*J. Physiol.* (1979), **294**, *pp.* 627–634
*With 2 text-figures and 2 plates*
*Printed in Great Britain*



# INTERCELLULAR SPACES IN CHEMICALLY FIXED CORNEAL ENDOTHELIA ARE RELATED TO SOLUTE PUMP ACTIVITY NOT TO SOLVENT COUPLING

BY STUART HODSON AND KENNETH R. MAYES

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

(*Received 29 January 1979*)

SUMMARY

1. Stereological analyses of the lateral intercellular spaces in the endothelium of rabbit corneas after chemical fixation were undertaken.

2. Immediately preceding chemical fixation the endothelia were in one of four transport modes: (*a*) bicarbonate pump activated and no coupled solvent flux, (*b*) bicarbonate pump activated and, coupled to it, a solvent flux, (*c*) no fluxes across the endothelia and (*d*) passive fluid fluxes across the endothelia.

3. In each transport mode, the intercellular spaces were measured after immersion in a series of fixatives in which the concentration of buffer (sodium cacodylate) was increased.

4. Lateral intercellular spaces were a function of buffer concentration in the fixative and activity of the bicarbonate pump. They were not a function of either solvent coupling or passive solvent flow.

5. It is concluded that the characteristic changes of lateral intercellular spaces, when transport is inhibited, do not indicate the site of local osmotic coupling.

## INTRODUCTION

Epithelia engaged in solute coupled water transport have intercellular spaces which, characteristically, are open at the basal end but terminate at a junctional complex on the apical side. Diamond & Bossert (1967) incorporated this observation into their 'standing-gradient osmotic flow' hypothesis in order to explain solute-solvent coupling. In epithelia such as gall-bladder where fluid flows from the mucosal to serosal sides, solute was supposed to be pumped into the intercellular spaces which then induced a flow of water in a partly transcellular route through the apical and lateral membranes around the junctional complex into the intercellular space and out into the serosal side of the epithelium. In what they termed 'backwards' fluid transporting epithelia, the same processes work in the reverse direction: the intercellular space fluid is made hypotonic by the action of solute pumps and, according to the standing-gradient hypothesis, fluid flows across the epithelia in the direction serosal to mucosal (Diamond & Bossert, 1968). As Diamond (1964) has emphasized, osmolarity of the emergent fluid is a critical test of any solute-solvent coupling hypothesis. Recently Hill (1975) has claimed that the geometric shapes of

0022-3751/79/5150-0001 $01.50 

the intercellular spaces of a variety of epithelia transporting isotonic or near-isotonic coupled solvent flows are incompatible with the standing-gradient hypothesis and Mills & Di Bona (1978) have indicated that the distribution of the solute pump along the lateral membranes of frog gall-bladder epithelium does not favour the standing-gradient hypothesis.

In spite of these criticisms, an important prediction of the standing-gradient hypothesis is frequently observed. In 'forwards' transporting epithelia, when the transport activities are operational the intercellular spaces are dilated and when the transport activities are inhibited the intercellular spaces contract (Diamond & Tormey, 1966; Tormey & Diamond, 1967). In 'backwards' transporting epithelia, the opposite is observed. If the transport mechanisms are switched on the spaces are narrow and when the transport mechanisms are inhibited the intercellular spaces broaden. It occurred to us that when these observations are made, as they usually are, in tissue which has been chemically fixed, it is possible that these intercellular spaces could have been altered at the time of initial fixation. Although it would not be relevant if such an alteration were present in equal degree in both the 'on' and 'off' transport modes, misinterpretation could easily arise if there were a differential effect in the two modes. For example, if the osmotic pressure in either the intercellular or intracellular spaces were different when the solute pumps were 'on' or 'off', independent of any solute coupling, then the observed alterations in chemically fixed tissue might not reflect a physiological phenomenon.

To test this possibility, the problem resolves into one of finding a fluid transporting epithelium where it is possible to achieve the two usual states (*a*) solute pumps on and an accompanying coupled solvent flow and (*b*) solute pumps off and no solvent flow, plus a third state (*c*) solute pumps on with no solvent coupling. The third state would help to distinguish whether the alterations in the intercellular spaces of chemically fixed tissue were due to the activity of the solute pumps alone or whether they did reflect a site of local osmotic coupling as described by the standing-gradient hypothesis. These three states, plus an extra one, may readily be achieved with the endothelial cells lining the posterior surface of the rabbit cornea.

