J. Physiol. (1974). **241**, *pp.* 647–675
With 20 text-figures
Printed in Great Britain

ROLE OF CATIONS, ANIONS AND CARBONIC ANHYDRASE IN FLUID TRANSPORT ACROSS RABBIT CORNEAL ENDOTHELIUM

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(*Received 28 December 1973*)

SUMMARY

1. A small electrical potential difference ($541 \pm 48\ \mu V$, aqueous side negative) across rabbit corneal endothelium has been recently found. Its dependence on ambient [Na^+], [K^+], [H^+] and metabolic and specific inhibitors was examined.

2. Changes in concentration of the ions above either were known or were presently shown to affect the rate of fluid transport across this preparation (normal value: $5{\cdot}2 \pm 0{\cdot}4\ \mu l./hr.cm^2$). Ionic concentration changes were also found here to influence potential difference in the same way as fluid transport. In the cases tested, the effects on both fluid transport and potential difference were reversible.

3. Fluid transport and potential difference were both decreased or abolished in absence of Na^+, K^+ and HCO_3^-, and when [H^+] was decreased. Fluid transport and potential difference were saturable functions of [HCO_3^-] and half-saturation occurred in both cases at about 13 mM-HCO_3^-. The potential difference was also a saturable function of [Na^+] (half-saturation around 15 mM). There was a pH optimum for potential difference in the range 7·4–7·6. Lower pH values decreases the potential difference and the fluid transport, and a small ($-100\ \mu V$) reversed potential was observed in the range of 5·3–5·5.

4. Total replacement of Cl^- by HCO_3^- or SO_4^{2-} produced no impairment on either fluid transport or potential difference.

5. Carbonic anhydrase inhibitors (ethoxyzolamide 10^{-5} or 10^{-4} M and benzolamide 10^{-3} M) produced a 40–60% decrease in the rate of fluid pumping. In contrast, ethoxyzolamide 10^{-4} M or acetazolamide 10^{-3} M did not produce any change in the potential difference. NaCN and Na iodoacetate (both 2 mM) eliminated the potential difference in 1–1·5 hr while in controls it lasted for 5–6 hr.

6. Ouabain (10^{-5} M) abolished the potential difference in less than 10 sec when added to the aqueous side, which suggests the existence of an electrogenic pump. This extremely fast time transient can be accounted for by the accessibility and simple geometry of the present monocellular layer. Ouabain abolished also the reversed potential difference observed at low pH.

7. The data are interpreted in terms of a scheme similar to that advanced for other epithelia and in which (*a*) H^+ would be pumped into the intercellular spaces, while Na^+ and CO_2 would enter into the cells, and (*b*) Na^+ would be subsequently pumped into the aqueous humour, producing as a result the fluid movement observed. The actual origin of the potential difference is further discussed in terms of two contrasting possibilities: (i) one or more electrogenic pumps, and (ii) a neutral pump which would create a diffusion potential across 'leaky' intercellular junctions.

INTRODUCTION

The maintenance of the relatively dehydrated and therefore transparent condition of the corneal stroma was postulated years ago to be due to active transport processes residing in the corneal limiting epithelial layers (Davson, 1949). This school of thought received important experimental support (Schwartz, Danes & Leinfelder, 1954; Davson, 1955; Harris & Nordquist, 1955; Langham & Taylor, 1956) and a consensus emerged that active transport across the endothelium was likely to be responsible for the transparency of the cornea. This view has been challenged at times by proponents of an active epithelial role in the maintenance of corneal transparency (cf. Green, 1969), but relatively recent findings (Mishima & Kudo, 1967; Trenberth & Mishima, 1968; Maurice, 1972; Dikstein & Maurice, 1972) left no doubt that the endothelial layer transports fluid. The most telling piece of evidence in this connexion was the finding by Maurice (1972) that the endothelium moves fluid across it against a head of pressure. Even after his finding, however, the fact that an electrical potential difference across the endothelium had not been consistently detected (Kikkawa, 1966*a*, *b*; Green, 1967; Hodson, 1971) still gave rise to questions about the true nature of the fluid movements observed. This matter was answered only recently (Fischbarg, 1972*a*, *b*; Barfort & Maurice, 1972; Fischbarg, 1973; Fischbarg & Lim, 1973) when such potential difference was indeed found, even if very small (0·5 – 1·0 mV, aqueous negative). The work presently reported was undertaken in an attempt to learn which ions are involved and which is likely to be their role in the inner workings of this transport mechanism. The results are discussed with reference to similar fluid transporting systems in other epithelia.

METHODS

Dissection and mounting. Albino rabbits weighing 2·5–3 kg were used throughout these studies. Most experiments were performed on female animals, but no difference was noticed if males were used instead. The animals were killed by an overdose of Na pentobarbitone injected into the marginal vein of the ear, and their two eyes were excised. One of the eyes was immediately subject to further dissection and mounting, while the second eye was placed temporarily in a moist, refrigerated (at 7–8° C) atmosphere. The epithelia were scraped off with a razor blade until the bare stroma presented typical 'ground glass' appearance. The isolation of endothelia (supported by the stromal layer) was done with the extremely effective technique developed by D. M. Maurice (Maurice, 1969; Dikstein & Maurice, 1972). After the isolation, the aqueous humour in contact with the endothelium was washed out and replaced by the artificial medium used.

Experimental solutions. The artificial medium had the following composition (in mM): NaCl 110, $KHCO_3$ 3·8, $NaHCO_3$ 39, $MgSO_4$ 0·8, KH_2PO_4 1·0, $CaCl_2$ 1·7, glucose 6·9, adenosine 5·0 and glutathione (GSH) 0·24. Except for the addition of glucose, it was similar to the medium developed by Dikstein & Maurice (1972). In the present case, the concentrations of some components were slightly varied so as to approximate values reported for the aqueous humour from several pooled sources (cf. Kinsey & Reddy, 1964). Stock solutions which had all the same osmolarity (300 m-osmole; cf. Levene, 1958) were prepared and were mixed in the required proportions. An adequate volume of distilled water was added to the mixture, which was subsequently filtered through glass paper. Before each experiment, preweighed amounts of GSH and adenosine were dissolved directly into this solution, and the pH was adjusted to 7·4 with CO_2. When needed, the ionic composition was changed by either varying the proportions of the mixture or by replacing a given salt by sucrose on an osmolal basis.

Measurement of fluid movements. After mounting fresh corneas (usually around 350 μm thick) in the experimental chamber (Fig. 1), they were made to swell up to a thickness of some 500 μm by placing a saline solution in contact with the denuded stroma. In the early experiments the procedure adopted to produce stromal swelling had been the classical one of placing the excised eyes in a moist refrigerated atmosphere for one or two days (under these conditions, the fluid transport is reversibly arrested and the stroma swells). Since no obvious difference in experimental behaviour was apparent when fresh rather than refrigerated corneas were used, the later and more convenient experimental procedure was adopted. After the corneal stroma was made to swell to the desired thickness, the fluid in contact with it was replaced by silicone oil (Dow Corning 200 fluid, viscosity 20 cs at 25° C; cf. Dikstein and Maurice, 1972). The corneal thickness was then measured by focusing alternately with a microscope on the stromal and endothelial interfaces, and noting the excursion of the micrometric dial (MacRobbie & Ussing, 1961; Maurice, 1968, 1969). Under those conditions, thickness changes correspond to fluid movements across the endothelium (Maurice, 1972). The microscope used at first was a Bausch and Lomb model DM metallurgical one, with a water immersion (cf. MacRobbie & Ussing, 1961) lens (Carl Zeiss, 40×). This setup was later duplicated by the addition of a 'specular reflexion' (cf. Maurice, 1968) modified B & L microscope (Oliver Instruments, Sunnyvale, California); no differences were apparent between the measurements done with either setup. The perfusion chambers and warm jacket enclosures were analogous to those described earlier (Dikstein & Maurice, 1972). In later experiments, the perfusion chambers were modified by the addition of a stirring device (Fig. 1) which allowed for fast exchange of perfusing

solutions. A troublesome problem found was that of the gas bubbles which after forming occasionally in the warm (37° C) perfusion chamber could lodge in its uppermost portion and, if not immediately flushed, would destroy upon contact the endothelial cells located in the optical axis. One excellent solution (S. Dikstein, personal

