# Effect of Bicarbonate-free Balanced Salt Solutions on Fluid Pump and Endothelial Morphology of Rabbit Corneas In-vitro

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**Abstract**—Corneas from young adult albino rabbits were exposed, in-vitro, to various bicarbonate-free balanced salt solutions under an applied hydrostatic pressure of 20 cmH$_2$O at 35°C for up to 4·5 h. The solutions were buffered with a HEPES-MOPS mixture, phosphate salts or acetate-citrate salts (pH 7·1–7·4). All of these solutions support a net fluid pump activity attributed to the corneal endothelium although the net fluid pump rates were less than those measurable with a bicarbonate-buffered, CO$_2$-equilibrated Ringer solution. Evaluation of the endothelia by scanning electron microscopy revealed no evidence of any acute toxic effect of the balanced salt solutions. Morphometric analyses of cell size and shape revealed a normal mosaic for endothelia exposed to commercial balanced salt solution for 5 h. The results further indicate that exogenous bicarbonate is not required for the net fluid pump function of the mammalian corneal endothelium.

Intraocular irrigating solutions are routinely used to fill and irrigate the anterior chamber of the eye during surgical procedures (Glasser & Edelhauser 1989; Closson et al 1990), basically because their use provides a superior post-operative prognosis when compared with the use of air to maintain the shape and volume of the anterior chamber (Apsitis & Ziangirova 1979; Samuelly et at 1989). Any such solutions could have a toxic potential and so a number of laboratory investigations and clinical studies have been carried out to assess the effects of irrigating solutions on the endothelial cell layer of the cornea.

Laboratory investigations on the effects of balanced salt solutions on the mammalian corneal endothelium have used a specular microscope perfusion system (Dikstein & Maurice 1972) that allows measurements of central corneal thickness along with qualitative assessment of the morphology of the cells in the corneal endothelial monolayer (e.g. Edelhauser et al 1976). The rationale of such studies is based on a concept that links the integrity of the corneal endothelium to the hydration balance of the corneal tissue. Any toxic effect of intraocular solutions on the corneal endothelium may result in passive fluid movement into the corneal tissue with a resultant increase in corneal thickness (Maurice 1984). The structural integrity of the corneal endothelium is commonly evaluated by scanning electron microscopy of the endothelium after in-vitro perfusion with the balanced salt solutions (Edelhauser et al 1975, 1981, 1983; Gonnering et al 1979; Glasser et al 1988). The specular microscope has also been used to evaluate the corneal endothelium after intracameral injection of commercially available balanced salt solutions (Frezzotti et al 1985). Similarly, scanning electron microscopy has been used to assess the effects of total immersion of isolated corneas in the same solution in-vitro (Bonafonte et

al 1985/1986). In either type of study, the scanning electron microscopy evaluation of the endothelium has been qualitative in that the region of the cornea evaluated was not defined and no numerical (morphometric) assessment of the endothelium was carried out.

In contrast, a number of animal (Glasser et al 1985; Samuelly et al 1989) and human (Komine 1982; Kline et al 1983/1984; Leonardi et al 1989) studies have been reported where the same balanced salt solution was used to irrigate the anterior chamber and the endothelium evaluated by clinical specular microscopy in-vivo. Such endothelial assessment has been quantitative. Common morphometric parameters and measures include assessments of endothelial cell density, endothelial cell surface area (and its variation) and cell shape (the percentage of 6-sided cells (hexagons) (Doughty 1989b)). Significant, but reversible, changes in these morphometric parameters were reported in these studies.

Since even single intracameral injections (Samuelly et al 1989) or a 30 min anterior chamber irrigation with balanced salt solutions (Glasser et al 1985) has been reported to produce changes in the endothelial morphometric parameters within 2 days, it is logical to consider how rapidly such changes may occur and whether such morphometric changes are detectable in acute exposure experimental in-vitro studies e.g. with exposure to commercial balanced salt solutions. Several bicarbonate-free balanced salt solutions were compared, since it has been stated that endothelial fluid pump function of the cornea can be adversely affected by a lack of extracellular bicarbonate (Glasser et al 1988) or even that such solutions are toxic to the endothelium (Matsuda et al 1984).

## Materials and Methods

*Balanced salt solutions*
Commercial balanced salt solutions were obtained from Iolab Corporation (Claremont, CA) (Iocare BSS, BSS-1) and Cooper Vision Pharmaceuticals (Mountain View, CA)

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Table 1. Composition of endothelial irrigating solutions (mM).

| | Commercial balanced salt solution | Phosphate Ringer[c] | HEPES-MOPS Ringer[d] | Bicarbonate Ringer[e] |
|---|---|---|---|---|
| NaCl | 156 | 138 | 78 | 93 |
| KCl | 10 | 2·7 | 5·5 | 5·5 |
| MgCl$_2$ | 1·5 | 1·1 | 0·9 | 0·9 |
| CaCl$_2$ | 3·3 | 1·2 | 1·8 | 1·8 |
| NaHCO$_3$ | — | — | — | 35 |
| Na-citrate | 5·8 | — | — | — |
| Na-acetate | 28·6 | — | — | — |
| Na$_2$HOP$_4$ | — | 8·1 | — | — |
| KH$_2$PO$_4$ | — | 1·5 | — | — |
| Na-MOPS buffer | — | — | 25 | — |
| Na-HEPES buffer | — | — | 25 | — |
| Glucose | — | 6·9 | 5·5 | 5·5 |
| Adenosine | — | — | 1·0 | 1·0 |
| Glutathione | — | — | 0·1 | 0·1 |
| pH | 7·2[a] | 7·35 | 7·45 | 7·55 |
| Osmolarity (mOsm kg$^{-1}$) | 300[b] | 302 | 307 | 297 |

[a] The pH was not stable at 35°C. A freshly opened bottle had pH values close to 7·0 but these could rise to close to 7·4 within 30 min. Therefore all solutions were used 15 min after placement at 35°C (pH 7·15–7·5). [b] Osmolarity range, 298–306 mOsm kg$^{-1}$. [c] Kuang et al (1990). [d] Doughty & Maurice (1988). [e] Doughty (1985); this solution was equilibrated with 5% CO$_2$–air before use.

