Apical Electrogenic NaHCO₃ Cotransport

A Mechanism for HCO₃ Absorption across the Retinal Pigment Epithelium

BRET A. HUGHES, JOSEPH S. ADORANTE, SHELDON S. MILLER, and Hai Lin

From the School of Optometry and Department of Biophysics, University of California, Berkeley, California 94720

ABSTRACT Intracellular microelectrode techniques and intracellular pH (pHi) measurements using the fluorescent dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) were employed to characterize an electrogenic bicarbonate transport mechanism at the apical membrane of the frog retinal pigment epithelium (RPE). Reductions in apical concentrations of both [HCO₃]₀ (at constant PCO₂ or pH₀) or [Na]₀ caused rapid depolarization of the apical membrane potential (Vₐp). Both of these voltage responses were inhibited when the concentration of the other ion was reduced or when 1 mM diisothiocyano-2-2 disulfonic acid stilbene (DIDS) was present in the apical bath. Reductions in apical [HCO₃]₀ or [Na]₀ also produced a rapid acidification of the cell interior that was inhibited by apical DIDS. Elevating pHi at constant PCO₂ (and consequently [HCO₃]ᵢ) by the addition of apical NH₄ (20 mM) produced an immediate depolarization of Vₐp. This response was much smaller when either apical [HCO₃]₀ or [Na]₀ was reduced or when DIDS was added apically. These results strongly suggest the presence of an electrogenic NaHCO₃ cotransporter at the apical membrane. Apical DIDS rapidly depolarized Vₐp by 2–3 mV and decreased pHi (and [HCO₃]ᵢ), indicating that the transporter moves NaHCO₃ and net negative charge into the cell. The voltage dependence of the transporter was assessed by altering Vₐp with transepithelial current and then measuring the DIDS-induced change in Vₐp. Depolarization of Vₐp increased the magnitude of the DIDS-induced depolarization, whereas hyperpolarization decreased it. Hyperpolarizing Vₐp beyond −114 mV caused the DIDS-induced voltage change to reverse direction. Based on this reversal potential, we calculate that the stoichiometry of the transporter is 1.6–2.4 (HCO₃/Na).

INTRODUCTION

The retinal pigment epithelium (RPE) is a single layer of cells that forms an important part of the blood–retina barrier and provides the major transport pathway...
responsible for the exchange of fluid, ions, and metabolites between the photoreceptors and the choroidal blood supply. The RPE transports fluid in the retina-to-choroid direction (Miller et al., 1982; Tsuboi, 1987), and this is believed to be an important factor in retinal adhesion (Marmor et al., 1980). In bullfrog RPE, a major driving force for fluid absorption is active HCO₃ transport (Hughes et al., 1984, 1988) but the membrane mechanisms that mediate transepithelial HCO₃ movement have not yet been identified. These transporters are likely to be important in two ways: (a) for driving fluid out of the extracellular space that separates the photoreceptor outer segments and the apical membrane and (b) for controlling the pH in this space.

It has been shown in several species that the apical membrane potential of the RPE is sensitive to changes in extracellular bicarbonate concentration (Lasansky and De Fisch, 1966; Miller and Steinberg, 1977a; Steinberg et al., 1978; Tsuboi et al., 1986; Joseph and Miller, 1986). In the simplest case, this effect could be due to a HCO₃ conductance in the apical membrane (Miller and Steinberg, 1977a; Saito and Wright, 1984; Kaila and Voipio, 1987), or it could be mediated by an electrogenic NaHCO₃ cotransporter similar to those found in the basolateral membrane of other epithelia (Boron and Boulpaep, 1983; Curci et al., 1987). The observed voltage responses might also be produced, at least in part, by other mechanisms, such as pH-sensitive channels or pumps.

In the present study, we characterized the electrogenic HCO₃ transport pathway using conventional electrophysiological techniques and fluorescence microscopy measurements. We provide evidence that the apical membrane of the RPE has an electrogenic NaHCO₃ transport mechanism that moves Na, HCO₃, and net negative charge into the cell under control conditions and is inhibited by diisothiocyanato-2-2 disulfonic acid stilbene (DIDS). From its apparent reversal potential of −114 mV, we calculate the HCO₃/Na stoichiometry of this transporter to have an upper bound of 2.4 and a lower bound of 1.6. The voltage dependence of this transporter has important physiological implications because the RPE responds electrically to changes in retinal activity (Steinberg et al., 1985). Some of this work has been presented previously in a preliminary form (Hughes et al., 1987a; Adorante et al., 1988).

**METHODS**

Medium-size bullfrogs, *Rana catesbeiana*, of both sexes were obtained from Central Valley Biologicals (Clovis, CA) or Western Scientific (Sacramento, CA) and maintained in running tap water at room temperature for at least 1 wk before use. The frogs were fed live crickets daily during captivity. The RPE-choroid was isolated from dark-adapted eyes as previously described and mounted as a flat sheet in a chamber that allowed the two sides of the tissue to be perfused independently (Hughes et al., 1988). Solutions were delivered to the chamber by gravity via CO₂-impermeable tubing (Saran; Clarkson Equipment and Controls, Detroit, MI). Separating the solution reservoirs and chamber was an open manifold (~250 μl) that served to maintain a constant hydrostatic head during solution changes (Joseph and Miller, manuscript submitted for publication). Since solution changes were made at the manifold, there was a 10–40 s delay before the dead space cleared and the new solution reached the tissue. In all figures, the horizontal bar indicates when solution changes were made at the manifold.

The composition of the apical perfusion solutions is listed in Table I. All chemicals were
obtained from Sigma Chemical Co. (St. Louis, MO). Bicarbonate-containing solutions were
gassed with 95% \( O_2/5\% \ CO_2 \), except where noted in text. Solutions buffered with \( N^\ominus 2\)-
hydroxyethylpiperazine-N'\(-2\)-ethanesulfonic acid (HEPES) were gassed with 100% \( O_2 \). The
pH of solutions 1, 3, 5, 6, 8, 9, 11, and 12 was 7.4 \( \pm \) 0.01, solutions 2, 7, and 10 had a pH of
6.4 \( \pm \) 0.02, and the pH of solution 4 was 7.8 \( \pm \) 0.02. Unless otherwise noted in text, the basal
side of the isolated RPE-choroid was perfused continuously with solution 1.

**Electrophysiology**

Conventional microelectrodes were drawn from thick-walled, fiber-filled borosilicate glass
tubing having an inside diameter of 0.5 mm and an outer diameter of 1 mm (Omega Dot;
Glass Company of America, Bargaintown, NJ). Micropipettes were pulled on a horizontal
puller (model P-77; Sutter Instrument Co., San Francisco, CA) and had a tip resistance of
80–120 MΩ when filled with 150 mM KCl.

The techniques used for electrophysiological recordings with conventional microelectrodes
were similar to those used in previous studies (Hughes et al., 1988). All experiments were
carried out in a newly designed chamber (Joseph and Miller, manuscript submitted for pub-
llication) that allowed more rapid exchange of perfusion fluid (10–20 chamber vol/min). In
both the apical and basal compartments of the recording chamber, a calomel electrode made
electrical contact with the perfusion solution via a Ringer-agar bridge. In Na-substitution
experiments, chlorided silver wires were used as reference electrodes to avoid liquid junction
potentials. The transepithelial potential (TEP) (apical-side positive) was recorded differentially
between the two calomel electrodes. Membrane potentials were recorded using micropipette
electrodes reference to either the apical or basal calomel electrode. When referred to
the apical calomel electrode, the voltage measured was the apical membrane potential, \( V_{ap} \), and
when referred to the basal calomel electrode, the voltage measured was the basal membrane
potential, \( V_{ba} \). The transepithelial resistance (\( R_t \)) and the apparent ratio of the apical to baso-
lateral membrane resistance, \( R_{ap}/R_{ba} \) (a value), were obtained by passing 2-\( \mu \)A current pulses
across the tissue and monitoring the voltage responses across the apical and basolateral mem-
branes (Miller and Steinberg, 1977a; Hughes et al., 1988). All voltage signals were digitized at
a rate of 4 Hz and stored on a microcomputer for subsequent analysis. In several figures
(Figs. 2, 5, 6, and 8 A), a slight ripple is present in the voltage records. This is due to the
digital subtraction of superimposed voltage deflections caused by the bipolar current pulses
used to measure $R_t$ and the apparent $a$ value.