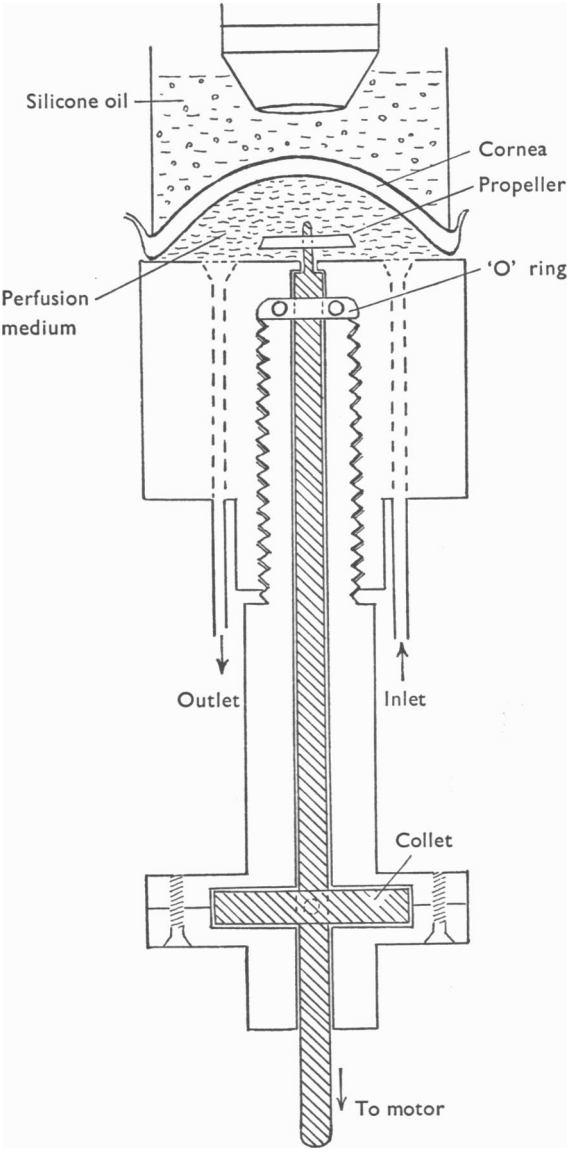

Fig. 1. Diagram showing a vertical section across the experimental setup used to measure fluid transport. An external vice (not shown) helped clamp the cornea between the two halves of the chamber.

communication) consisted of tilting the microscope away from the vertical by about 20° C, so that the bubbles would stay out of the optical path. Other refinements presently introduced consisted of heated (37° C) bubble traps and jackets for the perfusion lines, so as to avoid temperature transitions for the fluid entering the perfusion chamber. All these precautions combined were extremely effective. The endothelial side of the preparation carried always a pressure head of 20 cm H_2O obtained by adjusting the level of the perfusion outlet. The artificial medium used was perfused at a rate of 49 μl./min; when the perfusing solution was changed, the new solution was usually perfused at a faster (250 μl./min) rate for 25 min. Carbonic anhydrase inhibitors (ethoxyzolamide and benzolamide or CL-11,366) originated from the Upjohn Co. and Lederle Laboratories respectively.

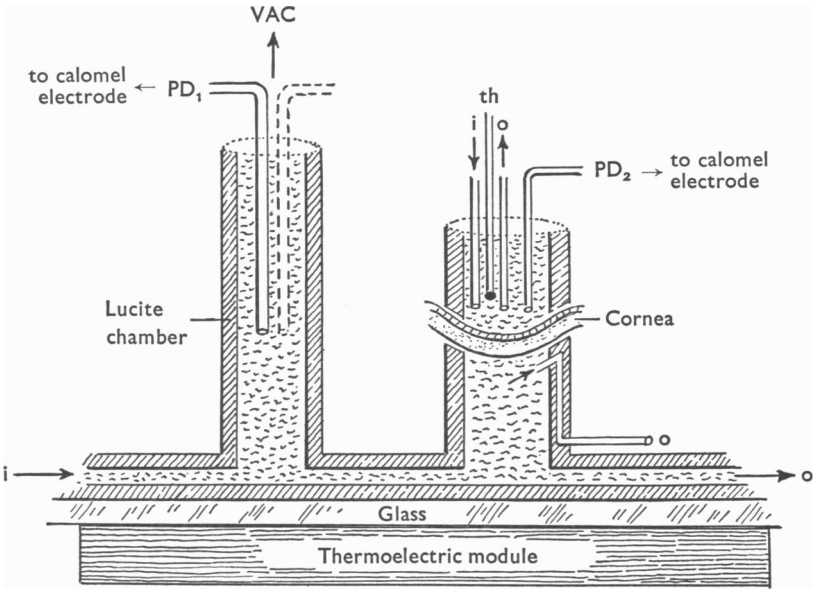

Fig. 2. Schematic vertical section across the setup used to measure electritrical potential differences. $PD_{1,2}$, movable agar-solution or double-junction bridges; i, o, inlets and outlets for perfusion: th, thermistor; VAC, vacuum. A lamp (not shown) heated the chamber from above.

Measurement of potential difference. The measurement of the very small d.c. potential differences required definite precautions to exclude errors due to the bridges. Several types of bridges were tried before a final choice was made. Chloridized Ag bridges (prepared in the laboratory) were not stable enough, were light sensitive and were not suited for Cl^- replacement experiments. The usual 3M-KCl bridges (micro-electrodes with coarse tips) were seen to produce artifacts of nearly 1 mV due to lack of stirring, much in agreement with the predictions formulated by Barry & Diamond (1970). On the other hand, a satisfactory solution was to employ the following arrangement on each side of the preparation: calomel cell: 3 M-KCl; agar-saline; saline; cornea. The agar-saline bridges were mounted in micromanipulators and could be moved swiftly from one hemichamber to the other so as to be able to correct their small deviations from the zero level with an adjustable series battery.

The need to have easy access to the solution pools dictated the shape of the chamber (Fig. 2). In later experiments, the agar-saline bridges were replaced by 'double-junction' bridges (D. M. Maurice, personal communication) which were as follows: calomel cell; saturated KCl:*saline (bridge):* saline (chamber); cornea. The liquid junctions denoted:* were established across fritted glass and, in so far as possible, the solutions placed inside the bridges were the same as those used in the experimental chamber. In both cases (agar or double-junction bridges) the bridges were immersed for long periods of time (*ca.* 24 hr) in saline solutions in order to insure good stability. Although the experimental results were the same with both procedures, the double-junction bridges appeared to be more stable than the agar bridges. The potential difference was detected with an electrometer (Keithley 610 C) whose output was fed to a recorder. The instrument could read 1 mV at full scale, and individual readings were judged accurate to $\pm 10\ \mu V$. Due to its particular shape, the chamber was heated from below by a thermoelectric plate, and from above by a lamp. This procedure sufficed to keep the preparation between 33 and 37° C, as determined with a thermistor immersed in the inside (endothelial) pool. In order to counter the evaporation from the chamber, the solution pools were renewed at regular intervals. When the solutions on the inside half of the chamber were changed, fresh solutions were passed through a polyethylene coil immersed in a bath at 37° C so as to avoid thermal transients. Normally there was no hydrostatic pressure difference across the preparation; in order to prevent it from wrinkling, however, a 20–40 cm H_2O vacuum was exerted on the outside while fluid on that side was being replaced. For the same purpose, when the fluid on the inside was being replaced, the fluid level on the outside was lowered.

RESULTS

Ionic substitutions and fluid transport

Bicarbonate replacement. The fact that the presence of HCO_3^- was needed in order to insure normal functioning of the endothelial fluid pump was noted first by Dikstein & Maurice in a paper belatedly published in 1972. This was confirmed and expanded by Hodson (1971). In the present work this effect was explored further, and the effects of different HCO_3^- concentrations were tested, so as to determine whether the different $[HCO_3^-]$ were numerically related to the rate at which fluid was being transported. Under the present experimental conditions there is good evidence (Maurice, 1972; Fischbarg, 1973) that the measured 'rate of deturgescence' of a previously swollen cornea is the result of two concomitant processes, (*a*) transport of fluid by the endothelium, and (*b*) passive leak of fluid into the stroma caused by the stromal imbibition pressure. The stromal imbibition pressure is, however, not linearly related to corneal thickness (cf. Hedbys & Mishima, 1966). Therefore, in order to obtain curves of thickness versus time whose linear slopes could be related directly to transport rates, it was judged necessary to restrict the analysis of the slopes to the corneal thickness range above some 450 μm (epithelium absent). In this range the change in imbibition pressure with thickness is not so pronounced (Hedbys & Mishima, 1966). Fitting a straight line

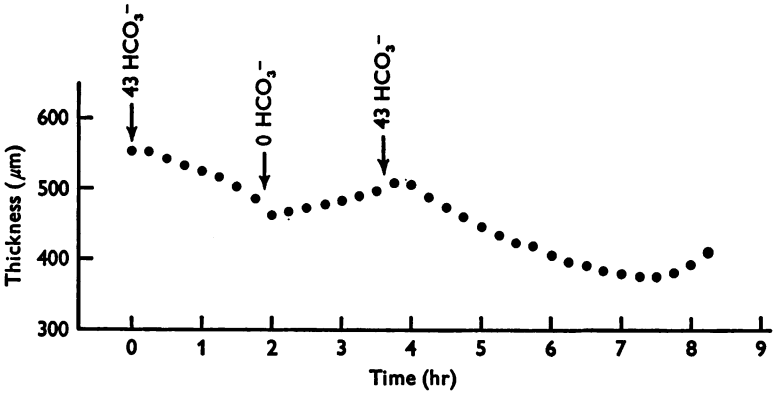

Fig. 3. A plot of stromal thickness (epithelium scraped off) *vs.* time depicts the rate of endothelial fluid transport out of a swollen stroma at normal (43 mM) and zero [HCO_3^-] perfused on the inside. The outer, denuded surface of the stroma was in contact with silicone oil (other details as in Fig. 5). 10 μm/h = 1 μl./hr.cm^2. T = 37° C, P = 20 cm H_2O.

