

The Roles of Bicarbonate and CO₂ in Transendothelial Fluid Movement and Control of Corneal Thickness

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Purpose. To determine whether maintenance of corneal hydration is dependent on bicarbonate ions and whether these ions can be derived from metabolic or exogenous CO₂, and to investigate the relationship of transendothelial fluid movement to control of hydration.

Methods. The thickness of intact or deepithelialized rabbit corneas was measured while superfused on the endothelial surface with either 33 mM HCO₃⁻/5% CO₂ buffered media or 10 mM HPO₄⁻ buffered media in the presence and absence of inhibitors of ion transport and respiration. The corneal surface was covered with either silicone oil ("normal" corneas) or with the same media used for superfusion ("swollen" corneas). ATP and Na⁺,K⁺-ATPase activity were measured in endothelia scraped from the tissues after superfusion.

Results. Intact and deepithelialized corneas covered with oil swelled at a negligible rate (4 to 8 μm/hour) in 33 mM HCO₃⁻ medium but at 45 to 60 μm/hour in HPO₄⁻ medium. Antimycin A altered neither of these swelling rates, but ethoxzolamide (0.1 mM) caused swelling in HCO₃⁻/CO₂ (\approx 12 μm/hour above controls) with no change of rate in HPO₄⁻. Ouabain (0.1 mM) increased swelling to 45 to 50 μm/hour in HCO₃⁻/CO₂ but had no effect in HPO₄⁻. Saturating the oil on deepithelialized corneas with 5% CO₂, or putting HCO₃⁻/CO₂ medium on the epithelial surface of intact corneas, did not alter the swelling rates seen with HPO₄⁻ superfusion. The equilibrium thickness of deepithelialized corneas swollen with HCO₃⁻/CO₂ on both surfaces was 35 μm less than that of corneas swollen in HPO₄⁻. The difference was abolished by ouabain, which caused corneas in HCO₃⁻/CO₂ to swell an additional 30 μm but did not alter the equilibrium thickness of corneas swollen in HPO₄⁻. Ethoxzolamide and DIDS (0.2 mM) increased the thickness in HCO₃⁻/CO₂ but not in HPO₄⁻. Na⁺,K⁺-ATPase activities of endothelia were similar after HCO₃⁻/CO₂ and HPO₄⁻ superfusions, but the concentration of ATP in the HPO₄⁻-superfused tissues was increased 35%.

Conclusions. Normal corneal thickness can be maintained in vitro only in media that contain HCO₃⁻ at concentrations of more than 20 mM. Neither metabolic CO₂ nor CO₂ present in air-equilibrated, nominally HCO₃⁻-free media can supply this requirement for HCO₃⁻, even though these sources support the presumably related processes of transendothelial fluid movement and intracellular pH regulation. Invest Ophthalmol Vis Sci. 1995;36:103–112.

After the pioneering measurements by Mishima,¹ Maurice,^{2,3} Hodson,^{4,5} and Fischbarg^{6,7} of the thickness of the in vitro superfused rabbit cornea, it has been accepted for almost 20 years that without exogenous bicarbonate ions, the endothelial transport mechanism fails. However, this concept has recently come to be questioned by Doughty and Maurice,⁸ who

showed that net transendothelial fluid movement from stroma to aqueous, measured by a technique originally developed by Maurice,^{2,9} was unchanged when HCO₃⁻ and CO₂ were eliminated from the perfusion solutions. The authors state that these results "require reconsideration of the widely accepted view that the fluid pump in the corneal endothelium is bicarbonate dependent." The issue was subsequently examined by Kuang et al,¹⁰ who used essentially the same method as Maurice² and concluded that the fluid pump did require HCO₃⁻, which, in the absence of exogenous HCO₃⁻, was supplied intracellularly by metabolically derived CO₂ through the action of carbonic anhydrase. This conclusion is in direct conflict

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with results obtained by measuring corneal thickness rather than water flow. In the former studies,³⁻⁷ an increase in corneal thickness or a failure of swollen corneas to deturgescce were observed when the tissues were superfused on the endothelial surface with media deficient in HCO_3^- (<20 to 30 mM) and covered anteriorly with silicone oil. Thus, there is an apparent dichotomy between conditions necessary to maintain a freshly isolated cornea at normal thickness or to support deturgescence and those necessary to support a net flux of fluid across the endothelium from a pool anterior to the swollen stroma.

We have attempted to resolve this issue by using a uniform measure of physiological function of the cornea, its thickness, in a single superfusion chamber, but under two different paradigms: the normal cornea, which swells as a result of the imbibition pressure of the stroma when pump mechanisms fail,¹¹ and the swollen cornea, which, with identical media on the endothelial and bare stromal surfaces, reaches a steady state thickness¹² and approximates closely the conditions of the net fluid movement techniques.^{2,8-10,13} Our results show that regardless of the experimental paradigm, the maintenance of corneal thickness and the ability of swollen corneas to deturgescce requires HCO_3^- in the external solution superfusing the endothelial surface or stroma. We further show that the intracellular production of HCO_3^- from metabolic or exogenous CO_2 is unable to activate the fluid pump to the extent necessary for the maintenance of normal corneal thickness or deturgescence from a swollen condition.

METHODS

Animals

New Zealand white rabbits, each weighing approximately 2 kg, were used in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. They were killed by intracardiac injection of T-61 Euthanasia solution (Hoechst-Roussel, Somerville, NJ), and the eyes were enucleated with the lids.

Superfusion

Corneas were mounted for superfusion of the endothelial surface, either intact or after removal of the epithelium, by scraping with a scalpel blade, as described previously.³ The composition of the media used is shown in Table 1. The bicarbonate medium ($\text{HCO}_3^-/\text{CO}_2$) was equilibrated with 7% O_2 /5% CO_2 /88% N_2 , and the other media were equilibrated with air. This medium has 33 mM HCO_3^- to allow a closer pH to that of the HPO_4^{2-} medium than do 37 mM HCO_3^- or 43 mM HCO_3^- media normally used for

TABLE 1. Composition of Media for Superfusion and Overlay of Stroma (mM)

	$\text{HCO}_3^-/\text{CO}_2$	HPO_4^{2-}	HEPES
NaCl	119 (132)	138	132
NaHCO ₃	33 (20)	—	—
Na ₂ HPO ₄	0.8	8.1	0.8
KH ₂ PO ₄	—	1.5	—
HEPES	—	—	25
KCl	3.8	2.7	3.8
MgCl ₂	1.2	1.2	1.2
CaCl ₂	1.2	1.2	1.2
Glucose	5.5	5.5	5.5
pH	7.5	7.4	7.4

40 µg/ml gentamycin was included in all media.

rabbit studies, but it does not completely maintain normal hydration of the isolated cornea (see Fig. 1). The phosphate medium (HPO_4^{2-}) is the same as that used by Kuang et al.¹⁰ All media had osmolarities of 289 mOsm/l to 292 mOsm/l.

Additions to the media were made either by dissolving the reagents directly in the appropriate media or, as indicated, by dissolving in ethanol, using the addition of vehicle as a control. When HCO_3^- was lowered from 33 mM to 20 mM, 13 mM NaCl was added as replacement. Superfusion was at 3 ml/hour and 37°C, with the exit port maintained at a height of 18 cm to ensure a constant "intraocular" pressure in all experiments. Solution changes were made as previously described,¹⁴ with four turnovers of chamber volume (250 µl) within 1 minute. Corneal thickness was measured in the specular microscope³ every 20 or 30 minutes. Starting thicknesses of deepithelialized corneas measured under silicone oil ranged from 330 µm to 390 µm and intact corneas from 370 µm to 410 µm. Only representative contralateral pairs (*n* of 3 or more), whose initial thickness differed by less than 10 µm, were used when comparing experimental conditions, except for Figures 1 and 3B. In the first procedure (see Results), corneas were covered throughout with silicone oil (200 fluid; 20 centistokes viscosity; Dow Corning, Midland, MI). When corneas were covered with aqueous solutions, the solutions were changed every 10 minutes¹² and covered with a glass coverslip (second procedure). When aqueous solutions were replaced by silicone oil (Fig. 5), the fluid was aspirated from the surface, and the peripheral cornea was gently touched with a filter paper strip before the oil was applied. The new fluid surrounding the objective lens caused an apparent increase in measured corneal thickness of approximately 5% because of the difference in refractive indices of the aqueous solutions (1.334) and silicone oil (1.40).³ All measurements made under aqueous solutions, therefore, are reported after correction for this optical effect.

