Boquet, P., Silverman, M. S., Pappenheimer, A. M., & Vernon, W. B. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4449–4453.
Borochov-Neori, H., Yavin, E., Staerz, U., & Montal, M. (1983) *Biophys. J. 41*, 381a.
Burgen, A. S. V., Dickens, F., & Zalman, L. J. (1949) *J. Physiol. (London) 109*, 10–24.
Collier, R. J. (1975) *Bacteriol. Rev. 39*, 54–85.
Dolly, J. O., Black, J., Williams, R. S., & Melling, J. (1984) *Nature (London) 307*, 457–460.
Donovan, J. J., Simon, M. I., Draper, R., & Montal, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 172–176.
Donovan, J. J., Simon, M. I., & Montal, M. (1982) *Biophys. J. 37*, 256A.
Dorland, R. B., Middlebrook, J. L., & Leppla, S. H. (1979) *J. Biol. Chem. 254*, 11337–11342.
Dorland, R. B., Middlebrook, J. L., & Leppla, S. H. (1981) *Exp. Cell Res. 134*, 319–327.
FitzGerald, D., Morris, R. E., & Saelinger, C. B. (1980) *Cell (Cambridge, Mass.) 21*, 867–873.
Hoch, D. H., Romero-Mira, M., Erlich, B. E., Finkelstein, A., DasGupta, B. R., & Simpson, L. L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1962–1966.
Kagan, B. L., Finkelstein, A., & Colombini, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4950–4954.
Kagawa, Y., & Racker, E. (1971) *J. Biol. Chem. 246*, 5477–5487.
Kitamura, M., Iwamori, M., & Nagai, Y. (1980) *Biochim. Biophys. Acta 628*, 328–335.
Leppla, S., Dorland, R. B., & Middlebrook, J. L. (1980) *J. Biol. Chem. 255*, 2247–2250.
Middlebrook, J. L., Dorland, R. B., & Leppla, S. H. (1978) *J. Biol. Chem. 253*, 7325–7330.
Miller, C., & Racker, E. (1976) *J. Membr. Biol. 26*, 319–333.
Montal, M. (1974) *Methods Enzymol. 32b*, 545–554.
Neher, E., & Stevens, C. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 345–381.
Schantz, E. J., & Kautter, D. A. (1978) *J. Assoc. Off. Anal. Chem. 61*, 98–99.
Simpson, L. L. (1981) *Pharmacol. Rev. 33*, 155–188.
Simpson, L. L. (1984) *J. Pharmacol. Exp. Ther. 225*, 546–552.
Sugiyama, H. (1980) *Microbiol. Rev. 44*, 419–448.
Syuto, B., & Kubo, S. (1977) *Appl. Environ. Microbiol. 33*, 400–405.
Zalman, L. S., & Wisnieski, B. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3341–3345.

Anomalous Driving Force for Renal Brush Border H^+/OH^- Transport Characterized by Using 6-Carboxyfluorescein†

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ABSTRACT: The pH, ΔpH, and membrane potential dependences of H^+/OH^- permeability in renal brush border membrane vesicles (BBMV) were studied by using the entrapped pH indicator 6-carboxyfluorescein (6CF). Quantitative H^+/OH^- fluxes (J_H) were obtained from a calibration of the fluorescence response of 6CF to intravesicular pH using vesicles prepared with varying intravesicular and solution pHs. Intravesicular buffer capacity, determined by titration of lysed vesicles, increased monotonically from 140 to 260 mequiv/L in the pH range 5–8. J_H was measured by subjecting voltage-clamped BBMV (K^+/valinomycin) to preformed pH gradients over the pH range 5–8 and measuring the rate of change of intravesicular pH. For small preformed pH gradients (0.4 pH unit) J_H [6 nequiv s^{-1} (mg of protein)$^{-1}$] was nearly independent of pH (5–8), predicting a highly pH dependent H^+ permeability coefficient. J_H increased in a curvilinear manner from 6 to 104 nequiv s^{-1} (mg of protein)$^{-1}$ as ΔpH increased from 0.4 to 2.5. J_H increased linearly [1.6–7.3 nequiv s^{-1} (mg of protein)$^{-1}$] with induced K^+ diffusion potentials (21–83 mV) in the absence of a pH gradient. These findings cannot be explained by simple diffusion of H^+ or OH^- or by mobile carrier models. Two mechanisms are proposed, including a lipid diffusion mechanism, facilitated by binding of H^+/OH^- to fixed sites in the membrane, and a linear H_2O strand model, where dissociation of H_2O in the membrane fixes H^+ and OH^- concentrations in strands, which can result in net H^+/OH^- transport.