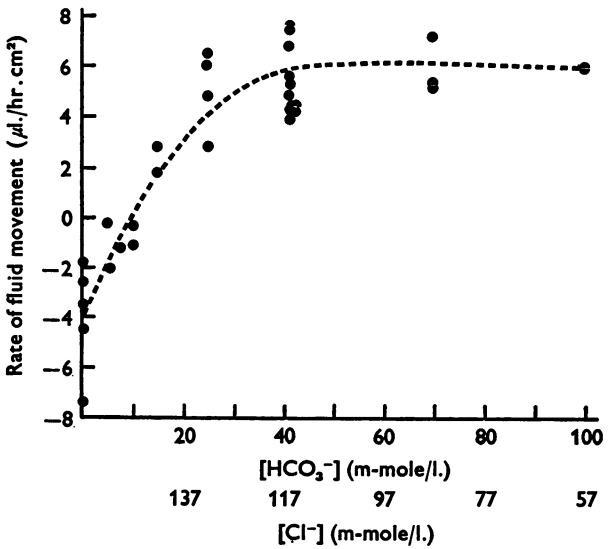

Fig. 4. Rates of fluid movement across the endothelium are plotted *vs.* HCO_3^- (or Cl^-) in the perfusing solution. Positive rates correspond to stromal deturgescence; negative rates to stromal swelling.

to the set of experimental points was relatively simple in most cases (cf. Fig. 12). In other cases, when the slope seemed to change almost continuously, the straight line was arbitrarily chosen so that it would pass through the experimental point at or near a thickness of 475 μm. When the

slope seemed to change in more irregular ways, an average slope was fitted, also centred on that point.

The effect of decreasing the HCO_3^- concentration from 43 mM (which is the amount normally present in the aqueous humour) to 0 mM is shown in Fig. 3, which depicts a representative experiment. The effect of the HCO_3^- replacement is apparent without much delay. Points obtained half

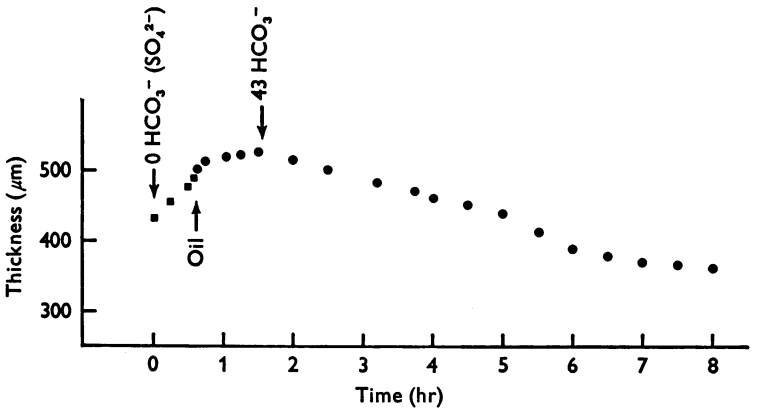

Fig. 5. Stromal thickness *vs.* time in the absence and presence of HCO_3^- (SO_4^{2-} substituted). Here and elsewhere, for experimental convenience, fluid transport was measured in swollen preparations. Initially (squares) saline was placed over the denuded stroma and swelling ensued; at the arrow below the curve, this saline was replaced by silicone oil, and subsequent fluid movements (circles) took place only across the endothelium. Arrows and legends above the curve refer to solutions perfusing the inside.

an hour after perfusion with the new solution was started already denoted a change in rate. The effects of changes in HCO_3^- concentration were reversible, provided that the endothelium was not exposed to HCO_3^- free solutions for more than some 60 min. Five such experiments showed this to be the case. In other experiments different [HCO_3^-] were tested, as summarized in Fig. 4. The rates of fluid transport shown there appear to reach a maximum of nearly 6 μl./hr.cm^2 at HCO_3^- concentrations close to the one normal for rabbit aqueous humour (40–44 m-mole/l.). Taking the value at zero HCO_3^- as the origin, half of the maximum rate of fluid transport is reached at 10 mM-HCO_3^-, which is also the approximate concentration needed to prevent swelling.

Since the HCO_3^- provided most of the buffering capacity of the solution, its total replacement might have resulted in pH shifts. This undesirable effect was not judged easy to correct, since counter measures such as addition of Tris buffer have been reported to have deleterious effects on this preparation (Hodson, 1971). Therefore, in the absence of HCO_3^-,

the solutions were practically unbuffered. Small pH shifts in the perfusing solution could then possibly occur; however, direct measurements of small samples taken from the chamber when no bicarbonate was present showed that the pH was about 6·9–7·0. Moreover, the use of pH 7·4 Tris buffer (10 mM) also in the absence of bicarbonate did not stop the swelling. As another control, SO_4^{2-} was used (instead of Cl^-) to replace HCO_3^-; as can be seen in Fig. 5, after a transient, the corneal thickness increased, in agreement with the experiments in which Cl^- replaces HCO_3^-. The SO_4^{2-} ion per se did not seem to have a detrimental effect, since the cornea could deturgesce in the usual manner even if Cl^- was totally replaced by SO_4^{2-} in the solution perfusing the aqueous side (Fig. 10). As one more precaution, the solution was made HCO_3^- free by removing 43 mM-$NaHCO_3$ and replacing them with sucrose. The result of this manipulation was again the arrest of the transport mechanisms (Fig. 6) and consequent

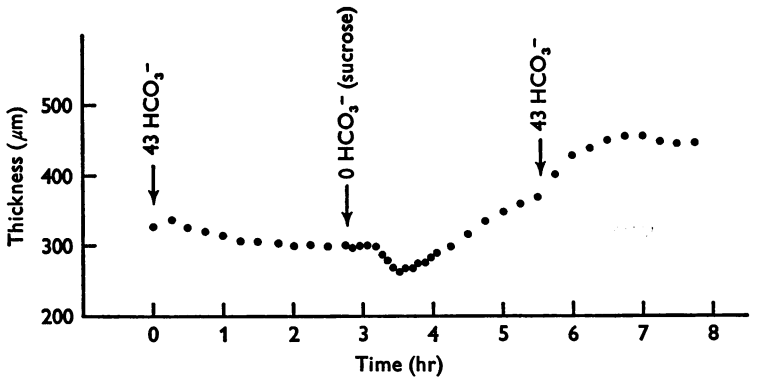

Fig. 6. Stromal thickness *vs.* time in the presence and absence of HCO_3^- (sucrose substituting 43 mM-$NaHCO_3$). Stroma not swollen initially.

swelling of the preparation. Replacement of only 40–50 mM-Na^+ by choline has not been reported to be detrimental (Hodson, 1971; Dikstein & Maurice, 1972) for the fluid transport mechanism, and caused only a 10% decrease of the electrical potential difference across the endothelium (this paper). The present results therefore support previous contentions as to the need of HCO_3^- for the transport mechanism. They also show that the $[HCO_3^-]$ has a continuous and saturable effect on the rate of fluid transport. As will be seen below, the effect of the HCO_3^- on the electrical potential difference closely parallels its effect presently described on fluid transport.

Sodium replacement. When Na^+ was replaced by choline fluid pumping was impaired, as shown in Fig. 7. When Na^+ was totally replaced by K^+, the transport mechanism was also arrested (Fig. 8). The corneas began to swell after a short delay, and swelled at a high rate suggestive of cellular

damage. The replacement of Na^+ by Li^+ in the perfusing solution produced also a marked impairment on the rate of deturgescence, and arrest of the transport mechanism also resulted when all of the NaCl present was replaced by sucrose. These results support previous observations (Dikstein & Maurice, quoted by Maurice, 1969; Hodson, 1971) linking the need for Na^+ with the fluid transport; moreover, as will be seen later, the electrical potential difference across the endothelium was also a function of the $[Na^+]$.

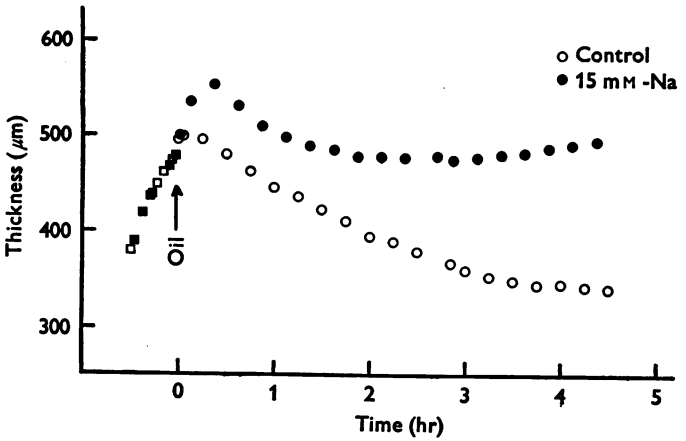

Fig. 7. Stromal thickness *vs.* time at regular (150 mM) and low (15 mM) $[Na^+]$ (choline substituted). The two curves correspond to two simultaneous experiments with the two corneas of a same rabbit (paired experiments); both preparations were perfused from the beginning with the given solutions. Other details as in Figs. 3 and 5.