**Cell pH Measurement**

The RPE-choroid was mounted in a specially designed chamber on the stage of an inverted
microscope (Diaphot-TMD; Nikon, Inc., Garden City, NY) equipped for epifluorescent illu-
mination. This chamber allowed constant perfusion of the apical and basolateral bathing solu-
tions and the measurement of TEP and $R_t$. The RPE cells were brought into focus through a
water immersion objective lens (Achromat 40/0.75; Carl Zeiss, Inc., Thornwood, NY) with a
working distance of 1.6 mm. This objective faced the apical bathing solution, which was 0.35
mm deep, and covered with a glass coverslip =0.1 mm thick.

The pH-sensitive dye 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular
Probes, Inc., Eugene, OR) was used along with a ratioing fluorimetry technique (Heiple and
Taylor, 1982) to measure the fluorescence intensity ratio of intracellular dye (Rink et al.,
1982). BCECF has a peak excitation at 504 nm that is pH sensitive, and an isoexcitation point
at 436 nm, where fluorescence excitation is independent of pH (Alpern, 1985). These prop-
erties of the dye were used to measure the fluorescence intensity ratio (Ex) 480 nm/440 nm,
which is linear over a wide pH range (6.4–7.5) and invariant with respect to changes in optical
path length and dye concentration (Heiple and Taylor, 1982). The excitation wavelength was
alternated at 1 Hz between 440 and 480 nm using interference filters with a bandwidth of 10
nm (Omeka Optical, Inc., Brattleboro, Vermont) and the epifluorescent emission was mea-
sured between 520 and 560 nm. The voltage and fluorescence signals were digitized and
stored on a microcomputer for later analysis. A more detailed description of this technique
and the data acquisition system is available from Dr. Chester Regan, 203 S. Coler Ave. No. 3,
Urbana, IL 61801.

At the beginning of each experiment, fluorescence was measured from the unloaded tissue
and was later subtracted from the total emission signal. Control Ringer (Table I, solution 1)
containing 13 μM of the acetomethyl derivative of BCECF (BCECF-AM) was then perfused
through the apical bath for 20–30 min (flow rate > 50 chamber vol/min). Fluorescence was
monitored continuously during this time. The impermeant dye (BCECF) was presumably gen-
erated intracellularly by the action of cytoplasmic esterase (Rink et al., 1982). After this
period of BCECF loading, control Ringer was perfused through the chamber for 10–15 min
to wash out the extracellular dye.

It seemed possible that some of the fluorescence signal originated from cells located in the

![Figure 1. In situ calibration curve for intracellular BCECF. Closed circles represent the mean steady-state fluorescence ratio for five tissues. Error bars represent SEM. F, fluorescence.](image-url)
choroid on the basal side of the tissue. This could have occurred by leakage of dye around the edge of the tissue or through the junctional complex that surrounds each cell. This possibility was tested by perfusing the dye (BCECF-AM) into the basal chamber and continuously monitoring the fluorescence. This procedure should produce a choroidal signal much larger than that due to leakage from the apical side. After 25 min the extracellular dye was washed out from the basal chamber. For comparison, we then apically perfused the same tissue with dye, again for 25 min. This produced a normal signal, which was 13 times larger than that produced by basal loading. Therefore, the contribution from the choroidal cells must be considerably <$7\%$ of the total signal.

At the end of each experiment a calibration was performed by the method of Thomas et al. (1979). The calibration solutions contained 10 mM HEPES-NaOH, 105 mM KCl (to match the RPE intracellular K activity; Oakley et al., 1978; La Cour et al., 1986; Hughes et al., 1988), 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM glucose, and 13 $\mu$M nigericin (Sigma Chemical Co.), a K-H antiporter. The pH of these solutions was varied by HCl addition, and under these conditions intracellular and extracellular pH should be equal. The in situ calibration curve for five tissues is shown in Fig. 1.

During and after BCECF perfusion, TEP and $R_t$ remained constant. The loading of BCECF did not alter the TEP and $R_t$ responses to apical changes of $[\text{Na}]_o$, $[\text{K}]_o$, $[\text{HCO}_3]^o$, NH$_4$, or DIDS. This indicates that incorporation of dye did not alter the transport or electrical properties of the RPE.

**RESULTS**

*Response of $V_{ap}$ to Changes in Apical $[\text{HCO}_3]^o$*

Fig. 2A shows a typical membrane voltage response to a decrease in bicarbonate concentration outside the apical membrane. Decreasing apical $[\text{HCO}_3]^o$ from 27.5 to 2.75 mM at constant $\text{PCO}_2$ caused $V_{ap}$ to rapidly depolarize and then slowly repolarize after reaching a peak. When apical $[\text{HCO}_3]^o$ was returned to 27.5 mM, $V_{ap}$ underwent a sharp hyperpolarization and then slowly repolarized back to its initial level. These results are compatible with the presence at the apical membrane of a simple bicarbonate conductance but as shown below, they are actually mediated by a more complex transport mechanism.

Presumably, the initial $V_{ap}$ depolarization was a direct result of the change in the transmembrane HCO$_3$ driving force on an electrogenic transport mechanism. The slower repolarization that followed the peak depolarization was probably due to secondary effects on the cotransporter and/or other transport pathways caused, for example, by changes in $\text{pH}_i$, $[\text{HCO}_3]^i$, or $[\text{Na}]_i$ (see below). Although limited by secondary effects, the initial peak voltage changes most likely reflect the changes in activity of the HCO$_3$-sensitive electrogenic transporter.

Similar but smaller voltage changes also occurred at the basal membrane and these were mainly the result of current shunted from the apical membrane via the paracellular pathway (Miller and Steinberg, 1977a).

The effect of increasing apical $[\text{HCO}_3]^o$ from 27.5 to 55 mM at constant $\text{PCO}_2$ is shown in Fig. 2B. The increase in apical $[\text{HCO}_3]^o$ caused $V_{ap}$ to rapidly hyperpolarize and then slowly repolarize to a value more negative than the control value. When the apical perfusion solution was returned to 27.5 mM HCO$_3$, $V_{ap}$ underwent a sharp depolarization and then repolarized to the control level.
FIGURE 2. Effects of changing apical [HCO₃]₀. TEP, Vₑₐ, and Vₑₐ denote transepithelial potential, basal membrane potential, and apical membrane potential, respectively. Values in front of each trace indicate voltages at the beginning of the record. (A) Reducing apical [HCO₃]₀ 10-fold at constant PCO₂, caused a rapid depolarization of Vₑₐ followed by a partial repolarization. See Table II. Horizontal bar at bottom indicates when control Ringer (solution 1, Table I) was replaced with 2.75 mM HCO₃ Ringer (solution 2) at the manifold (see Methods). (B) Increasing apical [HCO₃]₀ from 27.5 (solution 3) to 55 mM (solution 4) at constant PCO₂ caused a rapid hyperpolarization, followed by a partial repolarization. See Table II. (C) Reducing apical [HCO₃]₀ at constant pH caused a rapid depolarization of Vₑₐ. Horizontal bar indicates when HCO₃-buffered Ringer (solution 1) was replaced with HEPES-buffered Ringer gassed with 100% O₂ (solution 5). See Table III.
The results of several similar experiments are summarized in Table II. A 10-fold reduction in apical [HCO₃]₀ produced a peak $V_{ap}$ depolarization that averaged 12 ± 0.2 mV (±SEM, n = 14), whereas increasing apical [HCO₃]₀ twofold caused a peak $V_{ap}$ hyperpolarization that averaged 6.9 ± 0.3 mV (n = 4). Table II also shows that changes in apical [HCO₃]₀ significantly altered the ratio of apical to basolateral membrane resistances ($a$ value). The $a$ value doubled when apical [HCO₃]₀ was reduced by a factor of 10 and decreased by 21% when apical [HCO₃]₀ was doubled.