(Cooper Vision BSS, BSS-2). The compositions of salt solutions are shown in Table 1.

## Animals and tissues

Female New Zealand White rabbits, 2·1–2·3 kg, were used as the source of corneal tissue. The animals were housed in Canadian Council for Animal Care (CCAC)-approved quarters and had free access to food and water. All procedures were approved by the University of Waterloo, CCAC-supervised animal care committee. The animals were routinely monitored for 6–8 days to allow adequate recovery from shipment and adaptation to an artificial light:dark cycle of 14:10 h (with the light cycle starting at 0600 h). After having their eyes checked by biomicroscopy (Doughty & Cullen 1989), the animals were killed between 1400 and 1530 h with 120 mg kg$^{-1}$ pentobarbitone sodium (Euthanyl), intravenously. The eyes were enucleated with their lids as previously detailed (Doughty 1985) and the corneas prepared for experiments within 20 min.

## Experimental procedures

The external corneal dimensions in the horizontal and vertical meridian were measured with a vernier caliper (to 0·25 mm) and two measurements of central corneal thickness made using an ultrasound pachometer (Doughty 1989a). Two procedures were then used. In the first procedure, after removal of the corneal epithelium (Doughty 1985), the resultant stroma-endothelium preparations were mounted between two half-chambers as previously detailed (Doughty & Maurice 1988). Over the next 4–4·5 h, transendothelial net fluid flow was assessed at 35±1°C under an applied hydrostatic pressure of 20 cmH$_2$O by making observations of the position of a meniscus on a capillary line every 10 min. Both chambers were filled with pre-warmed experimental salt solution and the contents of both chambers exchanged for fresh solutions every 60 min (Doughty & Maurice 1988). At the end of the experiment, the endothelial-side chamber was flushed with pre-warmed fixative solution and then filled

with the fixative solution (see below). The second procedure involved similar measurements of corneal thickness and dimensions before the epithelium was coated with Dow Corning medical grade 200 dielectric fluid (silicone oil, 20 cs viscosity) and the cornea prepared for in-vitro perfusion using a laboratory specular microscope following published procedures (Dikstein & Maurice 1972; Doughty 1985, 1989a). The endothelium was perfused at 35°C with the balanced solution under an applied hydrostatic pressure of 20 cmH$_2$O for 3·5–4 h. The epithelium was kept covered with silicone oil. At the end of the perfusion with balanced salt solution, the perfusion solution was changed to pre-warmed fixative. Six to eight separate corneal preparations were studied for most solutions. With both methods, after 60–90 min exposure to the fixative solution, the cornea was removed from its support ring, thickness and limbal dimensions measured and a 9 mm diameter button removed using a surgical trephine.

## Measurements and observations

The fixative was a freshly prepared solution of 2% w/v high purity glutaraldehyde dissolved in 80 mM sodium cacodylate buffer, pH 7·2–7·4 (with dilute HCl). The total bulk solution osmolarity, determined by vapour pressure (Wescor 5500 osmometer, Wescor Inc., Logan, VT, USA) was 330–340 mOsm kg$^{-1}$. The solution, in an amber glass bottle, was warmed to 35°C shortly before use. Ultraviolet spectroscopy was routinely used to ensure that the solutions were nominally free of polymers (Tashima et al 1988). The 9 mm corneal button (see above) was placed in a vial of fresh fixative and refrigerated. After 7–10 days, the button was post-fixed by immersion in a fresh solution of 1% w/v OsO$_4$ in NaCl (total osmolarity of 330–340 mOsm kg$^{-1}$) at room temperature (21°C) for 1 h under subdued lighting. The button was rinsed thoroughly with 1% NaCl and then progressively transferred through a series of 1% NaCl: methanol mixtures (50:50, 25:75, 10:90 v/v) at 1 day intervals before being immersed in methanol. Half of the

Table 2. Fluid pump activity ($\mu$L h$^{-1}$) of stroma-endothelium preparations in-vitro ($\pm$s.e.m. for (n) preparations).

| | Net fluid pump in different time periods after start of experiment | | |
|---|---|---|---|
| | 2nd h | 3rd h | 4th h |
| BSS-1 (8) | 0·76±0·32 | 1·99±0·41 | 1·92±0·32 |
| BSS-2 (4) | 1·14±0·56 | 1·94±0·29 | 2·40±0·73 |
| Phosphate-buffered Ringer (6) | 2·56±0·27 | 3·21±0·22 | 3·56±0·29 |
| HEPES-MOPS buffered Ringer (6) | 4·26±0·24 | 4·76±0·21 | 4·66±0·46 |
| Bicarbonate-Ringer (8) | 5·65±0·72 | 5·43±0·12 | 5·87±0·23 |

button was then critical-point dried out of $CO_2$ via propylene oxide at 55°C. The dimensions of the dried preparation was measured. After gold-coating, the samples were viewed in a Hitachi S570 scanning electron microscope using an accelerating voltage of 15 kV at a working distance of 8 mm. A set of micrographs (on PAN F film) were taken normal to the corneal surface at × 100 to × 5000 at-stage magnification at central, mid-peripheral and peripheral sites on the button.

