), (A 3) and (A 4) give

$$[C]_o\Delta\mu_s = \Delta\pi - \frac{RTQ^2}{4[C]_o}. \tag{A 5}$$

Eqn. (A 5) is of great importance in physiological studies. It is quite different from the relationship for free-free solutions separating a membrane, i.e. $[C]_o\Delta\mu_s = \Delta\pi$. As a result it modifies the flux equations given by Kedem & Katchalsky (1958) to slightly different ones (derived below) if there is an ion-exchange resin on one side of the membrane. For these purposes, the contents of a cell would act as an ion exchange resin. The additional term $RTQ^2/4[C]_o$ has a simple physical interpretation. If the separating membrane is removed or, alternatively, if all its 'pumping' activities are neutralized the $RTQ^2/4[C]_o$ is numerically equal to the surface pressure which has to be applied to the resin to prevent it physically swelling. In corneal physiology, this pressure is called the stromal swelling pressure,

SP, and has been widely investigated (e.g. Hedbys & Dohlman, 1963). Eqn. (A 5) may be rewritten

$$[C]_0 \Delta\mu_s = \Delta\pi - SP. \tag{A 6}$$

The passive salt and water fluxes

The following treatment follows as closely as possible that given in Katchalsky & Curran (1965) for non-electrolytes separated by a membrane. All definitions will be found in their treatment.

The dissipation function may be written

$$\Phi = J_s \Delta\mu_s^T + J_w \Delta\mu_w^T. \tag{A 7}$$

Substituting (A 6) and the definitions of $\Delta\mu_s^T$ and $\Delta\mu_w^T$ in (A 7) gives

$$\Phi = (\Delta P - SP)J_v + (\Delta\pi - SP)J_D. \tag{A 8}$$

Incorporating the Onsager reciprocal relations, the phenomenological equations may be written

$$J_v = L_1(\Delta P - SP) + L_{12}(\Delta\pi - SP), \tag{A 9}$$

$$J_D = L_{12}(\Delta P - SP) + L_2(\Delta\pi - SP). \tag{A 10}$$

It can readily be shown that $L_1 = L_p$ and $L_{12} = -\sigma L_p$. Also

$$\frac{\omega(\Delta P - SP)}{\sigma} = (L_2 - \sigma^2 L_p)(\Delta\pi - SP).$$

The phenomenological equations become

$$J_v = L_p(\Delta P - SP) - \sigma L_p(\Delta\pi - SP), \tag{A 11}$$

$$J_s = [C]_0(1-\sigma)J_v + \frac{\omega}{\sigma}(\Delta P - SP). \tag{A 12}$$

The corneal model

The cornea consists of connective tissue, the stroma, bounded on its anterior surface by the epithelium and on its posterior surface by the endothelium. At its circumference, the stroma merges into the opaque and vascularized sclera. Fluxes into and out of the stroma may therefore occur across three routes, but significant fluxes are fewer. For instance, inter and extra-scleral fluxes may be ignored (Maurice, 1951). Similarly the epithelium may be considered a sealed barrier because 'temperature reversal phenomena' show similar kinetics whether the epithelial surface is covered by oil or Ringer solution (Riley, 1971) probably because both sodium permeability (Maurice, 1951) and hydraulic conductivity (Mishima & Hedbys, 1967) are much lower in the epithelium than the endothelium. All significant changes in the stromal fluid are therefore caused by fluxes

across the endothelium and the model may be reduced to a two compartment type. When the stroma swells or shrinks, its area remains constant and only its width alters (Mishima & Hedbys, 1967). Further, changes in its thickness follow that of a simple osmometer (Mishima & Hedbys, 1967) or, it may be considered as a non-adsorbing cation exchanger exhibiting no cross-linkage through its width (Hodson, 1971*a*).

Even with tissue exhibiting such simple properties, the rigorously correct flux equations cannot at present be solved.

Further approximations are listed below.

1. The actively transported flux of salt does not interact with the passive salt flux. In corneal endothelium, this is probably true. The passive flux of salt courses through the intercellular spaces (Maurice, 1961); the active flux would pass through the cells in the monolayer.

2. The active salt flux produces no non-conjugated flow of water.

3. A passive flux of water does not affect the phenomenological coefficients of the endothelium. It does not affect the hydraulic conductivity or the reflexion coefficient of a particular solute (Mishima & Hedbys, 1967), nor does it affect the intercellular spacing (Hodson, 1968).

4. The stroma is well stirred at all times. This approximation has previously been justified in osmotically induced fluid flows across the endothelium, where the time courses of changes are more than ten times faster than in the present treatment (Mishima & Hedbys, 1967).

5. The cornea is immersed in an ideal solution of $NaHCO_3$, molar concentration $[C]_0$ in the bathing medium. Considering only one salt in the Ringer greatly simplifies the algebra. In practice, HCO_3^- may be substituted for Cl^- without affecting either the temperature reversal phenomenon (Fig. 11) or the electrical potential across the endothelium (Fig. 4). The reverse is not true. The ideal solution approximation predicts the passive swelling properties of corneal stroma well (Hodson, 1971*a*).

The adopted convention is that positive fluxes flow across the endothelium from the stroma to the bathing medium. Chemical potential differences are positive when greater in the stroma. Hydrostatic pressures are positive when greater on the stromal side of the endothelial membrane. The model is illustrated in Fig. 14.