These results agree with those reported previously (Miller and Steinberg, 1977a) and are compatible with the presence at the apical membrane of an electrogenic bicarbonate transport mechanism. Since the alterations in apical [HCO₃]₀ were made at constant PCO₂ (Fig. 2, A and B), there were accompanying changes in extracellular pH that could have affected a membrane conductance to bring about the observed voltage changes. To address this possibility, we replaced the HCO₃/CΟ₂-buffered Ringer (solution 1) at constant extracellular pH with nominally HCO₃ and CO₂-free Ringer (solution 5). This caused $V_{ap}$ to undergo a biphasic change in voltage whose magnitude and time course was similar to that produced by the reduction in apical [HCO₃]₀ at constant PCO₂ (Fig. 2 C). The results of several similar experiments are summarized in Table III. Removing apical [HCO₃]₀ at constant pH produced a peak depolarization that averaged 13.9 ± 1.2 mV (mean ± SEM, n = 5). As

| Condition | $V_{ap}$ | $V_{ma}$ | TEP | $a$ | $R_t$ | Ω · cm² |
|-----------|---------|---------|-----|----|-------|--------|
| 27.5 mM HCO₃ | $-83.6 ± 1.7$ | $-74.5 ± 0.7$ | $9.0 ± 1.5$ | $0.40 ± 0.04$ | $238 ± 35$ |
| 0 mM HCO₃ | $-69.7 ± 0.6$ | $-68.4 ± 0.5$ | $1.2 ± 0.8$ | $1.26 ± 0.16$ | $252 ± 31$ |
| $|\Delta|$ | $13.9 ± 1.2\dagger$ | $6.1 ± 0.9\dagger$ | $7.8 ± 1.0\dagger$ | $0.85 ± 0.12\dagger$ | $13 ± 5\ast$ |
| (5) | (5) | (5) | (4) | (4) |

Symbols and protocol are the same as in Table II. *Not significant; †P < 0.01; ‡P < 0.001.
in the reduction of \([\text{HCO}_3^-]_o\) at constant \(\text{PCO}_2\). \(\text{HCO}_3\) removal resulted in a significant increase in the apparent \(a\) value.

When apical \([\text{HCO}_3^-]_o\) was decreased at either constant \(\text{pH}\) or constant \(\text{PCO}_2\) (Fig. 2, A and C), an inward-directed proton electrochemical gradient was established and, if the apical membrane had a large proton conductance, this could have contributed to the observed voltage responses. Therefore, the effects of extracellular \(\text{pH}\) changes were examined on RPE-choroid preparations bathed on both sides with HEPES-buffered Ringer equilibrated with 100% \(\text{O}_2\) (solution 5). Decreasing the \(\text{pH}\) of the apical solution from 7.4 to 6.4 caused a 2–4 mV depolarization of \(V_{ap}\) (\(n = 3\)), whereas increasing the apical \(\text{pH}\) from 7.4 to 8.4 hyperpolarized \(V_{ap}\) by ~2 mV (\(n = 3\); not shown). These results strongly suggest that changes in extracellular \(\text{pH}\) make a relatively minor contribution to the \(\text{HCO}_3\)-induced voltage changes.

**K Conductance and Na/K Pump Contributions to the \(\text{HCO}_3\) Response**

The RPE apical membrane contains two other mechanisms that determine \(V_{ap}\), a relatively large \(K\) conductance (\(T_K > 0.5\)) and an electrogenic \(Na/K\) pump (Miller and Steinberg, 1977b; Miller et al., 1978). It is possible that one or both of these mechanisms could underlie the voltage changes that immediately follow alterations in apical \([\text{HCO}_3^-]_o\). For example, decreasing apical \([\text{HCO}_3^-]_o\) could cause a \(\text{pH}\)-dependent decrease in \(K\) conductance, which would shift \(V_{ap}\) away from \(E_K\) and thereby depolarize \(V_{ap}\). To exclude any possible contribution from the \(K\) conductance, we measured the voltage response to changes in apical \([\text{HCO}_3^-]_o\) before and after blocking the \(K\) conductance with barium (Hughes et al., 1988; Joseph and Miller, 1986). Fig. 3 shows that the apical perfusion of 3 mM \(\text{BaCl}_2\) (substituted for 4.5 mM \(\text{NaCl}\)) caused \(V_{ap}\) to depolarize by ~8 mV. This concentration of barium is
sufficient to block the apical membrane voltage response to step changes in apical [K]o (Griff et al., 1985; Hughes et al., 1988). The second part of Fig. 3 shows that a 10-fold reduction in apical [HCO₃]o caused Vₑp to depolarize by ~18 mV. The magnitude of this HCO₃ response is significantly larger than the control response for the same tissue (10 mV; not shown) and this is probably due to a barium-induced increase in apical membrane resistance (Hughes et al., 1988). Therefore, the initial phase of the HCO₃-induced voltage change is not mediated by the apical K conductance.

It is also possible that changes in apical [HCO₃]o lead to intracellular ion activity changes that modulate Na/K pump rate to produce the observed voltage changes. This possibility was tested by examining the voltage response to changes in apical [HCO₃]o in the presence of apical ouabain. The inset of Fig. 4 shows that apical ouabain (0.1 mM) caused a rapid depolarization of Vₑp, which is due to inhibition of the Na/K pump current (Miller et al., 1978). Approximately 30 min later, when the membrane voltages had decreased even further, a 10-fold decrease in apical [HCO₃]o caused Vₑp to transiently depolarize by ~17 mV. Since the amplitude of the HCO₃-induced voltage change was undiminished by ouabain, we conclude that the Na/K pump does not underlie the voltage response to changes in apical [HCO₃]o.

**Effect of HCO₃ Transport Inhibitors**

The results presented thus far are compatible with the presence of an electrogenic bicarbonate transport system at the apical membrane. To characterize this mecha-

---

1 Additional experiments were performed with solutions in which apical [K]o was lowered from 2 to 0.1 mM to inhibit the Na/K pump (Oakley et al., 1978; Miller and Steinberg, 1982). These maneuvers, which were performed in the presence of 1 mM Ba in order to block the K channels, did not reduce the size of the HCO₃ responses.
nism more fully, we investigated the effect of several inhibitors of HCO₃⁻ transport. Apical acetazolamide (0.1 mM), which inhibits an electrogenic HCO₃⁻ transport mechanism in the proximal tubule (Biagi and Sohtell, 1986), had no effect on the HCO₃⁻-induced voltage response in the RPE (not shown). On the other hand, the disulfonic stilbene DIDS significantly attenuated the response to changes in apical [HCO₃⁻]ₒ.

Fig. 5 shows that adding DIDS (1 mM) to the apical bathing solution caused an immediate depolarization of Vₛₚ, consistent with the inhibition of a hyperpolarizing mechanism. When a 10-fold decrease in apical [HCO₃⁻]ₒ (constant Pco₂) was made after ~5 min exposure to apical DIDS, the peak depolarization of Vₛₚ was ~50% smaller than the control response in the same tissue (7 vs. 15 mV). The degree of inhibition depended on how long the tissue was exposed to DIDS. For example, in another tissue, a 5-min exposure to DIDS inhibited the voltage response to a [HCO₃⁻]ₒ decrease by ~60%. However, after 14 min of apical DIDS exposure, the HCO₃⁻ response was completely blocked. DIDS was also effective in inhibiting the voltage response to increases in apical [HCO₃⁻]ₒ. After 5 min of exposure to apical DIDS, the amplitude of the peak hyperpolarization induced by elevating apical [HCO₃⁻]ₒ from 27.5 to 55 mM averaged 1.3 ± 0.1 mV (n = 3), an 80% inhibition (compared with the data in Table II).

If the electrogenic HCO₃⁻ transport mechanism is conductive, then its inhibition by DIDS should produce an increase in apical membrane resistance. This prediction was tested in the experiment illustrated in Fig. 6. The addition of apical DIDS (t = 1 min) caused rapid increases in Rₛ and the apparent ratio of Rₛפג/Rₘₕ (a value), and the
time course of these resistance changes coincided with the depolarization of $V_{ap}$. In 10 experiments, DIDS increased the apparent $a$ value from $0.55 \pm 0.04$ to $1.07 \pm 0.11$ ($P < 0.001$, paired t test) and increased $R_t$ from $272 \pm 23$ to $288 \pm 22 \, \Omega \cdot \text{cm}^2$ (not significant). These results suggest that the primary effect of DIDS is to block the $\text{HCO}_3^-$-dependent apical membrane conductive mechanism (see below).

