The fluorescence quenching of acridine orange has been used to study the formation and dissipation of acid interior pH gradients in brush-border membrane vesicles from rabbit renal cortex. Acidic interior pH gradients were produced by 1) outwardly directed gradients of Na⁺ or K⁺, and 2) the addition of vesicles equilibrated at pH 8.0 to 7.5 buffer. The rate of pH gradient dissipation was stimulated 6.3-fold by the replacement of tetramethylammonium gluconate by tetramethylammonium chloride. A further increase, of 2-fold, was seen upon the addition of carboxyl cyanide-m-chlorophenylhydrazine, demonstrating the existence of a Cl⁻ conductance pathway. In the presence of valinomycin, the replacement of tetramethylammonium gluconate by K gluconate increased the rate of ΔpH dissipation by 11-fold, demonstrating the existence of a conductive pathway for protons. This pathway for protons was also shown by the formation of an acidic interior space by an outwardly directed K⁺ gradient in the presence of valinomycin. The parallel conductive pathways for H⁺ and Cl⁻ may dissipate pH and chloride gradients across the luminal membrane of the proximal tubule.

Brush-border membrane vesicles from renal cortex have been shown to possess an electroneutral Na⁺/H⁺ antiporter (1, 2). This system is believed to be responsible for Na⁺ absorption and H⁺ secretion in the proximal tubule (3). In addition, these membranes are known to have a conductance pathway for chloride (4–6). Previous work with these transport systems has relied on either the production of pH changes in the exterior space, or measurement of the flux of radiolabeled ions in the presence of valinomycin, the replacement of tetramethylammonium gluconate by K gluconate, or the addition of vesicles equilibrated at pH 6.0 to 7.6 buffer. The rate of pH gradient 42 dissipation was fitted with a 27-gauge needle. Salt solutions were added to make the resulting decrease in the concentration of dye in the acidic intravesicular space; the fluorescence at time 0 was monitored immediately after vesicle addition. General Methods—Protein concentration was determined by the method of Lowry et al. (12), using bovine serum albumin as a standard. Alkaline phosphatase was obtained from Eastman Kodak; all chemicals were the highest purity commercially available. Stock solutions of tetramethylammonium gluconate were made by titrating tetramethylammonium hydroxide pentahydrate in pH 7.5 with d-gluconic acid lactone.

RESULTS
Fluorescence Quenching by pH Jumps—The addition of BBM vesicles equilibrated at pH 6.0 to 7.5 buffer (pH-jump) caused quenching of acridine orange fluorescence (Fig. 1). This quenching has previously been shown to be caused by the accumulation of dye into an acidic intravesicular space; the resulting decrease in the concentration of dye in the external space causes a decrease in the observed fluorescence (8). As shown in Fig. 1 and Table I, the stability of pH gradients formed by pH jumps was affected by the

**Experimental Procedures**

Membrane Preparation—Female New Zealand white rabbits, 1–2 kg in weight, were killed by decapitation. Each kidney was perfused via the renal artery with 35 ml of ice-cold 50 mm sucrose, 0.5 mm EDTA, 10 mm Hepes/Tris, pH 7.5 (HIS). The renal cortical tissue was dissected and homogenized in HIS (60 ml/kidney) with an Omni-mixer (Sorval, DuPont Instrument Co.) for 4 min. Brush-border membrane fractions were prepared by precipitation with 12 mm MgSO₄, (6, 9–11). Subsequent homogenization and harvesting by differential centrifugation were performed at 4°C in 100 mm sucrose, 10 mm Hepes/Tris, pH 7.5. Final membrane pellets were suspended to 30 mg of protein/ml in the indicated buffers with a 1-ml syringe fitted with a 27-gauge needle. Salt solutions were added to make the vesicle suspension 150 mm in the desired salt, and the suspension incubated at room temperature for at least 2 h prior to use. A 7–to 10-fold purification of the brush-border marker enzyme alkaline phosphatase was obtained (6).