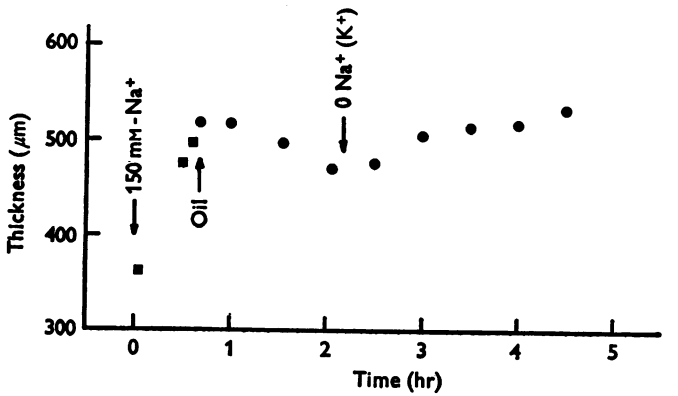

Fig. 8. Stromal thickness *vs.* time in presence and absence of Na^+ (K^+ substituted). Details as in Figs. 3 and 5.

K replacement. When the K^+ in the artificial aqueous (4·8 mM) was totally replaced by Na^+, the pumping mechanism was impaired in a short time (20–30 min after exposure), and the corneas slowly swelled. Fig. 9 shows a representative experiment out of five in which this substitution

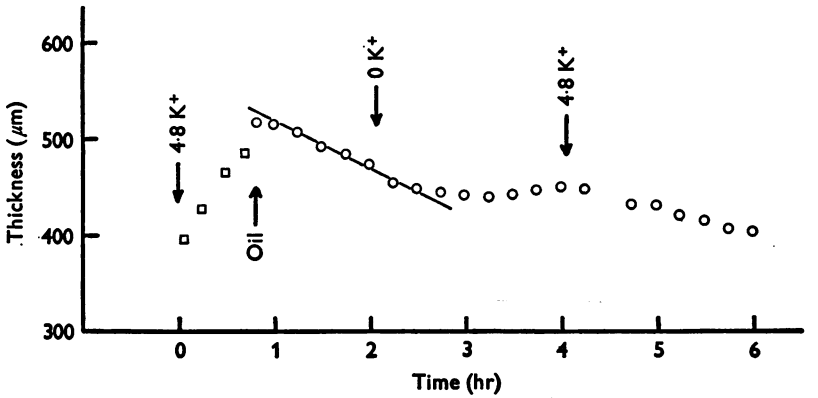

Fig. 9. Stromal thickness *vs.* time in the presence and absence of K^+ (Na^+ substituted) in the perfusing solution. Other details as in Figs. 3 and 5.

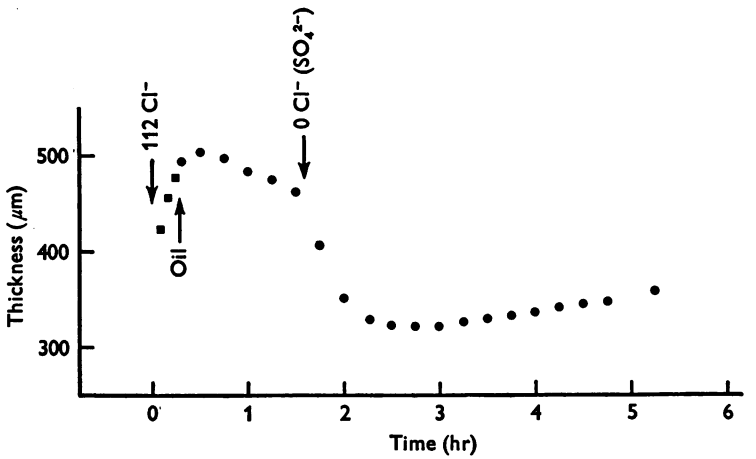

Fig. 10. Stromal thickness *vs.* time in the presence and absence of Cl^- (HCO_3^- substituted) in the perfusing solution. Other details as in Figs. 3 and 5.

was tried. In that particular experiment, and in another one, the swelling could be reversed by returning to 4·8 mM-K^+, provided that the exposure to K^+-free solution had not been maintained for more than some 45 min. From these results and the ones detailed later on [K^+] effects on potential

difference, it appears quite likely that K^+ is also involved in the transport mechanism.

Cl replacement. Total replacement of ambient Cl^- by SO_4^{2-} (Fig. 10), HCO_3^- (Fig. 11) or Br did not appear to affect the transport mechanism.

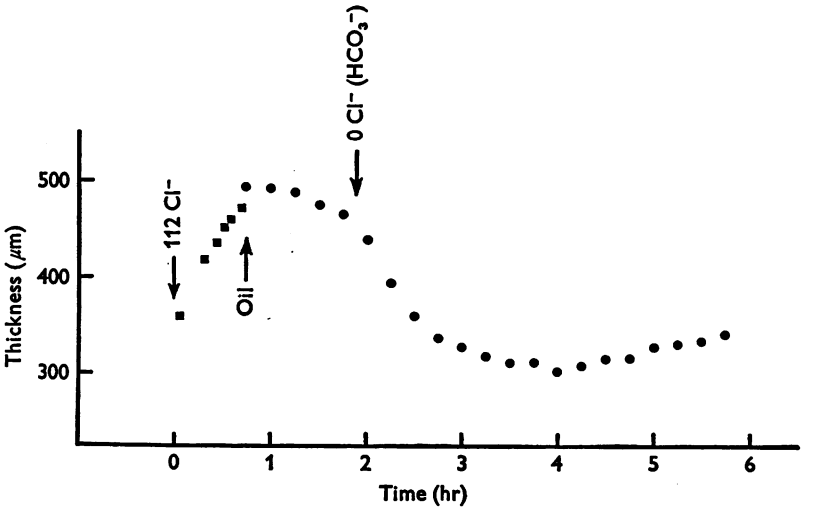

Fig. 11. Stromal thickness *vs.* time in the presence and absence of Cl^- (SO_4^{2-} substituted). Other details as in Figs. 3 and 5.

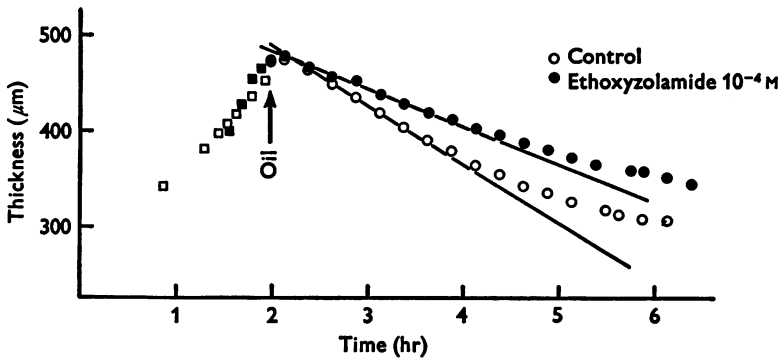

Fig. 12. Stromal thickness *vs.* time in presence of ethoxyzolamide 10^{-4} M compared to a control in paired experiments. Other details as in Figs. 3 and 5.

Carbonic anhydrase inhibitors. At first, experiments performed using acetazolamide were not conclusive because, when individual preparations were subject to the drug, a clear arrest of fluid transport was seen only at concentrations so high (10 mM) that the effect could also be termed a toxic one. When this matter was examined more in detail, however, it became

clear that the use of carbonic anhydrase inhibitors at more meaningful concentrations resulted in only a decreased average rate of fluid transport. In contrast to some of the more easily observable results presented so far, this decrease in rate required paired experiments to be ascertained with a reasonable degree of confidence. In this way, a dose-dependent inhibitory effect was seen in eight out of the nine experiments performed.

TABLE 1. Effect of carbonic anhydrase inhibitors on the rate of fluid transport

Inhibitor and concentration	Rate in control experiment μl./hr.cm^2	Rate with inhibitor μl./hr.cm^2	Rate with inhibitor/ control
Benzolamide 10^{-3} M	5·7	2·4	0·42
Benzolamide 10^{-3} M	7·1	3·3	0·46
Benzolamide 10^{-3} M	4·1	2·5	0·61
Ethoxyzolamide 10^{-4} M	6·2	4·0	0·64
Ethoxyzolamide 10^{-4} M	4·7	3·4	0·72
Ethoxyzolamide 10^{-4} M	4·2	4·2	1·00
Ethoxyzolamide 10^{-4} M	6·6	4·0	0·61
Ethoxyzolamide 10^{-5} M	4·8	2·6	0·54
Ethoxyzolamide 10^{-5} M	5·8	4·0	0·69
Ethozyzolamide 10^{-5} M	3·8	3·4	0·89
Average ± S.E. of mean	5·3 ± 0·4	3·4 ± 0·2	0·66 ± 0·06

The inhibitors employed were ethoxyzolamide (10^{-4} and 10^{-5} M) and benzolamide (10^{-3} M). Fig. 12 depicts one of those experiments. The results are summarized in Table 1; as can be seen, the inhibition is more pronounced with the higher concentrations of inhibitors, and even in the presence of these inhibitors, transport can still proceed at a sizable residual rate. These results suggest that catalysed cellular CO_2–HCO_3^- interconversion is a rate-limiting step in the chain of events resulting in fluid transport.