The passive transport of H^+/OH^- across biological membranes and phospholipid bilayers has been the subject of considerable interest. Passive H^+/OH^- transport is important biologically because it causes the dissipation of pH gradients required for normal cell function; it is of mechanistic interest because H^+ behaves anomalously when compared to the passive transport of other monovalent cations across membranes. The H^+ permeability of biological membranes and phospholipid bilayers is many orders of magnitude larger than would be predicted from the permeabilities of other monovalent cations. In addition, H^+ conductances measured in pure lipid bilayers do not vary linearly with applied voltage, suggesting that the mechanism for passive H^+/OH^- transport across membranes is not a simple diffusional process (Cafiso & Hubbell, 1983; Deamer & Barchfield, 1984; Gutknecht, 1984; Nichols & Deamer, 1980).

Fluorescence methods have been used to study H^+/OH^- fluxes in a wide variety of membranes. Many of these studies have been based on the partitioning of the fluorescent dye

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acridine orange across the brush border membrane in response to a pH gradient (Ives, 1985; Reenstra et al., 1981; Warnock et al., 1982). Since acridine orange can only be used to study the collapse of relatively large (>0.75 pH unit), outwardly directed H^+ gradients ($H^+_{in} > H^+_{out}$), it is not possible to study the symmetry of H^+ transport systems. Because acridine orange measurements are based on partitioning of dye across the membrane, the maximal rate of transport that can be measured is restricted by the rate of movement of acridine orange.[1] Entrapped fluorescein derivatives (including 6CF[2] and BCECF) do not have these limitations, although their use for H^+ flux measurement in vesicle systems is potentially limited by dye leakage and by relatively small signal intensity compared to acridine orange.

We describe here the use of 6CF to measure H^+/OH^- fluxes (J_H) in brush border membranes isolated from rabbit renal proximal tubule. One the basis of calibrations of 6CF fluorescence reponse to intravesicular pH and of vesicle buffer capacity, the dependence of passive H^+/OH^- transport rates on intravesicular pH, transmembrane pH gradients, and induced membrane potentials is examined. We find that the rate of H^+ transport for small pH gradients is almost independent of pH, similar to reported findings in lipid bilayers. Furthermore, H^+ flux does not appear to obey a transport law in which either the concentration ($\Delta[H^+]$ or $\Delta[OH^-]$) or the chemical potential (ΔpH) is the appropriate driving force.

Materials and Methods

Materials. Unless otherwise specified, all chemicals were of reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO). 6CF was obtained from Molecular Probes (Junction City, OR) and BCECF from Research Development Corp. (Toronto, Canada).

Vesicle Preparation. BBMV were prepared as previously described (Booth & Kenny, 1974). Briefly, the renal cortex was dissected from the kidneys of 1–2-kg female New Zealand white rabbits and homogenized at 4 °C in 50 mM sucrose, 50 mM Hepes/Tris, and 5 mM EGTA (pH 6) with a Sorvall Omni mixer. After exposure of the homogenate to 12 mM $MgCl_2$ for 30 min at 4 °C, brush border membranes were isolated by differential centrifugation. Purity of the preparation was examined by maltase and Na,K-ATPase activities, which exhibited enrichments of 15- and 0.3-fold over the crude homogenate, respectively. Vesicles were sometimes frozen and maintained for up to 1 month at −70 °C without loss of enzyme activity. The freezing process had no effect on H^+/OH^- fluxes. Protein concentration and maltase and Na,K-ATPase activities were assayed as described previously (Ives et al., 1983).

Buffer Capacity Measurement. BBMV buffer capacity was determined by the titration of lysed vesicles. BBMV (100 μL; 30 mg of protein/mL) were added to 10 mL of 0.1% Triton X-100 in deionized, doubly distilled water in a CO_2-free environment bubbled with 100% N_2. Titration was performed by using either HCl or NaOH. BBMV buffer capacity was calculated from the difference in milliequivalents of OH^- added to increase pH by 1 unit in parallel titrations preformed in the presence and absence of vesicle protein. Sequential acid and alkaline titrations revealed no hysteresis.