The fluorescence quenching of acridine orange was used to monitor changes in the transmembrane ΔpH (6). In all the experiments, 10 μl of BBM vesicles were added to 2 ml of buffer containing 5 μM acridine orange at 25°C. Subsequent changes in the fluorescence were monitored with a Perkin-Elmer MFP-44A spectrofluorometer (excitation, 493 nm; emission, 520 nm). The addition of vesicles under conditions where no ΔpH is formed causes a small fluorescence quenching (presumably, binding of acridine to membranes). Therefore, all quantitative measurements of fluorescence change are based on the total fluorescence measured after pH gradient dissipation (addition of either 2 μM nigericin when external K⁺ was present, or 25 mM NaCl). Ionophores, when present at initial time, were added from ethanol stocks to the buffer at the same time as vesicles. Samples were mixed with a single pass of a Bioblock cuvette mixer immediately after vesicle addition. General Methods—Protein concentration was determined by the method of Lowry et al. (12), using bovine serum albumin as a standard. Alkaline phosphatase was obtained from Eastman Kodak; all chemicals were the highest purity commercially available. Stock solutions of tetramethylammonium gluconate were made by titrating tetramethylammonium hydroxide pentahydrate in pH 7.5 with d-gluconic acid lactone.
composition of the media. The replacement of either external TMA by K' (Fig. 1, trace B) or internal gluconate by Cl- (Fig. 1, trace C) increased the rate of fluorescence recovery in pH-jump studies. Thus, mechanisms other than the Na+/H+ antipporter (1, 2) may also translocate protons across the brush-border membrane.

Dissipation of \( \Delta pH \) by Cl- and K'-The rate of \( \Delta pH \) dissipation \( (k_{\text{obs}}) \) following the addition of vesicles at pH 6.0 to 7.5 buffer was measured using various combinations of TMA gluconate, TMA Cl-, and K gluconate in the vesicles and in the external buffer (Table I). Taking the rate of \( \Delta pH \) dissipation seen when TMA gluconate-equilibrated vesicles were added to TMA gluconate buffer as the control rate, the presence of equal internal and external TMA Cl (150 mM) caused a 6.2-fold increase in the rate of \( \Delta pH \) dissipation. An outwardly directed Cl- gradient (150 mM TMA Cl inside, 150 mM TMA gluconate outside) increased the rate 9.4-fold (Table I). Both rates were further increased by the addition of the protonophore, CCCP, but addition of valinomycin was without significant effect. The rate of \( \Delta pH \) dissipation was also increased in the presence of external K', the rate being increased 4.4-fold by a K' gradient (150 mM K gluconate outside, 150 mM TMA gluconate inside) and 3.8-fold with 150 mM K gluconate on both sides. Both of these rates were further increased by the addition of the K' ionophore, valinomycin, while CCCP had a somewhat lesser effect on accelerating \( \Delta pH \) dissipation. The stimulation of \( \Delta pH \) dissipation by CCCP in the presence of internal Cl-, as compared to gluconate, is consistent with the presence of a Cl- conductance pathway. Furthermore, the stimulation of the rate of \( \Delta pH \) dissipation by valinomycin in the presence of external K' requires that a protonic conductance pathway also be present.

The effect of acridine orange, per se, on the rate of \( \Delta pH \) dissipation was examined. At initial time, vesicles equilibrated with 150 mM TMA gluconate buffer at pH 6.0 were added to K gluconate buffer at pH 7.5, with valinomycin. Acridine orange was either initially present or added at various times after addition of the vesicles. Curves for acridine orange fluorescence versus time were superimposable whether acridine orange was initially present or subsequently added. Therefore, the rate of \( \Delta pH \) dissipation was unaffected by the presence of acridine orange.

**Table I**  
Relative rates of \( \Delta pH \) gradient dissipation

| Internal salt\(^c\) (pH 6.0) | External salt\(^d\) (pH 7.5) | Relative rate |
|-----------------------------|-------------------------------|---------------|
| TMA gluconate | TMA gluconate | 1.00 | 0.99 ± 0.15 | 1.02 ± 0.14 |
| TMA Cl | TMA Cl | 6.2 ± 1.7 | 5.3 ± 1.4 | 12.7 ± 2.5 |
| TMA Cl | TMA Cl | 9.4 ± 1.9 | 9.3 ± 1.6 | 17.8 ± 3.2 |
| TMA gluconate | K gluconate | 4.4 ± 0.9 | 19.4 ± 2.9 | 6.2 ± 0.5 |
| K gluconate | K gluconate | 3.8 ± 0.1 | 11.0 ± 1.0 | 7.6 ± 1.9 |

\(^c\) Vesicles equilibrated in 10 mM Hepes/Tris, 100 mM sucrose at pH 6.0, and 25 °C with 150 mM of the indicated salt.