The over-all flux equation

At physiological values of Q it can be shown that within 1%

$$[C]_i[A]_i \simeq [C]_o[A]_o = [C]_o^2. \qquad \text{(A 13)}$$

Subscript i and o denote inside and outside the stroma. The approximation is numerically evaluated by solving for $[C]_i$ and $[A]_i$ from eqn. (A 5) (either $\Delta\mu_s = 0$ or $\Delta\pi = 0$) and eqns. (5), (6), (7) and (10) (Hodson, 1971*a*).

As the cornea swells the approximation improves. Consequently, stromal salt concentration in normal or swollen corneas remains nearly constant. The measured sodium concentrations in normal and swollen cornea (Otori, 1967) are consistent with the approximation. Generally

$$[C]_o M_w = M_s, \tag{A 14}$$

where M is the stromal mass of (subscript w) water and (subscript s) salt. Differentiating with respect to time, t

$$[C]_o \frac{dM_w}{dt} = \frac{dM_s}{dt} \tag{A 15}$$

or as the partial molar volume of water is much greater than that of salt

$$[C]_o J_v = J_s^t, \tag{A 16}$$

where J_v is the rate of flow of fluid out of the stroma across unit area of the endothelium ($cm.min^{-1}$) and J_s^t is the net rate of flow of salt out of the stroma across unit area of the endothelium.

From approximation (1) listed above

$$J_s^t = J_s + J_s^p, \tag{A 17}$$

where J_v is the passive salt flux and J_s^p is the actively transported salt flux. Also

$$J_v = -\frac{dx}{dt}, \tag{A 18}$$

where x denotes stromal thickness.

The relationship between stromal thickness and its ion exchange capacity

The cation exchange capacity of the stroma, Q, may be related to its hydration H (water weight:dry weight ratio) by (Hodson, 1971*a*)

$$Q_p H_p = QH, \tag{A 19}$$

where subscript p denotes the standard physiological state. The volume of the stroma is partly occupied by water and the remainder by solid substances. The volume occupied by the solid substances is independent of stromal hydration (Hedbys & Mishima, 1966).

Consider the thickness dimension only, then

$$H_p(x - x_s) = H(x_p - x_s), \tag{A 20}$$

where as before x is stromal thickness, x_p is normal physiological stromal thickness and x_s is the residual thickness occupied by the solid matter.

Combining (A 19) and (A 20) to eliminate H

$$Q = Q_p \frac{x_p - x_s}{x - x_s}. \tag{A 21}$$

The temperature reversal phenomenon

Eqns. (A 12), (A 16), (A 17), (A 18) and (A 21) may be combined to give

$$-[C]_o \frac{dx}{dt} = J_s^p + \frac{\omega \Delta P}{\sigma} - \frac{RTQ_p^2}{4(C)_o}\left(\frac{x_p - x_s}{x - x_s}\right)^2 \qquad \text{(A22)}$$

and this equation may be integrated so that

$$-(x - x_s) + (x_p - x_s)\coth^{-1}\left(\frac{x - x_s}{x_p - x_s}\right) = \left(J_s^p + \frac{\omega \Delta P}{\sigma}\right)\frac{t}{[C]_o} + \alpha, \quad \text{(A 23)}$$

where α is determined by the boundary condition at $t = 0$.

Now according to Fig. 12 (and see Friedmen, 1972), the hydrostatic pressure across the endothelium is zero. According to Fig. 13, x_s, the thickness of the stroma occupied by the collagen fibres, is 70 μm. The reflexion coefficient of $NaHCO_3$ in corneal endothelium equals that of NaCl at 0·6 (Mishima & Hedbys, 1967). Only J_s^p is unknown in eqn. (23).

The equilibrated cornea

At equilibrium all the trans-endothelial fluxes balance. $J_v = 0$ and the net salt flux $J_s^T = 0$. According to the model, in a physiologically equilibrated cornea the salt in the stroma and the aqueous humour are at dynamic equilibrium. The passive flux into the stroma is offset by the active flux out of the stroma or, according to eqn. (A 17), $J_s^T = 0$ gives

$$J_s = -J_s^p. \qquad \text{(A 24)}$$

Substituting in eqn. (A 12) when $J_v = 0$ (and recollecting that $\Delta P = 0$)

$$J_s^p = \frac{\omega RTQ^2}{4\sigma[C]_o}. \qquad \text{(A 25)}$$

The equation cannot be used for an independent estimate of J_s^p because ϖ, the endothelial permeability to $NaHCO_3$ is another immeasurable quantity. Eqn. (A 24) predicts ϖRT to be $4{\cdot}9 \times 10^{-5}$ cm.sec^{-1} Maurice (1951) measured the sodium permeability at $2{\cdot}1 \times 10^{-5}$ cm.sec^{-1}. The permeabilities are of the same order of magnitude.

Equilibrium does not imply that the osmotic pressure difference across the endothelium is zero. Eqn. (A 11) shows that when $J_v = 0$ (remember $\Delta P = 0$)

$$\Delta\pi = -SP\left(\frac{1-\sigma}{\sigma}\right). \qquad \text{(A 26)}$$

In vivo, if a cannula is implanted into a cornea it should demonstrate a suction pressure of about 40 mmHg (Hedbys, Mishima & Maurice, 1963).

This suction pressure should not be confused with either of the properties of an isolated cornea with a 'non-pumping' endothelium, variously called the swelling pressure *SP* (Hedbys & Dohlman, 1963) and the imbibition pressure *IP* (Hedbys, Mishima & Maurice, 1963).

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