Ionic substitutions and electrical potential difference

The small potential difference (p.d.) across the endothelium previously reported was here characterized in terms of its dependence on the ambient ionic composition. The results correlate extremely well with the dependence of fluid transport on ionic concentrations and allow to advance a consistent explanation to be discussed below.

Bicarbonate replacement and potential difference. In this series of experiments, the preparations (corneas whose epithelium had been scraped off) were mounted in the chamber described and bathed with regular solution on both sides. The p.d. usually increased after mounting for some half hour and reached a steady level, most often between 500 and 800 microvolts (μV). At this point, the [HCO_3^-] was decreased on both sides of the

preparation by multiple washings. Typically, a transient followed (Fig. 14*c*) and the p.d. decreased to a new level in 20–30 min. After a stable value had been reached, the [HCO_3^-] was again increased to its normal (43 mM) value, and the potential difference usually recovered. The effect was expressed in percent (ratio of the p.d. reached at the test [HCO_3^-] over the p.d. before the test). These tests could be repeated two or three times during a typical experiment before the preparation would deteriorate (as shown by a gradual decrease from the initial 500–800 μV to 200–300 μV).

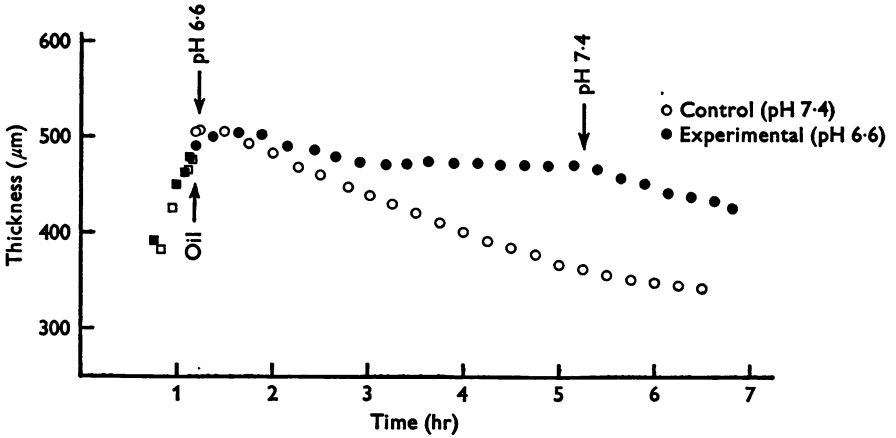

Fig. 13. Stromal thickness *vs.* time at regular (7·4) and low (6·6) pH. Curves are from paired experiments. Other details as in Figs. 3 and 5.

The effect of several concentrations of HCO_3^- were tested, and the results of thirty-five experiments are summarized in Fig. 15. As can be seen the p.d. is a saturable function of [HCO_3^-], with the half maximal value reached at 13 mM-[HCO_3^-]. This curve is similar to that depicting the behaviour of fluid transport at different [HCO_3^-] (Fig. 4). Lastly, it should be noted that even with HCO_3^--free solutions the p.d. does not fall to zero and residual values still amount to some 47 % of the control. The question of possible endogenous production of HCO_3^- raised by these findings was not pursued further in this study.

Sodium replacement and potential difference. The procedure for the replacements was essentially the same one detailed above, except that Na^+ was here replaced by choline. The time transient (an example is shown in Fig. 14*a*) was much faster than when HCO_3^- was replaced. Total Na^+ deprivation reduced the p.d. to zero (with no residual p.d. left), and the decrease was very fast; a new stable value was usually reached after 2–3 min. The values of p.d. as a function of [Na^+] fell on a smooth curve (Fig. 16); half of the maximum value was reached at 15 mM-Na^+.

Effect of ouabain on potential difference. The abolition of p.d. by ouabain (10^{-5} M) was immediate and irreversible (Fig. 17*a*) and took place as soon as the first exchange of the solution containing the ouabain had been

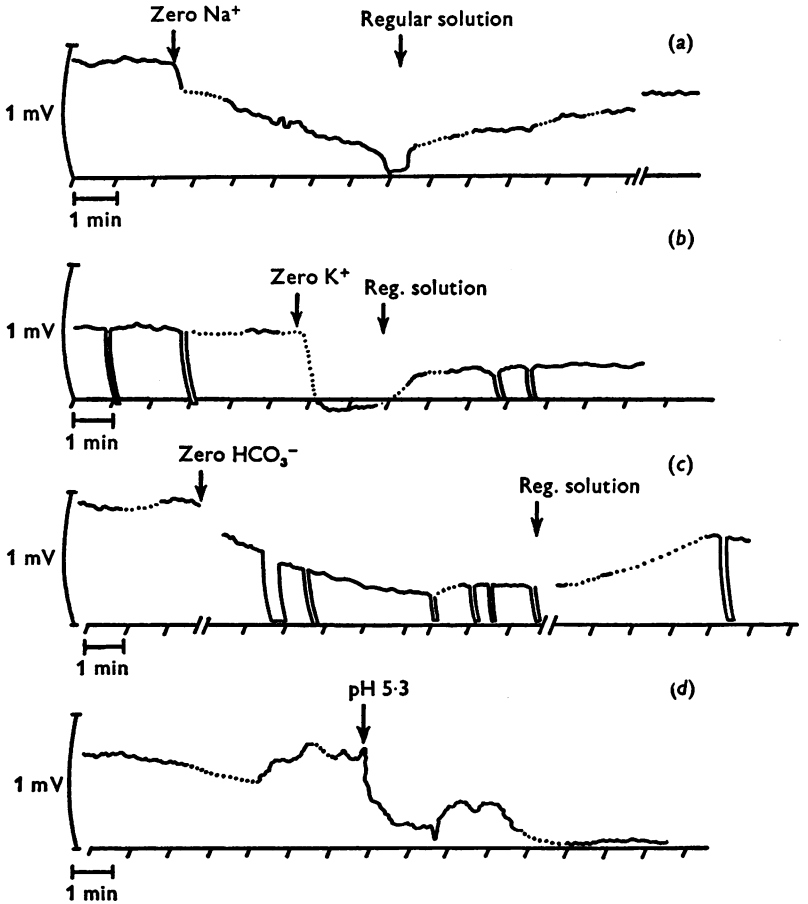

Fig. 14. Electrical potential difference across the endothelium *vs.* time after the ionic replacements shown: (*a*) Na^+ by choline; (*b*) K^+ by Na^+; (*c*) HCO_3^- by Cl^-; (*d*) Regular solution (pH = 7·4) by a solution of pH 5·5. Curves were traced from the recorder's chart. Base line calibrations are indicated; dotted lines correspond to electrode zero checks.

performed on the inside pool. It seems worth emphasizing that the endothelial cells are directly in contact with the inside solution pool, with only their surface coat and an unstirred aqueous layer interposed between them and the bulk of the solution. This circumstance, which is unusual for other epithelia, seems to account for the swiftness of the ouabain effect,

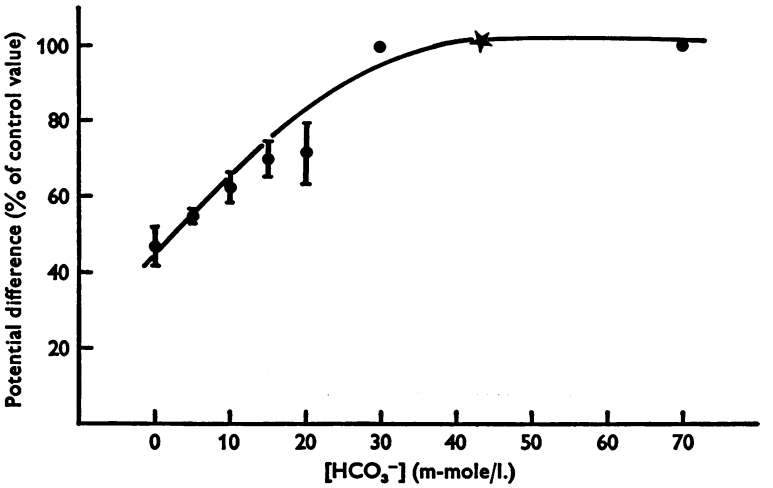

Fig. 15. Electrical potential difference across the endothelium *vs.* HCO_3^- on the inside (Cl^- substituted). Control values (at 43 mM-HCO_3^-) were recorded before test values. Graph depicts average ± S.E. of mean of six values per point. Single points represent average of two experiments.