![Figure 6](image)

**Figure 6.** Time course of DIDS-induced changes in membrane voltage and resistance. (Upper panel) $V_{ap}$ (continuous trace) and $R_{ap}/R_{ba}$ (●). (Lower panel) $R_t$. Vertical interrupted line indicates the start of apical DIDS perfusion (1 mM).

![Figure 7](image)

**Figure 7.** Effects of reducing apical $[\text{HCO}_3^-]$ in the absence of apical Na. Apical side had been perfused with Na-free Ringer (solution 6) for 2 min before the start of this record. Reducing apical $[\text{HCO}_3^-]$ from 27.5 to 2.75 mM (solution 7) in Na-free Ringer produced no significant voltage change. Abbreviations as in Fig. 2.
Figure 8. Effects of apical Na removal on membrane voltage. (A) Complete Na removal from the apical side in the presence of apical HCO₃⁻ (solution 6) produced an immediate depolarization of $V_{ap}$. See Table IV. (B) The effects of low [HCO₃⁻]o on the Na-induced voltage changes. The reduction of apical [HCO₃⁻]o from 27.5 to 2.75 mM (solutions 1 and 2) produced typical depolarizations of $V_{ap}$ and $V_{bc}$. When a Na-free solution (solution 7) was subsequently perfused to the apical side, there was no immediate depolarization of $V_{ap}$. (C) The effect of DIDS on the Na-induced voltage changes. Perfusion of apical DIDS (1 mM) depolarized $V_{ap}$, consistent with the inhibition of a hyperpolarizing mechanism. The perfusion of Na-free Ringer (solution 6) plus DIDS failed to cause an immediate depolarization. Abbreviations as in Fig. 2.
Ionic Dependence of the HCO₃ Response

In some cell types, it has been shown that the membrane voltage response to step changes in external [HCO₃]₀ is dependent on the presence of Na (Jentsch et al., 1984; Curci et al., 1987; Lopes et al., 1987). To test whether the electrogenic HCO₃ mechanism in the RPE is Na dependent, we examined the effect of apical [HCO₃]₀ reduction after apical Na had been removed. Fig. 7 shows that in the absence of apical Na, the apical membrane voltage response to a 10-fold reduction in apical [HCO₃]₀ was completely blocked, indicating that the RPE HCO₃ transporter is in fact Na dependent. Similar results were obtained in two other tissues.

If Na and HCO₃ transport are coupled in an electrogenic fashion, then several predictions can be made: (a) the removal of Na from the apical bathing solution should depolarize Vₐp; (b) the voltage response to Na removal should require the presence of HCO₃; and (c) the voltage response to Na removal should be inhibited by preincubation with apical DIDS. Fig. 8 A shows that perfusion of the apical side of the RPE with Na-free Ringer (solution 6) caused Vₐp to depolarize by ~8 mV and then repolarize toward the initial baseline. In nine experiments, the average peak depolarization of Vₐp was 7.5 ± 0.5 mV (Table IV). The polarity of this initial voltage change is opposite to that expected for a Na diffusion potential across either the apical membrane or the paracellular pathway.

The HCO₃ dependence of the 0 Na response was tested in a series of experiments similar to that illustrated in Fig. 8 B. The solution perfusing the apical side was first switched from control to 2.75 mM HCO₃ Ringer (solution 2), causing a transient depolarization of Vₐp. When Na was subsequently removed from the apical bathing solution (solution 6), the initial depolarization of the apical membrane seen under

\[
\begin{array}{|c|c|c|c|c|}
\hline
\text{Condition} & Vₐp & Vₐm & TEP & a & Rₙ \\
\hline
110 mM Na & -79.6 ± 1.0 & -68.2 ± 1.6 & 11.3 ± 1.0 & 0.62 ± 0.09 & 204 ± 8 \\
0 mM Na & -72.0 ± 1.3 & -58.6 ± 1.8 & 13.4 ± 1.0 & 1.44 ± 0.09 & 239 ± 15 \\
|Δ| & 7.5 ± 0.5¹ & 9.6 ± 0.4¹ & 2.1 ± 0.2² & 0.82 ± 0.13* & 36 ± 8* \\
(9) & (9) & (9) & (4) & (4) \\
\hline
\end{array}
\]

Data shown are means ± SEM obtained under steady-state conditions for 110 mM Na or at the peak voltage after a change to 0 Na. *P < 0.02; ²P < 0.001.

³ Because the paracellular pathway of the RPE is highly permeable to Na (Miller and Steinberg, 1977a; Hughes et al., 1988), the replacement of Na in the apical bath with the relatively impermeable cation N-methyl-o-glucamine creates a diffusion potential that hyperpolarizes Vₐp and depolarizes Vₐm. Therefore, the magnitude of the Vₐp depolarization induced by Na removal is attenuated by the Na diffusion potential across the paracellular shunt. In Fig. 8 A, the basal membrane depolarized even more than the apical membrane because of the combined effect of current shunted from the apical membrane and the Na diffusion potential across the shunt.
control conditions was completely absent. The hyperpolarization of $V_{sp}$ and depolarization of $V_{sa}$ that did occur were most likely caused by a Na diffusion potential across the shunt pathway.  

Fig. 8 C shows the effect of DIDS on the depolarization of $V_{sp}$ induced by apical Na removal. The perfusion of control Ringer containing 1 mM DIDS to the apical side caused its usual depolarization of $V_{sp}$. The subsequent substitution of control Ringer with Na-free Ringer (plus DIDS) 2 min later failed to depolarize $V_{sp}$. Again, the voltage changes that did occur at the two membranes were in opposite directions and most likely were caused by a Na diffusion potential across the paracellular shunt pathway. This result was corroborated in two other tissues. The interdependence of Na and HCO$_3$ in generating voltage changes at the apical membrane, and the inhibition of both voltage responses by apical DIDS, strongly suggest an electrogenic NaHCO$_3$ cotransporter.

NaHCO$_3$ transport in some tissues is coupled to Cl in an obligatory fashion (Thomas, 1977; Boron and Russell, 1983; Guggino et al., 1983; Boron, 1985). We found, however, that normal voltage responses to changes in apical [HCO$_3$]$_o$ could be elicited in the RPE even after it had been bathed on both sides with Cl-free Ringer for 1 h or more (n = 2, not shown). Therefore, it seems unlikely that the electrogenic NaHCO$_3$ transporter in the RPE is coupled to Cl.

**Origin of the DIDS-induced Change in $V_{sp}$**

The application of apical DIDS produced an apical membrane depolarization that averaged 2.1 ± 0.2 mV (mean ± SEM; n = 20). Since DIDS partially blocks the apical membrane voltage responses to reduction in [HCO$_3$]$_o$ and [Na]$_o$ (Figs. 5 and 8 C), this depolarization could result directly from an inhibition of cotransporter current. It is unlikely that the DIDS-induced depolarization was generated by a change in another transport pathway, such a decrease in $g_K$ or an increase in $g_{Na}$ and/or $g_C$ (Biagi, 1985; Inoue, 1985, 1986). The former possibility is eliminated because of the observation that 3 mM barium had no effect on the DIDS response (not shown). The latter possibility also seems unlikely because: (a) the apical membrane has very little conductance to either ion (Miller and Steinberg, 1977a); (b) $E_{Na}$ and $E_{Cl}$ are more positive than the resting membrane potential (Fong et al., 1988); and (c) DIDS appears to decrease, not increase, apical membrane conductance (Fig. 6).