Central cornea micrographs taken at × 500 at-stage magnification were printed at 10 × 8 in, the cell borders highlighted with a fine black marker pen and the areas of 200 to 300 tesselated cells (on the same micrograph) measured using a digitizer tablet (Doughty 1990a). Area data were entered into a computer program written in CBASIC to convert digitizer pad values into absolute values by use of the micrograph scale marker and applying corrections for tissue shrinkage due to fixation and drying (based on the measurements made on the samples—see above). The assembled files

were then read into SYSTAT (Systat Inc, Evanston, IL, USA) for sorting, evaluation of cell area variance and statistical analyses (Doughty 1990b). A level of significance for differences between samples was set at the 95% confidence level ($P < 0.05$).

### Results

#### Fluid pump activity

Stroma-endothelium preparations were mounted between half-chambers and assessed for their ability to translocate fluid against an applied hydrostatic pressure—the net fluid pump activity. The changes in meniscus position in each 10 min interval were converted to equivalent volume h$^{-1}$ values and the values averaged (Fig. 1). For all solutions tested, net fluid flow was initially into the corneal tissue in the same direction as the applied hydrostatic pressure. By convention, these fluid flow values are given negative values because they reflect fluid leakage into the cornea. Within 40 to 70 min, a positive flow out of the corneal tissue (the stroma to aqueous humour direction) developed for most preparations. This net fluid pump activity against the applied hydrostatic pressure was sustained apart from some fluctuations occurring shortly after changing of the chamber contents for fresh solutions.

Several experimental solutions (Table 1) were evaluated in the same study to permit objective assessment of the ability of two commercial balanced salt solutions (BSS) to support net fluid pump activity. Exposure of stroma-endothelium preparations to balanced salt solution 1 (BSS-1) resulted in a substantial negative fluid flow in the first 30 min ($-7$ $\mu$L h$^{-1}$) but only a slight negative fluid flow between 40 and 60 min (average values over this period of $-2.74 \pm 1.33$ $\mu$L h$^{-1}$). Over the second hour after exposure to BSS-1, the net fluid pump averaged $0.76 \pm 0.32$ $\mu$L h$^{-1}$ and the net fluid pump rates rose to nearly three times this value over the third and fourth hours (Fig. 1, Table 2). Very similar values were obtained for another commercial balanced salt solution, BSS-2, which has the same nominal composition (Table 2). The use of a phosphate-buffered, glucose-supplemented balanced salt solution gave slightly higher net fluid pump values compared with BSS-1 and BSS-2 which are citrate-acetate buffered (Table 2). Another bicarbonate-free balanced salt solution was also evaluated for comparison but in this case a solution buffered with a mixture of HEPES and MOPS buffers was used. This HEPES-MOPS-buffered Ringer solution was also supplemented with glucose, adenosine and glutathione and produced average net fluid pump rates of close to 5 $\mu$L h$^{-1}$ at all three time periods of evaluation: these rates were all significantly higher than those
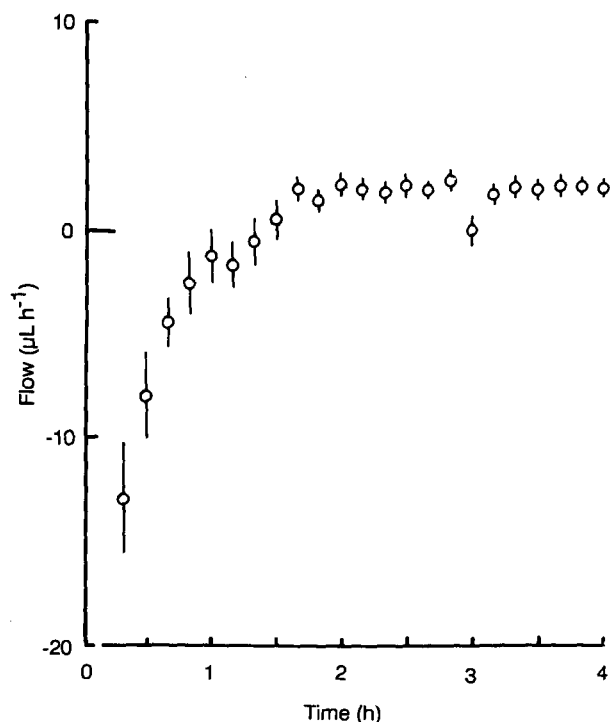


FIG. 1. Fluid flow across stroma-endothelium preparations in-vitro with commercial balanced salt solution (BSS-1) at 35°C with an applied hydrostatic pressure of 20 cmH$_2$O. The calculated fluid flow values in each 10 min interval are presented as the mean values ±s.e. for eight separate preparations. A positive value indicates net fluid pumping against the hydrostatic pressure.
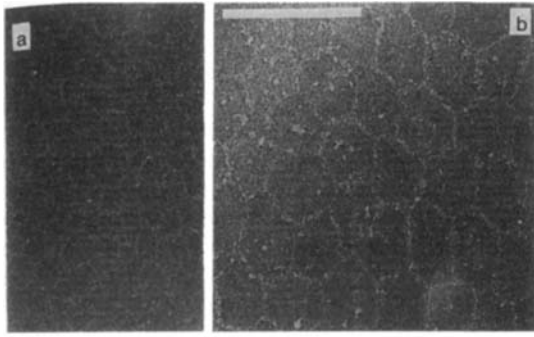
FIG. 2. Representative scanning electron micrographs of central endothelium taken at × 500 (a) and × 2000 (b) at-stage magnification after exposure of corneal stroma-endothelium preparations to balanced salt solution (BSS-1) in half-chambers to measure fluid flow. Bar indicates 60 μm (a) and 15 μm (b) (uncorrected for tissue shrinkage).