The hydration of the corneal stroma is maintained constant by a pump-leak mechanism operating across the endothelium. The solute species pumped is the bicarbonate ion (Hodson & Miller, 1976; Hull, Green, Boyd & Wynn, 1977; Mayes & Hodson, 1978). In the normal physiological state of the cornea, the endothelial electrogenic bicarbonate pump is not coupled to any solvent flow and condition (*c*) described above is operational. If the epithelium lining the anterior surface of the cornea is rubbed off the preparation, the endothelial bicarbonate pump remains just as active (Hodson & Miller, 1976) but now a fluid flow may be observed to pass across the endothelium (Maurice, 1972). The solvent flow is not driven by electrochemical gradients in the bulk phases on either side of the tissue but is coupled to the bicarbonate pump (Mayes & Hodson, 1978). Condition (*a*) described above is operational. Condition (*b*) may readily be achieved by bathing the cornea in a bicarbonate and carbon dioxide free Ringer (Hodson & Miller, 1976; Mayes, Graham & Hodson, 1978). In the absence of its substrate (for metabolic $CO_2$ is at relatively low concentrations), the pump is reversibly switched off (Hodson & Miller, 1976). Condition (*b*) described above may be achieved. Additionally a fourth condition of

transendothelial fluxes is found when the pump is inhibited and initially there is a passive fluid flow into the stroma across the endothelium.

We examined the effects of a system of fixatives with graded buffer concentrations on all four of these transport modes. Intercellular space volumes were recorded in the electron microscope using stereological techniques. A preliminary report of these observations has been communicated to the Society (Hodson & Mayes, 1978).

## METHODS

Three months old Dutch rabbits were killed by I.V. injections of sodium pentobarbitone and the corneas were dissected immediately for study *in vitro*.

*Incubation and fixation media.* In order to activate the bicarbonate ion pump of the endothelium, corneas were incubated in: NaCl, 106 mM; $NaHCO_3$, 37 mM; KCl, 6·7 mM; $MgSO_4$, 0·6 mM; $NaH_2PO_4$, 5·55 mM; $CaCl_2$, 0·56 mM; glucose, 5 mM; reduced glutathione, 0·5 mM bubbled to equilibrium with a gas mixture of 87% $N_2$, 8% $O_2$ and 5% $CO_2$. This solution is referred to as normal Ringer in the text. To inhibit the bicarbonate pump corneas were incubated in: NaCl, 140 mM; KCl, 6·7 mM; $MgSO_4$, 0·6 mM; $Na_2HPO_4$, 5·55 mM; $CaCl_2$ 0·56 mM; glucose, 5 mM; reduced glutathione, 0·5 mM bubbled to equilibrium with a gas mixture of 92% $N_2$ and 8% $O_2$. This solution is called BCFR (bicarbonate and carbon dioxide free Ringer) in this text. Both BCFR and normal Ringer had a pH of 7·40 ± 0·08 and a measured osmolarity of 287 ± 2 m-osmole. After suitable incubation the corneas were fixed by rapid and total immersion in solutions of one of the following compositions: 1% $OsO_4$ buffered at pH 7·40 by sodium cacodylate of molarity 120, 130, 140, 150 or 160 mM. Corneal endothelia were fixed in solutions of osmium tetroxide for two reasons. First, this was the fixative used in the original report of Tormey & Diamond (1967). Second, aldehyde fixatives always destroy the ultrastructure of the corneal endothelium (Hodson, 1968, 1969*a*).

The methods of achieving the four transport modes are fully described in Hodson (1971*a*), Dikstein & Maurice (1972), Maurice (1972), Hodson (1974) and Hodson & Miller (1976). For purposes of convention the active bicarbonate flux is nominated $j_B$ and positive values indicate a current of bicarbonate ions out of the cornea across the endothelium into the lens-side. Fluid flux is nominated $J_v$ and when positive it is coupled to $j_B$ and flows in the same direction. When $J_v$ is negative, there is a passive flow of fluid in the opposite direction into the stroma which is driven by the passive swelling forces of the corneal stroma (e.g. Hodson, 1971*b*). The four modes of transport with the measured mean transport rates are as follows.