**Fig. 2.** K' gradient-dependent quenching of acridine orange fluorescence. At the indicated time (1) 10 μl of BBM vesicles (30 mg of protein/ml) equilibrated in 150 mM K' gluconate, 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, were added to 2.0 ml of 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, containing 6 μM acridine orange and either 150 mM K' gluconate (A), 150 mM TMA gluconate (B-D), or 150 mM Na gluconate (E-G). Ionophores, 4.8 μM valinomycin (C, D, F, G) and 4.3 μM CCCP (D, G), were added from ethanol stocks immediately prior to vesicle addition. Ethanol concentrations were less than 0.2%. At the indicated times (1), 50 μl of 1 M NaCl and 50 μl of 1 M KCl were added to collapse the pH gradient and return the fluorescence to the base-line value.

**Generation of ΔpHs from K' Concentration Gradients**—In these experiments, vesicles equilibrated in 150 mM K gluconate buffer at pH 7.5 were added to pH 7.5 buffer containing K, Na, or TMA gluconate. Control experiments showed that the addition of vesicles to 150 mM K gluconate did not generate a ΔpH (Fig. 2A). The small quenching seen was due to binding of acridine to the added vesicles and was equal to the maximum fluorescence obtained after pH gradient dissipation (see below). The addition of K gluconate vesicles to 150 mM TMA gluconate buffer produced a small quenching of acridine orange fluorescence (Fig. 2B) which slowly returned toward the maximal value. The return was much faster after the addition of 25 mM Na gluconate and 25 mM K gluconate to the external buffer (vertical arrow, Fig. 2B). The same K' gradient in the presence of valinomycin (Fig. 2C) produced a much larger fluorescence quenching. The formation of a ΔpH by an outwardly directed K' gradient and valinomycin demonstrates an inward flux of protons through a membrane potential sensitive path. A still larger fluorescence quenching was seen if the K' gradient was formed in the presence of valinomycin and CCCP (Fig. 2D) or in the presence of nigericin (data not shown).

Finally, it was important to show that the fluorescence
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Na\(^+/\)H\(^+\) exchange is presumed to be the result of Na\(^+/\)H\(^+\) exchange by the Na\(^+/\)H\(^+\) antiporter (i.e. inhibition by external Li\(^+\) and amiloride). As shown in Fig. 3, the magnitude and stability of pH gradients formed by Na\(^+/\)H\(^+\) exchange are affected by the ionic composition of the media. The presence of either internal Cl\(^-\) (Fig. 3C) or external K\(^+\) (Fig. 3B) decreased the magnitude of Na\(^+/\)H\(^+\) gradient-dependent fluorescence quenching.

The effects of both CCCP and valinomycin on ΔpH generated by the Na\(^+/\)H\(^+\) antiporter were measured. All experiments were performed in the absence of chloride. As shown in Fig. 4A, the addition of vesicles equilibrated in 75 mM Na gluconate and 75 mM K gluconate at pH 7.5 to 75 mM TMA gluconate and 75 mM K gluconate buffer at pH 7.5 caused a large quenching of acridine orange fluorescence. The addition of vesicles in the presence of either CCCP (Fig. 4B) or valinomycin (Fig. 4C) significantly decreased the quenching of acridine orange. The addition of both valinomycin and CCCP (Fig. 4D) caused fluorescence quenching to be reduced to the base-line response caused by vesicle addition.

**DISCUSSION**

Several studies have shown that a vesicular suspension maintaining a pH gradient, acid interior, causes the fluorescence of acridine orange to be quenched (8, 13). It has been observed that the magnitude of the fluorescence quenching does not correspond to that predicted wholly on the basis of pH-dependent concentration of amines, but can be explained by a combination of 1) pH-dependent concentration into the internal space and 2) binding to internal sites (8). The combination of these effects increases the sensitivity of acridine orange to small pH gradients and makes it a useful probe for these studies (8). With this probe, we have confirmed the ability of BBM vesicles to produce a pH gradient by Na\(^+/\)H\(^+\) antiport, i.e. to couple downhill Na\(^+\) efflux to uphill H\(^+\) influx (1, 2). In addition, we have shown that BBM vesicles can maintain pH gradients imposed by pH jumps of the external pH. Furthermore, it is clear that the rate of pH gradient dissipation (H\(^+\) or OH\(^-\) leakage) varies with the ionic composition.