FIG. 4. Representative scanning electron micrographs of central endothelium taken at × 5000 at-stage magnification after exposure to balanced salt solution (BSS-1) in half-chambers (a) or by perfusion (b). Arrows indicate apical flaps of endothelial cell-cell junctions. Bar on micrograph equals 6 μm (uncorrected for tisssue shrinkage).

seen with the first three solutions. For comparison, the net fluid pump activity was measured in a 35 mM bicarbonate-containing balanced salt solution that was also supplemented with glucose, adenine and glutathione. This bicarbonate Ringer clearly produced higher fluid pump values compared with all other solutions with average values close to 6 μL h$^{-1}$ (Table 2).

*Morphology*

The primary object of this study was the assessment of simple balanced salt solutions to sustain fluid pumping and to assess the morphological appearance of the corneal endothelium after exposure to such solutions. Since the fluid pump function has not been reported for these solutions previously, the perfusion method used before other electron microscopic studies was also used. Thus, a set of corneas was also perfused with BSS-1 rather than simply incubated in these solutions. The perfusion time was the same as that for the incubation studies in the half-chambers so that the results of scanning electron microscopy could be compared.

The fixed endothelium was free of any gross physical artifacts beyond the occasional pieces of microscopic adherent debris. For all samples, there were no creases, undulations or other distortions of the endothelial cell layer.
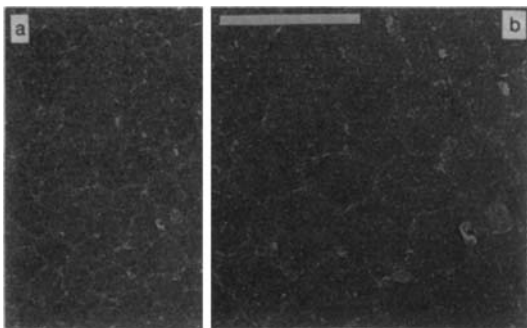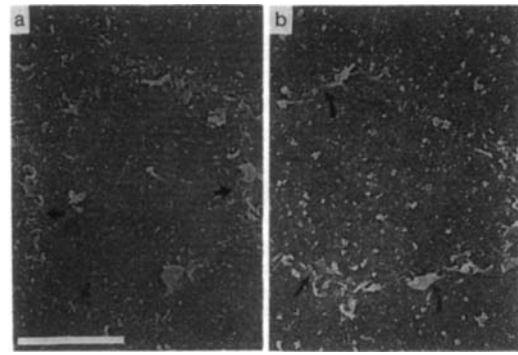
At × 500 at-stage magnification, the endothelial mosaic is resolved with the cell-cell borders being visible as thin white lines on the scanning electron micrographs (Figs 2, 3). The appearance of the endothelium was qualitatively similar regardless of the experimental method used, i.e. non-perfused (Fig. 2) vs perfused (Fig. 3) although the size of the cells appears slightly smaller with the former method (compare Figs 2a and 3a). In addition, following perfusion exposure to BSS-1, there was a greater incidence of small bleb-like flaps at the cell-cell borders (Fig. 3b) while the cell-cell borders appear more complex in the non-perfused endothelia (Fig. 2b). This complexity is better evident at higher magnification ( × 5000) (Fig. 4). Comparisons of the two methods indicates that there are more interdigitated flaps for the apical cell-cell borders for the non-perfused endothelia (Fig. 4a) compared with perfused endothelia (Fig. 4b). With the perfused endothelia, the apical flap-like interdigitations often seemed to be longer. In both cases, the endothelial surface was decorated with a modest number of stubby microvilli (Fig. 4). In evaluation of over 100 micrographs at × 500, × 2000 and × 5000, there was no evidence of cell pitting, substantial cell blebs, or cellular oedema or a central bulge in the region of the cell nucleus.
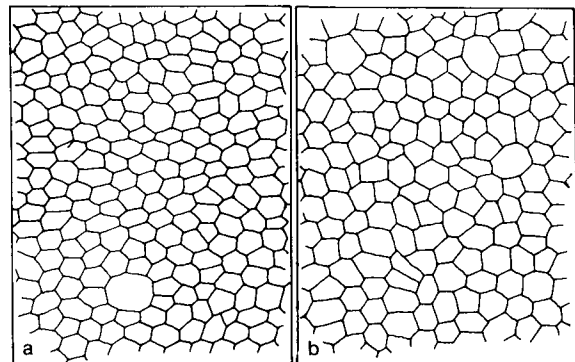


FIG. 3. Representative scanning electron micrographs of central endothelium taken at × 500 (a) and × 2000 (b) at-stage magnification after exposure of whole corneas to balanced salt solution (BSS-1) by perfusion. Bar indicates 60 μm (a) or 15 μm (b) (uncorrected for tissue shrinkage).