(*a*) Solute pumps on and solvent coupling. $j_B = 2{\cdot}8 \times 10^{-10}$ equiv.$cm^{-2}$.$sec^{-1}$, $J_v = 1{\cdot}1$ nl.$cm^{-2}$ $sec^{-1}$.

(*b*) Transport activities arrested. $j_B = 0$, $J_v = 0$.

(*c*) Solute pumps on with no net solvent flow. $j_B = 2{\cdot}8 \times 10^{-10}$ equiv. $cm^{-2}$.$sec^{-1}$, $J_v = 0$.

(*d*) Solute pump inhibited, passive flow into the cornea. $j_B = 0$, $J_v = -1{\cdot}1$ nl.$cm^{-2}$.$sec^{-1}$.

The magnitude of the fluid flow, $J_v$, is equivalent to the endothelial cells pumping through their own volume about every 6 min.

*Specimen preparation.* Freshly dissected corneas were incubated in the appropriate conditions for 1 hr 30 min at 35 °C and then plunged into fixative where they remained for a further 1 hr. They were dehydrated for 10 min each in an ethanol series (30, 50, 70, 90%, absolute × 2) and then exchanged into propylene oxide for 20 min (× 2). After an over-night period in a 1:1 Araldite (Ciba), epoxy propylene mixture, the corneas were dissected into 1 mm squares and transferred into uncured Araldite for a further 6 hr period. The blocks were then cured at 58 °C over-night.

*Stereological technique.* Sections of nominal thickness 70 nm and oriented orthogonally to the plane defined by the mosaic of the endothelial cells (Hodson, 1969*b*) were cut in the direction stroma to endothelium, in order to standardize any compression phenomenon. They were stained with uranyl acetate and lead citrate and examined at a fixed but nominal magnification of × 5600 in a Philips EM 300 electron microscope. Inevitable fluctuations in the nominal magnification caused by slight alterations in the vertical plane of different specimens, which required an alteration of the objective lens current in order to focus the image, were corrected by noting

the lens current (or, a more accurate marker with the Philips EM 300, the set positions on the potential dividers which regulate lens current) and calibrating these positions against one grating graticule. The magnifications may therefore all be in error by a particular factor (the error of the grating graticule) but that factor is systematically reproduced in all the results reported here. For each experimental protocol (four conditions of incubation fixed under five different conditions of osmotic pressure) six specimens were processed. From each specimen, four sample areas were sectioned into four ribbons. Each ribbon from each sample was separated by at least 50 $\mu$m (i.e. a ribbon of sections was prepared and collected and a further 50 $\mu$m was trimmed off the specimen before another ribbon was prepared). Sample areas to be photographed were selected at very low (scan mode) magnifications, in order to avoid regions of excessive cutting damage which is commonly seen in this tissue but where it was not possible to see any morphological detail. Two sample areas on each graticule were photographed. The electron microscope operator was not aware of the purpose of the experiments nor the coding of the specimens. Negatives were printed on to Ilfospeed paper and the prints were analysed by two methods.

Stereological lattices of the multipurpose test system design (Weibel, Kistler & Scherle, 1966) were laid over the prints. Lattice line intersection frequencies of the basal, lateral and apical membranes of the corneal endothelial cells were recorded in order to determine the relative surface areas of these three components. Additionally, end point frequencies in the intercellular and intracellular spaces were noted. Cell width in these orthogonal sections was measured in each print at five equal intervals and averaged. The prints were then cut with scissors to separate the inter- and intracellular spaces. All the spaces from each print were pooled and weighed. This method gave an independent estimate of the intercellular space volumes and the cellular volumes.

## RESULTS

Increasing the osmotic pressure of the fixative causes the width of the endothelial mosaic (including both cellular volumes and intercellular spaces) to diminish (Fig. 1). This is a result familiar to electron microscopists: hypertonic fixatives cause cells to shrink. At this level of analysis, there seemed to be no differences in the responses of endothelia acting in any of the four transport modes at the time of chemical fixation. When the minority elemental volume in the mosaic, namely, the intercellular spaces, was measured the responses were more complicated.