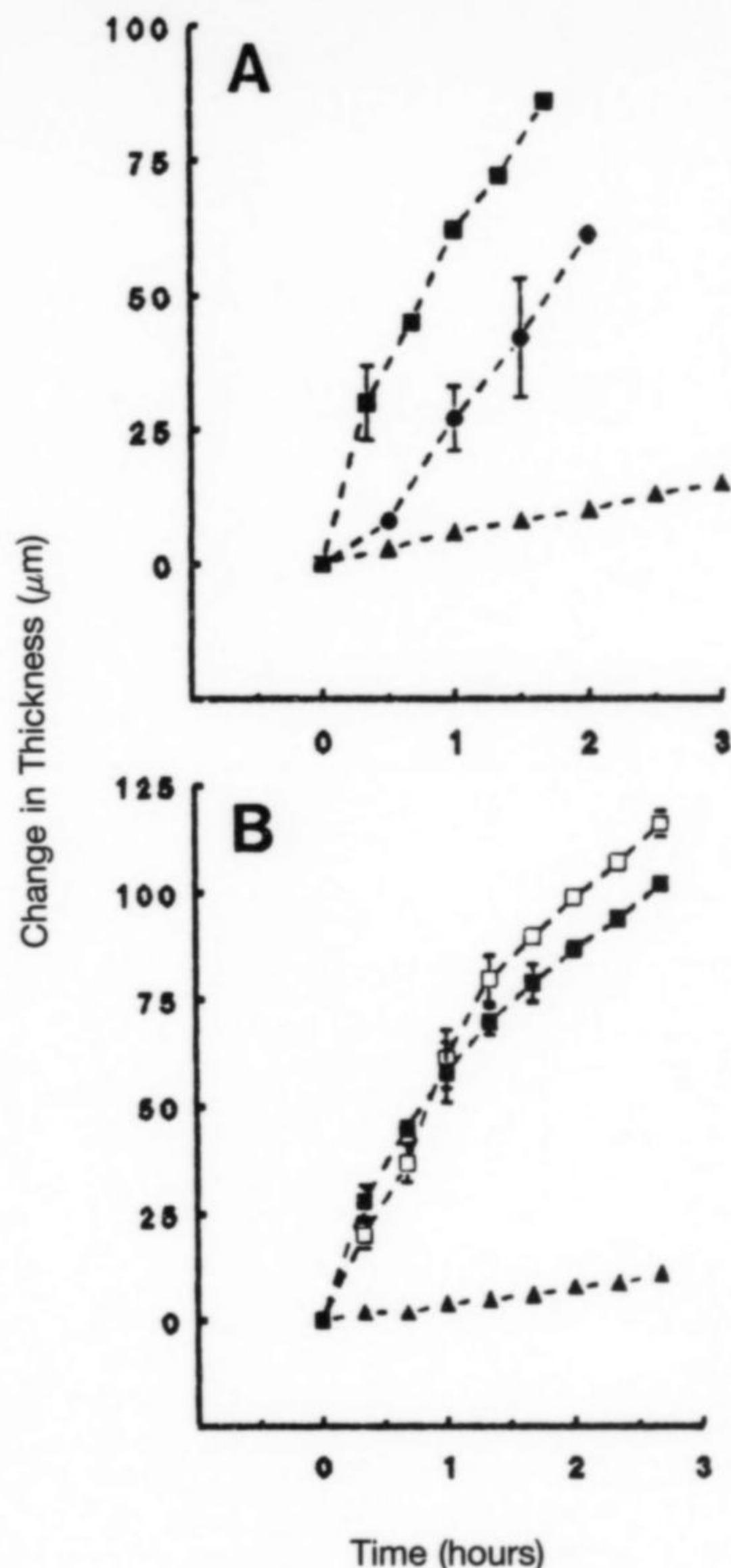


FIGURE 1. Change in thickness of corneas superfused with the different media listed in Table 1. (A) Deepithelialized corneas covered on the anterior stromal surface with silicone oil. HCO₃⁻/CO₂ medium (▲); HPO₄⁻ medium (■); Hepes medium (●). Starting thickness value of each cornea was defined as 0. The swelling rate of the controls in 33 mM HCO₃⁻ was 4.7 ± 2 μm/hour. Values are means of four or more experiments, with error bars showing SD where this value is greater than the symbol size. (B) Intact corneas covered on the anterior surface with fluid saturated with 5% CO₂. HCO₃⁻/CO₂ on endothelium and epithelium (▲); HPO₄⁻ superfusion with HCO₃⁻/CO₂ on epithelium (■); HPO₄⁻ superfusion with CO₂ equilibrated oil on stromal surface (□). *n* = 4 for each condition.

Ouabain, antimycin A, and 4,4' diisothiocyanostilbene-2,2'disulfonic acid (DIDS) were from Sigma (St. Louis, MO), and ethoxzolamide was from Upjohn (Kalamazoo, MI). Antimycin A was used in 0.1% EtOH and ethoxzolamide in 0.25% EtOH.

Assays

ATP and ATPase activity were measured by previously described methods^{14,15} in endothelium plus Descemet's membrane scraped from corneas at the end of the superfusion period. Bare stromata were placed in respective media overnight at 4°C in filled, closed vessels that were allowed to reach room temperature 18 hours later before weighing the tissues. These were then dried to constant weight at 60°C.

RESULTS

Procedure One: The Normal Thickness Cornea

Figure 1A compares the behavior of freshly isolated, deepithelialized corneas, covered on the anterior surface with silicone oil, when superfused with isosmotic media buffered with 33 mM HCO₃⁻/5% CO₂, 10 mM HPO₄⁻, or 25 mM Hepes, all at pH 7.4 to 7.5. Under these conditions, corneas in the HCO₃⁻/CO₂ medium swelled slowly at a rate between 4 μm/hour and 8 μm/hour. This is considered the control medium in the present experiments (see Methods), and it yielded identical results with intact corneas (not shown). By contrast, in the Hepes or phosphate buffer, corneas swelled at rates between 45 and 60 μm/hour. (The consistently observed delay before swelling seen in Hepes is attributed to the osmotic effects of substituting a large anion for HCO₃⁻, similar to that seen when gluconate replaces chloride¹⁶). Similar rates of swelling were observed in media buffered to pH 7.4 with 25 mM Tris-HCl, 50 mM Hepes (used by Doughty and Maurice⁸), and with citrate-acetate (5.8 and 28.6 mM, respectively; BSS, Alcon Laboratories, Fort Worth, TX). Subsequently, all experiments have compared HCO₃⁻/CO₂ with HPO₄⁻ buffer only.

Figure 1B shows the thickness of superfused intact corneas when the anterior surface was covered with CO₂-saturated fluids and a glass coverslip to prevent any excessive loss of metabolic CO₂ from the tissue that might occur in the absence of epithelium or because of high gas solubility in the silicone oil. Because corneas with intact epithelium allow negligible access of fluid or ions to the stroma across the cell layer,¹⁷ HCO₃⁻/5% CO₂ medium could be placed directly on the epithelial surface. When both corneal surfaces were exposed to this same HCO₃⁻ medium, virtually no swelling occurred, but when HPO₄⁻ superfused the endothelium, again with HCO₃⁻/CO₂ medium on the epithelium, corneas swelled at the same high rates as

those seen in Figure 1A. Similarly, saturation of the silicone oil overlying deepithelialized corneas by constant bubbling with 5% CO₂ did not alter the high swelling rates found in HPO₄⁻ medium. The possibility that metabolic CO₂ might rapidly diffuse out of the endothelial cells and be swept away by the high superfusion rate used in this study, which contrasts with the stagnant fluid conditions used in the transendothelial flux experiments,⁸⁻¹⁰ was tested in two further procedures. In one, the flow rate of the superfusing HPO₄⁻ medium was reduced to that of aqueous humor in vivo, 150 μ l/hour, and to zero (stagnant), and in the second, the superfusing HPO₄⁻ medium was itself bubbled with 5% CO₂ (causing the pH to fall to 6.8), while in each condition the epithelial surface was covered with HCO₃⁻/CO₂. In every case, corneas with HPO₄⁻ medium at the endothelium swelled continuously for up to 5 hours (data not shown). With the slow and zero superfusions, swelling was at about half the rate seen at 3 ml/hour, but was little changed by the addition of ouabain (see below). In HCO₃⁻/CO₂ media, corneas maintained normal thickness at both 150 μ l/hour and zero flow rates.