H^+/OH^- Transport Measurements. 6CF was introduced into BBMV by two methods. BBMV (20 mg of protein/mL) were incubated for 12 h with 100 μM 6CF or for 10 min with 10 μM 6-carboxyfluorescein diacetate (6CF-AM). The latter compound is more permeable than 6CF and is probably cleaved by intravesicular esterases to 6CF, which becomes trapped in the BBMV. Solution 6CF or 6CF-AM was removed by two washes in 50 volumes of buffer (250 mM sucrose, 50 mM Hepes/Tris, 150 mM potassium gluconate) not containing the dyes, followed by centrifugation at 300000*g* for 10 min. BBMV containing entrapped 6CF were maintained as a packed pellet at 0 °C until the time of the experiment (<4 h) in order to minimize dye leakage.

To determine the dependences of J_H on pH and ΔpH, the dissipation of preformed pH gradients (0.4–3 pH units) was studied as solution pH was varied from pH 5 to pH 8. Ten microliters of the 6CF-loaded vesicle suspension (40 mg of protein/mL containing 50 μg of valinomycin/mg of protein) was added to buffer at a preset pH, and base line 6CF fluorescence was determined by using an SLM 8225 fluorometer (Urbana, IL). Small volumes of acid (gluconic acid) or alkali (NMG) were then added to cause a specified pH change. The time course of fluorescence intensity (excitation at 486 nm, emission at 512 nm) consisted of a rapid change in fluorescence due to titration of extravesicular 6CF (<0.2-s instrument mixing time), followed by a slower fluorescence change in the same direction, due to collapse of the initial pH gradient.

For measurements of the dependence of J_H on induced membrane potential, 6CF-loaded BBMV containing valinomycin were stirred in a buffer containing 250 mM sucrose, 50 mM Hepes/Tris, and 5 mM potassium gluconate. 6CF fluorescence remained constant under these conditions. Small aliquots of 1 M potassium gluconate or *N*-methylglucamine–gluconate were added to induce a membrane potential, causing intravesicular alkalinization. J_H was determined from the linear fluorescence increase over the first 20 s following K^+ and NMG addition. Control experiments using the potential-sensitive dye 3,3′-dipropylthiodicarbocyanine iodide [diS-C_3-(5)] showed that there was no dissipation of the induced membrane potential at 20 s.

For some experiments the stopped-flow method was used. Equal volumes of vesicles loaded with 6CF were mixed rapidly (<1 ms) with buffers at a different pH in a Dionex-130 stopped-flow apparatus (Sunnyvale, CA). Fluorescence was excited at 485 nm by using a Leitz double monochromator and detected with a 415-nm high-pass filter. The time course of fluorescence intensity was recorded on a MINC/23 computer (Digital Equipment Corp., Maynard, MA). The instrument has a 2-ms dead time and maximum rate for data acquisition of 512 data points in 40 ms.

Calculation of J_H. J_H was calculated from initial rate of fluorescence change estimated from a single exponential fit to the 6CF fluorescence time course as in Figure 1, top. J_H in units of nequiv s^{-1} (mg of protein)$^{-1}$ is related to the exponential time constant τ, the 6CF fluorescence vs. pH calibration data [f_{CF}(pH), Figure 1, bottom], and the intravesicular buffer capacity [B(pH), Figure 2] by

$$J_H = B(pH_i)[f_{CF}(pH_i) - f_{CF}(pH_f)]/[\tau f_{CF}'(pH_i)] \quad (1)$$

where pH_i and pH_f are the initial and final pH values measured with a pH meter and $f_{CF}'(pH_i)$ is the derivative of

[1] Movement of acridine orange into brush border membranes occurs over a 0.25–0.5-s time course at 23 °C and an ~30-s time course at 4 °C. Movement out of the vesicles appears to be much faster. This rate of dye motion is adequate for studies of the endogenous H^+ permeability or Na^+/H^+ exchange at 25 °C. Under conditions where a pH gradient would collapse in times shorter than the time course of acridine orange uptake, another method is required.

[2] Abbreviations: BBMV, brush border membrane vesicle(s); 6CF, 6-carboxyfluorescein; BCECF, 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NMG, *N*-methylglucamine; Tris, tris(hydroxymethyl)aminomethane.

$f_{CF}(pH)$ with respect to pH evaluated at pH_i. For experiments involving the $\Delta\psi$ dependence of J_H, the normalized initial slope of 6CF fluorescence vs. time data $[F'(t)/F(0)]$ was converted to nequiv s^{-1} (mg of protein)$^{-1}$ units by

$$J_H = B(pH_i)[F'(t)/F(0)]f_{CF}(pH_i)R/f_{CF}'(pH_i) \quad (2)$$

where R is the fraction of 6CF present in the intravesicular space. R is measured from the ratio of the amplitude of the slower exponential process to the total signal as described below.

