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THE LOCATION OF THE FLUID PUMP IN THE CORNEA

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SUMMARY

1. Fluid transport across rabbit corneal tissue has been investigated by observing the movement of fluid interfaces under the microscope, or by mounting the tissue between two chambers and observing the displacement of menisci within capillary tubes.

2. In both cases, the endothelial layer supported on a thin sheet of connective tissue is capable of pumping fluid in a direction out of the cornea, against a head of pressure. The volume of fluid moved may amount to twelve times the thickness of the endothelial cells in an hour.

3. This active fluid movement accounts for the prevention of swelling of the normal corneal stroma. The hypothesis that corneal hydration is regulated by the sodium pump in the epithelial layer is not supported by these experiments.

INTRODUCTION

Davson (1955) and Harris & Nordquist (1955) showed that the cornea of the enucleated rabbit's eye would swell if the eye was refrigerated, but that the tissue would return to its original thickness on incubation. In both studies it was found that the reversal was dependent upon metabolic energy, and the fact that it operates against the swelling pressure of the corneal stroma indicates that it is an energy-requiring process.

Since that time, several workers have confirmed the existence of the temperature reversal effect both in the intact eye as well as in the isolated, perfused cornea. On the other hand, there has been disagreement as to the general mechanism by which fluid is removed from the oedematous tissue on incubation. The only pump that has been identified in the rabbit cornea is an active transport of sodium located at the outer, epithelial surface (Donn, Maurice & Mills, 1959; Green, 1965). This, however, pumps ions from the tear film into the tissue and cannot be the prime mover in a system which moves fluid out of the tissue.

While many workers believe that the fluid pump lies in the inner cellular

layer, the endothelium, Green (1969) considers this layer to be inactive. He has provided indirect evidence to incriminate the epithelial sodium pump through a mechanism which involves changes in the sodium ion concentration in the stroma which in turn modify the swelling properties of the polysaccharide of this connective tissue layer.

The difficulties of demonstrating fluid transport in the endothelium have probably stemmed from the unusual sensitivity of this layer to damage. Recent techniques of isolation and perfusion have overcome these difficulties, and the experiments described here are intended to demonstrate the existence of an endothelial fluid pump directly.

METHODS

Corneas were taken from 5–6 lb New Zealand albino rabbits of either sex. The procedure for isolating the rabbit's eyeball complete with conjunctiva and lids, and for excising the cornea while held firm by light suction, was used (Dikstein & Maurice, 1972). In the first two experimental series the cornea was mounted in the perfusion chamber and placed in a water-jacketed enclosure under a microscope used to measure its thickness, as described by the same workers.

In a further set of experiments, the cornea was mounted in a chamber constructed of methacrylate, in which both surfaces could be perfused (Fig. 1). The cornea is held in the tube with a suction of about 15 cm water, and the eyeball is anchored in place by tying the conjunctiva in a groove on the tube with a thread. The lids and conjunctiva may be trimmed away up to the thread. The posterior three-quarters of the sclera is then cut away and the root of the ciliary body is gripped in fine forceps and pulled away in one piece with the iris and lens. The outer mounting block is slid over the tube and the inner block is tightened down on it with four screws so as to clamp the edge of the cornea. The aqueous humour is then washed out of the inner chamber with a few ml. of the perfusion fluid, the chamber is filled with the same fluid, and the inner plug is inserted with its bore hole open so as to avoid abrupt pressure changes. When it has entered sufficiently, the hole is blocked with a screw, and fluid is passed through the chamber and up the outlet tube so as to establish the normal intra-ocular pressure. The suction is then released, the mounting rod withdrawn and the outer plug is inserted in a similar manner after inverting the system. The plugs are smeared with silicone grease (Dow Corning, Stopcock Grease) on their cylindrical surfaces before assembly.

Care is taken at all times to maintain a physiological pressure gradient across the cornea. The chamber is submerged in a constant temperature water-bath when assembled, and the cornea may be observed from the side by cutting away the conjunctival and scleral frill against the face of the inner mounting block with a scalpel.

RESULTS

Three experimental approaches were used, two with the measuring microscope and one with the double chamber.

