Bicarbonate Sensitivity of Rabbit Corneal Endothelium Fluid Pump In Vitro

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Stroma-endothelium preparations from rabbit corneas were mounted between two chambers and incubated with identical media on either side which contained different bicarbonate levels and, in some experiments, organic (Good’s) buffers. Active fluid flow across the preparations was measured by means of a capillary tube attached to the stroma-side chamber. With media containing 2 to 50 mM bicarbonate (pH 6.2 to 7.8 in equilibrium with 5% CO₂-air at 37°C), the fluid pump was constant for at least 3 hr at a rate of 5 μl/hr cm² and was not significantly affected by the bicarbonate level. Over the same range of pH and bicarbonate but supplemented with 50 mM organic buffer, fluid pump was 8 μl/hr cm² for all bicarbonate concentrations used. Using Ringer solutions supplemented with 50 mM buffer (pH 6.3 to 8.4) but without added bicarbonate and in equilibrium with air, fluid pump was observed at approximately 4 μl/hr cm² at pH 6.3 and increased to 8 μl/hr cm² at pH 7.8. In all cases, fluid pump persisted for at least 5 hr. Invest Ophthalmol Vis Sci 29:216–223, 1988

The corneal endothelium will thin a swollen cornea or maintain it at its normal thickness by an active mechanism of deturgescence, which is thought to be associated with an active fluid pump in the endothelium. The sensitivity of corneal deturgescence to a lowering of the level of bicarbonate ions in the anterior chamber was noted more than 10 years ago,¹ ² and the effect has been confirmed and examined in some detail.³ ⁴ Recently, it was subjected to a more thorough analysis, paying particular attention to the control of CO₂-HCO₃⁻ equilibria in the anterior chamber.⁵ In that study, the deturgescence of isolated rabbit cornea was measured under a specular microscope when the anterior, epithelium-free, surface was covered with silicone oil after a period of pre-equilibration. However, because of the permeability of the oil to CO₂, it was found that gas equilibria in the stroma could not be adequately controlled. Since two previous studies concerning the bicarbonate sensitivity of the endothelial fluid pump (ie, direct measurement of fluid flow across the endothelium) also employed techniques where a layer of silicone oil was used to cover a solution bathing the anterior corneal surface,⁶ ⁷ the present experiments were undertaken to check their conclusions. The bicarbonate sensitivity of the endothelial fluid pump (with adequate control of stromal HCO₃⁻-CO₂ equilibria) was evaluated by mounting the endothelium-stroma combination between two chambers containing regularly changed Ringer’s solution and then directly measuring fluid flow along a capillary. Some of this work has been published in abstract form.⁸

Materials and Methods

Animal care and usage followed the guidelines established in the ARVO Resolution on the Use of Animals in Research. New Zealand White rabbits (mostly female; 1.6 to 3.0 kg; average external corneal diameter of 10.75 to 11.75 mm) were killed by decapitation or an intravenous overdose of Euthanyl® (M.T.C. Pharmaceuticals, Mississauga, Ontario, Canada) or T-61® (Hoecht Corp., Sommerville, NJ) followed by division of the neck blood vessels. The globes were enucleated, together with their lids and conjunctiva within 15 min of death and, if they could not be used immediately, they were stored for up to 23 hr in sealed, moist containers at 4°C in the dark, with the lids closed over the cornea, which was facing downwards.

Immediately before use, the corneal surface of the enucleated eye was flushed with 150 mM NaCl at room temperature and the corneal epithelium totally removed by firm abrasion with a dull razor blade. The scraped surface was then thoroughly washed.
first with saline and then with the incubation solution of choice at 37°C. The completion of the epithelial removal was routinely checked by light microscope observation of gluraldehyde-fixed sections prepared after the fluid flow studies were completed.

The stroma-endothelium preparation was mounted in a chamber similar in essentials to those used in both fluid pump and solute transport studies on the rabbit cornea9,10 (Fig. 1). The stromal surface of the rabbit eye was then sealed to an acrylic plastic mounting tube (OD, 14 mm; wall thickness, 1.5 mm; length, 20 mm) by applying a light suction in the tube and tying the conjunctiva to a groove near its end. To provide mechanical stability to the preparation, a 100 mesh polypropylene grid had been molded to a 6.25 mm radius of curvature and glued to the end of the mounting tube. After cutting away the back of the globe, the vitreous and crystalline lens, iris and ciliary body were carefully removed after folding a scleral lip back over the edge of the mounting tube.