Results

Previous quantitative work on H^+/OH^- transport in BBMV has been done with acridine orange. Since acridine orange cannot be used to detect small or inwardly directed ($H^+_{in} > H^+_{out}$) pH gradients, it was necessary to develop a different method. BCECF-AM has has been used recently to measure intracellular pH in living cells (Moolenaar et al., 1983). We found however, that BCECF-AM cannot be used to measure pH in BBMV since it was cleaved entirely in the extravesicular space and unesterified BCECF does not measurably enter the vesicles. Therefore, experiments were carried out to examine the usefulness of the simpler compound, 6CF, in the measurements of H^+/OH^- fluxes in BBMV.

Dye Calibration. 6CF was trapped in BBMV by either of the two methods described under Materials and Methods. The use of of an acetoxymethyl derivative of 6CF was not necessary to obtain adequate intravesicular dye concentrations. To demonstrate H^+ transport across the BBMV membrane, solution pH was changed rapidly by addition of an impermeant acid or alkali (gluconic acid or *N*-methylglucamine). In a typical acid jump (Figure 1), a biphasic change in 6CF fluorescence was observed. An initial, rapid decrease in fluorescence (<1 s, labeled "out", Figure 1) was followed by a slower further decline (labeled "in", Figure 1). The initial rapid phase appeared to result from titration of extravesicular dye, while the slow phase results from H^+/OH^- transport across the membranes, resulting in titration of intravesicular dye. Three different experiments were performed to prove that this interpretation is correct.

First, vesicles were washed with buffer not containing 6CF repeatedly prior to assay by using experimental conditions given in the legend to Figure 1, top. The ratio of the amplitude of the slower fluorescence decline to the total signal amplitude (R) was 0.12, 0.21, and 0.22 with 1, 2, and 3 washes, respectively, consistent with removal of extravesicular dye with washing. Despite repeated washings, it was not possible to achieve total elimination of the extravesicular component of 6CF. This may be due to binding of 6CF to the outside membrane surface. Second, vesicles were diluted into the assay buffer (23 °C) for increasing times prior to addition of acid or base. The preincubation resulted in a decrease in R from 0.31, without preincubation, to 0.18, 0.12, 0.08, and 0.04 with incubation times of 10, 20, 30, and 60 min, respectively. Therefore, leakage of intravesicular 6CF into the extravesicular space occurs with a time constant of ~20 min at 23 °C. Lastly, the experiment shown in Figure 1 was repeated in a stopped-flow fluorescence apparatus; the time course of the rapid phase was less than 2 ms (not shown). If this rapid phase represented H^+ transport, it would be 10000 times more rapid than H^+ transport across BBMV as measured by acridine orange. Taken together, these three experiments provide strong evidence that the initial rapid change in 6CF fluorescence is due to titration of extravesicular dye and that the slower phase is due to H^+ transport across the membrane with titration of intravesicular dye as labeled in Figure 1.