If the voltage response to apical DIDS is due to inhibition of the cotransporter, then it should be possible to alter the magnitude or direction of the DIDS response by the appropriate alteration in [Na]$_o$ or [HCO$_3$]$_o$. Fig. 9 A shows that when apical HCO$_3$ was elevated to 55 mM, DIDS caused a 5-mV depolarization of $V_{sp}$. This response (5.8 ± 0.2 mV; mean ± SEM, n = 3) was significantly larger than the DIDS-induced depolarization in control Ringer, as would be expected if the cotransporter were stimulated by high apical [HCO$_3$]. In contrast, Fig. 9, B and D, shows that DIDS caused $V_{sp}$ to hyperpolarize when either HCO$_3$ or Na were removed from the apical bathing solution (n = 3). These results are consistent with a reversal of the cotransporter. More importantly, Fig. 9, C and E, shows that when either substrate was removed from both bathing solutions, DIDS had no effect on $V_{sp}$ (n = 3). From these results we conclude that the DIDS-induced voltage change can be attributed solely to inhibition of the NaHCO$_3$ cotransporter.
Relationship between NaHCO₃ Cotransport and pHᵢ

It seems likely that an important function of the NaHCO₃ cotransporter is to provide the main driving force for active HCO₃⁻ (and fluid) absorption across the RPE (Hughes et al., 1988; Hughes et al., 1987). Therefore, this cotransporter should provide a constant influx of HCO₃⁻ into the cell, elevating its intracellular concentration above electrochemical equilibrium. Consequently, any alteration in cotransporter rate should change [HCO₃⁻]ᵢ and thus intracellular pH (pHᵢ). These expectations were tested directly by using the fluorescent dye BCECF to measure pHᵢ.

Fig. 10 shows simultaneous recordings of (A) pHᵢ and (B) TEP and Rs. In control Ringer, pHᵢ was 7.21 ± 0.03 (mean ± SEM; n = 20). Removal of apical Na or a 10-fold reduction in apical [HCO₃⁻]₀ (constant PCO₂) produced normal changes in TEP (compare with Figs. 2 A and 8 A) and also caused rapid and reversible acidification of the cell interior. Both of these pHᵢ responses were inhibited by the application of apical DIDS (1 mM). In the absence of DIDS, the initial rates of acidification (measured over the first 30 s) after Na removal or HCO₃⁻ reduction averaged 0.49 ± 0.04 (mean ± SEM; n = 6) and 0.64 ± 0.01 (n = 7) pH U/min, respectively. After 15 min of exposure to DIDS, these rates of acidification were reduced by 80–90%. In the case of Na removal, the initial rate of acidification was reduced to 0.05 ± 0.01 pH
U/min (n = 4) and in two tissues, the acidification rate produced by HCO₃ reduction was decreased to 0.10 and 0.15 pH U/min.

It is worth noting that DIDS itself caused a rapid acidification of the cell interior (0.11 ± 0.06 pH U/min; n = 5). This result and the ion substitution experiments summarized above strongly support the notion of a NaHCO₃ cotransporter that moves HCO₃ and net negative charge into the cell.

**Effect of NH₄**

Extracellular NH₄ addition causes intracellular alkalinization due to the nonionic diffusion of NH₃ (Boron and De Weer, 1976), and in the presence of CO₂, this will cause an increase in [HCO₃]. Thus, NH₄ can be used to investigate the effects of changes in [HCO₃], and/or pH, on the electrogenic NaHCO₃ transporter. Fig. 11 shows that elevating NH₄ in the apical bath leads to a rapid alkalinization, 0.2 pH U, followed by a slower acidification. In seven tissues, the peak pH increase averaged 0.25 ± 0.03 (±SEM). Since these pH increases took place at constant Pco₂, one can calculate that [HCO₃] increased from 17 to 31 mM. On thermodynamic grounds, this increase in [HCO₃] would be expected to reduce the net inward driving force on the electrogenic NaHCO₃ cotransporter and thereby depolarize the apical membrane and decrease the TEP (see bottom of Fig. 11).
A typical intracellular voltage response to apical NH$_4$ is shown in the first part of Fig. 12 A. NH$_4$ (20 mM, solution 8) caused $V_{ap}$ to depolarize rapidly by $\approx 13$ mV and then partially repolarize. When control Ringer was restored, $V_{ap}$ underwent a sharp hyperpolarization and then slowly repolarized toward its initial level. In 11 experiments, the peak depolarization produced by 20 mM NH$_4$ averaged 16.3 $\pm$ 0.8 mV (Table V).

If the NH$_4$-induced depolarization of $V_{ap}$ is mainly due to the slowing of the electrogenic cotransporter, then one would expect the voltage response to NH$_4$ to be reduced by the reduction of apical HCO$_3^-$ or Na or by the addition of apical DIDS. The second part of Fig. 12 A shows that the NH$_4$ response can be inhibited by the reduction of apical [HCO$_3^-$]$_o$ at constant Pco$_2$. In this cell, the peak of the NH$_4$-induced apical membrane depolarization was reduced by 50% to 7 mV after apical [HCO$_3^-$]$_o$ was lowered to 2.75 mM. Fig. 12 B shows that in the absence of apical Na, a 20 mM pulse of NH$_4$ (solution 9) produced a relatively small depolarization of $V_{ap}$. In five experiments on three cells, the mean peak depolarization produced by NH$_4$ in the absence of apical Na was 3.6 $\pm$ 0.7 mV ($\approx 78\%$ inhibition). Finally, the initial voltage response to NH$_4$ was significantly inhibited by DIDS, as illustrated in Fig. 12 C. After preincubation with apical DIDS (1 mM) for 5 min, the addition of apical NH$_4$ caused $V_{ap}$ to rapidly depolarize by $\approx 6$ mV and then slowly depolarize another 10 mV over the next few minutes. The mechanism(s) underlying the slower, secondary depolarization has not been studied but probably depends on changes in pH$_i$ (see Fig. 11). In four experiments on DIDS-treated tissues, the initial peak depolarization induced by NH$_4$ averaged 6.0 $\pm$ 0.6 mV, a 62% smaller response than that obtained in the absence of DIDS. Inhibition of the NH$_4$-induced $V_{ap}$ depolarization transient by Na or HCO$_3^-$ reduction or by DIDS addition strongly suggests that this voltage response is mediated by the apical membrane electrogenic NaHCO$_3$ cotransporter.

---

3 This depolarization is not due to the permeation of NH$_4$ through the apical K conductance because the voltage response to NH$_4$ was not diminished by barium (2 mM).
Relationship between Apical Membrane Potential and the DIDS-induced Voltage Change

If membrane voltage is a determinant of the overall driving force for this electrogenic transporter, then it should be possible to alter or even reverse the direction of net transport by the appropriate variation in membrane voltage. To test this hypothesis, we altered the apical membrane voltage by passing DC current across the epi-

\[ \text{FIGURE 12} \]
TABLE V
Effects of Apical NH₄

| Condition | $V_{ap}$ | $V_{ba}$ | TEP | $a$ | $R_e$ |
|-----------|---------|---------|-----|-----|-------|
| Control   | -82.4 ± 1.3 | -73.6 ± 1.0 | 8.8 ± 0.9 | 0.51 ± 0.05 | 226 ± 15 |
| 20 NH₄    | -66.1 ± 1.0 | -63.4 ± 1.0 | 2.7 ± 0.7 | 1.38 ± 0.10 | 222 ± 18 |
| $|\Delta|$ | 16.3 ± 0.8$^i$ | 10.1 ± 0.3$^j$ | 6.2 ± 0.7$^l$ | 0.87 ± 0.05$^l$ | 4 ± 6$^*$ |

Data shown are means ± SEM obtained under control conditions or at the peak voltage after a change to 20 mM NH₄ Ringer. *Not significant; $^iP < 0.001$.

FIGURE 13. Reproducibility of DIDS-induced voltage changes. Two successive 1-min exposures to 1 mM apical DIDS produced nearly identical voltage responses. (B) DIDS-induced voltage changes recorded from a single cell under open-circuit conditions and while apical membrane was polarized by transepithelial current clamp. Baselines of these traces have been superimposed to facilitate comparison. Abbreviations as in Fig. 2.