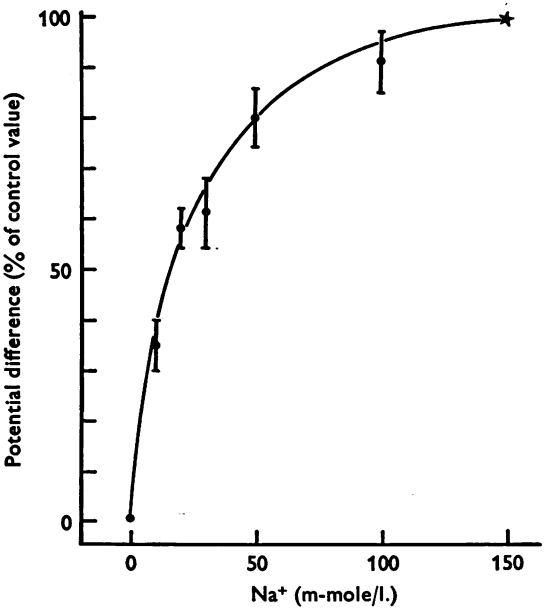

Fig. 16. Electrical potential difference across the endothelium *vs.* Na^+ on the inside (choline substituted). Control values (at 150 mM-Na^+) were recorded before test values. Graph depicts average ± S.E. of mean of five values per point. The point at zero Na^+ was identically zero p.d. with no spread.

which, with the limitations of the methods used, was estimated to take place in less than 10 sec and possibly in as short a time as 3 sec or less. The ouabain effect depicted in Fig. 17*a* was so fast that an artifact was suspected after the initial measurements; it was hence reassuring to find out that, if the drug was added slowly to the top of the inside pool so as to lengthen the diffusion path to about 1 cm, the ouabain effect could now be seen to take place more gradually (Fig. 17*b*).

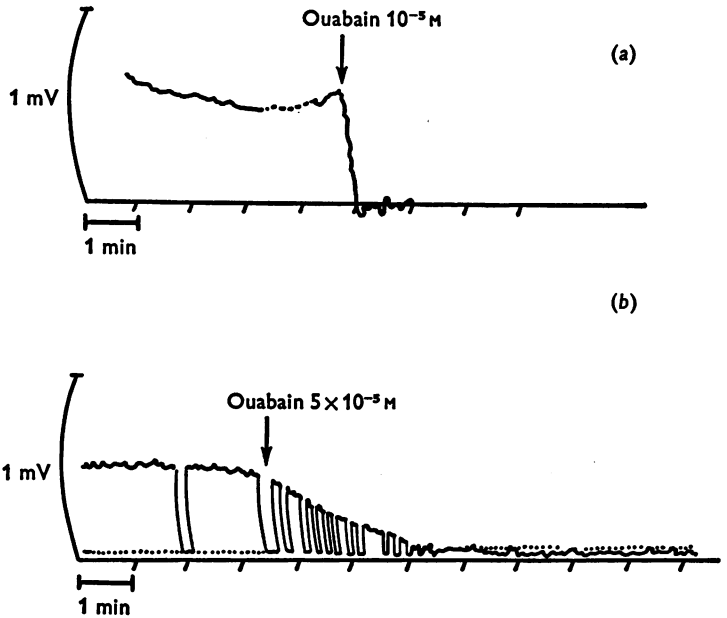

Fig. 17. Electrical potential difference across the endothelium *vs.* time; at the arrow the inside was flushed with a ouabain-containing solution. In (*a*) the solution exchange was done as fast as possible; in (*b*) the exchange was done more slowly (see text).

Effect of pH on potential difference. By analogy with other tissues in which HCO_3^- role is linked to an acidification mechanism, here too the H^+ was presumed to be an important parameter, and the results below lend support to this notion. The $[H^+]$ was changed by gassing the solutions with CO_2 (or with air) immediately prior to use. In order to obtain pH values below 6·5, the solutions were acidified further with 1·0 N-HCl. During a typical experiment the preparations were allowed to stabilize at pH 7·4 for approximately half an hour, after which the pH was changed on both sides by multiple washings. The effect on p.d. was immediate; in less than a minute a new p.d. value could be read, which remained fairly stable. This value was reached after merely exchanging the solution in the

inside pool; however, in order to preserve a symmetrical arrangement, the outside solutions were subsequently also exchanged. The new p.d. value was expressed as percent of the p.d. at pH 7·4 immediately preceding the exchange. Upon returning to pH 7·4 the p.d. regained its former value, except for the normal slow decay of the preparation with time. The time

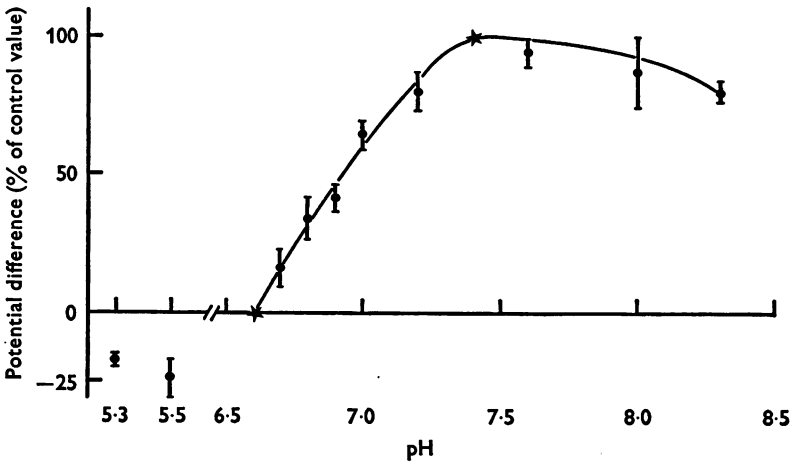

Fig. 18. Electrical potential difference across the endothelium *vs.* pH. Control values (at pH 7·4) were recorded before test ones. Graph depicts average ± S.E. of mean of eight values per point.

TABLE 2. Potential difference at low pH

pH	p.d. (μV)	pH	p.d. (μV)
5.3	−200	5·5	−200
	−200		0
	0		−150
	0		−200
	−100		−100
	0		
	−200		
	−80		
	0		
	0		
	−200		
Average ± S.E. of mean	−89 ± 11		−130 ± 37

course of one of the experiments is shown in Fig. 14*d*, and Fig. 18 summarizes the results. The p.d. is seen to reach an optimum around pH values from 7·3 to 7·5. At pH 6·6 the p.d. is reduced to zero, and at the lower pH values examined (5·3 and 5·5) the p.d. is actually reversed in direction (Table 2) although much smaller in magnitude (−80 to −200 μV). In general, the absolute value of the reversed p.d. was proportional to the

value of the p.d. before the reversal. Although in six out of the sixteen experiments (in Table 2) the p.d. remained at zero without reversing, when it reversed the values observed were well within the range which could be detected with the sensitive instrument used. In order to ascertain which was the origin of this p.d. in four experiments carried out at pH 5·5 ouabain (10^{-5} M) was added to the inside and in all four it caused immediate abolition of the reversed p.d., which became zero. Using the Henderson–Hasselbach equation, [HCO_3^-] (normally 43 mM) can be calculated to be 16 and 13 mM at pH values of 5·5 and 5·3 respectively; this shift in [HCO_3^-] does not appear to be enough to account for the reversed p.d. observed (cf. Fig. 15). The implications of these findings are discussed below.

K replacement and potential difference. The [K^+] in the regular solutions used was 4·8 mM. Total replacement of this ambient K^+ (substituted by Na^+) resulted in decrease or abolition of the p.d. in eight experiments. The time course of the effect was erratic; in some experiments, the decrease in p.d. followed immediately the washing of the inside (Fig. 14*b*), while in others prolonged periods of time (up to 1 hr) and repeated washings on both sides were necessary to observe the effect. In contrast to the long periods of washing which were sometimes needed, the K^+-free effect on p.d. could be reversed almost immediately after returning to regular solution. From these results, it would appear likely that minimal amounts of K^+ would suffice to maintain the transport mechanism operative. The proposed role of K^+ is discussed below.

Cl replacement. A possible effect of total Cl^- replacement on p.d. was looked for and not found in twelve experiments. Sulphate was substituted for Cl^-. Even after repeated washings performed during periods of several (2–3) hours the p.d. was not measurably affected. It should be noted that the solutions used for these experiments contained HCO_3^- at all times. Since absence of HCO^- alone sufficed to arrest fluid transport and decrease the p.d., replacement of both Cl^- and HCO_3^- simultaneously was not attempted. It may be interesting to note that this preparation seems to differ from the other limiting corneal layer, the epithelium, where Cl^- is actively transported (Zadunaisky, 1966; Klyce, Neufeld & Zadunaisky, 1973).

Inhibitors and potential difference. Aside from the effects of ouabain reported here and those of cytochalasin-B reported elsewhere (Fischbarg, 1972*b*; Kaye, Fenoglio, Hoefle & Fischbarg, 1974), other inhibitors had a noticeable effect on the potential difference. Both cyanide and iodoacetate have been known for years to cause corneal swelling when injected into the anterior chamber (Philpot, 1955) and have been recently shown to arrest the endothelial fluid pump (Dikstein & Maurice, 1972). The inhibitory

effects of NaCN and Na iodoacetate (both 2 mM) on p.d. are contrasted to the behaviour of an untreated control in Fig. 19. Carbonic anhydrase inhibitors (ethoxyzolamide 10^{-4} M or acetazolamide 10^{-3} M) added to the regular solution employed had no effect on the p.d. even after exposure for 0·5–1 hr.