Reversal without epithelium. The enucleated eye was covered with silicone oil (Dow Corning, 200 dielectric fluid, viscosity grade 20 cs), and the thickness of the stromal layer was measured immediately after enucleation by

focusing in turn on the endothelium and the epithelial-stromal boundary with the measuring microscope. Two alternative plans were then followed: in the first, the eye was refrigerated overnight as for a normal temperature reversal experiment, but in the morning the corneal epithelium was carefully and completely scraped away with a scalpel blade. The cornea was then mounted, covered with silicone oil and incubated under the microscope in the normal manner (Dikstein & Maurice, 1972).

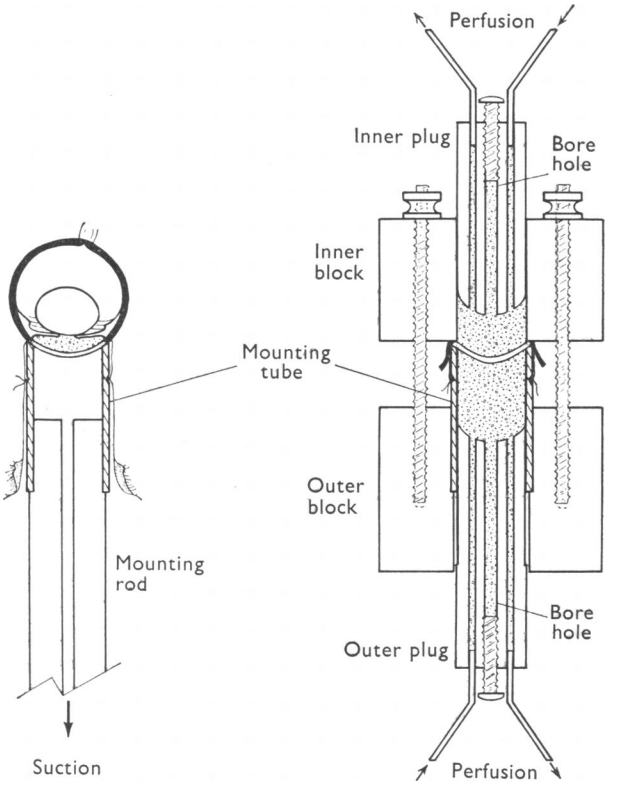

Fig. 1. Method of mounting cornea in perfusion chamber, and chamber assembly.

In the second alternative, the epithelium was scraped off the fresh eye and the cornea was mounted and covered with the perfusion medium. When the stroma had swollen by 100–150 μ, oil was placed on the surface instead of the aqueous solution and the incubation was proceeded with as normally.

In both cases the bare stroma thinned as in a normal temperature reversal experiment with an intact cornea (Fig. 2). The minimum thickness

reached was sometimes equal to that of the fresh stroma, but more often it was not quite as thin.

Loss of overlying fluid. In this experiment either the epithelium was scraped off the cornea of a fresh eye, or a deep cut was made in the cornea with a guarded razor blade, the stroma split over the entire surface area at the bottom of the cut and the anterior layers of tissue excised and discarded. In this way a preparation of the endothelium supported on about

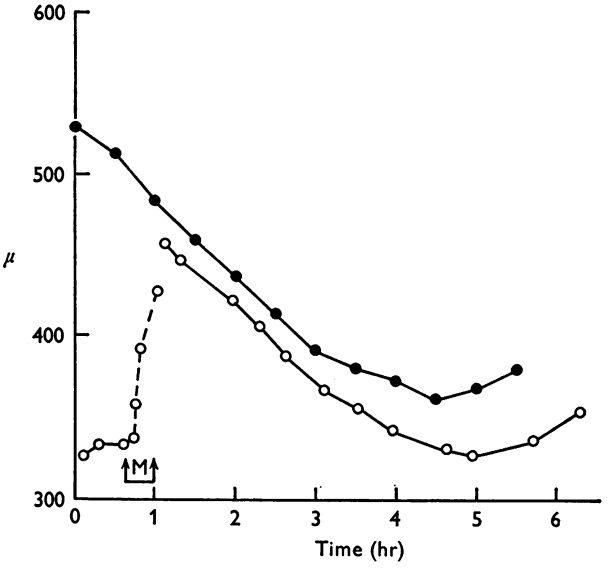

Fig. 2. Thinning of a cornea in the absence of epithelium. Open circles: cornea, without epithelium, swollen by application of perfusion fluid to bare surface of cornea at time M, first arrow. At second arrow perfusion fluid replaced by oil. Filled circles: temperature reversal of intact second eye of pair on next day. Vertical displacement of curves corresponds to thickness of scraped-off epithelial layer, 40μ.

one-quarter of the stromal thickness could readily be achieved. This preparation was mounted for observation under the microscope in the normal manner, but instead of being covered directly with oil, a layer of perfusion fluid was run over the cornea so as to cover its apex to a depth of a few hundred micra. This layer of fluid was in turn immediately covered with oil to the top of the supporting ring, a depth of 5–10 mm (Fig. 3).