A double chamber was designed to stand upright in a water bath at 37°C with the cornea facing downwards. In the bottom (stromal) half of the chamber, one fluid connection (PE-100 tubing) was made to its base and another reached up to the edge of the corneal stroma, where it was cut so as to fit into the angle of the supporting mesh. This arrangement made for more efficient replacement of fluid contained in the chamber and served also to facilitate removal of any gas bubbles trapped in it.

To assemble the system, the lower tube (L) was dipped into a reservoir of the incubation solution and gentle suction applied to the upper tube (U) by means of a 5 ml syringe. The mounting tube with corneal preparation attached was inserted into the bottom chamber and was slowly drawn down to its seating by the suction. At the same time, the chamber was filled (~2 ml) by fluid from the reservoir. The assembly was then completed by carefully clamping the top onto the bottom chamber while maintaining a slight suction on the cornea. The top chamber (endothelial side) was filled with incubation solution and its opening plugged with a rubber stopper pierced with a 19 gauge syringe needle, into the barrel of which was inserted a 22 gauge needle. This closure served to prevent evaporation from the upper chamber and minimize loss of CO₂ in the solutions in the upper chamber. The assembled apparatus was immersed in a water bath up to the lip of the top chamber. Using the syringe on line U, approximately 3 ml of incubation solution was drawn from the reservoir through the bottom chamber and then the line was clamped. Exit line L was then removed from the reservoir and connected to a horizontal capillary tube M (PE-100 tubing), 20 cm below the endothelium. Line U was then briefly unclamped and a few microliters of colored 0.9% NaCl containing 0.5% Photoflow® (Eastman Kodak, Rochester, NY), drawn into line M and the meniscus position adjusted to be around 10 cm from its open end. Line U was then clamped again. A millimeter scale was attached to the benchtop next to the meniscus.

At any point in the experiments (usually every 60 min), the solution in the lower chamber could be changed by clamping line M, uncoupling the connection and replacing the free end of line L into the freshly-replenished reservoir. Fresh solution was then drawn through the apparatus by the syringe on the unclamped line U without contamination of the bottom chamber with Photoflow. At the same time, the solution in the top chamber also was replaced by removing the stopper, sucking out its contents with a Pasteur pipette, quickly refilling the chamber with fresh solution from the reservoir and replacing the stopper.

The incubation solutions were prepared and used as described previously. Details of the solutions are provided in Table 1. The solutions in series 1 and 2 contained various levels (2 to 50 mM) of bicarbonate, which were exchanged on an isomolar basis for NaCl.
Table 1. Composition of corneal incubation solutions

<table>
<thead>
<tr>
<th>Series</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl (mM)</td>
<td>80–128</td>
<td>30–78</td>
<td>78</td>
</tr>
<tr>
<td>NaHCO₃ (mM)*</td>
<td>2–50</td>
<td>2–50</td>
<td>zero†</td>
</tr>
<tr>
<td>KCl (mM)</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>MgCl₂ (mM)</td>
<td>0.9</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>CaCl₂ (mM)</td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
</tr>
<tr>
<td>Glutathione (mM)‡</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Adenosine (mM)</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>pH</td>
<td>6.2–7.8</td>
<td>6.3–7.9</td>
<td>6.0–8.4</td>
</tr>
<tr>
<td>Good’s buffers (mM)§</td>
<td>0</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

* All solutions containing added NaHCO₃ were equilibrated with 5% CO₂-air at 37°C to ensure maintenance of bicarbonate levels and pH.
† No special precautions were taken to eliminate CO₂ and carbonate from the solutions so that a trace of bicarbonate (0.03 mM) may be present.
‡ Added as its reduced form but no precautions were taken to maintain the glutathione in this form.
§ All buffer ingredients were prepared, prior to addition to the corneal incubation solutions, to contain a titratible equivalent of 50 mM Na (OH) along with 50 mM buffer concentration.

Thus, with the slight difference in dissociation of the two salts in aqueous solution,¹¹,¹² the osmolarity of the solutions fell slightly as the bicarbonate level was raised.⁵ All solutions were prepared in double distilled water, and used within 8 hr of preparation.