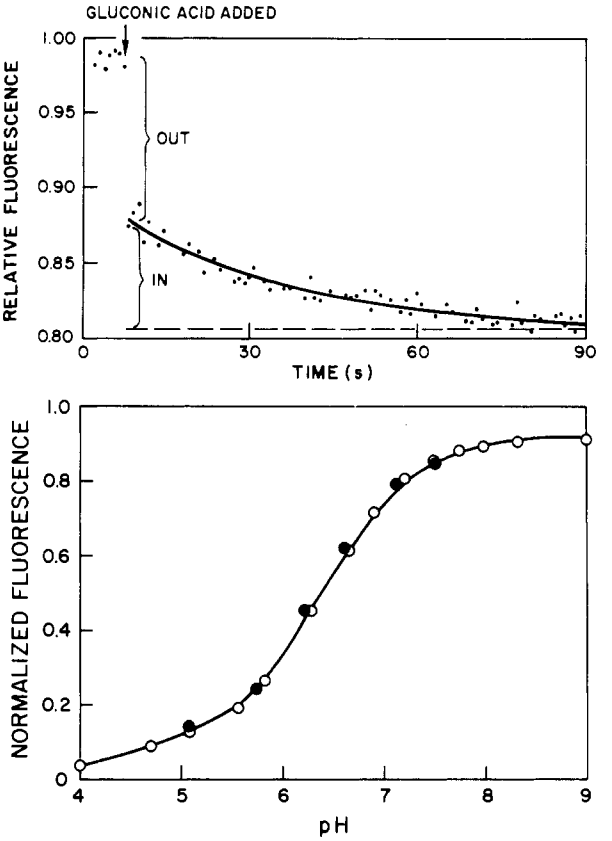


FIGURE 1: Calibration of the fluorescence response of 6CF. (Top) Gluconic acid was added to 6CF-loaded BBMV (0.2 mg of protein/mL) at pH 7.3 to change external pH rapidly to 6.9. The fast fluorescence decrease is due to titration of solution 6CF. The slower decrease, due to collapse of the 0.4-unit pH gradient, was fitted to an exponential with $\tau = 31$ s. (Bottom) Solution and intravesicular calibration curves for 6CF were constructed from the amplitudes of the fast (solution) and slow (intravesicular) fluorescence responses of 6CF as pH was decreased from 8 to 4.5 by serial additions of gluconic acid. Curves were normalized to give identical F values at pH 4.5 and 8. Normalized fluorescence $[f_{CF}(pH)]$ data were fit empirically to a cubic equation with $f_{CF}(pH) = 0.0018(pH)^3 - 0.00104(pH)^2 + 0.00018(pH) - (9.5 \times 10^{-6})$.

To calibrate the fluorescence response to intravesicular 6CF to pH, small aliquots of gluconic acid (sufficient to reduce the solution pH by ~0.5 pH units) were added to vesicles that were initially equilibrated at a pH between 5 and 8. After each addition of gluconic acid, the slow component of the fluorescence response was fitted to an exponential, and its amplitude was determined as in Figure 1. The amplitude of the rapid response was determined from the difference between the total amplitude and that of the slow phase. A titration curve for intravesicular and extravesicular dye was constructed from the measured fast and slow amplitudes at each pH (Figure 1, bottom). This titration revealed a pK of 6.5 for both intravesicular and extravesicular 6CF.

To demonstrate that 6CF could be used to study the collapse of outwardly directed pH gradients, vesicles at pH 5 were repeatedly alkalinized with small additions of *N*-methylglucamine. At each pH, the amplitudes were similar to those found when vesicles were titrated in the acid direction (data not shown).

In order to determine the H^+/OH^- flux (J_H) across BBMV membranes after addition of acid or alkali to the extravesicular medium, it is necessary to use the vesicle buffer capacity [B(pH)] to convert the pH changes measured by 6CF to J_H. Ives (1985) has reported previously the buffer capacity of brush border vesicles at pH 6 (135 mequiv/pH unit) and has

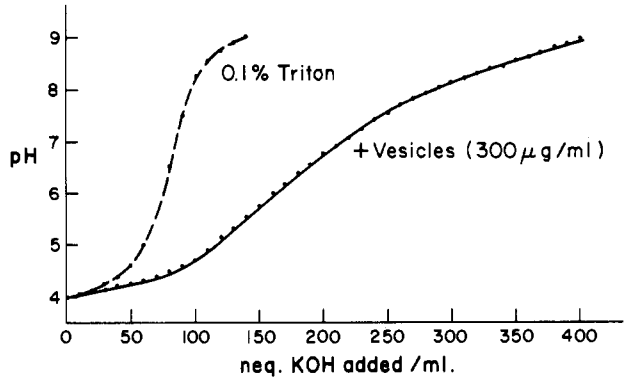


FIGURE 2: Titration of lysed vesicles with KOH. The pH response to additions of KOH to BBMV (0.3 mg of protein/mL) in 0.1% Triton X-100 and to 0.1% Triton X-100 alone is shown. BBMV buffer capacity, B(pH), was calculated as described in the text: $B(pH) = 20.8(pH)^2 - 231(pH) + 779$ nequiv of OH^- (pH unit)$^{-1}$ (mg of vesicle protein)$^{-1}$.