The high swelling rates observed in Figure 1 in HPO₄⁻ medium were also seen in the HCO₃⁻/CO₂ buffer when the endothelial Na⁺,K⁺-ATPase was inhibited by 10⁻⁴ M ouabain.¹⁴ Figure 2 shows that paired corneas superfused with HCO₃⁻/CO₂ plus 10⁻⁴ M ouabain, or with HPO₄⁻ (no added ouabain), swelled at identical rates. When ouabain was added to the HPO₄⁻ medium, the two corneas continued to show identical swelling patterns. Ouabain also had no effect on the high swelling rates of HPO₄⁻ superfused corneas, covered as in Figure 1B with CO₂-gassed fluids, and increased swelling at the slow and zero superfusion rates by only 6 μ m/hour to 10 μ m/hour (data not shown). The inset to this figure demonstrates that on replacement of the HPO₄⁻ medium with one containing 20 mM HCO₃⁻ or 33 mM HCO₃⁻, the swelling ceased and deturgescence occurred. The rate and extent of thinning were greater as the HCO₃⁻ concentration was increased: The initial rates in 33 and 20 mM HCO₃⁻ were 38 \pm 2 and 12 \pm 6 μ m/hour, respectively (\pm SD, n = 3). Readdition of HCO₃⁻ was without effect if corneas were first incubated with HPO₄⁻ medium containing ouabain.

Kuang et al¹⁰ reported that the rate of fluid pumping in 10 mM HPO₄⁻ was reduced by 35% to 50% when buffering was provided by only 1 mM HPO₄⁻, and that this rate was further reduced by the addition of the carbonic anhydrase inhibitors acetazolamide or ethoxzolamide. We found no difference in swelling rates between 1 mM and 10 mM HPO₄⁻ superfusions (data not shown), nor was a difference detectable when 0.1 mM ethoxzolamide was added to 10 mM HPO₄⁻ (Fig. 3A). The carbonic

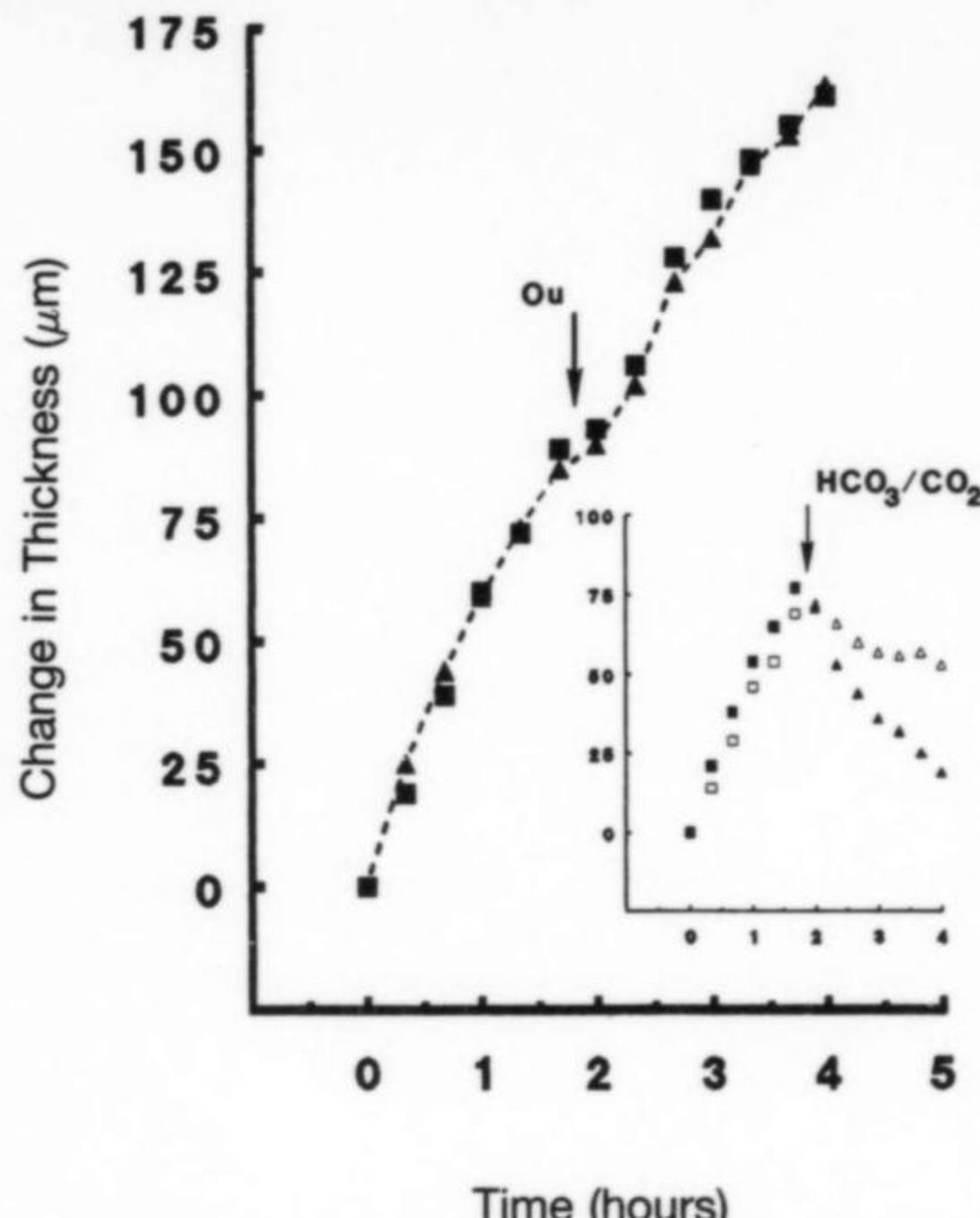


FIGURE 2. Change in thickness of paired corneas superfused under silicone oil with: HPO₄⁻ (■); HCO₃⁻/CO₂ + 10⁻⁴ M ouabain (▲). At arrow, 10⁻⁴ M ouabain (Ou) was added to the HPO₄⁻ medium. Dashed line connects the HCO₃⁻/CO₂ data. (inset) A pair of corneas, both superfused with HPO₄⁻ medium (■, □) for 2 hours. The medium was then changed to 33 mM of HCO₃⁻/5% CO₂ (▲), or 20 mM HCO₃⁻/3% CO₂ (△).

anhydrase inhibitor did, however, cause swelling of corneas in HCO₃⁻/CO₂ medium at about 12 μ m/hour. To detect this swelling reliably in the face of vehicle-induced effects (0.25% ethanol), we used in this instance a 33 mM HCO₃⁻/CO₂ medium with 0.1 mM adenosine, providing an entirely flat baseline for the control.¹⁵

To determine whether HCO₃⁻ generated from metabolic CO₂ is relevant to the behavior of corneas superfused with HPO₄⁻ media, antimycin A was added to the media at 5.10⁻⁶ M. This compound increases corneal glycolysis as a result of the inhibition of respiration¹⁵ but was without effect on the swelling rates of paired corneas superfused with HPO₄⁻ or HPO₄⁻ plus inhibitor (Fig. 3B). Moreover, the subsequent addition of 10⁻⁴ M ouabain to the medium with antimycin A caused no deviation from the rate seen in HPO₄⁻ alone. Antimycin A had a negligible effect on corneas superfused with HCO₃⁻/CO₂, consistent with a previous finding¹⁵ that this inhibitor did not alter normal hydration in deepithelialized corneas.