On being gassed to equilibrium with 5% CO₂-air at 37°C, series 1 solutions exhibited a range of pH values from 6.2 to 7.8.³,¹³,¹⁸ Loss of CO₂ resulted in a either a rapid or slow increase in pH, depending on the rate of outgassing,¹³,¹⁹ and careful monitoring of the pH of the solutions is a convenient way to check the CO₂ tension. The series 2 solutions were similar to series 1 but an organic buffer system was added in order to stabilize the pH. The effectiveness of the buffer was evaluated by observing the change in pH that occurred while CO₂ was allowed to leave these solutions under standard conditions. The best results were obtained with the following Good’s buffers²⁰,²¹ at 50 mM concentration: MES (pKₐ = 5.95; 37°C adjusted), PIPES (6.65), Bis-Tris-Propane (6.50), MOPS (7.10), TES (7.16), HEPES (7.30), Tricine (7.8) and Bicine (8.04). Using a combination of two buffers with slightly different pKₐ values enhanced the control of pH.²¹,²² Each buffer was prepared as a 10 × stock in distilled water, its pH adjusted to the pKₐ with NaOH and then they were added to the incubation solutions in that proportion that was found empirically to give a pH corresponding to that of any of the bicarbonate solutions when saturated with 5% CO₂-air at 37°C. These solutions were then gassed with 5% CO₂-air. The final pH was within 0.1 pH unit of the designated values (Fig. 2).

It should be noted that Tris buffer,²³ while it has been used in corneal research,¹¹,²⁴ is not satisfactory both because it has very limited buffer capacity below pH 7.5 and because it is rather reactive with CO₂ and bicarbonate.¹⁵,²⁰,²¹ It behaved poorly in controlling the pH in comparison with the buffers listed above.

A third series of solutions (series 3) was also used. The NaHCO₃ was totally replaced with NaCl and the solutions equilibrated with air. The pH was set by the use of the same combinations of buffers used in series 2 thus permitting the assessment of the pH dependence of fluid flow in the nominal absence of bicarbonate and CO₂. It is acknowledged that the tissue is capable of producing CO₂ and that the atmosphere contains a small quantity of CO₂ that could produce a [bicarbonate + CO₂] level of 0.03 mM²⁶. No effort was made during the preparation of solutions to purge them of atmospheric CO₂ or dissolved carbonate.

Results

Fluid Flow Measurements

The chambers were constructed of thick acrylic, and errors could occur as a result of the uptake of water by the plastic or of slow thermal expansion of the chamber²⁷ and its fluid contents. In previous experiments with a similar chamber, capillaries were attached to both sides of the cornea and the loss of fluid on one side was seen to be compensated by a gain on the other, which showed that these errors were negligible.¹⁰,⁲⁹ With a single capillary system, they need to be reconsidered.
The chamber was filled with water at room temperature and immersed in the incubator bath while the temperature at the level of the cornea was measured with a digital thermometer. It was found to rise half-way to its final value in 10 min and show no appreciable change after 30 min. Therefore, in the experimental situation where the chambers are filled with 37°C solution, temperature changes are not likely to be a cause of trouble after this time. In other tests, the cornea was replaced with a 1 mm thick silicone rubber disc and the chamber was assembled and filled in the normal manner. The movements of the meniscus were found to be negligible after 60 min. The initial velocity of the meniscus, which was away from the chamber, was directly related to the time that the equipment was allowed to dry between experiments: it varied from 5 to 37 µl/hr for drying periods of 1 to 21 days. Apparently the volume of the plastic, as it absorbs water, is greater than the sum of its constituents.

Evaporation, which must be very small through the needle hole in the stopper, would have a miniscule effect on the tonicity of the fluid in the upper chamber and then could only change the meniscus position indirectly by osmosis across the cornea.

Where a stroma-endothelial preparation is perfused in a similar manner under a specular microscope, the stroma is seen to swell immediately after mounting. Since the anterior surface is fixed by the grid, such swelling would displace the endothelium into the upper chamber, and this could move the meniscus as if the cornea was pumping fluid into the top chamber. However, the extent of swelling is limited, and with a pressure head of 20 cm H₂O, it is virtually complete within 60 min, although it may continue for 90 min (observed in five of 13 preparations) in very low bicarbonate solutions.

For many reasons, therefore, the movements of the meniscus over the first hour were ignored, and readings were carried out over the next 4 hr.

**Effect of Bicarbonate Concentration on Pump With Bicarbonate Buffer/CO₂**

The fluid flow corresponded to a leak for the first 35 to 40 min in most preparations but later reversed its direction and moved against the applied hydrostatic pressure of 20 cm H₂O (Fig. 3). Part of the initial leak is attributed to the chamber equilibration (which could persist for as long as 60 min), and the time of initiation of the net fluid pump activity is uncertain. However, once established, it continued for at least 5 hr in all 107 preparations examined. The slightly longer starting times were generally observed in those preparations incubated with 2 to 10 mM bicarbonate.