The oil–water interface, the anterior surface of the corneal stroma, and the endothelial layer could all be clearly seen on focusing down through the system. The working distance of the objective, 1·6 mm, ensured that it remained immersed in the oil layer even when focused on the endothelial surface. Fluid movement across this surface was made evident by

a movement of the oil–water interface relative to it. Passing ice-cold water through the water-jacket surrounding the chamber allowed fluid to leak passively through the endothelium and resulted in a slight rise in the level of the oil–water interface (Fig. 4). Increasing the temperature to 37° C caused the interface to drop, showing that water was passing into the

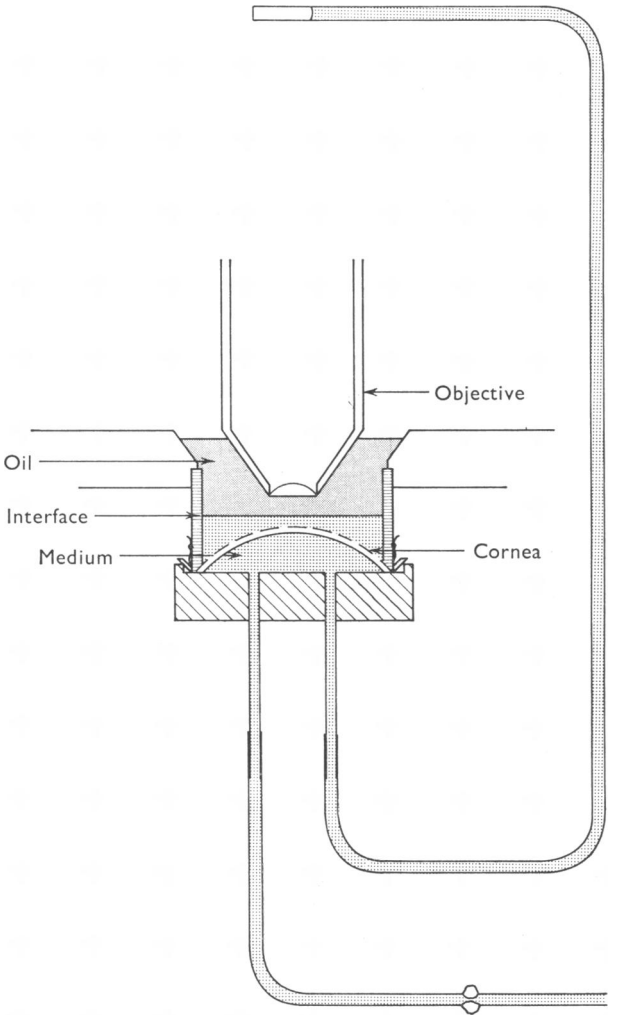

Fig. 3. Method of determining flux of fluid across corneal endothelium. The cornea is mounted after the removal of its epithelium and is just covered with the same medium that bathes its inner surface. A thick covering of silicone oil is then poured over the watery layer. Fluid movement is determined by focusing the microscope in turn on the oil–water interface and on the endothelial surface.

perfusion chamber against the intra-ocular pressure. The endothelial layer itself sometimes underwent a slow drift which was always in the upward direction; this movement occurred principally during incubation and appeared to be more marked in preparations where the stroma was preserved intact. The anterior face of the cornea swelled quickly to a value that corresponded to a constant thickness of the stroma.