FIGURE 12. (Opposite) The effects of apical [NH₄]o on membrane voltage. (A) The first horizontal bar indicates the substitution of control Ringer (solution 1) with 20 mM NH₄ Ringer (solution 8) on the apical side. NH₄ produced a rapid depolarization of $V_{ap}$ followed by a partial repolarization. See Table V. Next, apical [HCO₃]o was decreased from 27.5 to 2.75 mM (solution 2), and the apical side was again exposed to 20 mM NH₄ (solution 10). The amplitude of the NH₄-induced depolarization was significantly reduced. (B) The effects of increasing apical [NH₄]o in the presence of DIDS. Apical side had been perfused with Na-free Ringer (solution 6) for 5 min before the start of this record. In the absence of apical Na, an increase in [NH₄] to 20 mM (solution 9) failed to produce a significant depolarization of $V_{ap}$. (C). Effects of increasing apical [NH₄]o in the presence of DIDS. Apical side had been perfused with control Ringer plus DIDS for 3 min before the start of this record. The perfusion of NH₄ Ringer (20 mM; solution 8) plus DIDS produced a rapid depolarization of $V_{ap}$ that was ~60% smaller than that observed in the absence of DIDS (compare with Fig. 10). Abbreviations as in Fig. 2.
thelium, either inward across the apical membrane (hyperpolarizing) or outward across the apical membrane (depolarizing). These current steps were kept on for several minutes in order to maintain a steady level of membrane hyperpolarization or depolarization before DIDS was applied. The degree to which changes in apical membrane voltage altered the NaHCO₃ transport rate was assessed by measuring the magnitude of the DIDS-induced voltage change at each level of polarization. In these experiments, the exposure of the tissue to DIDS was limited to 1 min. This length of time was chosen because it was long enough for the DIDS-induced change in voltage to reach its peak value and yet short enough to ensure reversibility. Fig. 13A shows two sequential DIDS responses from the same cell. These two responses are practically indistinguishable, indicating that the effects of short exposures to apical DIDS are reversible and that the degree of inhibition is constant from one response to the next.

Fig. 13B shows four DIDS responses recorded from a single cell with the apical membrane potential first at open circuit and then at three current-clamped potentials. The baselines of these traces have been superimposed to facilitate comparison. In the open circuit condition, $V_{ap}$ of this cell was $-82$ mV and apical DIDS caused a 2-mV depolarization. When $V_{ap}$ was depolarized to $-48$ by transepithelial current clamp, DIDS caused a larger depolarization of 7.5 mV. On the other hand, when $V_{ap}$ was hyperpolarized to $-122$ mV, DIDS had essentially no effect on $V_{ap}$, and when $V_{ap}$ was driven even more negative to $-157$ mV, the DIDS-induced voltage change reversed polarity and was $-2.5$ mV.

Fig. 14 summarizes the results of similar experiments in which $V_{ap}$ was polarized by either transepithelial current (○) or by elevating apical $[K]_o$ to 10 mM (△) or

![Figure 14](image)

**Figure 14.** Relation between the DIDS-induced change in $V_{ap}$ and the initial value of $V_{ap}$. DIDS responses were recorded either under open-circuit conditions (○), while $V_{ap}$ was depolarized by 10 mM $[K]_o$ (solution 11, △) or hyperpolarized by 0.2 mM $[K]_o$ (solution 12, ■), or while $V_{ap}$ was polarized by transepithelial current clamp (○). The straight line indicates the best fit of the data by least squares analysis. $R^2 = 0.84$; X intercept = $-114$ mV.

4 These transepithelial currents also polarized the basolateral membrane in the opposite direction and could have influenced the DIDS response. This possibility seems unlikely because similar DIDS-induced voltage changes were produced when both membranes were polarized in the same direction by alterations in apical $[K]_o$ (Fig. 14).
reducing it to 0.2 mM (A). There is a clear correlation between membrane voltage and the DIDS-induced voltage response. When \( V_{ap} \) was depolarized, the DIDS-induced depolarization tended to be larger than it was at the resting potential (C). Conversely, when \( V_{ap} \) was hyperpolarized, the voltage response tended to be smaller than it was at resting potential. Most importantly, the DIDS-induced voltage change reversed polarity and became negative-going at \(-114 \text{ mV}\). This reversal potential is most likely the equilibrium potential of the cotransporter (see below).

**DISCUSSION**

Previous work by Miller and Steinberg (1977a) established that the apical membrane potential of the frog RPE is affected by changes in extracellular [HCO_3^-]. Because HCO_3^- absorption across the RPE is an important determinant of fluid transport across the RPE (Hughes et al., 1984; Hughes et al., 1987b), the present study was undertaken to characterize the underlying mechanism. Evidence is now provided that the apical membrane of the RPE has an electrogenic NaHCO_3 cotransporter that moves Na, HCO_3^- and negative charge into the cell.

In agreement with a previous study (Miller and Steinberg, 1977a), decreases in apical [HCO_3^-] caused rapid depolarization of \( V_{ap} \), whereas increases caused hyperpolarization (Fig. 2, A–C). The possibility that an electrogenic NaHCO_3 cotransport mechanism underlies these voltage responses was suggested by four observations. First, the HCO_3^- induced voltage changes were blocked by the removal of Na from the apical bath (Fig. 7), which indicates that the electrogenic HCO_3^- transporter is Na dependent. Secondly, apical Na removal itself caused an immediate depolarization of \( V_{ap} \) (Fig. 8 A). This finding is incompatible with a simple Na conductance. Thirdly, the Na-induced voltage change was blocked by low apical [HCO_3^-] (Fig. 8 B). Finally, the initial apical membrane depolarizations produced by [HCO_3^-] or [Na] reduction were significantly attenuated by the addition of the anion transport inhibitor DIDS (Figs. 5 and 8 C). These electrophysiological results, which indicate that Na and HCO_3^- are transported across the apical membrane along with net negative charge, were corroborated by intracellular pH measurements (Fig. 10).

DIDS significantly increased the apparent \( a \) value and \( R_i \), which is consistent with an increase in apical membrane resistance and provides evidence that the cotransporter is a conductive mechanism. Reducing apical [Na] or [HCO_3^-] also increased the apparent \( a \) value (Tables II–IV) and supports the notion that this cotransporter moves net (negative) charge.

The introduction of 20 mM NH_4 into the apical bath caused a rapid depolarization \( V_{ap} \) (Fig. 12 A). The peak NH_4 response was markedly reduced either by Na removal, HCO_3^- reduction, or the addition of DIDS, suggesting that it is mediated by a slowing of the electrogenic NaHCO_3 transporter. Measurements of pH using the fluorescent dye BCECF showed that extracellular NH_4 (20 mM) alkalinizes the RPE cell interior by 0.2 pH U (Fig. 11). Since this alkalinization, which occurred at constant Pco_2, must also increase [HCO_3^-]_i, it is likely that a change in the HCO_3^- chemical gradient is a principal cause of the cotransporter inhibition. The present experiments do not exclude the possibility that the transporter might be also affected kinetically by the increase in pH_1.
Direction of Cotransport

The disulfonic stilbenes DIDS and SITS have been shown to inhibit electrogenic NaHCO₃ cotransport in a variety of tissues (Boron and Boulpaep, 1983; Jentsch et al., 1984; Biagi, 1985; Curci et al., 1987; Sasaki et al., 1987). For example, in the proximal tubule, electrogenic NaHCO₃ transport mediates the efflux of HCO₃ across the basolateral membrane and SITS causes both a membrane hyperpolarization and an intracellular alkalinization (Boron and Boulpaep, 1983; Lopes et al. 1987). In contrast, we found in the RPE that apical DIDS acidified the cell interior (Fig. 10) and caused a rapid depolarization of Vₐₚ (Figs. 5, 6, and 8 C). This effect of DIDS on Vₐₚ was mediated by the cotransporter since it was absent when the tissue was bathed in either Na-free or HCO₃-free solutions (Fig. 9, C and E). Therefore we conclude that this cotransporter must hyperpolarize the apical membrane by carrying Na, HCO₃, and net negative charge inward across the apical membrane.