Intercellular space volume as a function of the molarity of the buffer in the fixative is presented for the four modes of endothelial transport in Fig. 2. When the bicarbonate ion pump is switched off, $j_B = 0$, whether or not there is a passive flow of water across the mosaic, the intercellular space volume diminishes approximately linearly with increasing molarity over the range tested. When $j_B$ is activated, the response is shifted but once again the response is not dependent upon solvent flow. Besides the osmolarity of the fixative solution, the intercellular space volume is dependent upon the activity of the bicarbonate ion pump, $j_B$. In contrast, under the conditions tested the intercellular spaces do not reflect either the degree of solvent coupling or the magnitude of the passive flow of solvent.

Illustration of the structural features associated with the alterations in intercellular space volumes are given in Pls. 1 and 2. Pl. 1 illustrates that the concentration of the buffer in the fixative causes an alteration in the perceived intercellular spaces of the corneal endothelium. The particular cornea illustrated in Pl. 1 was divided into three segments immediately after dissection from a freshly killed rabbit and plunged into the various buffer strength fixatives. Clearly, intercellular spaces can be expanded or diminished during fixation. Pl. 2 shows intercellular space volumes of

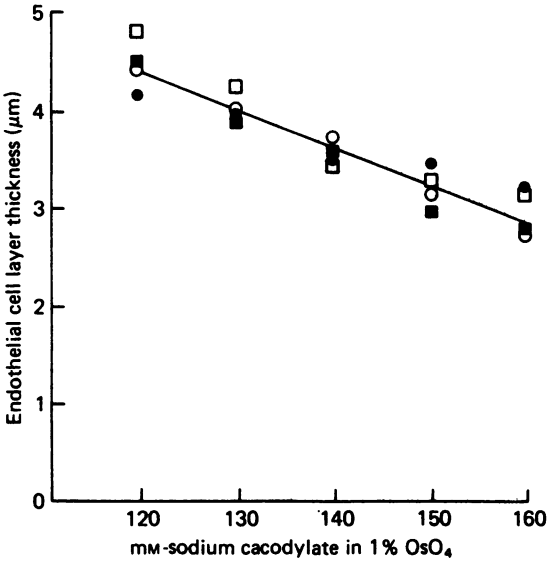



Fig. 1. The corneal endothelial cell layer thickness is observed to narrow as the concentration of buffer in the fixative increases, no matter what the transport mode of the tissue at the time of chemical fixation. Please see legend to Fig. 2 for details of the transport modes.

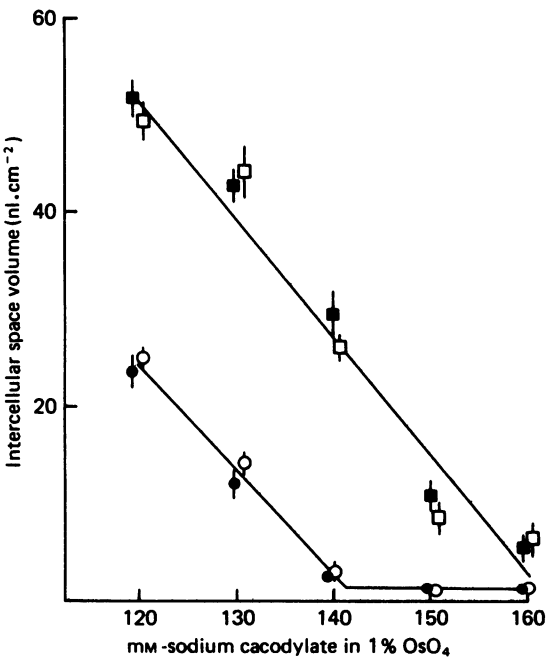



Fig. 2. Intercellular space volume is a function not only of the concentration of buffer in the fixative but also of the activity of the bicarbonate ion pump in the endothelium at the time of chemical fixation. Circles indicate that the bicarbonate pump ($j_B$) is switched on. Squares indicate that the $j_B = 0$. Open circles indicate that whilst $j_B$ is active there is no fluid flow across the endothelium ($J_v = 0$). Filled circles indicate that a solute flow is coupled to $j_B$. Open squares indicate both $j_B = 0$ and $J_v = 0$. Filled squares indicate that whilst $j_B = 0$, there is a passive back flow of fluid across the endothelium. Average values of $j_B$ and $J_v$ are reported in the text.

