Effects of $[\text{Na}^+]$, $[\text{Cl}^-]$, carbonic anhydrase, and intracellular pH on corneal endothelial bicarbonate transport

Keith Green, Stuart Simon, Gordon M. Kelly, Jr., and Karen A. Bowman

Sodium removal from the solution bathing the isolated rabbit corneal endothelium caused a reduction in both unidirectional and net flux of bicarbonate, whereas chloride withdrawal from the solution had no effect on the net bicarbonate flux but increased the unidirectional fluxes. These data correlate with previously published data on the effects of similar solution manipulations on both fluid movement and potential difference across the endothelium and strongly implicate bicarbonate as the primary ion involved in the maintenance of corneal hydration. Carbonic anhydrase (1 mg/ml) added to the solution bathing both sides of the endothelium markedly increased unidirectional and net bicarbonate fluxes, possibly by maintaining a high bicarbonate/CO$_2$ concentration close to the membrane and thereby eliminating chemical gradients in the unstirred layer adjacent to the membrane. Determinations of intracellular pH with the 5,5-dimethyl-2,4-oxazolidine-dione method indicate that at more acid ambient conditions there is a lesser gradient between cell and bathing medium for H$^+$; similar ambient conditions in other experiments resulted in larger unidirectional bicarbonate fluxes than at neutral pH. The data are suggestive of a nonevectorial H$^+$-HCO$_3^-$ exchange occurring across the endothelial cellular membranes. (INVEST OPHTHALMOL VIS SCI 21:586-591, 1981.)

Key words: rabbit, cornea, endothelium, bicarbonate transport, unidirectional fluxes, ionic dependence, intracellular pH, carbonic anhydrase

Regulation of rabbit cornea hydration by the endothelium has been demonstrated to be both bicarbonate- and sodium-sensitive$^{1-4}$

From the Departments of Ophthalmology (K. G., S.S., G.M.K., K.A.B.) and Physiology (K. G.), Medical College of Georgia, Augusta.

Supported in part by Public Health Research Grant EY 01413 from the National Eye Institute, by the J. B. Hall Foundation, and by a Research to Prevent Blindness, Inc., grant. Stuart Simon was the recipient of an American Medical Association Education and Research Foundation Studentship during the course of this work. Keith Green was the recipient of a Research Manpower Award from Research to Prevent Blindness, Inc.

Submitted for publication Nov. 17, 1980.

Reprint requests: Keith Green, Department of Ophthalmology, Medical College of Georgia, Augusta, Ga. 30912.

and to show no dependence on chloride.$^3$ The measurement of a net bicarbonate movement across the endothelium$^{1-6}$ has provided impetus to the concept that a bicarbonate transport system is directly linked to fluid movement across the endothelium.$^7$ Certainly the link between fluid movement, thickness control, and potential difference across the endothelium and the ionic content of the bathing medium appears strong,$^3$ but a similar dependence between bicarbonate transport and the composition of the bathing medium has not been established. Such a relationship would support the theory of a direct link between endothelial fluid and bicarbonate transport.

The endothelial net bicarbonate flux has been shown to be dependent on the bicarbon-
ate concentration in the bathing medium, yet other studies have shown that carbonic anhydrase inhibitors influence both corneal thickness and net bicarbonate fluxes. These two observations appear to be ambiguous but must be reconciled. Research using artificial bilayer lipid membranes has shown that the unstirred layers immediately adjacent to either side of the membrane are rate-limiting to the movement of CO$_2$. In the presence of carbonic anhydrase, which accelerates the hydration-dehydration of CO$_2$, the concentration of CO$_2$ in the unstirred layers was increased so that the membrane itself became rate-limiting. The participation of carbonic anhydrase in the process of endothelial net bicarbonate movement required further study to determine whether a similar process occurred in the endothelium as in the artificial membrane.

To determine the dependence of corneal endothelial bicarbonate movement on other ions, bicarbonate fluxes were measured after changes of [Na$^+$] and [Cl$^-$] in the bathing medium as well as in the presence of carbonic anhydrase. Measurements were made of endothelial intracellular pH under various ambient conditions to determine whether proton movement might be related to bicarbonate transport.

**Materials and methods**

Adult albino rabbits weighing 2-3 kg were used in three groups of procedures.