Na or HCO₃ removal from the apical bathing solution produced Vₐₚ depolarizations that were large relative to the DIDS response, presumably because these maneuvers reversed the direction of the cotransporter, whereas DIDS caused only its inhibition. Evidence that the cotransporter reverses direction when either HCO₃ or Na are removed from the apical bath is provided by the data shown in Fig. 9, B and D, where DIDS produced apical membrane hyperpolarizations.

Apparent Reversal Potential and Stoichiometry

The electrogenicity of the NaHCO₃ transporter suggests that its rate may be affected by membrane voltage as well as Na and HCO₃ chemical gradients across the apical membrane. Fig. 14 summarizes data which shows that the magnitude of the DIDS-induced voltage change is a function of Vₐₚ. When Vₐₚ was depolarized from its resting level, the magnitude of the DIDS-induced voltage change increased, and when it was hyperpolarized, the DIDS response became smaller. At values more hyperpolarized than -114 mV, the polarity of the DIDS-induced voltage change reversed. This reversal can be used to estimate the cotransporter's equilibrium potential, since the DIDS-induced voltage change results from inhibition of the electrogenic cotransporter.

This reversal potential estimate allowed us to calculate the stoichiometry of the NaHCO₃ cotransporter. From thermodynamic considerations (Aronson, 1984), it can be shown that:

\[ E_{rev} = \frac{2.3RT}{F(n-1)} \log \left( \frac{[\text{Na}]_i}{[\text{HCO}_3]_o} \right) \times \left( \frac{[\text{Na}]_o}{[\text{HCO}_3]_o} \right) \]  

where \( E_{rev} \) is the reversal (equilibrium) potential, \( n \) is the ratio of HCO₃ to Na ions that are carried by the cotransporter, and \( R, T, \) and \( F \) have their usual meaning. In control Ringer, \([\text{Na}]_o\) was 110 mM, \([\text{HCO}_3]_o\) was 27.5 mM. When \( V_{ap} \) was current-clamped to \(-114 \pm 10 \text{ mV}; \) see Fig. 14), \( \text{pH}_i \) was \(-6.9 (n=5; \text{Lin and Miller, unpublished observations}). Assuming that intracellular and extracellular Pco₂ are equal (5%), we calculate \([\text{HCO}_3]_i \) to be 9 mM. Intracellular \([\text{Na}]_i \) is assumed to lie between 5 and 30 mM, a range that encompasses virtually all epithelia.

These values can be used in Eq. (1) to calculate the upper and lower bounds for
the stoichiometric ratio \((n)\) of the cotransporter. The data imply that the \(\text{HCO}_3^-/\text{Na}\) ratio lies between 1.6 and 2.4.

**Relation to Other NaHCO\(_3\) Cotransport Mechanisms**

Evidence for electrogenic NaHCO\(_3\) cotransport mechanisms has been presented for a variety of other tissues, including amphibian and mammalian proximal tubule (Boron and Boulpaep, 1983; Guggino et al., 1983; Biagi and Sohtell, 1985; Alpern and Chambers, 1986; Sasaki et al., 1987), oxyntic cells (Curci et al., 1987) and cultured bovine corneal endothelium (Jentsch et al., 1984). These transporters mediate the net efflux of \(\text{HCO}_3^-\) and \(\text{Na}\) from the cell and acidify the cell interior. The electrochemical gradient for \(\text{Na}\) across the basolateral membrane requires that the \(\text{HCO}_3^-/\text{Na}\) stoichiometry of these cotransporters be approximately 3:1 (Alpern and Chambers, 1986; Lopes et al., 1987). In contrast, the RPE cotransporter mediates the influx of \(\text{HCO}_3^-\) and \(\text{Na}\) into the cell across the apical membrane and has an opposite effect on cell \(pH_i\).

**NaHCO\(_3\) Cotransport and RPE Physiology**

Fluid transport across the RPE is driven by active \(\text{HCO}_3^-\) and \(\text{Cl}\) absorption (Hughes et al., 1984, 1987b; Tsuboi, 1987). Although no direct measurement of \(\text{HCO}_3^-\) flux across the RPE has been reported to date, a considerable amount of evidence has accumulated that strongly supports the notion of net \(\text{HCO}_3^-\) absorption by this epithelium. Lasansky and De Fisch (1966) used a manometric technique and measured the disappearance of \(\text{HCO}_3^-\) from the solution bathing the apical membrane. Net \(\text{HCO}_3^-\) absorption has also been inferred from the analysis of net ionic fluxes measured across the frog RPE under both open and short-circuit conditions (Miller and Steinberg, 1977b; Miller and Farber, 1984; Hughes et al., 1984). Finally, fluid absorption across the frog RPE has been shown to be significantly inhibited by the removal of \(\text{HCO}_3^-/\text{CO}_2\) from the bathing media (Hughes et al., 1984).

It seems likely that transepithelial \(\text{HCO}_3^-\) absorption begins with active influx through the NaHCO\(_3\) cotransporter. The exit mechanism for \(\text{HCO}_3^-\) across the basolateral membrane is electroneutral since \(V_m\) is insensitive to changes in \([\text{HCO}_3^-]_o\) outside the basolateral membrane (Miller and Steinberg, 1977a). Recent studies provide strong evidence that this mechanism is a \(\text{HCO}_3^-/\text{Cl}\) exchanger (Fong et al., 1988; Edelman et al., 1988).

In the vertebrate eye, the \(K\) concentration of the subretinal space decreases from approximately 5 to 2 mM after the onset of light. This decrease in \([K]_o\) hyperpolarizes the apical membrane of the RPE, producing the RPE component of the \(c\)-wave of the DC electroretinogram (Steinberg et al., 1985). Since the cotransporter is voltage dependent (Fig. 14), one might expect the light-evoked decrease in \([K]_o\) to reduce the rate of \(\text{HCO}_3^-\) influx across the apical membrane and decrease intracellular \(pH\). These changes in \(pH_i/[\text{HCO}_3^-]_i\) could significantly affect other RPE transport processes. Recently, we have observed in frog RPE that increases in apical \([K]_o\) from 2 to 5 mM stimulate the net secretion of \(\text{Cl}\) (Edelman et al., 1988). This \(\text{Cl}\) secretion is blocked by apical or basal DIDS and by the removal of either apical \(\text{Na}\) or \(\text{HCO}_3^-\). If this secretory response is mediated by the electrogenic NaHCO\(_3\) cotransporter, then it provides an example of RPE function modulated by changes
in retinal physiology. It will be interesting to learn what other retina–RPE interactions are mediated by this cotransporter.

We thank Steven Bialek for carrying out some preliminary experiments and for providing the data shown in Fig. 4. It is our pleasure to thank Drs. Terry Machen and Jeffery Demerest for their helpful comments on this manuscript.

This work was supported by National Institutes of Health grants EY02205 and RCDA EY00242 to Dr. Miller, Core Grant EY02176, and National Research Service Award EY05968 to Dr. Adorante.

Original version received 31 March 1988 and accepted version received 7 February 1989.

REFERENCES

Adorante, J., B. Hughes, and S. Miller. 1988. Electrogenic NaHCO₃ transport at the apical membrane of the frog retinal pigment epithelium. FASEB J. 2:A1722. (Abstr.)

Alpern, R. J. 1985. Mechanism of basolateral membrane H⁺/OH⁻/HCO₃⁻ transport in the rat proximal convoluted tubule. J. Gen. Physiol. 86:613–636.

Alpern, R. J., and M. Chambers. 1987. Basolateral membrane Cl⁻/HCO₃⁻ exchange in the rat proximal convoluted tubule: Na-dependent and -independent modes. J. Gen. Physiol. 89:581–598.

Aronson, P. S. 1984. Electrochemical driving forces for secondary active transport: kinetics of Na⁺-H⁺ exchange and Na-glucose cotransport. In Electrogenic Transport: Fundamental Principles and Physiological Implications. Volume 38. M. B. Blaustein and M. Liberman, editors. Raven Press, New York. 49–70.

Biagi, B. A. 1985. Effects of the anion transport inhibitor, SITS, on the proximal straight tubule of the rabbit perfused in vitro. J. Membrane Biol. 88:25–31.