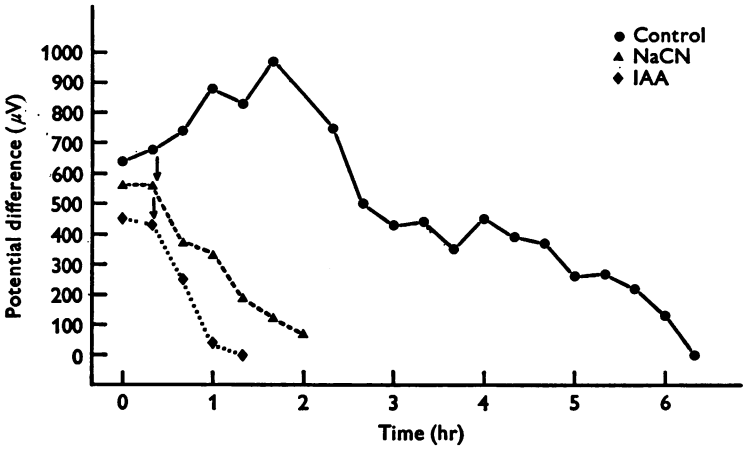

Fig. 19. Electrical potential difference across the endothelium *vs.* time. At the arrows the chambers were flushed with solutions containing NaCN or IAA (2 mM), whose pH had been adjusted to 7·4.

Intracellular potential of endothelial cells. Upon penetration with conventional 3 M-KCl filled micro-electrodes (40–80 MΩ resistance), the endothelial cells exhibited a resting potential of about −14 mV with respect to the reference electrode placed in the solution on the inner (aqueous) side of the cells (range: −9 to −40 mV). Similar results have been obtained in another study (Di Ulio & Edelhauser, personal communication). Although no effort was made here to ascertain the actual location of the micro-electrode tip, the slow movement downwards of a tip placed in the immediate neighbourhood of the endothelial cells produced the characteristic shift in potential difference which accompanies intercellular penetration in other systems; this potential difference was considerably decreased by slight lateral movements or vibration of the micromanipulator, as if due to cell injury, and disappeared upon further downward penetration by some 5–10 μm with the micro-electrode, or upon withdrawing it upwards. After each penetration, the potential difference initially read decreased rapidly to nearly zero (in 2–3 min). Measurements of these potentials could be carried out for several hours in a given preparation with little variation in the values read.

DISCUSSION

Potential difference and fluid transport. The likelihood of a connexion between the potential difference and the transport of fluid across the endothelium has been previously proposed (Fischbarg, 1972*b*, 1973) and is reinforced by data presented here. A summary of past and present findings related to this subject is given in Table 3. As can be seen, under most conditions the p.d. behaves in the same way as the fluid pump mechanism. There are some exceptions, however, namely (*a*) after 5–6 hr

TABLE 3. Relationship between potential difference and fluid transport

Factor	Effect on p.d.	Effect on fluid transport
$[HCO_3]$ decrease	Reversibly decreases following behaviour similar to that of fluid pump (1, 2)	Decreases or arrests (3, 4); reversibly decreases (1, 2)
$[Na^+]$ decrease	Reversibly decreases (5, 1, 2)	Arrests (6, 3, 4, 1, 2)
K^+ absence	Reversibly decreases (5, 1, 2)	Reversibly arrests (5, 1, 2)
$[H^+]$ increase	Reversibly decreases (2)	Arrests (2, 7)
Temperature decrease	Decreases (8)	Arrests (9)
Ouabain	Abolishes (5, 1, 2)	Arrests (10)
Carbonic anhydrase inhibitors	No effect (2)	Reduces by half (2); reduces (7)
Cyanide	Abolishes (5, 2)	Arrests (3)
Iodoacetate	Abolishes (5, 2)	Arrests (3)
Cytochalasin B	Reversibly decreases (5)	Arrests ? (5, 1, 11)

Key to sources: (1) Fischbarg, 1973; (2) this paper; (3) Dikstein & Maurice, 1972; (4) Hodson, 1971; (5) Fischbarg, 1972*b*; (6) Maurice & Dikstein, quoted by Maurice, 1969; (7) D. M. Maurice, personal communication; (8) Barfort & Maurice, 1972; (9) Maurice, 1972; (10) Trenberth & Mishima, 1968; (11) Kaye, Fenoglio, Hoefle & Fischbarg, 1974.

of *in vitro* perfusion, the p.d. disappears before the pumping ceases (D. M. Maurice, in the discussion of Fischbarg, 1973*b*), (*b*) after addition of cytochalasin-B at relatively low concentrations (5–10 μg/ml.) the p.d. is immediately affected while the effect on fluid movement may appear with a delay (J. Fischbarg, unpublished observations), (*c*) carbonic anhydrase inhibitors (see Table 1) affect fluid pumping but not p.d. These examples of divorce between p.d. and fluid pump can be explained if

(cf. Fischbarg, 1973 and discussion below) the p.d. would be normally shunted to a great extent across 'leaky' intercellular junctions which could become even 'leakier' (case of prolonged perfusion or cytochalasin-B treatment), and if the electrogenic step of the pump would precede the step involving carbonic anhydrase.

A scheme for the ionic roles. On obvious problem that appeared after finding the endothelial p.d. was that of its polarity. The fluid was being pumped from outside (stroma) to inside (aqueous), and yet the p.d. was negative on the inside, opposite to what would be expected simply of an electrogenic Na^+ transporting mechanism. The polarity could not be conveniently explained by an anion transport either, since total Cl^- replacement had no effect on p.d. or fluid pump and total HCO_3^- replacement arrested the fluid pump but left a residual p.d. (cf. Fig. 15). Admittedly, endogenously produced CO_2 could be converted to HCO_3^- and serve as substrate for such pump even in the absence of ambient HCO_3^-. However, the fact that carbonic anhydrase inhibitors decreased the rate of fluid pumping without affecting p.d. was taken to suggest that an electrogenic HCO_3^- pump was not an adequate explanation for all the findings. By exclusion, since both p.d. and fluid transport were so markedly dependent on $[Na^+]$ and $[H^+]$, cation pumps appeared to provide a better explanation for the p.d. observed. In this framework, one of them would have to operate in a direction opposite to that of fluid transport, and this condition would be met by a H^+ pump from cell to intercellular spaces. A Na^+ pump would on the other hand operate from cell to aqueous, resulting in the fluid transport observed. A particular combination of geometrical factors together with density of sites and rate of pumping of them could result in an electrical asymmetry in favour of the pump located in the lateral area (the H^+ pump), which would explain the direction of the observed p.d. The ouabain-inhibitable inversion of p.d. observed at high $[H^+]$ could thus be explained as due to the electrogenic Na^+ pump predominating under those conditions over the impaired H^+ pump. At least one important assumption has to be invoked, however, for the sake of consistency of this argument. The very fast ouabain effect on p.d. observed should in principle be attributed to the inhibition of a Na^+ pump, but in the proposed scheme such inhibition would produce an increase rather than the observed abolition of p.d. One is led therefore to postulate that either ouabain would also inhibit the H^+ pump or that some link between the Na^+ and the H^+ pumps would be expected to exist.

There are other findings which are consistent with this general line of reasoning. ATPase activity presumably related to transport has been found by histochemical methods to be localized in the lateral and apical endothelial cell membranes (Kaye & Tice, 1966; Leuenberger & Novikoff, 1974).

The cornea (Scott, 1971) and specifically, its endothelium show carbonic anhydrase activity (Lönnerholm, 1972; Silverman & Gerster, 1974), and a HCO_3^- dependent ATPase is present in endothelia of calves and rabbits (E. I. Anderson, personal communication). Consequently, the scheme presented in Fig. 20 suggests itself as an explanation consistent with the line of thought advanced so far. The scheme as presented is admittedly based on indirect evidence; there are however technical reasons