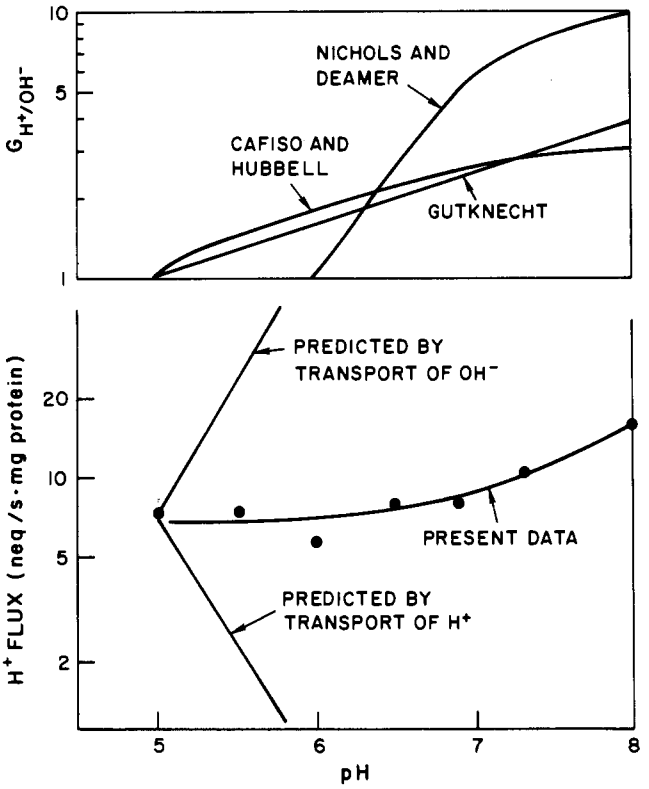


FIGURE 3: Dependence of the rate of H^+/OH^- transport on pH. (Top) Three reported dependences of H^+/OH^- conductance (G_{H^+/OH^-}) on pH are shown. Absolute conductances have been arbitrarily normalized. (Bottom) BBMV J_H is plotted against pH for 0.4-unit outwardly directed pH gradients. The predicted dependences of G_{H^+/OH^-} on pH are shown for diffusive transport of either H^+ or OH^- alone.

shown that titration of lysed vesicles is an adequate method to determine intravesicular buffer capacity in this system. BBMV were lysed and titrated in a CO_2-free environment as described under Materials and Methods (Figure 2). B(pH) was determined from the difference in nequiv of KOH added mL^{-1} (pH unit)$^{-1}$ between the curves obtained in the presence and absence of vesicles.

Dependence of H^+/OH^- Flux on pH. The rate of collapse of 0.4-unit pH gradients was measured as a function of solution pH (Figure 3, bottom). Surprisingly, J_H varied by less than a factor of 2 over the pH range 5–8, similar to the data of Cafiso and Hubell (1983) and Gutknecht (1984; Figure 3, top) obtained from electrical measurements in pure lipid bilayers.

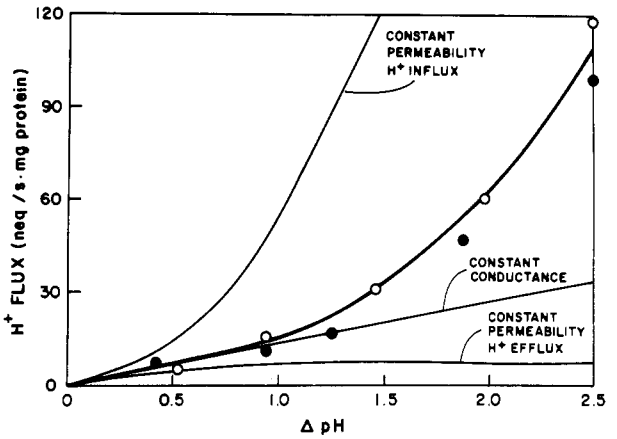


FIGURE 4: Dependence of the rate of H^+/OH^- transport on ΔpH. Open circles represent acid jumps with initial pH 7.5; closed circles represent alkaline jumps with initial pH 5.5. H^+ fluxes for alkaline jumps were each multiplied by a factor of 3 as discussed in the text. Data were fitted empirically to the cubic equation $J_H = 11.0(\Delta pH)^3 - 16.7(\Delta pH)^2 + 17.2(\Delta pH) + 1.3$. The predicted dependences of H^+ flux on ΔpH for constant H^+ permeability and constant H^+ conductance (see Results) are shown.

If the mechanism for pH equilibration is a simple diffusive pathway for H^+ or OH^-, the initial flux should either decrease significantly as pH is increased (if the transported species is H^+) or increase significantly with pH (if the transported species is OH^-). If both H^+ and OH^- are transported, then the flux vs. pH relation would be a composite, V-shaped curve. None of these simple diffusive models fits our data. These results indicate that the derived permeability coefficients, $P_H = J_H/\Delta[H^+]$ or $P_{OH} = J_H/\Delta[OH^-]$, are strongly pH dependent.