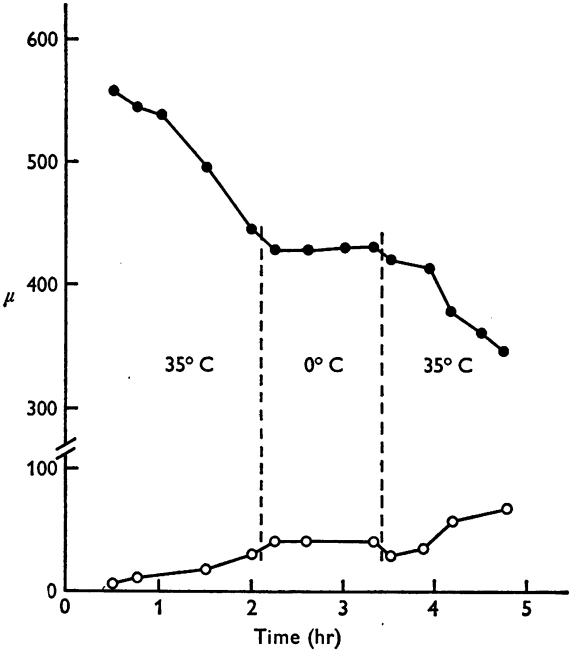

Fig. 4. Movements of the endothelial surface (○) and movements of the oil–water interface relative to the endothelial surface (●). Perfused cornea with epithelium and part of stroma excised. Temperature of water-jacket as indicated.

The only other possible mechanisms whereby the oil-water interface could drop were through leakage or evaporation. The results appeared to be too consistent for the former alternative, nor was evidence of leakage ever noted through the tight tissue seal at the periphery of the cornea. The possibility of evaporation was tested on two occasions by making a control run in which the cornea was replaced by a layer of Parafilm lying flat on the back of the perfusion chamber so as to provide a seal around the edge. This was covered by perfusion fluid and oil to levels similar to those obtaining in the normal experiments. The level of the oil–water interface was observed at 37° C and dropped inappreciably over a period of an hour.

As a further check on the fluid movement across the endothelium, one polyethylene tube leading to the perfusion chamber was clamped with a haemostat after the system was set up, so that the meniscus in the other tube was a few cm from its

tip, which was raised 15–20 cm above the chamber. Movements of this meniscus were found to correspond exactly to the movements of the oil–water interface (Fig. 5).

On the basis of the geometrical areas of the polyethylene tube and perfusion chamber, the movement of the interface appeared to be about twice too great. This disproportion was confirmed by separate calibrations in which rapid fluid movements were induced by changing the height of the outlet tube. It is probable that the circle

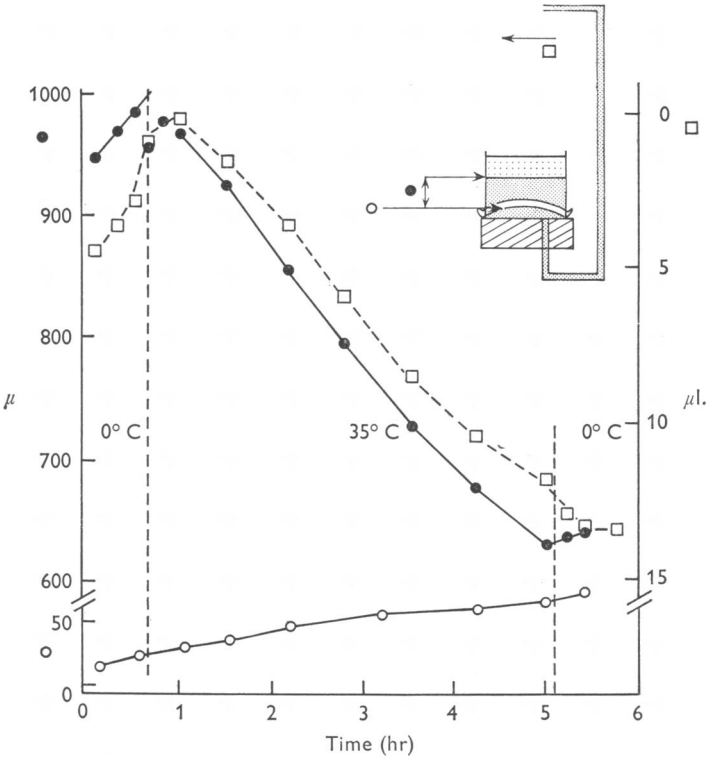

Fig. 5. Movements of the oil–water interface relative to the endothelium, and of the fluid meniscus in the outlet tube in a non-perfused system. Temperature of water-jacket as indicated.

of contact of the oil and water with the plastic ring of the perfusion chamber does not shift its position in accordance with the small changes in level encountered in these experiments. The behaviour of the oil–water interface would then be between that of a membrane clamped at its edges and that of a plunger sliding in a cylinder.

The rate of displacement of the oil–water interface relative to the endothelium when the cornea was incubated at 37° C was measured in eleven experiments. The maximum value measured over at least 90 min had a mean of 108 μ/hr (range 80–130). This corresponds to a fluid flux of about 5·0 μl./hr.cm^2 inward across the endothelial surface.