FIG. 5. Overlays of scanning electron micrographs taken at × 500 at-stage magnification to show cell border marking (for digitization) and identification of cells by their number of sides. (a) Half-chambers; (b) perfusion method.

Table 3. Evaluation of endothelial cell size by scanning electron microscopy after exposure to commercial balanced salt solution, BSS-1.

| | Average cell density (mm²) | Average cell area (μm²) | Average standard deviation | Coefficient of variation of cell area | Skewness (cell area) n = 8 | Kurtosis (cell area) n = 8 |
|---|---|---|---|---|---|---|
| Experiment 1 | 3437 | 307 | 67 | 21·5 | 0·776 | 2·823 |
| Experiment 2 | 2344 | 417 | 99 | 24·9 | 0·810 | 1·941 |

Experiment 1. Corneal stroma-endothelium preparations were exposed to BSS for 4–4·5 h in half-chambers. Experiment 2. Whole corneas perfused with BSS for 3·5–4 h in a specular microscope.

Cell density was calculated by dividing the 1 000 000 μm² by the cell area in each case and calculating the average value from eight preparations in each case. The cell densities are significantly lower for perfused cornea preparations as a result of the experimental procedures. Since the distribution of cell areas are within normal limits of a Gausian distribution (assessed by Kolgmogorov-Smirnov test), the coefficient of variation, (standard deviation of cell area/average cell area) × 100 is presented for comparison with other studies (Doughty 1990b).

Table 4. Morphometric analyses of rabbit corneal endothelium exposed to commercial balanced salt solution BSS-1, in-vitro.

| | % 4-sided cells | % 5-sided cells | % 6-sided cells | % 7-sided cells | % 8-sided cells | % 9-sided cells | % 10-sided cells |
|---|---|---|---|---|---|---|---|
| Experiment 1 (8) | 0·1 | 19·2±1·1 | 63·3±1·5 | 16·4±0·5 | 1·1±0·2 | 0·1 | 0·1 |
| Experiment 2 (8) | 0·2 | 19·3±1·3 | 63·4±2·1 | 15·9±1·0 | 1·3±0·3 | 0·3 | — |

Experiment 1. Corneal stroma-endothelium preparations were exposed to BSS for 4–4·5 h in half-chambers. Experiment 2. Whole corneas perfused with BSS for 3·5–4 h in a specular microscope.

Similar sets of micrographs were obtained after exposure of the endothelium to the other bicarbonate-free balanced salt solutions and also for the bicarbonate-Ringer solution (micrographs not shown).

*Cell measurements*

The cell sizes were quantitatively measured from micrographs taken at × 500. Tracing overlays of representative micrographs are given in Fig. 5. Cell area data from each endothelium were averaged to produce grouped frequency percent distribution plots (Fig. 6). While the distributions of cell areas are clearly close to that expected for normally distributed samples for both the non-perfused, half-chamber-incubated (Fig. 6a) and the specular microscope perfused endothelia (Fig. 6b), the cell areas were significantly larger for the perfused endothelia (Table 3). The variance in cell areas, as assessed by the average standard deviation of the cell areas was modest (Table 3). That the overall distributions of cell areas were reasonably close to that expected for a normally distributed sample is indicated by the modest coefficient of skewness and kurtosis values (Table 3). The morphometry thus indicates that the endothelial mosaic was relatively uniform and that sample-to-sample variance was modest.

The cells are also easily identified by the number of sides, e.g. 5-, 6- or 7-sides (or bordering cells). The relative percentages of each of these cell types in the samples are calculated by the computer program (Table 4). For endothelia exposed to commercial balanced salt solutions, the percentage of 6-sided cells ranged from 54·6 to 73·3% with average values close to 64% for both half-chamber-incu-
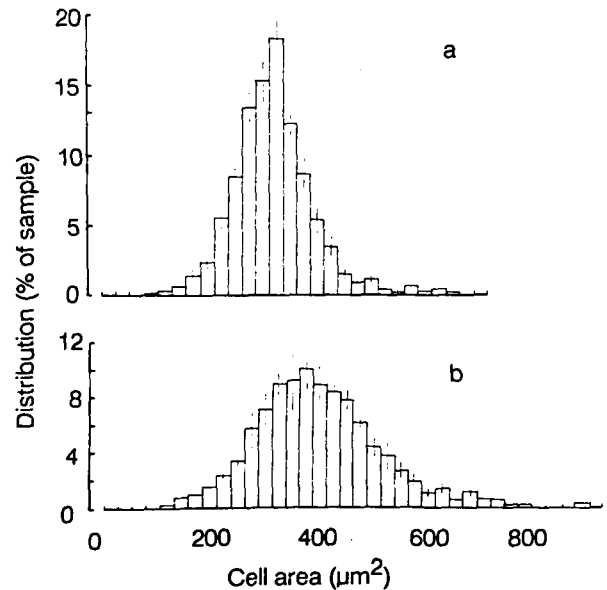


Fig. 6. Histograms to show distribution of cell areas at central region of corneal endothelium for corneas exposed to balanced salt solution (BSS-1) either in half-chambers (a) or by perfusion (b). The height of each bar reflects the mean value (±s.e.) for eight separate corneas.

bated and perfused endothelia (Table 4). Most of the rest of the cells were either 5-sided (range 14·4–25·8%) or 7-sided (range 13·3–22·1%). The occasional 4-, 8-, 9- or even 10-sided cell (e.g. Fig. 5a) was also evident but there were no obvious differences between the two sets of corneas.