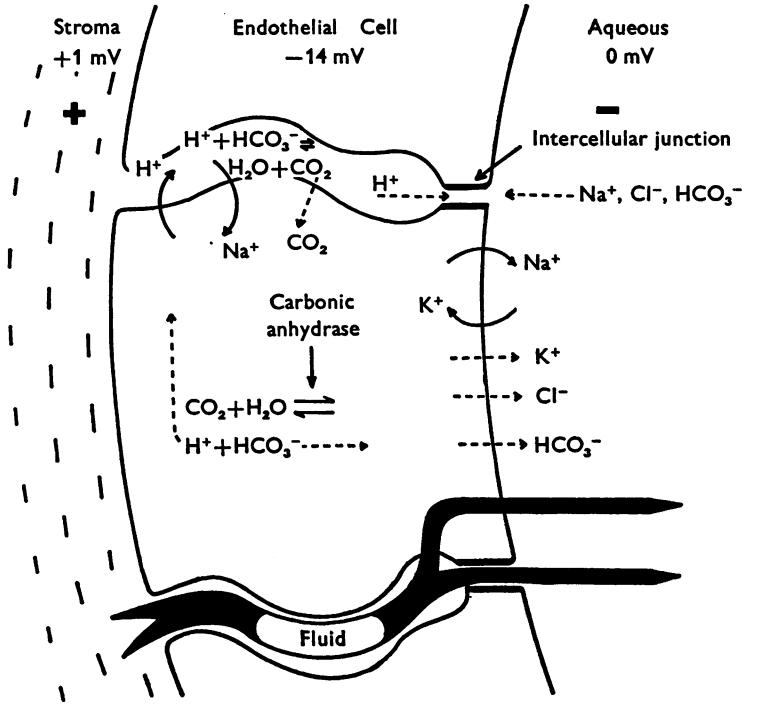

Fig. 20. Schematic diagram of endothelial cells, their electrical potential profile, and the proposed ionic movements. Solid curved arrows represent ionic pumps, while dashed arrows depict passive movements.

which have limited the type of information that could be obtained. Passive radioisotope fluxes (about 10 μ equiv/hr.cm^2 for ^{22}Na; Green, 1967) across this very permeable preparation are of the order of 10 times the expected net flux and a demonstration of net ionic movements by these means has not been possible yet. Due to similar reasons, procedures which are common in other epithelia have not been useful here so far. On the other hand, schemes similar to that presented in Fig. 20 have received strong experimental support when other preparations were examined. The same basic idea was advanced for kidney proximal tubules by Pitts &

Alexander (1945), and has been proposed for the choroid plexus in a recent example (Wright, 1972). Furthermore, some of the transport-related characteristics of the corneal endothelium are strikingly similar to the corresponding ones of a group of fluid-transporting epithelia. Table 4 depicts these similarities (the few references selected for that table are in many cases only a sample of a much larger body of knowledge). It should be noted that different authors have emphasized different aspects of the ionic 'involvement' shown. In spite of this, the existence of all these common characteristics cannot but raise the possibility that a similar general mechanism may underlie all these particular examples of transporting

TABLE 4. Evidence for involvement of ions, effects of inhibitors and existence of a potential difference in several fluid-transporting epithelia

	Na^+	K^+	HCO_3^-	H^+	Cardiac glycosides	Carbonic anhydrase inhibitors	Potential difference
Kidney proximal tubule	1	2	3, 4	3, 4	5, 2	6, 7	8, 9
Gall bladder	10,11	10	12	13	10	14	15, 16
Small intestine	17	18	19	19	20	21	17
Choroid plexus	22, 23	22, 23	24, 25	25, 26	27	25, 27	28, 29
Ciliary epithelium	30, 31	32	33, 34	35, 32	31	34, 35	36, 31
Corneal endothelium	37, 38, 39	40, 39	37, 38, 39	39	41, 40, 39	39	40, 39

Key to sources: (1) Windhager, Whittembury, Oken, Schatzman & Solomon, 1959; (2) Maude, 1969; (3) Pitts & Alexander, 1945; (4) Rector, Carter & Seldin, 1965; (5) Schatzman, Windhager & Solomon, 1958; (6) Clapp, Watson & Berliner, 1963; (7) Kunau, 1972; (8) Willbrandt, 1938; (9) Frömter, 1974; (10) Diamond, 1962; (11) Wheeler, 1963; (12) Diamond, 1964; (13) Whitlock & Wheeler, 1969; (14) Wheeler, Ross & King, 1969; (15) Whitlock & Wheeler, 1964; (16) Machen & Diamond, 1969; (17) Curran, 1960; (18) Riklis & Quastel, 1958; (19) Turnberg, Fordtran, Carter & Rector, 1970; (20) Schultz & Zalusky, 1964; (21) Turnberg, Bieberdorf, Morawski & Fordtran, 1970; (22) Rougemont, Ames, Nesbett & Hofmann, 1960; (23) Wright, 1972; (24) Maren, 1972; (25) Tschirgi, Frost & Taylor, 1954; (26) Mottschall & Loeschcke, 1963; (27) Welch, 1963; (28) Loeschcke, 1956; (29) Welch & Sadler, 1954; (30) Barany, 1947; (31) Cole, 1961; (32) Berggren, 1964; (33) Friedenwald, 1949; (34) Kinsey & Reddy, 1959; (35) Becker, 1959; (36) Lehmann & Meesmann, 1924; (37) Dikstein & Maurice, 1972; (38) Hodson, 1971; (39) this paper; (40) Fischbarg, 1972*b*; (41) Trenberth & Mishima, 1968.

tissues. It is also pertinent to point out that many other epithelia specialized in different transport functions share all or more of the characteristics presently shown (cf. H^+ secretion and presence of carbonic anhydrase in gastric mucosa) and that fresh examples are still being produced (i.e. finding of carbonic anhydrase in urinary bladder, Rosen, 1970; Scott, Shamoo & Brodsky, 1970).

Electrogenic vs. neutral pump. There is an alternative hypothesis that can be invoked to explain the magnitude and polarity of the p.d. observed. The fluid transport could conceivably be due to a 'neutral' pump which could move salt (NaCl and/or $NaHCO_3$) out of the intercellular spaces and, through the cells, into the aqueous. Such process could set up a diffusion potential across the intercellular (cf. Fischbarg & Lim, 1973) junctions; a similar explanation was advanced for the small p.d. observed across the gall bladder by Machen & Diamond (1969). In the corneal endothelium, the operation of a 'backwards' (Diamond & Bossert, 1968) pump out of the intercellular spaces would result in hypotonicity of the fluid contained in the spaces. The endothelium can be calculated to behave close to the fashion depicted in curve 2, page 696 of that paper, and on that basis the fluid at the apex of the spaces should be some 40 m-osmole hypotonic with respect to that in the aqueous. On the other hand, an unstirred layer of about 350 μm (Green & Otori, 1970) present at the cell-aqueous boundary would increase the local salt concentration only by about 1 m-osmole (the transendothelial net water flow of 5·2 μl./hr.cm^2, if isotonic, corresponds to some 0·8 μl./hr.cm^2 of salt). A concentration difference of 20 mM-NaCl across the 'leaky' intercellular junctions could thus be assumed and under those conditions a diffusion potential would appear across these junctions whose magnitude and polarity would depend on the properties of the junctions (from -750 μV for liquid junctions to $+3{\cdot}5$ mV for perfectly Na-selective junctions). The p.d. experimentally observed ($+0{\cdot}5$ mV) might thus be explained by the presence of fixed negative charges in the junctions. Many of the present findings could indeed be interpreted in terms of this diffusion potential; [Na^+], [HCO_3^-] and [K^+] changes might thus interfere with the rate of salt pumping and vary the concentration gradient across the junctions and therefore the p.d., while a reduction in [H^+] might decrease the selectivity for Na of the junctions up to the point where the p.d. would reverse, as experimentally observed here. In the same vein, cytochalasin-B, which affects endothelial p.d. and electrical resistance (Fischbarg, 1972; Fischbarg & Lim, 1973) might affect the salt gradient through an observed decrease in the length of the intercellular spaces (Kaye, Fenoglio, Hoefle & Fischbarg, 1974). The very fast action of ouabain, aside from affecting an electrogenic pump, might also be explained by a fast disappearance of the salt gradient, since diffusion of ouabain across the unstirred layer requires only 2 sec and ionic redistribution along the 12 μm long intercellular space would require only 20–30 msec more. There are some findings, however, which are difficult to account for in terms of such a diffusion potential. Total replacement of Cl^- by SO_4^{2-} does not affect p.d., while some change would be expected if Cl^- diffusion would play a role. Even more importantly, carbonic anhydrase

inhibitors cut the rate of fluid transport by 40–60 % while leaving the p.d. unaffected. Similar decreases in fluid pumping rate nearly always correspond to decreases in p.d. when pH, [HCO_3^-], [Na^+] or [K^+] were decreased, so it is not clear why the postulated salt gradient should be affected in some cases and not in the other. Lastly, in order to account for the results observed with pH changes in terms of a neutral pump, one would have to assume very peculiar simultaneous effects of pH on both the fluid pump mechanism and the fixed charges at the intercellular junctions, which, if conceivable, appears rather complicated. In balance, both the electrogenic and neutral mechanisms can be argued for and against; at present the evidence suggesting an electrogenic pump (fast ouabain effect) commands predominant attention, but the debate cannot be considered closed.

Corollary. The present preparation has experimental advantages over other epithelia and may be useful for the investigation of some general properties. This flat single layer of cells is in direct contact with the nutrient fluid, and its simple geometry allows some numerical conclusions to be reached with ease. The fact that the fluid pump here has characteristics similar to those of other epithelia again points to its possible usefulness for transport studies.

This work was done with the able technical assistance of Miss Pegri Varjabedian. It was supported by U.S.P.H.S. Research Grants EY-00727 and EY-01080. Dr J. J. Lim was supported by U.S.P.H.S. Training Grant EY-00029, and in part by Fight for Sight, Inc., New York.

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