Double chamber. The epithelium or the anterior corneal layers were removed, as described previously. Measurements of fluid transfer in the double perfusion chamber were made by clamping one tube leading to each of the chambers and observing the movements of the meniscus in the other two tubes. These tubes were placed equally above and below the level of the water-bath at a distance of about 20 cm from each other. Apart from occasional experiments, in which a leakage must be presumed, the movements of the two menisci went hand in hand (Fig. 6). The direction

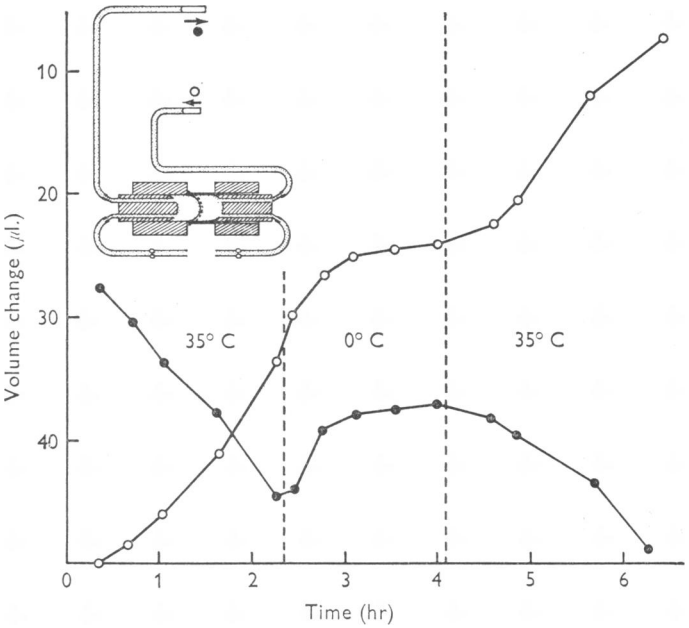

Fig. 6. Transfer of fluid from lower outlet tube ○, to upper outlet tube ●, in double chamber, with closed inlet tubes. Fresh cornea with epithelium and majority of stroma excised; medium gassed with 95 % O_2 5 % CO_2. Temperature of water-bath as indicated.

of movement in the cold and the incubated system corresponded to that found in the previous preparation. Since the fluid was stagnating in the chambers, it was gassed with O_2 rather than air, and the total volume was adjusted to be more than 2 ml. in order to ensure an extended activity of the preparation.

Under these conditions, the movement of the fluid against the pressure gradient could continue over more than 8 hr incubation, with a gradually diminishing velocity. In twelve experiments the maximum surface, flux, measured over at least 90 min, averaged 6·5 μl./hr.cm^2 (range 3·2–12·0).

It may be seen immediately in this preparation that fluid is being transferred from a lower to a higher level. The only circumstance which could achieve this, apart from a fluid pump in the tissue, would be if the remaining collagen lamellae contracted in the perfusion chamber. It should be pointed out that, in many hundreds of preparations observed under the microscope, the cornea invariably expanded under conditions corresponding to those in these experiments, and thus would tend to reduce the effectiveness of the pump. This was tested more directly by photographing the preparation in the water-bath so that the profiles of the corneal surface and of the mounting tube could be compared directly. The accuracy of this procedure was estimated to be about 50 μ, corresponding to a fluid shift of 10 μl. if it occurred over the whole surface. In four experiments, photographs taken with a fixed camera system showed no signs of shrinkage; the movement, if any existed, was slightly in the direction of expansion, as expected. Displacements of fluid of up to 80 μl. from the lower to the upper tube occurred between the photographs.

DISCUSSION

Location of pump. The experiments described above show directly that the endothelium of the rabbit cornea, supported on a layer of the underlying stroma, is capable of pumping fluid across its surface against a head of pressure. The stroma is a layer of connective tissue offering little resistance to the diffusion of low molecular weight solvents and a purely mechanical obstruction to the flow of water. The endothelium, on the other hand, is a continuous cellular layer with fully attached lateral cell membranes, which exhibits a relatively low permeability to salts and the hydraulic flow of water (Maurice, 1969). It is scarcely conceivable that any active process arising in the connective tissue could force fluid across the cell layers rather than across its open anterior surface. The endothelium remains, therefore, as the only possible site of the fluid transport mechanism.