The rate of fluid flow was measured by taking readings of the meniscus position every 10 min and then fitting (by linear regression, if necessary) the data points to a line over the intervals of 60 min between changes of the chamber solutions. The fluid flow is expressed as µl/hr cm² of endothelium, taking into account the area of exposed endothelium in the apparatus (1.17 mm²).

In most experiments, changing the solutions in the chambers every 60 min resulted in good control of their gas equilibria. This equilibrium was assessed by measuring the top chamber pH at the end of the 60 min period. The increase in pH was only 0.1 to 0.2 pH units, and often no change was detectable (ie <0.1 pH unit change). However, with longer periods between solution changes, increases of up to 0.5 pH units were observed.

Changing solutions every 60 min also resulted in a maintenance of fluid pump activity stable to within ±10% from hour to hour, at all bicarbonate concentrations tested. A few corneas were studied over a period of 9–10 hr with 2, 5, 35 and 50 mM bicarbonate and showed the same behavior. With longer intervals between changes (eg 2 hr), the variance from hour to hour increased to ±15%. Without such solution changes, the fluid pump declined to zero at a rate of approximately 30%/hr (see also refs. 29–31).

Using the same bicarbonate-Ringer solutions on both sides of the preparation, the effect of 11 different levels of bicarbonate (in equilibrium with 5% CO₂-air
At all 11 concentrations of bicarbonate and at all time periods evaluated, the fluid pump activity was significantly higher, almost double, than that seen with solutions in series 1 not containing the buffers. As with these unbuffered solutions, no dependence upon bicarbonate concentration was observed for the fluid pump (Fig. 5).

**Effect of pH on Fluid Pump Activity Without Bicarbonate**

In this series, the solutions were similar to those of series 2 except that no bicarbonate was added and the solutions were equilibrated with air at 37°C.

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**Effect of Bicarbonate Concentration on the Pump With 50 mM Organic Buffers in the Presence of CO₂**

The experiments described above were repeated but now using the series 2 solutions that were supplemented with a total concentration of 50 mM of the Good’s buffers.

In 56 preparations, the fluid pump started within 40 to 50 min, after an initial leak period, and persisted for at least 5 hr unchanged. With the use of these buffered solutions, no changes in the pH of the top chamber were detected after the 60 min period, demonstrating that the desired effect of the buffer addition had been achieved. It is evident, furthermore, that these buffers are not toxic to the endothelium.

Fig. 4. Fluid pump activity as a function of the bicarbonate levels (series 1, without added buffers) in the solutions incubating the stroma-endothelium preparations in the 2nd (A), 3rd (B) and 4th (C) hour after starting the experiments. Results are mean values ± SEM for seven to eight preparations incubated under each condition.

Fig. 5. Fluid pump activity as a function of the bicarbonate levels (series 2, containing 50 mM Good’s buffers) in the solutions incubating the stroma-endothelium preparations in the 2nd (A), 3rd (B) and 4th (C) hours after starting the experiments. Results are mean values ± SEM for four to six preparations incubated under each condition.
These results (Fig. 6) reveal that such "bicarbonate-free" solutions support fluid pump activity. However, the fluid pump was rather less predictable when these solutions were used and in most preparations (17 out of 21), the fluid pump in the third hour was higher than that in the second hour. There was also considerable variability in the fluid pump activity of different preparations and over time in the same preparation. While the pump persisted in all preparations for at least 5 hr, the rates observed in each hour could differ by as much as ±20% from those seen in the second hour. The data suggest that the pump rate increases slightly with pH from a value of about 4
\( \mu l/hr \) cm\(^2\) at pH 6.0 to about 8 \( \mu l/hr \) cm\(^2\) at pH 8.0 and falls at higher pH values.

**Discussion**

Several previous studies\(^ {5,28,32,33} \) have shown that the thickness of the stroma-endothelium preparation incubated in vitro, between two solutions, changes from its initial physiological value to a steady state (equilibrium) value which is dependent upon the hydrostatic pressure in the anterior chamber. The length of the time required for this stable thickness value to be achieved depends upon the hydrostatic pressure and is about 60 min for the 20 cm H\(_2\)O used in the present experiments. After this time, the movement of fluid into the top chamber of the apparatus must correspond to an equal movement of fluid across the corneal endothelium and out of the bottom chamber, and is against the hydrostatic pressure gradient. The rate found for this pump, in bicarbonate/C\(_2\)O\(_3\)-buffered solutions was around 5 \( \mu l/hr \) cm\(^2\) which is within the range found by other workers.