**Endothelial bicarbonate fluxes and ionic changes**

*Group I.* The methods and procedures were as described previously. Briefly, corneas were de-epithelialized and were mounted on specular microscope mounting rods after removal from the eyes. The mounted tissue was then placed in water-jacketed Lucite chambers with a volume of about 1.2 ml each. Paired corneas were used, with one unidirectional flux measured on one of each pair (stromal-to-aqueous [epend] on one cornea and aqueous-to-stromal [epend] on the other). Both corneal surfaces were bathed with Krebs-bicarbonate Ringer’s solution with added adenosine (0.34 gm/L) and glutathione (0.09 gm/L). The endothelial surface of one cornea of each pair and the stromal surface of the other cornea of the pair were equilibrated with Ringer’s solution containing H$^{14}$CO$_3$ on one side for 1 hr prior to sampling and the cold solution on the opposite endothelial surface; the latter was thereafter replaced at 30 min intervals. The solutions were sampled and counted in a Scarle Isocap 300 with the NaI filament scintillation system. The bathing solutions contained 143 mM NaCl, 50 mM NaCl, 10 mM NaCl, or 0 mM NaCl. Substitution of NaCl was made with choline chloride, and in Na-free and 10 mM NaCl solutions KHCO$_3$ was substituted for NaHCO$_3$; thus [Cl$^-$] was maintained constant and only [Na$^+$] changed. For chloride changes, solutions of 50 and 0 mM NaCl were used; substitution of Cl$^-$ was made with NaSO$_4$ and sucrose. All solutions were isosmotic at 305 mOsm, as determined by measurement in a Fiske osmometer, were equilibrated with 95% O$_2$, 5% CO$_2$, and were at pH 7.3. The pH was adjusted by adding either NaOH or HCl as needed, usually only minor adjustments were needed.

**Carbonic anhydrase effects**

*Group II.* Endothelia were allowed to equilibrate with H$^{14}$CO$_3$ for 1 hr with 1 mg/ml carbonic anhydrase in the bathing solution on both sides, and the appropriate solutions were sampled after replacement every 30 min after equilibration. The solutions used contained 25 mM bicarbonate at either pH 8 or 7.3.

**Intracellular pH**

*Group III.* The 5,5-dimethyl-2,4-oxazolidine-dione (DMO) method of Miller et al. was used with 14C-labeled material. Solutions were made containing, nominally, 25 or 60 mM bicarbonate. Solution variations, all at pH 7.5, included the presence or absence of 1 mg/ml carbonic anhydrase; the addition of 1 mM acetazolamide (carbonic anhydrase inhibitor), 10$^{-4}$M ouabain, 10$^{-3}$M furosemide, or 10$^{-4}$M amiloride; or a sodium-free solution. The effect of de-epithelialization was tested, as was the stability of the intracellular pH with time. Six or eight whole corneas that had been quickly removed from rabbits were placed into a scintillation vial containing Ringer’s solution and 14C-DMO (2 mCi/ml). The 25 mM bicarbonate solutions were at pH 7.5, and 8 without additional drugs and at pH 7.5 when containing drugs; the 60 mM bicarbonate solution was at pH 7.5. The vials were sealed without air space in order to maintain the fluid composition constant, and the corneas were incubated for 1 hr at 37°C, except for one set of incubations for 3 hr. At this time the corneas were removed and rinsed in nonlabeled Ringer’s solution of the same composition as that in which the cornea was bathed. The endothelium was lightly blotted with filter paper and was scraped off before placement in a tared homogenization tube. Three or four endothelia
Table I. Unidirectional and net bicarbonate fluxes across rabbit corneal endothelium as a function of solution [Na\(^+\)] and [Cl\(^-\)]

<table>
<thead>
<tr>
<th>Group</th>
<th>J(^{\text{endo}})</th>
<th>J(^{\text{epi}})</th>
<th>J(^{\text{endo}}) - J(^{\text{epi}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>I:A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>143 mEq Na (control)</td>
<td>3.24 ± 0.07</td>
<td>1.57 ± 0.07</td>
<td>1.67 ± 0.30</td>
</tr>
<tr>
<td>50 mEq Na</td>
<td>2.89 ± 0.07(^{\text{a}})</td>
<td>2.17 ± 0.11(^{\text{b}})</td>
<td>0.72 ± 0.15(^{\text{b}})</td>
</tr>
<tr>
<td>10 mEq Na</td>
<td>2.94 ± 0.10(^{\text{b}})</td>
<td>2.14 ± 0.07(^{\text{b}})</td>
<td>0.79 ± 0.15(^{\text{b}})</td>
</tr>
<tr>
<td>0 mEq Na</td>
<td>2.78 ± 0.02(^{\text{a}})</td>
<td>2.30 ± 0.04(^{\text{b},\text{e}})</td>
<td>0.52 ± 0.17(^{\text{b},\text{e}})</td>
</tr>
<tr>
<td>50 mEq Cl(^-)</td>
<td>3.67 ± 0.10(^{\text{a}})</td>
<td>2.15 ± 0.06(^{\text{b}})</td>
<td>1.50 ± 0.21</td>
</tr>
<tr>
<td>0 mEq Cl(^-)</td>
<td>3.62 ± 0.11(^{\text{a}})</td>
<td>2.22 ± 0.11(^{\text{b}})</td>
<td>1.40 ± 0.25</td>
</tr>
<tr>
<td>IIA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.32 ± 0.05</td>
<td>8.26 ± 0.24(^{\text{c}})</td>
<td>5.19 ± 0.29(^{\text{c}})</td>
<td>3.07 ± 0.33(^{\text{c}})</td>
</tr>
<tr>
<td>Pco(_2) 42.6 ± 1.4 mm Hg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCO(_3) 20.8 ± 1.4 mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
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</tbody>
</table>