## Discussion

These studies were designed to assess qualitatively and quantitatively if there was any evidence of a toxic effect of bicarbonate-free balanced salt solutions on the rabbit corneal endothelium in-vitro. It is acknowledged that caution should be exercised in applying these results to human tissue, especially to corneas from patients over 60 years of age. At the very least, further studies are needed on corneas from much older rabbits (i.e. 2-3 years of age; studies in progress). However, within the limits of the rabbit model, the results can be assessed from three perspectives—the measure of a physiological function assigned to the corneal endothelium (the fluid pump), gross morphological appearance of the endothelial mosaic and the individual cells and, finally, by morphometric methods (to permit a comparison with in-vivo morphometric data obtained by other investigators).

The measurement of transendothelial net fluid flow was chosen as a measure of corneal physiology since it is the most direct measurement of endothelial cell layer viability. While the technique only measures the net fluid flow, any significant deterioration of the endothelial cell layer (local or diffuse) will result in a net leakage of fluid in the direction of the applied hydrostatic pressure (Doughty 1989a). Net fluid pump activity has previously been found to be the same from 2-50 mM exogenous bicarbonate in equilibrium with 5% $CO_2$-air (Doughty & Maurice 1988), the net pump rate averaging 5 $\mu$L h$^{-1}$. Similarly, with bicarbonate-free Ringer solutions buffered with 50 mM organic buffers (e.g. HEPES), similar fluid pump rates were also found. A phosphate-buffered (bicarbonate-free) Ringer solution has also been reported to support a net fluid pump activity (Kuang et al 1990). Although bicarbonate-free, these Ringer solutions are different from commercial balanced salt solutions since they contain glucose (Table 1). The present studies clearly show that such glucose-free balanced salt solutions can support a net fluid pump activity at least over a few hours with the rate of net pump activity being only slightly lower than that observed with a glucose-supplemented and phosphate-buffered Ringer solution evaluated under the same hydrostatic pressure. This parameter is important since net fluid pump activity has been reported to decrease as the hydrostatic pressure is raised (Fischbarg et al 1977). Overall it would appear that the presence of glucose is beneficial but that the effect of its presence is not substantial in the short term. The endothelial fluid pump functions presumably via intracellular glycogen reserves (Maurice 1984). Notwithstanding, these studies are presented as further evidence that the net fluid pump activity of the mammalian corneal endothelium can persist in the absence of exogenous (extracellular) bicarbonate. Thus, while any bicarbonate-free solution can cause substantial swelling of the cornea (Fischbarg & Lim 1974; Edelhauser et al 1976; Weekers & De Thinne 1978; Glasser et al 1988), the stromal swelling cannot thus be obviously attributed to a failure of the endothelial fluid pump as a result of the absence of bicarbonate. Both corneal swelling rates (Doughty 1991a) and corneal deswelling rates (Doughty 1985, 1987) can change by at least an order of magnitude on lowering extracellular bicarbonate from 35 to 2 mM. In contrast, the net fluid pump decreases by only a factor of two over the same bicarbonate range. Therefore,

the role of the endothelial fluid pump in control of hydration levels of the cornea can only, at best, be supportive rather than being a pre-requisite for this control. As discussed in detail elsewhere (Doughty 1991a, b), current studies indicate that it is a separate mechanism responsible for stromal hydration itself that appears to be bicarbonate-sensitive.

The present studies provide no indication that bicarbonate-free balanced salt solutions elicit an obvious toxic effect on the endothelial cell layer. The morphometric assessments clearly show that the sample-to-sample reproducibility was good. On the basis of this standardization, the differences between the micrographs presented in this study and those reported by other workers can be addressed. Thus, the areas of necrotic cells seen by scanning electron microscopy of monkey endothelia after 5 h exposure to balanced salt solution in-vitro (Edelhauser et al 1975) are attributed to small air bubbles adhering to the endothelial surface during perfusion (micrographs not shown). Cellular bulges, blebs and pits were observed by scanning electron microscopy of rabbit corneal endothelia prepared for microscopy 2 h after anterior chamber perfusion in-vivo with balanced salt solution (Frezzotti et al 1985). Since such features were not seen in the present study, it seems reasonable to argue that they are not characteristic of the effects of balanced salt solutions on the corneal endothelium. The same lack of abnormal features has also been found for endothelia perfused with a very low (2 mM) bicarbonate-containing solution (Doughty 1991a). Bulges on individual cells can also be seen in micrographs of endothelia perfused with balanced salt solution in-vitro for 2-3 h (Edelhauser et al 1981). These morphological characteristics were also not evident in the present study and are perhaps either due to the perfusion procedure itself or the preparation of the samples for microscopy. That the balanced salt solutions do not elicit a substantial cytotoxic insult to the cells is also evident from scanning electron microscopy images reported by Gonnering et al (1979).

The clear lack of any overt toxic effect in the present study should not be confused with micrographs showing gross endothelial cell oedema and surface pitting and blebbing after exposure to balanced salt solution in-vitro for 2 h (Glasser et al 1988). Part of these effects must be attributed to fixation artifacts since in the same study, even endothelia perfused with a commercial bicarbonate-Ringer (BSS Plus) showed gross nuclear bulges. These are possibly the result of treatment with a fixative solution that was hypertonic to the cell cytoplasm (Hoffmann & Dumitrescu 1970). Such gross imbalance may have arisen because post-mortem human corneas were used in the study (Glasser et al 1988). Nevertheless, the gross damage observed with the balanced salt solution exposure is clearly not a simple effect that can be directly attributed to this solution. Further studies are, however, needed to define a possible post-mortem (or storage)-dependent alteration in endothelial cell response to balanced salt solution exposure.