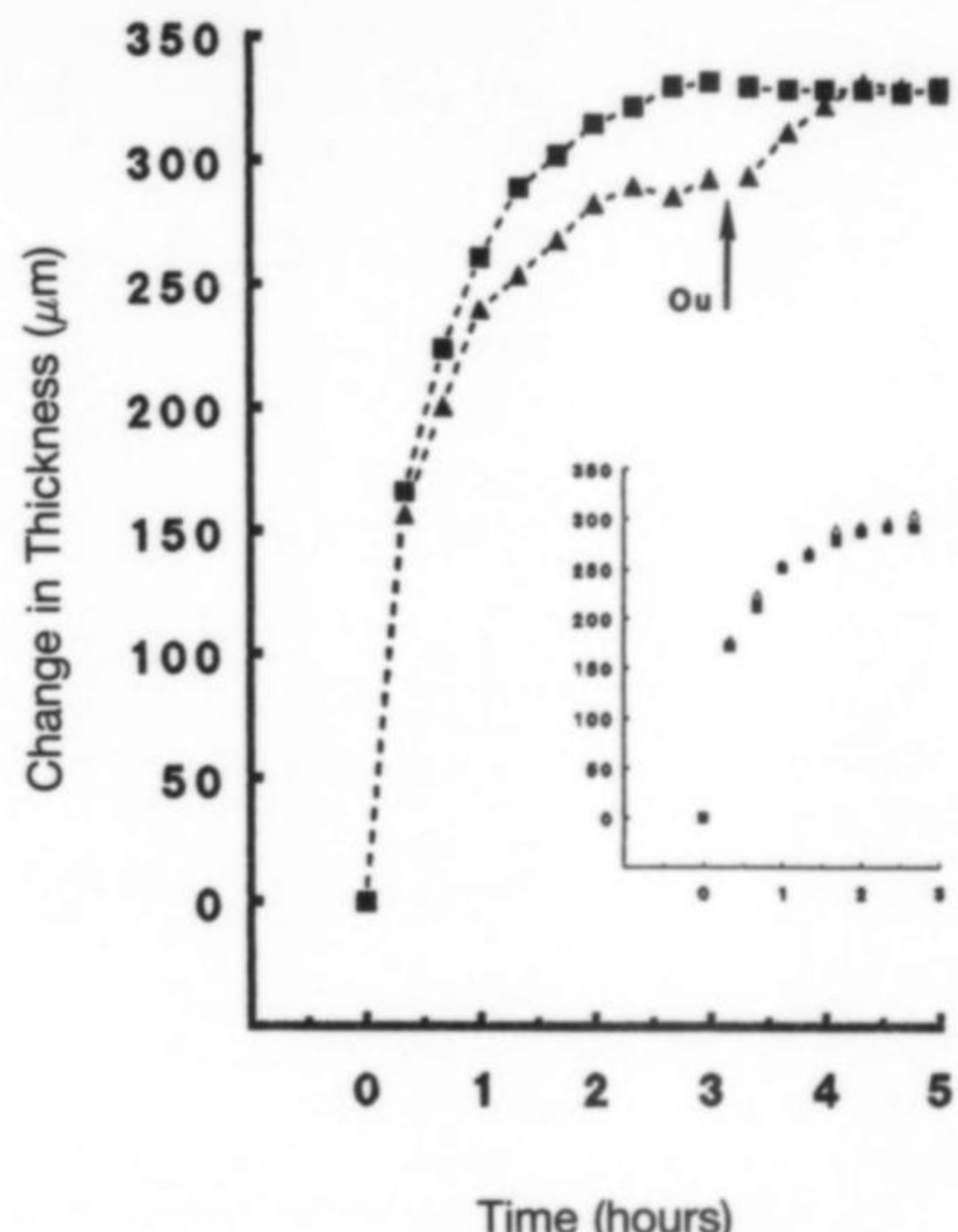
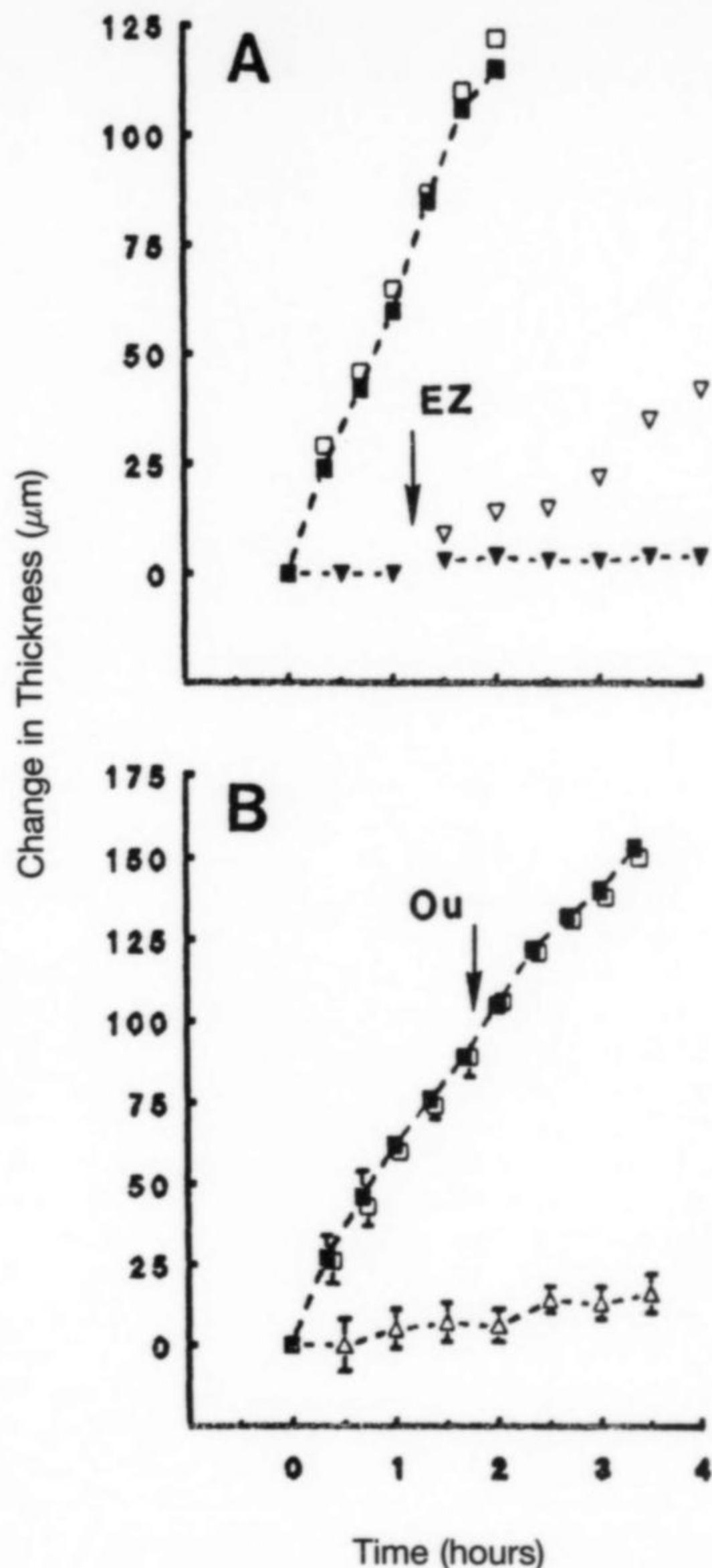


FIGURE 4. Change in thickness of paired corneas superfused with $\text{HCO}_3^-/\text{CO}_2$ or HPO_4^- and overlaid with these media on the anterior stromal surface (this fluid changed every 10 minutes). $\text{HCO}_3^-/\text{CO}_2$ (\blacktriangle); HPO_4^- (\blacksquare). At arrow, 10^{-4} M ouabain added to both media. (inset) Paired corneas superfused with $\text{HCO}_3^-/\text{CO}_2$ (\triangle) or HPO_4^- (\blacksquare), both with 10^{-4} M ouabain present from origin.