Under the experimental conditions used here, the fluid pump of the corneal endothelium was found not to be sensitive to bicarbonate regardless of whether the organic buffer supplement was present or absent. It is also noteworthy that, in the presence of these buffers, the fluid pump activity was almost twice as high as when the preparations are incubated in simpler bicarbonate/C\(_2\)O\(_3\)-buffered solutions. Similarly, the pump showed no significant pH sensitivity over the range tested (6.0 to 8.0); the alternative possibility that the sensitivity to a fall in [HCO\(_3^-\)] is exactly compensated for by that to a fall in pH is not probable. Furthermore, under the very unphysiological situation in which the levels of bicarbonate and CO\(_2\) are extremely low and the solution pH is controlled by 50 mM organic buffer, fluid pump activity is maintained over an extended time period. It does not appear, therefore, that this activity is controlled by the CO\(_2\) level.

Thus, the fluid pump of the endothelium does not show the marked bicarbonate sensitivity that has been observed for the phenomenon of corneal deturgescence as measured in the specular microscope when the cornea is covered with silicone oil.\(^ {1-5} \) During the course of the present investigations, measurements of the fluid pump with solution series 1 were carried out simultaneously with measurements of deturgescence\(^ 3 \) using the same solutions in a paired-eye protocol. The two phenomena show very different bicarbonate sensitivities: the former changed not at all and the latter 15-fold. At very low bicarbonate levels, no deturgescence was observed yet the fluid pump appeared intact. These results therefore require reconsideration of the widely accepted view that the fluid pump in the corneal endothelium is bicarbonate-dependent.\(^ {34-38} \)

The previous study in which the fluid pump (rather than deturgescence) had been assessed under several different levels of bicarbonate, claimed that the "pH was adjusted to be constant" in all solutions and used a method in which the stromal side of the preparation was covered with bicarbonate-Ringer solution overlaid by a layer of silicone oil.\(^6 \) It must be borne in mind that of the three variables—CO\(_2\) pressure, pH and HCO\(_3^-\) concentration—only two can be independently controlled.\(^ {13-19} \) In a second series of experiments, where the effects of incubating stroma-endothelium preparations with or without a single level of bicarbonate were reported,\(^ {38} \) it was noted that "in HCO\(_3^-\) and CO\(_2\)-free Ringer solution movements (of
fluid) generally cease.” The solution contained 5.5 mM Na₂HPO₄ and the pH was not specified.

In the deturgescence experiments, carried out under a specular microscope, the anterior stroma was covered with a layer of silicone oil whereas in the present experiments the stromal side of the preparation was in contact with a large volume of frequently renewed solution. Carbon dioxide is readily soluble in silicone oil (Dow Corning literature) as it is in other organic solvents¹⁴,³⁹ and can thus pass across a layer of silicone oil and be lost from any solution over which it is placed. The loss of CO₂ should be accompanied by an increase in pH (which was noted³) and a shift in the equilibrium to give a lower concentration of bicarbonate in the solutions immediately underlying the oil. Since the pump was found to be insensitive to pH, HCO₃⁻ concentration and CO₂, it may be that a difference in concentration on the two sides of the endothelium is responsible for the observation of an apparent bicarbonate sensitivity to endothelial fluid pump activity. Further studies are required to test this possibility.

When bicarbonate was present, the addition of an effective concentration of Good’s buffers considerably enhanced the pump. It is perhaps also significant that when bicarbonate was absent, the pump operated in our experiments when buffers were added but was largely inhibited in those of Mayes³⁸ where the solutions were scarcely buffered. In view of the varied chemical nature of these compounds, their action on the pump must be a direct response to their buffering capacity rather than some pharmacological action. There is only negligible change in pH in the bulk of the solution when bicarbonate and CO₂ alone are used, but it is possible that larger changes take place locally on the boundary layers that will readily form on either side of the endothelium, and the organic buffers may be more effective in neutralizing them. It is unknown, at this time, whether the added organic buffers serve to buffer the bulk solution hydrogen ion concentration or indirectly serve to buffer H⁺ or CO₂ equivalents related to cellular pH homeostasis and responsivity to extracellular or intracellular metabolic changes. In this connection, it can be noted that the basal surface of the endothelial cells is adjacent to a natural thick unstirred layer, the corneal stroma, and thermal gradients in the bottom chamber will be small. On the other hand, small but appreciable gradients, which might lead to convective stirring, are present in the upper chamber because its upper surface is not covered by the waterbath, as was confirmed by measurements with a digital thermometer.

Key words: cornea, endothelium, fluid pump, bicarbonate

References

24. Lim JJ: Effects of bicarbonate on the potential difference