In this baseline in-vitro study, the endothelia have been evaluated using the same morphometric procedures used in in-vivo studies so that some objective comparisons can be made between specular microscopy and scanning electron microscopy. Fronterre et al (1984) simply noted that no modification of the endothelial mosaic was evident by

specular microscopy during rabbit anterior chamber perfusion with balanced salt solutions. We confirm this observation, qualitatively, in that no cellular oedema or guttae were evident by specular microscopy in-vitro. However, with either intracameral injection of balanced salt solution in rabbits (Samuelly et al 1989) or a 30 min anterior chamber perfusion in cats (Glasser et al 1985), a significant reduction in the percentage of 6-sided cells (hexagons) in the endothelial mosaic was observed by specular microscope evaluation of the central cornea 2 days later. While no cell loss was evident from cell counts made in either of these studies, the variance (range) of endothelial cell areas increased slightly. The effects observed were reversed within a few days. It was therefore of interest to establish a set of standard values for in-vitro studies. In the present in-vitro study, the results for cell area variation (i.e. population standard deviation and coefficient of skewness values) and the percentage of 6-sided cells in the mosaic, fall well within the ranges for control rabbit endothelia evaluated by specular microscopy in-vivo (Samuelly et al 1989). Since a significant reduction in the number of 6-sided cells was observed 2 days after in-vivo treatment with balanced salt solution, it seems reasonable to consider that the mosaic change occurs between 5 h and 24–48 h after the in-vivo exposure to the balanced salt solution. However, two specific areas of further study are needed before further conclusions on the effects of balanced salt solution on endothelial morphometry can be made. Firstly, the in-vivo studies were presumably performed with room temperature balanced solution rather than 35–37°C solutions as in-vitro. Mosaic changes could be, in part, related to mild hypothermia. Secondly, and as a specific extension of the studies reported by Bonafonte et al (1985/1986), longer exposure times to balanced salt solution need to be evaluated to define the kinetics of any mosaic changes.

The present study has revealed one unexpected result in that the endothelial cell mosaic is quantitatively different with respect to actual cell size when comparing two very similar experimental methods. Since the endothelial cell areas for the half-chamber-incubated samples is very similar to that measured in-vivo (Samuelly et al 1989), we must conclude that the perfused endothelia either stretch in-vitro or that the intact cornea responds to fixation and drying in a different way to the non-perfused preparations. The modest increase in variance in cell areas may be a consequence of this stretching.

*Acknowledgements*

**References**

Apsitis, Yu. A., Ziangirova, G. G. (1979) Influence of various substances in replacing humor in the anterior ocular chamber on the corneal endothelium. (In Russian). Vestn. Ophthalmol. 96: 43–46

Bonafonte, S., Aguirre Vila-Coro, A., Aguirre Vila-Coro, A. (1985/

1986) Scanning electron microscopy study: the effect of balanced salt solution on rabbit corneal endothelium. Cornea 4: 195–201

Closson, R. G., Biggio, C. A., Childress, L., Cox, T., Leonard, J. H., Proffitt, D. F., Raymond, L. A., Schleider, E. M., Teeter, M. E., Yee, E. M. (1990) Multi-institutional drug-use evaluation of intraocular irrigating solutions. Am. J. Hosp. Pharm. 47: 2255–2259

Dikstein, S., Maurice, D. M. (1972) The metabolic basis to the fluid pump in the cornea. J. Physiol. (Lond) 221: 29–41

Doughty, M. J. (1985) New observations on bicarbonate-pH effects on thickness changes of rabbit corneas under silicone oil in vitro. Am. J. Optom. Physiol. Optics 62: 879–888

Doughty, M. J. (1987) Sided effects of bicarbonate on rabbit corneal endothelium in vitro. Clin. Exp. Optom. 70: 168–178

Doughty, M. J. (1989a) Physiological state of the rabbit cornea following 4°C moist chamber storage. Exp. Eye Res. 49: 807–827

Doughty, M. J. (1989b) Towards a quantitative analysis of corneal endothelial cell morphology: a review of techniques and their application. Optom. Vision Sci. 66: 626–642

Doughty, M. J. (1990a) Analyses of areas and shapes of cells on the corneal surface of the albino rabbit by scanning electron microscopy. Curr. Eye Res. 9: 295–306

Doughty, M. J. (1990b) The ambiguous coefficient of variation; polymegethism of the corneal endothelium and central corneal thickness. Int. Contact Lens Clinic 17: 240–247

Doughty, M. J. (1991a) Evidence for a direct effect of bicarbonate on the rabbit corneal stroma. Optom. Vision Sci. 68: 687–698

Doughty, M. J. (1991b) pH, ionized calcium and osmotic artifacts can affect determination on rabbit corneal deturgescence in vitro. Ophthalmic Res. 23: 246–258

Doughty, M. J., Cullen, A.P. (1989) Long-term effects of a single dose of ultraviolet-B on albino rabbit cornea I in vivo analyses. Photochem. Photobiol. 49: 185–196