Procedure Two: The Swollen Cornea at Equilibrium Thickness

Figure 4 shows the swelling patterns obtained in the second procedure, with the same medium as that superfusing the endothelium placed and renewed every 10 minutes on the anterior stromal surface, allowing the stroma to reach an equilibrium thickness.¹² Although swelling was rapid and extensive in both $\text{HCO}_3^-/\text{CO}_2$ and HPO_4^- , corneas superfused with HPO_4^- reached a steady state thickness 35 μm greater than that of the $\text{HCO}_3^-/\text{CO}_2$ superfused corneas (see Table 2). Reduction of the superfusion rate to 150 $\mu\text{l}/\text{h}$ and gassing the overlying HPO_4^- medium with 5% CO_2 to eliminate the possible "wash-out" or diffusional loss of metabolic CO_2 did not alter the HPO_4^- equilibrium thickness. With $\text{HCO}_3^-/\text{CO}_2$ on the stromal surface and HPO_4^- as the superfusing medium, the equilibrium thickness of corneas was 29 μm to 33 μm less than that in paired corneas treated with HPO_4^- medium on both surfaces. When ouabain was added after the corneas reached steady state thickness, the cornea in $\text{HCO}_3^-/\text{CO}_2$ swelled further to reach the same thickness as the one in HPO_4^- , which was unaffected by

TABLE 2. Differences in Thickness of Paired Stroma–Endothelial Preparations at Equilibrium With Various Media Superfusing and Overlying the Tissue

<i>Conditions</i>		<i>Difference in Thickness: B – A (μm)</i>
<i>A</i>	<i>B</i>	
$\text{HCO}_3^-/\text{CO}_2$	HPO_4^-	$35 \pm 6 (9)$
$\text{HCO}_3^-/\text{CO}_2$	$\text{HCO}_3^-/\text{CO}_2 + \text{ouabain}$	$29 \pm 4 (5)$
HPO_4^-	$\text{HPO}_4^- + \text{ouabain}$	$1 \pm 2 (6)$
$\text{HCO}_3^-/\text{CO}_2 + \text{ouabain}$	$\text{HPO}_4^- + \text{ouabain}$	$-2 \pm 5 (4)$
$\text{HCO}_3^-/\text{CO}_2$	$\text{HCO}_3^-/\text{CO}_2 + \text{ethoxzolamide}$	$13 \pm 7 (3)$
HPO_4^-	$\text{HPO}_4^- + \text{ethoxzolamide}$	$2 \pm 2 (5)$
$\text{HPO}_4^-:\text{HCO}_3^-/\text{CO}_2$	$\text{HPO}_4^-:\text{HPO}_4^-$	$29, 33 (2)$

For all conditions listed except those in the bottom row, the identical solution was used to superfuse the endothelium and to cover the stroma. For example, the top row compares the equilibrium thicknesses between corneas superfused and covered with $\text{HCO}_3^-/\text{CO}_2$ medium (A) versus corneas superfused and covered with HPO_4^- medium (B). In the bottom row, the conditions are as follows: In A, the superfusion medium was HPO_4^- , but the stroma was covered with $\text{HCO}_3^-/\text{CO}_2$ medium. In B, HPO_4^- was on both surfaces. Values are means \pm SD with number of experiments in parentheses.

ouabain. If ouabain was present throughout the perfusion period, both corneas reach the same plateau thickness (see inset and Table 2).

The experiments with ouabain present in both $\text{HCO}_3^-/\text{CO}_2$ and HPO_4^- media, where corneas reach the same equilibrium thickness, also show that these buffers do not alter the swelling properties of the stroma per se. This was confirmed by the observation that when stromal buttons, with endothelium and Descemet's membrane removed, were allowed to swell freely overnight in the two media (without ouabain), they imbibed equal amounts of fluid, with resulting wet weight/dry weight ratios of 15.9 ± 1.4 in $\text{HCO}_3^-/\text{CO}_2$ and 16.6 ± 1.1 in HPO_4^- (mean \pm SD, $n = 6$).

When the aqueous medium overlying the bare stroma was removed and was replaced with silicone oil, corneas superfused with $\text{HCO}_3^-/\text{CO}_2$ began to deturgescce, whereas those in HPO_4^- showed no change in thickness (Fig. 5A). However, if the HPO_4^- medium superfusing the endothelium was replaced by $\text{HCO}_3^-/\text{CO}_2$, then deturgescence ensued in the same manner as in the corneas constantly in $\text{HCO}_3^-/\text{CO}_2$ (Fig. 5B). No deturgescence was observed in the $\text{HCO}_3^-/\text{CO}_2$ medium in the presence of 10^{-4} M ouabain (Fig. 5B).

To resolve the question of why corneas in HPO_4^- reach an equilibrium thickness despite the fact that the null effect of ouabain in this medium suggests there is no functional active fluid transport, we superfused corneas in HPO_4^- medium lacking calcium ions. The endothelial cells of such corneas form clumps with intervening areas of exposed Descemet's membrane, which results in high rates of swelling even under oil.¹⁸ With the calcium-deficient HPO_4^- medium on the stroma, an equilibrium was established approximately 70 μm thicker (data not shown) than

that of paired corneas superfused with 1.2 mM calcium. Thus, even with the endothelial permeability barrier partly destroyed, corneas under these conditions do not swell to the maximum (1500 μm) reached by isolated stromal buttons.

Ethoxzolamide (0.1 mM) caused no change in equilibrium thickness of corneas superfused with 10 mM HPO_4^- but increased by approximately 13 μm that of corneas in $\text{HCO}_3^-/\text{CO}_2$ medium (Fig. 6A and Table 2). Similarly, DIDS, an inhibitor of $\text{HCO}_3^-/\text{Cl}^-$ exchange and $\text{Na}^+/\text{HCO}_3^-$ symport reactions,^{19,20} was without effect in HPO_4^- , but, again, increased the equilibrium thickness in $\text{HCO}_3^-/\text{CO}_2$ (Fig. 6B). Also shown (Fig. 6C) is the absence of an effect of 5.10^{-6} M antimycin A on the equilibrium thicknesses reached in either media. Superfusion with 1 mM HPO_4^- gave identical corneal swelling and equilibrium patterns (data not shown) to those seen with 10 mM HPO_4^- .

The data of Table 3 show that endothelia of corneas superfused with HPO_4^- had a higher ATP content than those from $\text{HCO}_3^-/\text{CO}_2$ medium ($P < 0.01$), and that the Na^+,K^+ -ATPase activities of the two sets of endothelia were equal.

DISCUSSION

Several studies suggest that measurements of net transendothelial fluid movement and corneal thickness are equivalent methods for assessing the regulation of corneal hydration, congruent conclusions having been reached from the effects of temperature,² ouabain,^{9,21} and adenosine^{3,22,23} in both assay systems. However, this is not true for studies of the effects of bicarbonate, which is actively transported by the endothelium.^{24,25} Although the apparent bicarbonate independence of fluid movement⁸ has been attributed to the role of