the cornea fixed in 1% $OsO_4$ buffered at pH 7·4 by 0·15 M-sodium cacodylate when it is engaged in three different transport modes and demonstrates a phenomenon germane to the discussion following on in this paper. In Pl. 2*A*, the active transport of bicarbonate ions was switched off and there was no flow of fluid across the tissue ($j_B = 0$ and $J_v = 0$) immediately before the cell mosaic was immersed in fixative. In Pl. 2*B*, the active transport of bicarbonate ions was activated and there was a coupled fluid flow across the endothelium. These images are consistent with Diamond & Bossert's theory for 'backward' transporting epithelia. In Pl. 2*C*, active transport of bicarbonate ions was taking place but there was no coupled fluid flow. The intercellular spaces remained (relatively) closed.

Relative areas of the membrane surfaces bounding the apical, lateral and basal planes of the cell were not measurably altered by any of the experimental protocols investigated here. They were 1·05:2·10:1·00.

## DISCUSSION

In the course of this investigation, two parameters were noted to have an effect upon intercellular space volumes in the corneal endothelium of rabbit after chemical fixation. They were (*a*) the concentration of buffer in the fixative and (*b*) the activity of the electrogenic bicarbonate ion pump, $j_B$. Two parameters had no measurable effect upon intercellular space volume. They were (*a*) passive fluid flow and (*b*) fluid flow coupled to the bicarbonate ion pump.

In our experiments, the opening and closing of the intercellular spaces cannot be used as evidence for or against the idea that this is the compartment where the solvent flow is coupled to the activity of the solute pumps. The results may be seen as a caution against using chemically fixed tissue as evidence to support the standing-gradient hypothesis. If only the two modes of transport (*a*) solute pumps on accompanied by a coupled solvent flow and (*b*) no transport activities are used then it is shown here that although it may seem that we have evidence in favour of the standing-gradient hypothesis (Pl. 2*A* and *B*), the third transport mode (Pl. 2*C* and Fig. 2) disposes of the evidence.

Tormey & Diamond (1967) working with 'forward' transporting gall-bladder state that 'the fact that the lateral spaces are open during transport and closed in the absence of transport suggests but does not prove that fluid transport proceeds through the spaces'. We propose that in the present work on 'backwards' transporting corneal endothelium this suggestion is negated by our experimental demonstration that the width of the intercellular spaces is not a function of fluid transport. We emphasize, however, that our work in no way disproves the standing-gradient hypothesis. Although it was not our intention, when we initiated this investigation, to indicate that the examination of chemically fixed tissue is irrelevant in this particular study, such does seem to be the case.

From our data (Fig. 2) it seems likely by extrapolation along the abscissa that there might be conditions of fixation where all four transport modes could show similar intracellular space volumes. It is possible that the two functions ($j_B$ on, $j_B$ off) are similar except for an abscissa displacement of 18 mM-sodium cacodylate which we have measured to equal 35 m-osmole. Data such as these suggest an interpretation

of the phenomena we record here but we do not wish to expand on these interim speculations because we do not know what property associated with buffer concentration causes the effect on intercellular spaces. Both osmotic pressure and ionic strength are candidates for investigations. Some preliminary investigations strongly suggest that the effects are independent of the chemical nature of the buffer and that they occur within the first few seconds of exposure to the chemical fixative but we do not propose to examine these phenomena further.

This investigation was supported by a grant from The Wellcome Trust. Mr R. Mitha rendered technical assistance in the preliminary studies.

## REFERENCES

DIAMOND, J. M. (1964). The mechanism of isotonic water transport. *J. gen. Physiol.* **48**, 15–42.

DIAMOND, J. M. & BOSSERT, W. H. (1967). Standing-gradient osmotic flow. *J. gen. Physiol.* **50**, 2061–2083.

DIAMOND, J. M. & BOSSERT, W. H. (1968). Functional consequences of ultrastructural geometry in 'backwards' fluid-transporting epithelia. *J. cell Biol.* **37**, 694–702.

DIAMOND, J. M. & TORMEY, J. M. (1966). Role of long extracellular channels in fluid transport across epithelia. *Nature, Lond.* **5038**, 817–820.

DIKSTEIN, S. & MAURICE, D. M. (1972). The metabolic basis to the fluid pump in the cornea. *J. Physiol.* **221**, 29–41.