Dependence of H^+/OH^- Flux on ΔpH. In general, ion flux is a linear function of the driving force (ΔC) for diffusion. This relationship was tested directly for BBMV H^+/OH^- transport (Figure 4). BBMV were loaded with 6CF, and acid or alkaline jumps (ΔpH) were created by the addition of aliquots of gluconic acid and *N*-methylglucamine, respectively. For acid jumps (H^+ entry), J_H was not a linear function of $\Delta[H^+]$ or ΔpH but increased with ΔpH in a curvilinear manner. For a given ΔpH the rate of collapse of the pH gradient was 2–3 times smaller for alkaline jumps (H^+ exit) than for acid jumps. This difference appeared to be a property of the assay system[3] rather than a property of the H^+ conductance pathway. When this 2–3-fold difference was corrected for, the curves relating J_H and ΔpH for acid and alkaline pH jumps were superimposable (Figure 4). The curve shape was also independent of the initial intravesicular pH_i, as tested for pH_i 5.5 and 6.5 (acid jumps) and pH_i 6.5 (alkaline jump, data not shown). Also shown in Figure 4 are the predicted dependences of H^+ flux on ΔpH for several types of driving forces. The line labeled "constant conductance" shows a linear relation between H^+ flux and ΔpH with slope arbitrarily set equal to the slope of the data at low ΔpH. The curves labeled "constant permeability H^+ influx" and "constant permeability H^+ efflux" were

[3] Asymmetry of H^+ transport as measured by 6CF was not due to inadequate voltage clamping of the vesicles, since increasing $[K^+]$ from 0 to 500 mM increased J_H from 8.9 ± 0.5 to 14.2 ± 1.0 nequiv s^{-1} (mg of protein)$^{-1}$ for 1 pH unit acid jumps and from 3.0 ± 0.4 to 7.3 ± 0.7 nequiv s^{-1} (mg of protein)$^{-1}$ for 1 pH unit alkaline jumps. Both processes were saturated above 100 mM K^+. Increasing valinomycin from 50 to 200 μg/mg of vesicle protein had no effect on the transport asymmetry. When the protonophore CCCP (0–200 μg/mg of protein) was added to the vesicles, J_H increased from 14 ± 2 to 123 ± 10 nequiv s^{-1} (mg of protein)$^{-1}$ for 1 pH unit acid jumps and from 6 ± 1 to 51 ± 8 nequiv s^{-1} (mg of protein)$^{-1}$ for 1 pH unit alkaline jumps.

calculated from assumed linearity between H^+ flux and $[H^+_{out}] - [H^+_{in}]$ for constant $[H^+_{in}]$ with increasing $[H^+_{out}]$ (acid jumps) and for constant $[H^+_{in}]$ and decreasing $[H^+_{out}]$ (alkaline jumps), respectively. The data do not fit any of these models adequately.

Dependence of H^+/OH^- Flux on Membrane Potential. The dependence of the magnitude of H^+/OH^- flux induced by K^+/valinomycin diffusion potentials was determined in the absence of an initial pH gradient. Increase in solution K^+ induces an interior positive membrane potential which causes H^+ efflux and intravesicular alkalinization. BBMV containing 50 μg of valinomycin/mg of protein in buffer consisting of 250 mM sucrose, 50 mM Hepes/Tris, and 5 mM potassium gluconate, pH 7.0, were loaded with 6CF, washed, and suspended in the same buffer. A 250-μL aliquot of 1 M potassium gluconate plus 1 M NMG–gluconate was added to give final $[K^+_{out}]$ ranging between 5 and 125 mM. J_H, determined as described under Materials and Methods, varied linearly (ohmic response) with ψ over the range 21–83 mV with slope 0.093 ± 0.002 nequiv s^{-1} (mg of protein)$^{-1}$ mV^{-1} and intercept 0.006 ± 0.003 nequiv s^{-1} (mg of protein)$^{-1}$. In addition, the magnitude of J_H induced by a 59-mV potential is comparable to that induced by a 1-unit pH gradient (Figure 4), which would have an equal electrochemical driving force. Driving forces >80 mV, where non-ohmic responses have been observed in other systems (Benz & McLaughlin, 1983; Clark et al., 1983; Krishnamoorthy & Hinkle, 1984), could not be studied in the renal BBMV system because of inability to maintain and measure these large potentials.