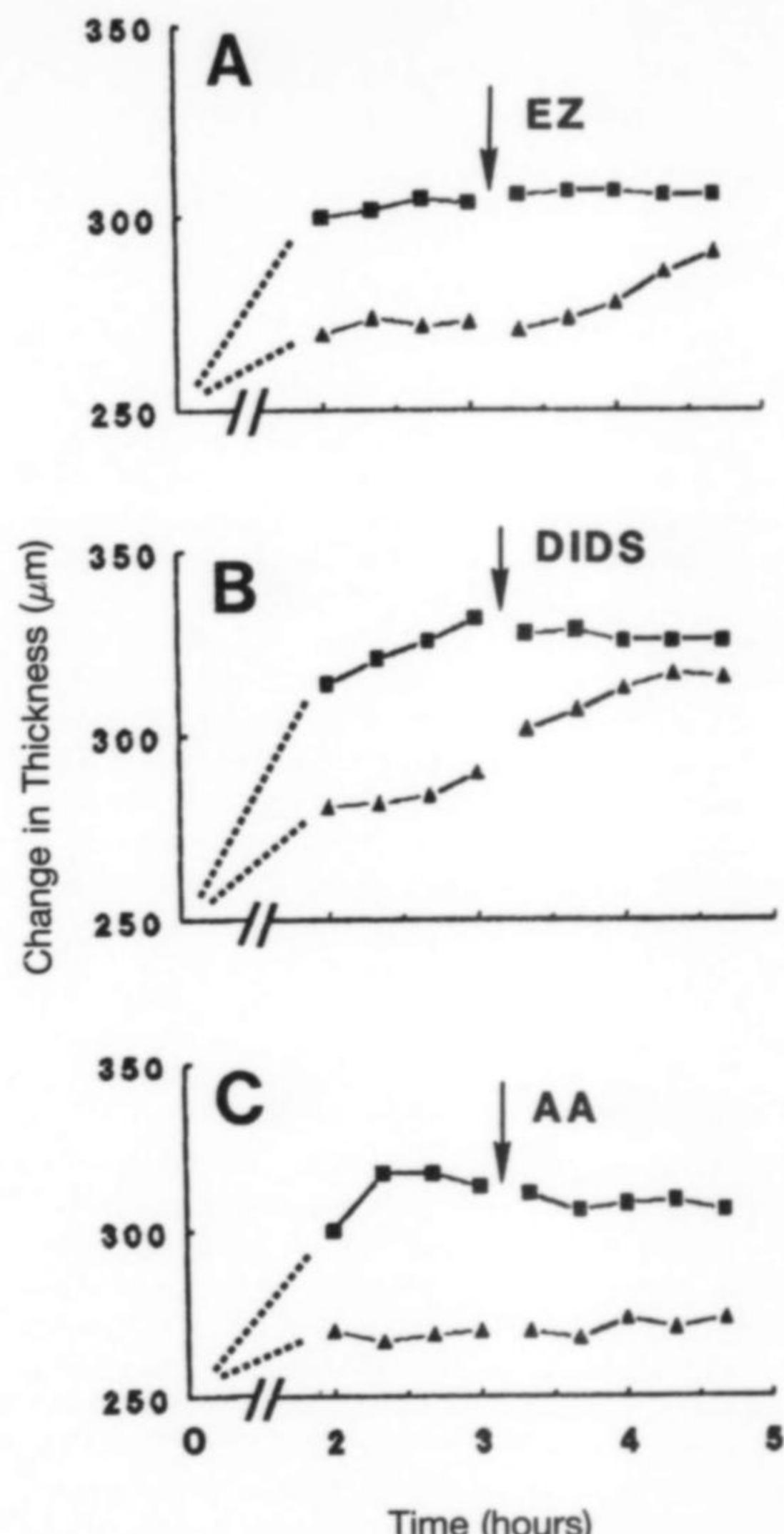
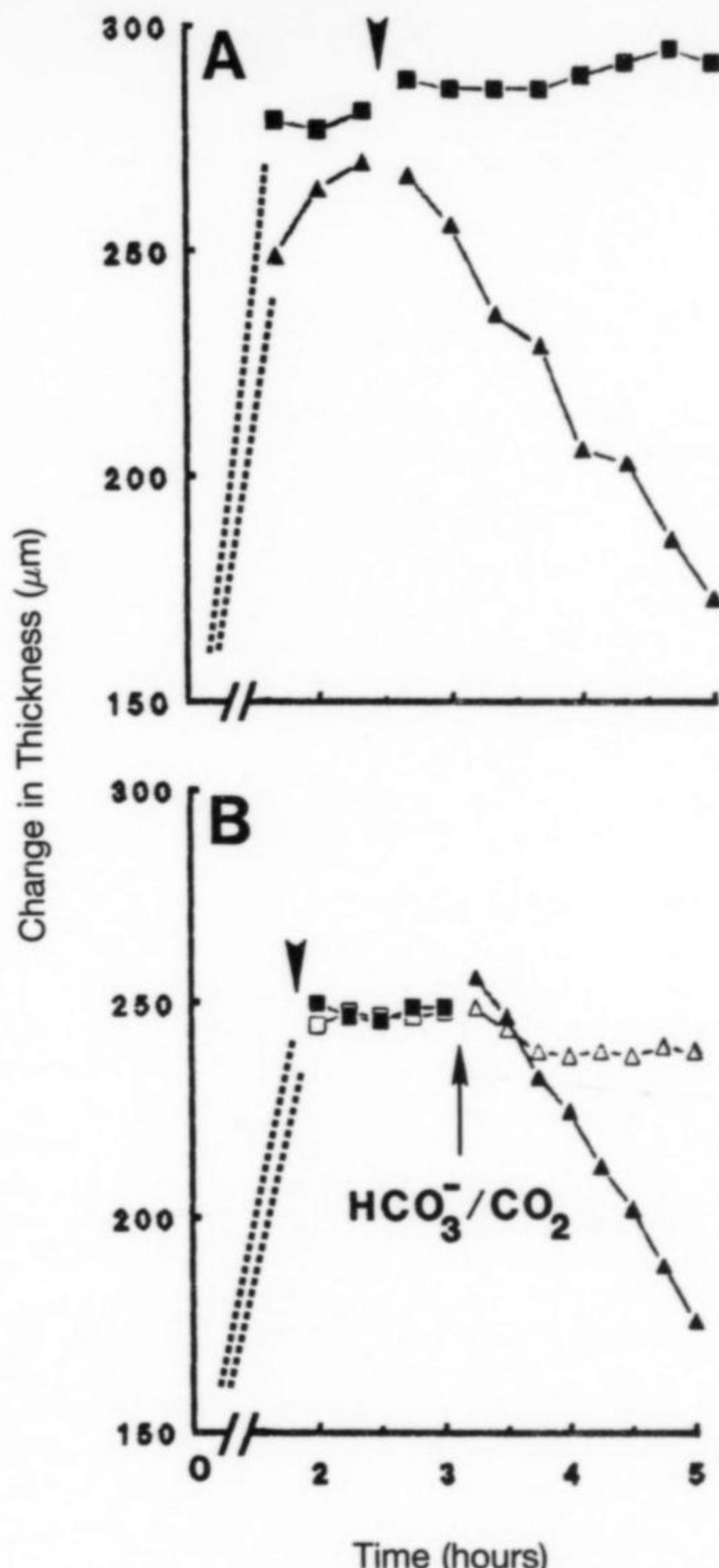


FIGURE 5. Change in thickness of corneas superfused and overlaid with $\text{HCO}_3^-/\text{CO}_2$ or HPO_4^- . Ordinate refers to change from initial thickness, the dotted line representing the rapid swelling phase seen in Figure 4; data are shown from the time corneas approached equilibrium thickness. (A) Paired corneas in $\text{HCO}_3^-/\text{CO}_2$ (\blacktriangle) or HPO_4^- (\blacksquare). At arrowhead, the aqueous media on the anterior surface were removed and replaced with silicone oil. (B) Paired corneas superfused from the origin with HPO_4^- (\blacksquare) and HPO_4^- with 10^{-4} M ouabain (\square). At equilibrium thickness, the anterior fluid was replaced with silicone oil (arrowhead). At arrow, the superfusing fluid of both corneas was changed to $\text{HCO}_3^-/\text{CO}_2$ medium (\blacktriangle , \triangle).

metabolic CO_2 ,¹⁰ there has been no evidence that normal thickness of rabbit³⁻⁷ or human^{26,27} corneas can be maintained in HCO_3^- -free media. The two experimental procedures used in this study attempt to resolve this issue. The first measures swelling or deturgescence of corneas under silicone oil.³⁻⁷ The second results in the stroma imbibing fluid from the anterior side and causes the cornea to reach an equilibrium thickness dependent on osmolarity and pH of the fluids and the imposed "intraocular" (hydrostatic) pres-

TABLE 3. ATP Content and $\text{Na}^+ \text{-K}^+$ ATPase Activity of Endothelia From Superfused Corneas

Conditions	ATP (nmol/cm ²)	$\text{Na}^+ \text{-K}^+$ -ATPase Activity ($\mu\text{mol Pi/mg protein per hour}$)
$\text{HCO}_3^-/\text{CO}_2$	1.20 ± 0.18 (6)	3.53 ± 0.27 (6)
HPO_4^-	1.62 ± 0.24 (6)	3.38 ± 0.21 (7)

Values are mean \pm SD with number of experiments in parentheses.

sure.¹² In this respect, the cornea is in a condition essentially similar to that pertaining in the studies of net fluid movement,^{2,8-10} that is, the tissue is exposed to the same media on both the bare stromal and endothelial surfaces, a defined hydrostatic pressure exists, the stroma (either full thickness or partially pared down) is swollen to a steady state thickness, and the endothelium is functional. However, in the present study, rather than measure net fluid movement across the endothelium by means of capillary tubes attached to the anterior and posterior compartments or by an automatic volume feedback system,²² we have assessed endothelial fluid transport in these corneas by observing shifts in equilibrium thicknesses and by their capability to deturgescce on replacing the anterior fluid with silicone oil.