HILL, A. E. (1975). Solute-solvent coupling in epithelia: a critical examination of the standing-gradient osmotic flow theory. *Proc. R. Soc.* B **190**, 99–114.

HODSON, S. (1968). Inadequacy of aldehyde fixatives in preserving the ultrastructure of corneal endothelium in rabbit and monkey. *Expl Eye Res.* **2**, 221–224.

HODSON, S. (1969*a*). Observations on the posterior membrane of the corneal endothelium. *Expl Eye Res.* **8**, 99–101.

HODSON, S. (1969*b*). Light-microscopic radioautography utilising araldite sections. *J. Microsc.* **89**, 113–120.

HODSON, S. (1971*a*). Evidence for a bicarbonate-dependent sodium pump in corneal endothelium. *Expl Eye Res.* **11**, 20–29.

HODSON, S. (1971*b*). Why the cornea swells. *J. theor. Biol.* **33**, 419–427.

HODSON, S. (1974). The regulation of corneal hydration by a salt pump requiring the presence of sodium and bicarbonate ions. *J. Physiol.* **236**, 271–302.

HODSON, S. & MAYES, K. R. (1978). Intercellular spaces and coupled water transport in the rabbit cornea. *J. Physiol.* **284**, 146*P*.

HODSON, S. & MILLER, F. (1976). The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. *J. Physiol.* **263**, 563–577.

HULL, D. S., GREEN, K., BOYD, M. & WYNN, H. R. (1977). Corneal endothelium bicarbonate transport and the effect of carbonic anhydrase inhibitors on endothelial permeability and fluxes and corneal thickness. *Invest. Ophthal.* **16**, 883–892.

MAURICE, D. M. (1972). The location of the fluid pump in the cornea. *J. Physiol.* **221**, 43–54.

MAYES, K. R., GRAHAM, M. V. & HODSON, S. (1978). The deleterious effects of exogenous bicarbonate on the rabbit cornea undergoing prolonged refrigerated storage. *Expl Eye Res.* **26**, 555–560.

MAYES, K. R. & HODSON, S. (1978). Local osmotic coupling to the active trans-endothelial bicarbonate flux in the rabbit cornea. *Biochim. biophys. Acta* **514**, 286–293.

MILLS, J. W. & DI BONA, D. R. (1978). Distribution of Na pump sites in the frog gall bladder. *Nature, Lond.* **271**, 273–275.

TORMEY, J. M. & DIAMOND, J. M. (1967). The ultrastructural route of fluid transport in rabbit gall bladder. *J. gen. Physiol.* **50**, 2031–2060.

WEIBEL, E. R., KISTLER, G. S. & SCHERLE, W. F. (1966). Practical stereological methods for morphometric cytology. *J. cell Biol.* **30**, 23–38.

## EXPLANATION OF PLATES

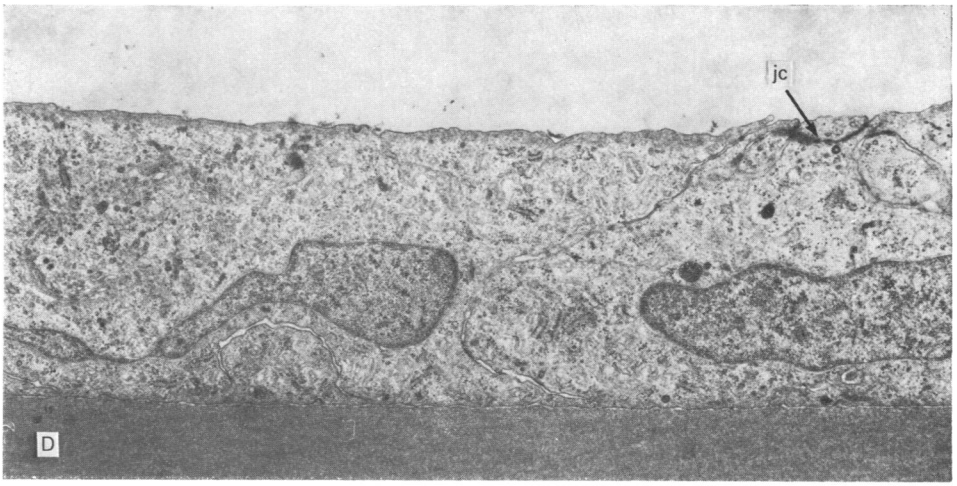
### PLATE 1

The endothelium of fresh rabbit cornea fixed in 1% $OsO_4$ buffered at pH 7·4 by (*A*) 160 mM, (*B*) 140 mM, (*C*) 120 mM-sodium cacodylate. Lower concentrations of buffer cause a progressive dilation of the intercellular spaces. The junctional complex, jc, remains intact. D = Descemet's membrane. Bar represents 5 $\mu$m.