It is readily seen that the presence of chloride accelerates the rate of pH-gradient dissipation (Table I). Previous studies of BBM vesicles have suggested the existence of both Cl\(^-\)/OH\(^-\) antiport and rheogenic Cl\(^-\) pathways (4–6, 15). The observation that CCCP increases the rate of ΔpH dissipation requires that an electrogenic path for some co-ion must also exist in these membranes in order to balance net charge movement. Acceleration is seen with TMA Cl\(^-\) but not with TMA gluconate (Table I); this result demonstrates that the conductance of chloride is greater than the conductance of gluconate. Likewise, the increase in the rate of pH dissipation by valinomycin in the presence of external potassium demonstrates that a protonic conductance exists across BBM vesicles. A protonic conductance pathway was also shown by the formation of a pH gradient from a K\(^+\) concentration gradient in the presence of valinomycin (Fig. 3C). Together, the proton and chloride conductance provide a mechanism for ΔpH dissipation by internal Cl\(^-\). However, the present results are not inconsistent with the existence of an electrically neutral Cl\(^-\)/OH\(^-\) exhanger in addition to rheogenic fluxes of H\(^+\) and Cl\(^-\).

Murer et al. (1) studied the Na\(^+/\)H\(^+\) antiporter in BBM vesicles prepared from rat small intestine and renal cortex. They concluded that Na\(^+/\)H\(^+\) exchange occurs by an electrically neutral mechanism rather than by electrically coupled fluxes of Na\(^+\) and H\(^+\). This result has been confirmed by other studies (2), as well as by the present results.

On the other hand, the present studies demonstrate a protionic conductance pathway in BBM vesicles prepared from

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**Proton Conductance in Renal BBM Vesicles**

**Fig. 3. Na\(^+\) gradient-dependent quenching of acridine orange fluorescence.** At the indicated time (t), 10 μl of BBM vesicles (30 mg of protein/ml) equilibrated in 75 mM Na gluconate, 75 mM K gluconate, 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, with 150 mM Na gluconate (A, B) or 150 mM NaCl (C, D) were added to 2.0 ml of 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, containing 6 μM acridine orange and either 150 mM TMA gluconate (A, C), 150 mM K gluconate (B) or 150 mM NaCl (D). At the indicated times (t), 50 μl of 1 M NaCl was added to collapse the pH gradient and return the fluorescence to the baseline.

**Fig. 4. Effects of CCCP and valinomycin on Na\(^+\) gradient-dependent quenching of acridine orange fluorescence.** At the indicated time (t), 10 μl of BBM vesicles (30 mg of protein/ml) equilibrated in 75 mM Na gluconate, 75 mM K gluconate, 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, were added to 2.0 ml of 75 mM TMA gluconate, 75 mM K gluconate, 100 mM sucrose, 10 mM Hepes/Tris, pH 7.5, containing 6 μM acridine orange, 4.8 μM valinomycin (C, D), and 4.3 μM CCCP (B, D). Ionophores, when present, were added from ethanol stocks immediately prior to vesicle addition; ethanol concentrations were less than 0.2%. At the indicated times (t), 50 μl of 1 M NaCl was added to collapse the pH gradient and return the fluorescence to the baseline.

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2 Warnock, D. G., Reenstra, W. W., and Yee, V. J., Am. J. Physiol., in press.
rabbit renal cortex. This finding is at odds with the results of Murer et al. (1), who did not observe any effect of CCCP or valinomycin on Na⁺/H⁺-dependent proton extrusion in BBM vesicles pre-equilibrated with K⁺. They monitored external pH changes in response to Na⁺/H⁺ exchange, whereas the present studies use acridine orange to follow changes in the pH gradient. At present, we have no explanation for this discrepancy. Although our results differ from Murer et al. (1) with regard to the separate effects of valinomycin and CCCP on the transmembrane pH gradient, the present results confirm their previous finding that the combined effects of valinomycin and CCCP will collapse any ΔpH generated by the Na⁺/H⁺ antiporter.