Thickness in $\text{HCO}_3^-/\text{CO}_2$ and HPO_4^-

The data in Figure 1A show a clear-cut difference in the ability of deepithelialized corneas to maintain normal thickness in $\text{HCO}_3^-/\text{CO}_2$ -buffered media and in media lacking $\text{HCO}_3^-/\text{CO}_2$. However, there is a potential drawback in the methodology of these and similar experiments of others³⁻⁷ in that the high solubility of CO_2 in silicone oil could cause an efflux of CO_2 from the tissue into this sink,²⁸ thereby depriving the endothelium of metabolic CO_2 to be used for production of HCO_3^- . Metabolic CO_2 might also be lost by diffusion into the rapidly superfusing fluid. These conditions do not prevail in the fluid movement studies because the stromal side fluid is stagnant in a virtually fully enclosed chamber.^{8,10,22} Nevertheless, when loss of CO_2 from the tissue was counteracted by leaving the epithelium intact and covering it with the HCO_3^- medium in equilibrium with 5% CO_2 , or by saturating the silicone oil covering the bare stroma with 5% CO_2 , corneal thickness was not maintained when the endothelium was superfused with HPO_4^- medium (Fig. 1B). Swelling rates were the same as those when CO_2 was absent from the anterior surface (Fig. 1A). In these conditions, given the diffusion characteristics of CO_2 across the epithelium and stroma of rabbit corneas,^{29,30} CO_2 should flux readily from the anterior surface to the CO_2 -free HPO_4^- medium. Moreover, reducing or stopping the flow of superfusate to minimize potential loss of CO_2 by this route also failed to

prevent swelling in HPO_4^- while not affecting normal thickness in $\text{HCO}_3^-/\text{CO}_2$. Because the swelling rates in HPO_4^- under the stagnant and slow flow conditions were lower than at 3 ml/hour, the fluid pump may be active to some degree, a possibility supported by the small, additive effect of ouabain on these swelling rates. However, under our several conditions designed to maximize CO_2 retention and availability, we have thus far failed to demonstrate that metabolic and ambient CO_2 can build up HCO_3^- to the concentration sufficient to prevent corneal swelling or to promote deturgescence.

Comparison With Ouabain Effects

Figure 2 shows that in the HPO_4^- medium, the rate of swelling is equal to that seen when active transport is inhibited by ouabain, and that swelling in HPO_4^- is not altered by the addition of ouabain. However, although ouabain-inhibitable active transport appears to be absent in corneas superfused with HPO_4^- , there is no irreversible damage to the endothelium, for, on replacement of HPO_4^- with $\text{HCO}_3^-/\text{CO}_2$, deturgescence begins immediately (Fig. 2, inset). When HPO_4^- and $\text{HCO}_3^-/\text{CO}_2$ media are compared in the second protocol, again marked differences are seen that are analogous to those described above. Equilibrium thicknesses are greater in HPO_4^- than in $\text{HCO}_3^-/\text{CO}_2$ (Fig. 4 and Table 2) but can be equalized by inhibiting active transport in $\text{HCO}_3^-/\text{CO}_2$ with ouabain, which itself does not affect the HPO_4^- equilibrium thickness. There is no evidence to suggest that the difference in equilibrium thickness in $\text{HCO}_3^-/\text{CO}_2$ and HPO_4^- is due to permeability differences, for ouabain does not alter the permeability of the endothelium.²¹ The reversible nature of the HPO_4^- effect is also clearly seen in Figure 5 in which, on covering the stroma with oil, deturgescence can be seen to ensue when superfusion continues in $\text{HCO}_3^-/\text{CO}_2$ medium but not in HPO_4^- or in $\text{HCO}_3^-/\text{CO}_2$ with ouabain. These results show that media lacking $\text{HCO}_3^-/\text{CO}_2$ cannot maintain the normal hydration of freshly isolated corneas nor promote deturgescence in swollen corneas, even though the energy level and sodium pump activity are unimpaired (Table 3).

These results all show that in nominally HCO_3^- -free media, the capacity for controlling corneal hydration is

abolished, with the result that corneas then behave in essentially the same manner as when the sodium pump is inhibited by ouabain, an unequivocal inhibitor of active transport. The recent studies^{8,10} of transendothelial fluid movement, which show no dependence on exogenous HCO_3^- concentration, have unfortunately not tested the effects of ouabain. In an earlier study,⁹ in which ouabain was used, its effect was uncharacteristically slow in one⁹ (contrast with reference 7); fluid movement did not cease until more than an hour after addition of ouabain, by which time the experiment had been in progress for 4 hours and control corneas also were failing. The simultaneous failure of controls and ouabain-treated corneas may have been due to the lack of glucose in the medium, a factor known to cause corneal swelling after 3 to 4 hours.¹⁵ In a second study,¹³ ouabain did inhibit transendothelial fluid movement, an effect which, in contrast to the recent studies,^{8,10} was also produced by reducing the bicarbonate concentration of the medium.

Ambient and Metabolic CO_2

The proposal of Kuang et al.,¹⁰ that metabolic CO_2 from the endothelial cells and keratocytes provides sufficient HCO_3^- to support fluid movement in 10 mM phosphate (but not 1 mM HPO_4^- or 20 mM Hepes) at a rate consistent with maintenance of normal corneal thickness, should, if valid, be demonstrable in the present study. They showed that carbonic anhydrase inhibitors reduced fluid movement to about 50% of that in 1 mM HPO_4^- and, by inference, to less than 30% of that in 10 mM HPO_4^- . The effect of ethoxzolamide on corneas in $\text{HCO}_3^-/\text{CO}_2$, in both procedures of the present study, does confirm a role for carbonic anhydrase in endothelial control of hydration, but the lack of an effect on either swelling rate or equilibrium thickness in HPO_4^- (Figs. 3A, 6A) suggests that the supply of metabolic CO_2 is unable to provide adequate HCO_3^- . In support of this conclusion, when respiration of the tissue was inhibited with antimycin A, thus curtailing the mitochondrial production of CO_2 (more than 60% of the total³¹), the swelling pattern of corneas in both $\text{HCO}_3^-/\text{CO}_2$ and HPO_4^- media was unaltered (Figs. 3B, 6B). Further evidence for the absence in HPO_4^- medium of a role for metabolically derived HCO_3^- is the lack of an effect of DIDS, which has been shown to disrupt HCO_3^- exchange reactions of both cultured and *in situ* corneal endothelial cells^{20,32} and to cause corneal swelling in $\text{HCO}_3^-/\text{CO}_2$ medium.¹⁶ Thus, the role of carbonic anhydrase evidenced by the effects of ethoxzolamide in $\text{HCO}_3^-/\text{CO}_2$ medium must be to catalyze reaction of exogenously supplied substrate rather than metabolic CO_2 . Its function may be to provide HCO_3^- at a specific membrane site³³ or, alternatively, to generate protons necessary to maintain coupled Na^+/H^+ and $\text{Cl}^-/\text{HCO}_3^-$ exchange essential to fluid transport.^{16,20}

Such a role for exogenous, but not metabolic, CO_2 was supported by a recent study of pH regulation in cultured endothelial cells.³⁴ However, in contrast to other studies of bicarbonate on corneal thickness,³⁻⁷ the reactions required only the low concentrations of CO_2 and HCO_3^- (6.8 μM and 172 μM , respectively) found in air-equilibrated buffers at pH 7.5.