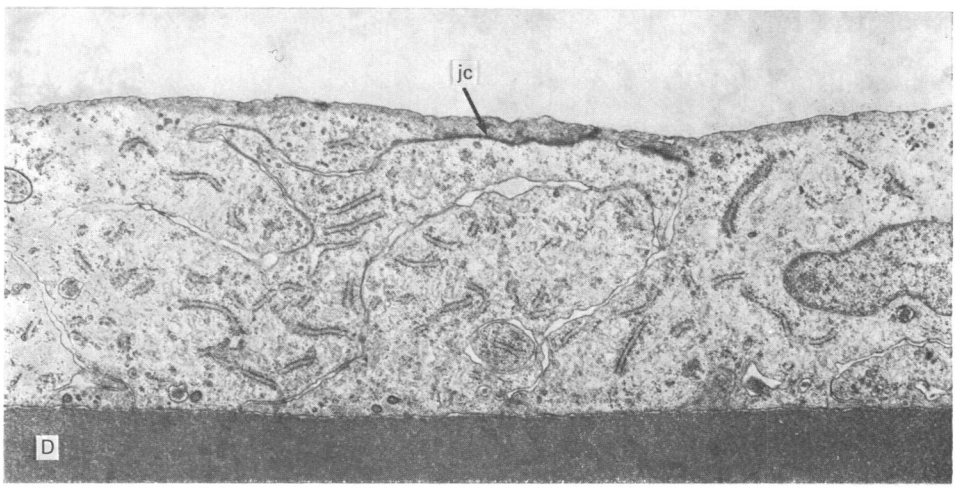
### PLATE 2

All these endothelia were fixed in 1% $OsO_4$ buffered at pH 7·4 by 150 mM-sodium cacodylate. In *A* both the bicarbonate ion pump ($j_B$) and trans-endothelial fluid flows ($J_v$) were zero. In *B*, the bicarbonate ion pump ($j_B$) of the cells was active and a coupled fluid flow ($J_v$) passed across the cells. The intercellular spaces contracted as predicted by the standing-gradient hypothesis for 'backwards' transporting epithelia. In *C*, the bicarbonate ion pump ($j_B$) was activated but there was no fluid flow across the cells. The intercellular spaces remained contracted suggesting that under the experimental conditions reported here, the intercellular space contraction does not represent the site of local osmotic coupling. D = Descemet's membrane. Bar represents 5 $\mu$m.