Biagi, B. A., and M. Sohtell. 1986. Electrophysiology of basolateral bicarbonate transport in the rabbit proximal tubule. Am. J. Physiol. 250:F267–F272.

Boron, W. F. 1985. Intracellular pH regulating mechanism of the squid axon: relation between the external Na⁺ and HCO₃⁻ dependences. J. Gen. Physiol. 85:325–345.

Boron, W. F., and E. L. Boulpaep. 1983. Intracellular pH regulation in the renal proximal tubule of the salamander: basolateral HCO₃⁻ transport. J. Gen. Physiol. 81:55–94.

Boron, W. F., and P. De Weer. 1976. Intracellular pH transients in squid giant axons caused by CO₂, NH₃, and metabolic inhibitors. J. Gen. Physiol. 67:91–112.

Boron, W. F., and J. M. Russell. 1983. Stoichiometry and ion dependencies of the intracellular pH regulating mechanism in squid giant axons. J. Gen. Physiol. 81:373–399.

Curci, S., L. Debellis, and E. Fromter. 1987. Evidence for rheogenic bicarbonate cotransport in the basolateral membrane of oxyntic cells of frog gastric mucosa. Pflügers Arch. 408:497–504.

Edelman, J. L., S. S. Miller, and B. A. Hughes. 1988. Regulation of chloride transport by frog retinal pigment epithelium. FASEB J. 2:A1722. (Abstr.)

Fong, C. N., S. Bialek, B. A. Hughes, and S. S. Miller. 1988. Modulation of intracellular chloride in bullfrog retinal pigment epithelium (RPE). FASEB J. 2:A1722. (Abstr.)

Guggino, W. F., R. London, E. L. Boulpaep, and G. Giebisch. 1983. Chloride transport across the basolateral cell membrane of the Necturus proximal tubule: dependence on bicarbonate and sodium. J. Membrane Biol. 71:227–240.

Griff, E. R., Y. Shirao, and R. H. Steinberg. 1985. Ba²⁺ unmasks K⁺ modulation of the Na⁺-K⁺ pump in the frog retinal pigment epithelium. J. Gen. Physiol. 86:853–876.

Heiple, J. M., and D. L. Taylor. 1982. An optical technique for measurement of intracellular pH in
single living cells. In Intracellular pH: Its Measurement, Regulation & Utilization in Cellular Functions: Proceedings. R. Nuccitelli and D. Deamer, editors. Alan R. Liss, Inc., New York. 21–54.

Hughes, B. A., S. S. Miller, J. Adorante, and S. Bialek. 1987a. Electrogenic Na-dependent HCO₃⁻ transport in the apical membrane of the frog retinal pigment epithelium. Investigative Ophthalmology and Visual Science. 28(Suppl.): 382 (Abstr.)

Hughes, B. A., S. S. Miller, and D. B. Farber. 1987b. Adenylate cyclase stimulation alters transport in frog retinal pigment epithelium. American Journal of Physiology. 252(Cell Physiology 21):C385–C397.

Hughes, B. A., S. S. Miller, D. P. Joseph, and J. E. Edelman. 1988. Cyclic AMP stimulates the Na/K pump in the retinal pigment epithelium. American Journal of Physiology. 254 (Cell Physiology 23):C84–C98.

Hughes, B. A., S. S. Miller, and T. E. Machen. 1984. Effects of cyclic AMP on fluid absorption and ion transport across frog retinal pigment epithelium: measurements in the open circuit state. Journal of General Physiology. 83:875–899.

Inoue, I. 1985. Voltage-dependent chloride conductance of squid axon membrane and its blockade by some disulfonyl stilbene derivatives. Journal of General Physiology. 85:519–537.

Inoue, I. 1986. Modification of K conductance of the squid axon membrane by SITS. Journal of General Physiology. 88:507–520.

Jentsch, T. J., S. K. Keller, M. Koch, and M. Wiederholt. 1984. Evidence for coupled transport of bicarbonate and sodium in cultured bovine corneal endothelial cells. Journal of Membrane Biology. 81:189–204.

Joseph, D., and S. S. Miller, 1986. Passive ionic properties of the bovine retinal pigment epithelium. Biophysical Journal. 49:399a. (Abstr.)

Kaila, K., and J. Voipio. 1987. Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. Nature. 330:163–165.

La Cour, M., H. L. Andersen, and T. Zeuthen. 1986. Potassium transport of the frog retinal pigment epithelium: autoregulation of potassium activity in the subretinal space. Journal of Physiology. 375:461–471.

Lasansky, A., and F. W. De Fisch. 1966. Potential, current, and ionic fluxes across the isolated retinal pigment epithelium and choroid. Journal of General Physiology. 49:913–924.

Lopes, A. G., A. W. Siebens, G. Giebisch, and W. F. Boron. 1987. Electrogenic Na/HCO₃ co-transport across basolateral membrane of isolated perfused Necturus proximal tubule. American Journal of Physiology. 252:F340–F350.

Marmor, M. F., A. S. Abdul-Rahim, and D. S. Cohen. 1980. The effect of metabolic inhibitors on retinal adhesion and fluid reabsorption. Investigative Ophthalmology and Visual Science. 19:893–903.

Miller, S. S., and D. Farber. 1984. Cyclic AMP modulation of ion transport across frog retinal pigment epithelium: measurements in the short-circuit state. Journal of General Physiology. 83:853–874.

Miller, S. S., B. Hughes, and T. E. Machen. 1982. Fluid transport across frog retinal pigment epithelium. Proceedings of the National Academy of Sciences. 79:2111–2115.

Miller, S. S., and R. H. Steinberg. 1977a. Passive ionic properties of the frog retinal pigment epithelium. Journal of Membrane Biology. 36:337–372.

Miller, S. S., and R. H. Steinberg. 1977b. Active transport of ions across frog retinal pigment epithelium. Experimental Eye Research. 25:235–248.

Miller, S. S., and R. H. Steinberg. 1982. Potassium transport across the frog retinal pigment epithelium. Journal of Membrane Biology. 67:199–209.
Miller, S. S., and R. H. Steinberg, and B. Oakley II. 1978. The electrogenic sodium pump of the frog retinal pigment epithelium. *Journal of Membrane Biology*. 44:259–279.

Oakley, B., S. S. Miller, and R. H. Steinberg. 1978. Effect of intracellular potassium upon the electrogenic pump of the frog retinal pigment epithelium. *Journal of Membrane Biology*. 44:281–302.

Rink, T. J., R. Y. Tsien, and T. Pozzan. 1982. Cytoplasmic pH and free Mg$^{2+}$ in lymphocytes. *Journal of Cell Biology*. 95:189–196.

Saito, Y., and E. M. Wright. 1984. Regulation of bicarbonate transport across the brush border of the bullfrog choroid plexus. *Journal of Physiology*. 350:327–342.

Sasaki, S., S. Shigai, N. Yoshiyama, and J. Takeuchi. 1987. Mechanism of bicarbonate exit across basolateral membrane of rabbit proximal straight tubule. *American Journal of Physiology*. 252:F11–F18.

Steinberg, R. H., R. A. Linesenmeier, and E. R. Griff. 1985. Retinal pigment epithelial cell contributions to the electroretinogram. In Progress in Retinal Research. Volume 4. N. Osborne, and G. Chader, editors. Pergamon Press, New York. 33–66.

Steinberg, R. H., S. S. Miller, and W. H. Stern. 1978. Initial observations on the isolated retinal pigment epithelium-choroid of the cat. Investigative Ophthalmology and Visual Science. 17:675–678.

Thomas, J. A., R. N. Buschbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry*. 18:2210-2218.

Thomas, R. C. 1977. The role of bicarbonate, chloride, and sodium ions in the regulation of intracellular pH in snail neurons. *Journal of Physiology*. 272:317–338.

Tsuboi, S., R. Manabe, and S. Iizuka. 1986. Aspects of electrolyte transport across isolated dog retinal pigment epithelium. *American Journal of Physiology*. 250:F1–F4.

Tsuboi, S. 1987. Measurement of the volume flow and hydraulic conductivity across the isolated dog retinal pigment epithelium. *Investigative Ophthalmology and Visual Science*. 28:1776–1782.