Doughty, M. J., Maurice, D. M. (1988) Bicarbonate sensitivity of rabbit corneal endothelium fluid pump in vitro. Invest. Ophthalmol. Vis. Sci. 29: 216–223

Edelhauser, H. F., Van Horn, D. L., Hyndiuk, R. A., Schultz, R. O. (1975) Intraocular irrigating solutions. Their effect on the endothelium. Arch. Ophthalmol. 93: 648–657

Edelhauser, H..F., Van Horn, D. L., Schultz, R. O., Hyndiuk, R. A. (1976) Comparative toxicity of intraocular irrigating solutions on the corneal endothelium. Am. J. Ophthalmol. 81: 473–481

Edelhauser, H. F., Hanneken, A. M., Pederson, H. J., Van Horn, D. L. (1981) Osmotic tolerance of rabbit and human corneal endothelium. Arch. Ophthalmol. 99: 1281–1287

Edelhauser, H. F., Antoine, M. A., Pederson, H. J., Hiddeman, J. W., Harris, R. G. (1983) Intraocular safety evaluation of ethylene oxide and sterilant residues. J. Toxicol.-Cut. Ocular Toxicol. 2: 7–39

Fischbarg, J., Lim, J. J. (1974) Role of cations, anions and carbonic anhydrase in fluid transport across rabbit corneal endothelium. J. Physiol. (Lond.) 241: 647–675

Fischbarg, J., Warshavsky, C. R., Lim, J. J. (1977) Pathways for hydraulically- and osmotically-induced water flows across epithelia. Nature (Lond.) 266: 71–74

Frezzotti, R., Pannini, S., Morocutti, A., Caporossi, A., Del Vecchio, M. T., De' Santi, M. M., Sforza, V., Luzi, P. (1985) Valutazione del danno dell'endotelio corneale dopo introduzione di adrenalina in camera anteriore. Boll. Oculist. 64: 967–974

Fronterre, A., Furiosi, G., Marongiu, A., Galeazzi, A. (1984) I fluidi sostitutivi dell'acqueo e l'endotelio corneale. Ann. Ottalmol. Clin. Oculist. 110: 331–335

Glasser, D. B., Edelhauser, H. F. (1989) Toxicity of surgical solutions. Int. Ophthalmol. Clin. 29: 179–187

Glasser, D. B., Matsuda, M., Ellis, J. G., Edelhauser, H. F. (1985) Effects of intraocular irrigating solutions on the corneal endothelium after in vivo anterior chamber irrigation. Am. J. Ophthalmol. 99: 321–328

Glasser, D. B., Matsuda, M., Edelhauser, H. F. (1988) Comparison of corneal endothelial structural and functional integrity after irrigation with bicarbonate-buffered and acetate-citrate-buffered solutions. In: Cavanagh, H. D. (ed.) The Cornea: Transactions of the World Congress on the Cornea III. Raven Press, New York, pp 101–106

Gonnering, R., Edelhauser, H. F., Van Horn, D. L., Durant, W. (1979) The pH tolerance of rabbit and human corneal endothelium. Invest. Ophthalmol. Vis. Sci. 18: 373–390

Hoffmann, F., Dumitrescu, L. (1970) Beobachtung der Kaninchen-Hornhaut des Auges unter dem Raster-Elektronenmikroskop. Beitr. elektronenmikroskop. Direktabb. Oberfl. 3: 307–318

Kline, O. R., Symes, D. J., Loronzetti, O. J., de Faller, J. M. (1983/1984) Effect of BSS Plus on the corneal endothelium with intraocular lens implantation. J. Toxicol.-Cut. Ocular Toxicol. 2: 243–247

Komine, T. (1982) Specular microscopic studies in corneal endothelium after intracapsular lens extraction. Effect of filling materials in the anterior chamber. (In Japanese). Jpn. J. Clin. Ophthalmol. 36: 223–227

Kuang, K., Xu, M., Koniarek, J. P., Fischbarg, J. (1990) Effects of ambient bicarbonate, phosphate and carbonic anhydrase inhibitors on fluid transport across rabbit corneal endothelium. Exp. Eye Res. 50: 487–493

Leonardi, E., D'Angelo, R., Ricci, A., Nistri, A., Onori, P. (1989) Risultati clinici dell'uso di una nuova soluzione irrigante nella chirurgia extracapsulare della cataratta. Boll. Oculist. 68: 225–231

Matsuda, M., Tano, Y., Edelhauser, H. F. (1984) Comparison of intraocular irrigating solutions used for pars plana vitrectomy and prevention of endothelial cell loss. Jpn. J. Ophthalmol. 28: 230–238

Maurice, D. M. (1984) Cornea and sclera. In: Davson, H. (ed.) The Eye. Vol. 1B, Accademic Press, New York, pp 1–158

Samuelly, R., Zuccheri, G., Grignolo, F. M., Peruccio, C. (1989) Experimental specular microscope study of the corneal endothelium: effects of the introduction of various substances into the anterior chamber. New Trends Ophthalmol. 4: 305–317

Tashima, T., Kawakami, U., Harada, M., Satoh, N., Nakagawa, T., Tanaka, H. (1988) Relationship between precipitation in aqueous solution of glutaraldehyde for chemosterilization and impurities detected by gas chromatography. Int. J. Pharmaceut. 42: 61–67

Weekers, J.-F., De Thinne, M. (1978) Effets de différentes solution d'irrigation intraoculaires sur l'endothélium de la cornée humaine. J. Fr. Ophthalmol. 1: 643–648