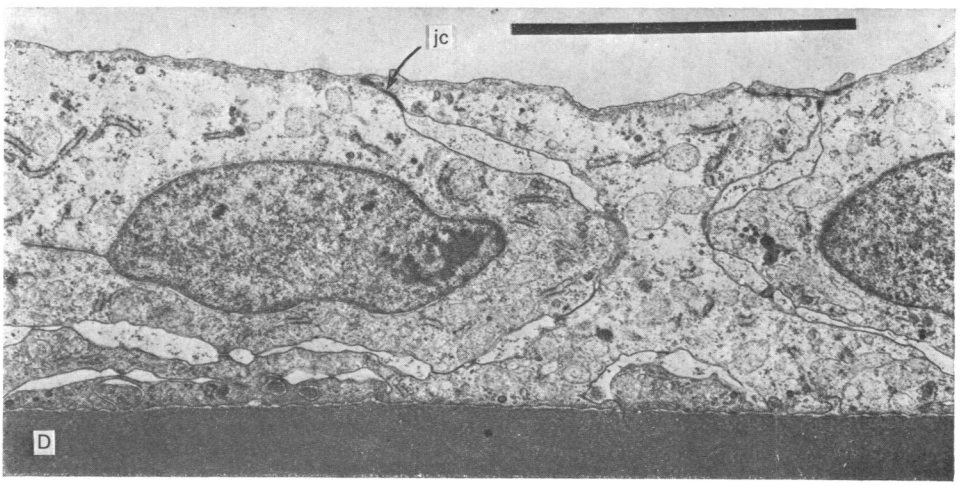
A

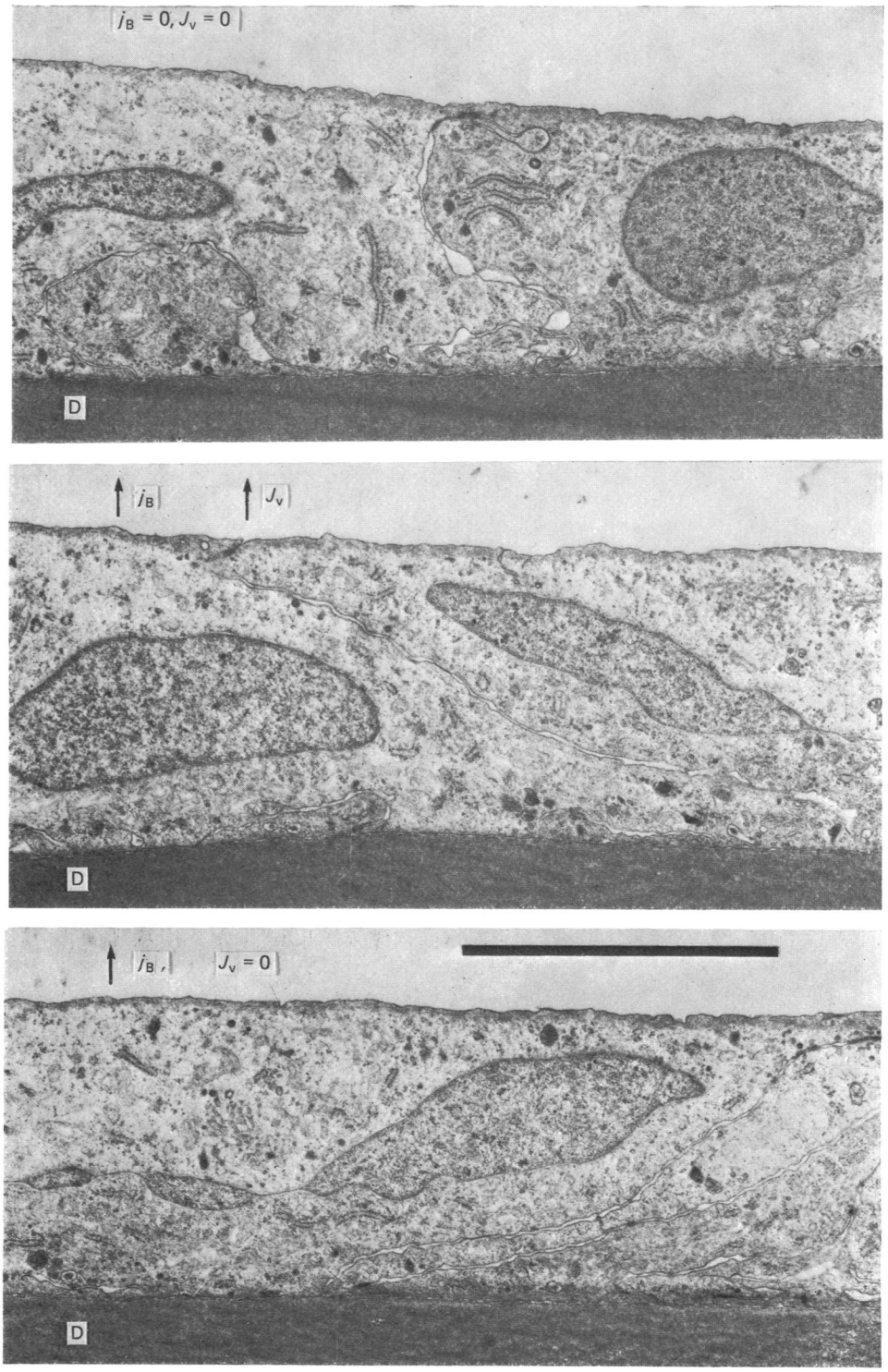



B




C






S. HODSON AND K. R. MAYES