\(^{\text{a}}\)All unidirectional flux values are \(\mu\)Eq/cm\(^2\)/hr and represent the mean ± S.E.M. of 36 measurements (no less than four per cornea).

\(^{\text{b}}\)Values are the mean ± S.E.M. of at least six experiments (each with at least four determinations per cornea) and the pH, Pco\(_2\) and HCO\(_3\) values are those of the solutions at the termination of the incubation.

\(^{\text{c}}\)Values are the mean ± S.E.M. of the arithmetic difference between the unidirectional fluxes of each corneal pair at each time interval (n = 36).

\(^{\text{d}}\)Significantly different from control values (p < 0.02).

\(^{\text{e}}\)Statistically different from 10 mEq Na\(^+\) values (p < 0.05).

...were pooled for each wet weight determination, and after weighing, 100 \(\mu\)l of distilled water was added to each tube and the tissues were homogenized. Duplicate 20 \(\mu\)l samples were taken, and 10 ml of Aquasol was added prior to counting in a Searle Isocap 300 scintillation counter.

Results

Group I. The unidirectional and net bicarbonate fluxes across the rabbit corneal endothelium as a function of solution [Na\(^+\)] and [Cl\(^-\)] are given in Table I. Reduction of [Na\(^+\)] caused a fall in \(J_{\text{endo}}\) (active) and an increase in \(J_{\text{epi}}\) (passive), with a combined effect to reduce \(J_{\text{endo}}\) net. Reduction from 143 mEq [Na\(^+\)] to 50 mEq [Na\(^+\)] caused a large proportion of the total change induced by Na\(^+\)-free solution. \(J_{\text{endo}}\) is not significantly different between 50, 10, or 0 mEq [Na\(^+\)] solutions. Although 50 and 10 mEq [Na\(^+\)] solutions resulted in statistically similar values of \(J_{\text{endo}}\), reduction to an Na\(^+\)-free solution caused a further statistically different increase in the passive flux (\(J_{\text{epi}}\)) and a consequent decrease in the net flux.

Changes in [Cl\(^-\)] appeared to have no detrimental effect on \(J_{\text{endo}}\) net. Both unidirectional fluxes were significantly increased, albeit the change induced by 50 mEq [Cl\(^-\)] solution was not further altered by Cl\(^-\)-free solution.

Group II. The addition of 1 mg/ml carbonic anhydrase to both sides of the endothelium approximately doubled the net and unidirectional fluxes of H\(^14\)CO\(_3\). The unidirectional fluxes were significantly greater at pH 7.3 than at pH 8, which corresponds to the data found previously in the absence of carbonic anhydrase.\(^6\) There was a trend in both passive and active fluxes at both pH values to decrease as time progressed; for example, at pH 8 the successive 30 min measurements of \(J_{\text{endo}}\) were 8.52 ± 0.27, 7.40 ± 0.22, 6.73 ± 0.17, and 6.12 ± 0.17, and for \(J_{\text{epi}}\) they were 5.03 ± 0.29, 4.23 ± 0.32, 3.92 ± 0.29, and 3.77 ± 0.34 (n = 6).