The suggestion that "fluid transport . . . is supported in air equilibrated, nominally HCO_3^- free Ringer's"³⁴ is not confirmed by the current experiments on corneal thickness, where swelling in air equilibrated HPO_4^- medium was equal to that seen with ouabain. Moreover, with even more stringent tests of the role of ambient CO_2 , with 5% CO_2 equilibrated oil or, for intact corneas, $\text{HCO}_3^-/\text{CO}_2$ medium to prevent CO_2 loss to the atmosphere, corneal swelling was not abolished. Even in the situations in which the flow rate was reduced or the HPO_4^- superfusing medium itself was bubbled with 5% CO_2 , corneal swelling persisted. Although the pH of the medium in the latter case was 6.8, this is within the range well tolerated by the endothelium,³⁵ and pH should not have contributed significantly to the swelling. In addition, from the Henderson-Hasselbach equation, it can be calculated that the medium contained approximately 8 mM HCO_3^- ; the failure to maintain normal hydration under these conditions is consistent with the observation (see Fig. 4) that more than 20 mM HCO_3^- is needed to support full deturgescence of swollen corneas. Supporting the primacy of HCO_3^- is the observation that, in contrast to manipulations of CO_2 alone, giving $\text{HCO}_3^-/\text{CO}_2$ instead of HPO_4^- direct access to the stroma (Table 2) allowed HPO_4^- -superfused corneas to equilibrate at lower thicknesses.

That control of corneal hydration has an absolute requirement for HCO_3^- in the external medium (from either aqueous or stromal sources), as it does for external Na^+ ,^{7,13} suggests that coupled $\text{Na}^+/\text{HCO}_3^-$ transport may be an important route of entry of these ions into the endothelial cells, presumably across the basolateral membranes. This mechanism would be in addition to the coupled cotransport of these ions already proposed for their exit into the aqueous humor²⁰ and similar to that proposed to be an initial event in fluid transport in the retinal pigment epithelium of the frog.³⁶

Key Words

corneal thickness, fluid transport, bicarbonate, metabolic CO_2 , endothelial function

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References

1. Mishima S, Kudo T. In vitro incubation of rabbit cornea. *Invest Ophthalmol*. 1967;6:329-339.
2. Maurice DM. The location of the fluid pump in the cornea. *J Physiol*. 1972;221:43-54.
3. Dikstein S, Maurice DM. The metabolic basis to the fluid pump in the cornea. *J Physiol*. 1972;221:29-41.
4. Hodson S. Evidence for a bicarbonate-dependent sodium pump in corneal endothelium. *Exp Eye Res*. 1971;11:20-29.
5. Hodson S, Miller F. The bicarbonate ion pump in the endothelium which regulates the hydration of rabbit cornea. *J Physiol*. 1976;263:563-577.
6. Fischbarg J. Active and passive properties of the rabbit corneal endothelium. *Exp Eye Res*. 1973;15:616-638.
7. Fischbarg J, Lim JJ. Role of cations, anions and carbonic anhydrase in fluid transport across rabbit corneal endothelium. *J Physiol*. 1974;214:647-675.
8. Doughty MJ, Maurice DM. Bicarbonate sensitivity of rabbit corneal endothelial fluid pump in vitro. *Invest Ophthalmol Vis Sci*. 1988;29:216-223.
9. Barfort P, Maurice DM. Electrical potential and fluid transport across the corneal endothelium. *Exp Eye Res*. 1974;19:11-19.
10. Kuang K, Xu M, Koniarek KP, Fischbarg J. Effects of ambient bicarbonate, phosphate and carbonic anhydrase inhibitors on fluid transport across rabbit corneal endothelium. *Exp Eye Res*. 1990;50:487-493.
11. Maurice DM. The cornea and sclera. In: Davson H, ed. *The Eye*. Vol. 1, 2nd ed. New York: Academic Press; 1969:489-600.
12. Doughty MJ. New observations on bicarbonate-pH effects on thickness changes of rabbit corneas under silicone oil in vitro. *Am J Optom Physiol Optics*. 1985;62:879-888.
13. Hodson S. The regulation of corneal hydration by a salt pump requiring the presence of sodium and bicarbonate ions. *J Physiol*. 1974;236:271-302.
14. Riley MV, Winkler BS, Peters MI, Czajkowski CA. Relationship between fluid transport and in situ inhibition of Na^+/K^+ adenosine triphosphatase in the corneal endothelium. *Invest Ophthalmol Vis Sci*. 1994;35:560-567.
15. Riley MV, Winkler BS. Strong pasteur effect in rabbit corneal endothelium preserves fluid transport under anaerobic conditions. *J Physiol*. 1990;426:81-93.
16. Winkler BS, Riley MV, Peters MI, Williams FJ. Chloride is required for fluid transport by the rabbit corneal endothelium. *Am J Physiol*. 1992;262:C1167-C1174.
17. Riley MV. The role of the epithelium in control of corneal hydration. *Exp Eye Res*. 1971;12:128-137.
18. Kaye GI, Hoefle FB, Donn A. Studies on the cornea: VIII: Reversibility of the effects of in vitro perfusion of the rabbit corneal endothelium with calcium-free medium. *Invest Ophthalmol*. 1973;12:98-113.
19. Knauf P, Rothstein A. Chemical modification of membranes: I: Effects of sulfhydryl and amino reactive agents on anion and cation permeability of the human red blood cell. *J Gen Physiol*. 1971;58:190-210.
20. Jentsch TJ, Keller SK, Koch M, Weiderhold M. Evidence for coupled transport of bicarbonate and sodium in cultured bovine corneal endothelial cells. *J Membr Biol*. 1984;81:189-204.
21. Trenberth S, Mishima S. The effect of ouabain on the rabbit corneal endothelium. *Invest Ophthalmol*. 1968;7:44-52.
22. Fischbarg J, Lim JJ, Bourguet J. Adenosine stimulation of fluid transport across rabbit corneal endothelium. *J Membr Biol*. 1977;35:95-112.
23. Riley MV, Winkler BS, Czajkowski CA, Peters MI. Role of adenosine receptors in cyclic AMP-dependent stimulation of corneal fluid pump. ARVO Abstracts. *Invest Ophthalmol Vis Sci*. 1994;35:1889.
24. Hull D, Green K, Boyd M, Wynn H. Corneal endothelial bicarbonate transport and the effect of carbonic anhydrase inhibitors on endothelial permeabilities and fluxes and corneal thickness. *Invest Ophthalmol Vis Sci*. 1977;16:883-892.
25. Mayes KR, Hodson S. Local osmotic coupling to the active trans-endothelial bicarbonate flux in the rabbit cornea. *Biochim Biophys Acta*. 1978;514:286-293.
26. Edelhauser HF, van Horn DL, Schultz RO, Hyndiuk RA. Comparative toxicity of intraocular irrigating solutions on the corneal endothelium. *Am J Ophthalmol*. 1976;81:473-481.
27. Edelhauser HF, Gonnering R, van Horn DL. Intraocular irrigating solutions: A comparative study of BSS plus and lactated Ringer solutions. *Arch Ophthalmol*. 1978;96:516-520.
28. Reuss L. Independence of apical membrane Na^+ and Cl^- entry in *Necturus* gallbladder epithelium. *J Gen Physiol*. 1984;84:423-445.
29. Fatt I, Hill RM, Takahashi GH. Carbon dioxide efflux from the human cornea in vivo. *Nature*. 1964;203:738-740.
30. Fatt I, Bieber MT. The steady state distribution of oxygen and carbon dioxide in the in vivo cornea: I: The open eye in air and the closed eye. *Exp Eye Res*. 1968;7:103-112.
31. Geroski DH, Edelhauser HF, O'Brien WJ. Hexose-monophosphate shunt response to diamide in the component layers of the cornea. *Exp Eye Res*. 1978;26:611-619.
32. Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium: II: $\text{Na}^+/\text{HCO}_3^-$ cotransport and $\text{Cl}^-/\text{HCO}_3^-$ exchange. *Invest Ophthalmol Vis Sci*. 1992;33:3068-3079.
33. Ridderstrale Y, Wistrand PJ, Brechue WF. Membrane-associated CA activity in the eye of the CA II-deficient mouse. *Invest Ophthalmol Vis Sci*. 1994;35:2577-2584.
34. Bonanno JA. Bicarbonate transport under nominally bicarbonate-free conditions in bovine corneal endothelium. *Exp Eye Res*. 1994;58:415-421.
35. Gonnering R, Edelhauser HF, Van Horn DL, Durant W. The pH tolerance of rabbit and human corneal endothelium. *Invest Ophthalmol Vis Sci*. 1979;18:373-390.
36. Hughes BA, Adorante JS, Miller SS, Lin H. Apical electrogenic NaHCO_3 cotransport: A mechanism for HCO_3^- absorption across the retinal pigment epithelium. *J Gen Physiol*. 1989;94:125-150.