The possibility that electrodifusional flux of protonated acridine orange could account for the observed protonic conductance and the differences between our results and those of Murer et al. (1) was considered. However, we view this as unlikely because 1) the flux of protonated acridine orange must be extremely small relative to the un-ionized form or else the ΔpH-dependent accumulation of acridine orange would be severely compromised (7, 14); 2) K⁺ gradients with valinomycin do not cause quenching of acridine orange fluorescence in vesicles known to have low intrinsic proton conductance; and 3) the rate of pH gradient dissipation is unaffected by the presence of acridine orange. These findings are readily explained by an intrinsic proton conductance in the BBM vesicles, and are not consistent with electrodifusional flux of protonated acridine orange.

The observation that CCCP increases the rate of ΔpH dissipation in the presence of K⁺ and the absence of Cl⁻ (Table I) is also noteworthy. This fact, as well as that of the inhibition of Na⁺ gradient-dependent ΔpH formation by CCCP in the presence of K⁺ (Fig. 4B) suggests that a K⁺ permeability also exists in these membranes. Electrical coupling of H⁺ and K⁺ transport via conductive pathways could also explain 1) the decreased ΔpH formed by Na⁺/H⁺ antiporter when Na⁺-loaded vesicles were added to K⁺ instead of TMA⁺ buffer (Fig. 3B), 2) the formation of a ΔpH by a K⁺ diffusion gradient in the absence of valinomycin (Fig. 2B), and 3) the increased dissipation of a preformed pH gradient by external K⁺ (Fig. 1B). It should be noted that under conditions where H⁺ flux was not limiting, the effects of K⁺ on pH-gradient dissipation were smaller than the effects of Cl⁻ (Table I). Therefore, the intrinsic permeability of K⁺ is less than that of Cl⁻.

Finally, these findings may be considered in reference to the physiology of the proximal tubule. Early in the proximal tubule, the Na⁺/H⁺ antiporter secretes protons into the lumen titrating the filtered bicarbonate (3). In the latter portions of the proximal tubule, after reabsorption of most of the bicarbonate, the luminal concentrations of Cl⁻ and H⁺ are elevated with respect to the original filtrate. Any mechanism which then effectively translocates H⁺ and Cl⁻ across the luminal membrane will allow continued operation of the Na⁺/H⁺ antiporter, and accomplish net NaCl absorption (6, 15). Therefore, the Na⁺/H⁺ antiporter may play a central role in Na⁺ absorption along the entire length of the proximal tubule (16).

Our results demonstrate parallel conductance pathways in the BBM for Cl⁻ and H⁺. Thus, these pathways suggest a mode of H⁺/Cl⁻ co-transport across the luminal membrane, which is electrically coupled. The elevated luminal Cl⁻ and H⁺ concentrations in the late proximal tubule could provide the driving force for HCl uptake. If Cl⁻ transport is rheogenic and coupled to rheogenic H⁺ transport, then the cytoplasmic activity of Cl⁻ should not be greater than its electrochemical equilibrium value. On the other hand, if the cytoplasmic Cl⁻ activity is greater than its electrochemical equilibrium value, then an electrically neutral, coupled transport system for Cl⁻, such as HCl cotransport (or Cl⁻/OH⁻ exchange), must exist in the luminal membrane. Recent measurements have given conflicting results; data from studies using Necturus proximal tubules indicate that cytoplasmic chloride activity exceeds its electrochemical equilibrium value by approximately 2-fold (17, 18), while measurements in bullfrog and rat proximal tubules indicate that Cl⁻ is distributed at its electrochemical equilibrium value (19-21). While the conductive pathways for H⁺ and Cl⁻ are clearly large in BBM vesicles, the importance of these paths in the proximal tubule cannot be presently determined.

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Note Added in Proof—An independent report of a protonic conductance pathway was recently made by Burnham, C., Muenzshemer, T., Rabon, E., and Sachs, G. (1981) Fed. Proc. 40, 462.

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Footnotes:
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