Group III. The intracellular pH was calculated according to equations given previously by Irvine et al.,\(^{11}\) in which tissue hydration and the ratio of intracellular to extracellular water are included. Tissue hydration was taken as 70% and extracellular water as 10% of total tissue water for all cases. Increased tissue hydration would reduce the calculated intracellular pH by about 0.03 to 0.04 pH.
Table II. Corneal endothelial intracellular pH as a function of ambient conditions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Intracellular pH</th>
<th>No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 mM bicarbonate, pH 7, 1 hr</td>
<td>6.85 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 1 hr</td>
<td>7.20 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 3 hr</td>
<td>7.23 ± 0.05</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, de-epithelialized, 1 hr</td>
<td>7.28 ± 0.09</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 1 mg/ml carbonic anhydrase, 1 hr</td>
<td>7.38 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 1 mM acetazolamide, 1 hr</td>
<td>7.36 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, Na&lt;sup&gt;+&lt;/sup&gt;-free, 1 hr</td>
<td>7.32 ± 0.02</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 10&lt;sup&gt;-6&lt;/sup&gt;M ouabain, 1 hr</td>
<td>7.34 ± 0.04</td>
<td>4</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 10&lt;sup&gt;-4&lt;/sup&gt;M amiloride, 1 hr</td>
<td>7.38 ± 0.02, 7.44</td>
<td>2</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 7.5, 10&lt;sup&gt;-4&lt;/sup&gt;M furosemide, 1 hr</td>
<td>7.28 ± 0.02, 7.32</td>
<td>2</td>
</tr>
<tr>
<td>25 mM bicarbonate, pH 8.0</td>
<td>7.70 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>60 mM bicarbonate, pH 7.5</td>
<td>7.36 ± 0.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are the mean ± S.E.M.

<sup>b</sup>Number of experimental determinations, each consisting of either three or four pooled endothelia (duplicate samples were taken of each pooled set of endothelia, and a mean was obtained and counted as one experimental determination). Where less than four determinations were made, the average of duplicate analyses are given.

<sup>c</sup>Statistically different from 25 mM bicarbonate, pH 7.5, 1 hr.

units per 5% increase in hydration; an increase in extracellular water to 20% would reduce the calculated intracellular pH by 0.01 pH unit, and a decrease to 5% extracellular water would increase intracellular pH by 0.01 pH unit. The choice of extracellular water value is therefore of no consequence in the calculation. Only if tissue hydration was significantly changed would an effect be seen on intracellular pH. The primary determinant of the distribution of DMO therefore is intracellular and extracellular pH.

The values obtained for intracellular pH are shown in Table II. The intracellular pH was close to that of the ambient solution at low pH (7.0), and the difference between extracellular and intracellular pH was greater as the external pH increased. The difference between intracellular and extracellular pH (ΔpH) appears to become constant above ambient pH of 7.5. Incubation for 1 or 3 hr under the same ambient conditions gave the same intracellular pH, indicating that equilibrium was complete within 1 hr, and the de-epithelialized cornea data showed that the presence of the epithelium in the majority of determinations had no effect. At 60 mM bicarbonate, pH 7.5, the intracellular pH was closer to that of the bathing solution than that found at the same pH and 25 mM bicarbonate. The intracellular pH increased with sodium removal from the bathing solution, with a concurrent decrease in net bicarbonate flux (Table I), but a similar trend was observed with both carbonic anhydrase, which increased net and unidirectional bicarbonate fluxes (see Table I), and acetazolamide, which decreased net bicarbonate flux. Ouabain, which inhibits (Na<sup>+</sup> + K<sup>+</sup>) adenosine triphosphatase (ATPase), also increased intracellular pH. Amiloride tended to increase the intracellular pH, but furosemide did not cause such a large increase.

Discussion

Previous research has shown that both the rate of fluid movement across the endothelium (calculated from the rate of stromal deturgescence) and the electrical potential difference across the endothelium are dependent on the sodium content of the bathing medium and that replacement of chloride has no effect on these parameters. Although bicarbonate transport has been implicated as a causative factor in transendothelial fluid movement, no studies thus far have shown that bicarbonate transport shows the same ionic dependence as fluid movement and potential difference. The results of this study indicate that there is a strong sodium dependence of both net and unidirectional bicarbonate movement and an absence of any chloride dependence of net bicarbonate transfer.

Sodium removal from the bathing solution
not only decreased \( \text{J}_{\text{endo}}^{\text{net}} \) but also increased \( \text{J}_{\text{endo}}^{\text{net}} \), implying that the passive properties of the membrane might also be affected by this maneuver. It appears that the effect of sodium reduction is almost fully realized by reduction to at least 50 mEq Na⁺, since the only other significant change in fluxes between 50 and 0 mEq Na⁺ was an increase in the passive \( \text{J}_{\text{endo}}^{\text{net}} \) flux.