The great activity of this transport mechanism is worthy of notice. Since the thickness of the endothelial cell layer is only about 5 μ and it is capable of displacing a column of fluid at a linear rate of over 60 μ/hr, it appears that in 5 min a cell can transfer its own volume of fluid across its surface.

Control of corneal thickness. Because of its swelling pressure, the corneal stroma would be expected to slowly but continuously absorb aqueous humour across the endothelium (Maurice, 1951, 1969). The cornea does not swell *in vivo* and so an active process is assumed to be responsible for the removal of this fluid. The only pump that has been characterized in the epithelium of the mammalian cornea is in the wrong direction (Donn *et al.*

1959). It is natural therefore to identify this active process with the fluid transport mechanism demonstrated in the endothelium.

Although some details remain to be explained (Maurice, 1969) in the pump-leak hypothesis of corneal thickness regulation outlined above, it may be noted that the average rate of the pump, equivalent to a fluid flux of 60 μ/hr, is similar to the rate of swelling observed by Trenberth & Mishima (1968), 45 μ/hr, when the active transport mechanism was inhibited by ouabain, without affecting the endothelial permeability.

Green's theory. Green (1966, 1968, 1969*a*, *b*, 1970) has suggested a mechanism by which the normal thickness of the cornea is maintained that is entirely different from that proposed above. According to his view, the sodium pump in the epithelium raises the stromal concentration of this ion, the endothelium playing an entirely passive role as a barrier in maintaining this concentration. As a result, the amount of sodium bound by the stromal polysaccharide is increased and the attraction of the polyanion for water decreased, possibly as a result of a conformational change.

The main support for this mechanism is indirect. The evidence that the sodium pump in the epithelium is related to the hydration lies in two series of experiments; in one he found the short circuit current was decreased, and in the other the corneal thickness was increased, as the sodium concentration in the fluid bathing the epithelial surface was lowered. The hypothesis concerning the reciprocal water and sodium binding of the stroma is based on the finding of a drop in the apparent excess of the ion in the stroma as the hydration of the tissue increased, as a result of various forms of experimental interference. In a recent paper (Green, 1969*b*) some more direct evidence is produced, namely that in four cases a swollen cornea thinned when the endothelial layer was removed and covered with oil while the epithelium was covered with Ringer solution, and in another four the cornea swelled when the endothelium was spared and the epithelium removed and the surface covered with oil.

Clearly, there is a direct conflict of evidence between the experiments of Green and those reported here, and it is difficult to do more than claim that the techniques described here are more developed and are less liable to traumatize the cellular layers than those employed by Green.

There is a further conflict of evidence as to the necessity for sodium in the solution bathing the epithelial surface. The author has observed no swelling in an isolated intact fresh cornea bathed on its anterior surface with an isotonic (10·3 g/100 ml.) sucrose solution, and found that swollen corneas give indistinguishable temperature reversal curves irrespective of whether they are bathed on their anterior surface with sucrose or the normal medium containing 143 mM sodium.*

Stronger objections can be raised against the part of Green's theory which requires that the hydration of the stroma is controlled by the sodium concentration within it. The magnitude of the active sodium flux across the epithelium is of the order of $2–5 \times 10^{-7}$ equiv/cm^2.hr (Donn *et al.* 1959; Green, 1965) and the endothelium has a permeability of 0·07 cm/hr for small ions of either sign. It follows that an excess of no more than 7 m-equiv/l. in the sodium ion concentration in the stroma may be expected from this mechanism. There is no evidence that this small increase has any effect upon the properties of the stroma, let alone prevent it absorbing water. On the contrary, the excised tissue is known to swell in salt solutions of concentrations much higher than those encountered in the body, and the swelling pressure is only diminished to about half its value even when soaked in 10% sodium chloride (Fatt, 1968).

Finally, Green's hypothesis seems to provide no mechanism whereby fluid can be excreted from the stromal tissue during a temperature reversal. The only force arising within the stroma itself that could effect this would appear to be a rise in the tissue fluid pressure; such a rise has not been observed (Hedbys, Mishima & Maurice, 1963) and, if present, it would be expected to separate the cellular layers of the cornea from the underlying stromal tissue. It would be necessary, on this basis, to suppose that the temperature reversal mechanism is separate from that which maintains the normal thickness of the cornea.

* *Note added in proof*

Since the preparation of this paper, an article by Riley (1971) has appeared giving a detailed account of experiments in which temperature reversal took place when the epithelium was bathed with sodium-free solutions.

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