Chloride removal from the bathing solution, on the other hand, had no effect on the net bicarbonate flux but caused a significant increase in both unidirectional fluxes. Perhaps the latter is indicative of either some regulatory role of Cl⁻ on bicarbonate, such as an exchange mechanism where, in the absence of Cl⁻, another anion can substitute, or a general effect on endothelial cell membrane permeability. The net flux is unaltered, however, and this correlates with the lack of effect on both potential difference and fluid movement.³

Both experiments with sodium and chloride changes of the bathing media support the hypothesis that bicarbonate is directly related to potential difference and fluid movement across the rabbit corneal endothelium. The correlation is striking, although with the sodium data the majority of response appeared to be caused by a change from 143 to 50 mEq Na⁺, whereas the potential difference showed a more gradual reduction as sodium concentration was decreased.³

The addition of carbonic anhydrase to the endothelial bathing solutions caused a large increase in net and unidirectional bicarbonate fluxes. The unidirectional fluxes were greatly increased, by between two and three times, over control values and still showed the same pH dependence as noted previously under normal conditions,⁶ i.e., greater unidirectional fluxes at lower ambient pH. The addition of carbonic anhydrase did not change the pH, PCO₂, or PO₂ of the bathing solution despite the marked increase in the unidirectional fluxes in the initial 30 min after drug application. Carbonic anhydrase could be maintaining a high concentration of HCO₃⁻ or CO₂ in the unstirred layer adjacent to the membrane, thereby providing substrate for the transport system, which normally would remove HCO₃⁻ or CO₂ from the unstirred layer and create a gradient from the membrane face to the bulk solution. Since carbonic anhydrase enhances the hydration-dehydration of CO₂, such a gradient could be minimized by the generation of HCO₃⁻ or CO₂ in the unstirred layer as fast as it was removed by the transport system. The latter could well explain the marked elevation of unidirectional fluxes and of the net flux, especially since the stroma is a large unstirred fluid volume.

Support for this concept is obtained from the slight increase in intracellular pH after carbonic anhydrase addition to the ambient solution (Table II). These findings with carbonic anyhdrase may also serve to explain how carbonic anhydrase inhibitors affect net movement of radiolabeled bicarbonate across the endothelium,⁵ since local inhibition of the cellular enzyme would lead to an inability to maintain a high bicarbonate or CO₂ concentration at the membrane surface for either exchange or transport function. That there is inhibition of measured bicarbonate fluxes by carbonic anhydrase inhibitors⁵ and acceleration of fluxes by extracellular carbonic anhydrase supports the concept that both bicarbonate and carbon dioxide are involved in the net transfer of bicarbonate.⁴

The relationship between intracellular and extracellular pH is that the intracellular pH is approximately equal to that of the ambient solution when the latter is at pH 7, whereas, with the external pH approaching 8, the intracellular pH does not increase equivalently. Above pH 7.5, however, the difference between intracellular and extracellular pH appears to become constant at 0.3 pH units. The results of a previous study showed that the unidirectional bicarbonate fluxes at pH 7, 25 mM bicarbonate, are greater than those at pH 8.⁶

As pH increases in the external medium the \( [\text{H}^+] \) gradient would tend to assist \( \text{H}^+ \) movement from the cell, which might decrease the fluxes of bicarbonate if there were not preferential directional movement of \( \text{H}^+ \). The measured values of intracellular pH at
various external pH values, when related to previous flux data, would appear to suggest that a proton-bicarbonate exchange mechanism plays some role in bicarbonate transfer across the corneal endothelium or in exchange at one or both cell surfaces. Nevertheless, the primary determinant of intracellular pH appears to be the pH of the environment. The data at 60 mM bicarbonate, where the unidirectional fluxes are greater, indicate that the intracellular pH more closely resembles that of the extracellular fluid, a situation that again (in combination with the elevated bicarbonate concentration) would provide a more suitable environment for enhanced bicarbonate transfer.

Data obtained with various perturbations of the endothelial bathing media indicate that removal of sodium causes an increase of intracellular pH (Table II) concurrent with a fall in net bicarbonate flux (Table I). Both carbonic anhydrase, which accelerates both unidirectional and net bicarbonate fluxes, and acetazolamide, which reduces net bicarbonate flux across the endothelium, increase intracellular pH. Ouabain, which inhibits (Na\(^+\) + K\(^+\))-dependent ATPase\(^{12,13}\), and causes corneal swelling without alterations of passive endothelial permeability,\(^{14}\) increased intracellular pH, which may relate to some alteration of bicarbonate fluxes. Amiloride tended to increase intracellular pH compared to controls, which may imply an involvement of sodium in intracellular H\(^+\) regulation. Furosemide, on the other hand, had little effect, implying that Cl\(^-\)-H\(^+\) exchange plays no role in regulation of intracellular pH or bicarbonate fluxes.

We thank Mrs. Ilene Prior for her valuable secretarial assistance.

REFERENCES