Discussion

Previous work from numerous laboratories has indicated that H^+/OH^- diffusion in aqueous solution and passive transport of H^+/OH^- across membranes do not conform to patterns observed for other monovalent cations. Both the magnitude of H^+/OH^- fluxes and their response to changes in pH are anomalous. For example, the diffusion coefficient for H^+/OH^- in H_2O is 3 times higher than that for self-diffusion of H_2O; the permeability for H^+ in lipid bilayers is 6 orders of magnitude higher than the permeability for Na^+. Where it has been studied, these characteristics are also found in biological membranes. In renal BBMV the H^+ conductance is of the same order of magnitude as the K^+ conductance (Wright et al., 1984), predicting a much larger H^+ permeability coefficient because of the low H^+ concentrations at pH 6 or 7.

Since H^+/OH^- flux cannot be measured directly by radioactive tracer methods, workers have relied on various indirect methods (pH meters, electrical conductances, NMR, and fluorescence probes) to study these fluxes. We have focused attention on fluorescence methods to obtain quantitative H^+/OH^- fluxes in a model biological membrane system, the renal brush border membrane vesicle, in order to examine the dependence of H^+/OH^- fluxes on pH, ΔpH, and $\Delta\psi$.

In the classical determination of membrane permeabilities, it is assumed that permeation obeys Fick's law of diffusion. For simple diffusive transport of a cation X^+ across a membrane, the electrochemical potential predicts that

$$J_X = P_X(\psi F/RT)([X^+]_1 - [X^+]_2 e^{-\psi F/RT})/(1 - e^{-\psi F/RT}) \quad (3)$$

where J_X is transmembrane X^+ flux, P_X is the permeability coefficient for X, F is faraday's constant, R is the gas constant, T is absolute temperature, and the subscripts 1 and 2 refer to different sides of the membrane. When $\psi = 0$, eq 3 reduces to

$$J_X = P_X[(X_1] - [X_2]) \quad (4)$$

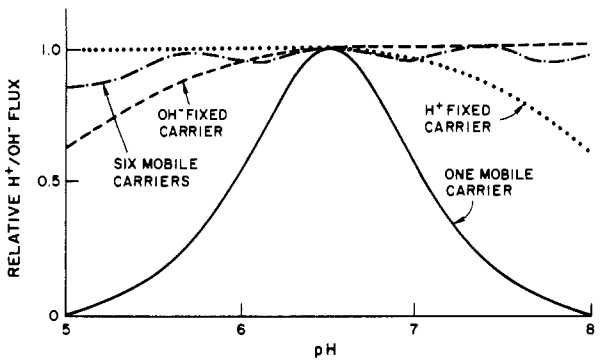
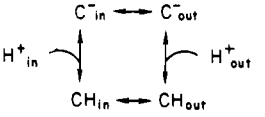


FIGURE 5: H^+/OH^- flux predicted by carrier models. The dependences of relative H^+/OH^- flux on pH are calculated for a single mobile carrier model (k_c'/k_c = 1000, pK = 5) and a multiple mobile carrier model (k_c'/k_c = 1000, pK's of 4, 5, 6, 7, 8, and 9) by using eq 5. The curve drawn for the fixed H^+ carrier model is calculated from eq 7 with pK = 8.5. The curve drawn for the fixed OH^- carrier model was drawn from the model complementary to that given in eq 7 with pK = 5. The maximum relative H^+/OH^- flux for each model has been normalized to unity.

where J_X is proportional to the product of the concentration difference across the membrane and a constant permeability coefficient. These concepts are valid for many ions and nonelectrolytes. However, eq 3 appears to break down in the case of H^+/OH^- transport across membranes.

In response to a small pH gradient, J_H changes little over a 3-unit range in solution pH (Figure 3, bottom), quite similar to observations made in lipid bilayers, where H^+/OH^- conductance is weakly dependent on pH (Figure 3, top). Since $\Delta[H^+]$ or $\Delta[OH^-]$ changes by 3 orders of magnitude under these conditions, a 3 order of magnitude change in J_H is predicted by eq 3. In order to explain this unusual behavior, we have examined a number of "carrier" models for passive H^+/OH^- transport (Figure 5). If H^+ transport is facilitated by a membrane-bound mobile carrier, the carrier could bind H^+ on one side of the membrane, translocate it, and then return across the membrane in the unbound state.



(5)

CH is bound carrier and C^- is unbound carrier present at the inner or outer membrane surface. In the limit of small $\Delta[H^+]$, J_H(pH) for such a mobile carrier is[4]

$$J_H(\text{pH}) = \frac{C_t k_c'(10^{-pK})\Delta[H^+]}{2[10^{-2pK} + k_c'(10^{-2pH})/k_c]} \quad (6)$$

where k_c and k_c' are translocation rates for the bound and unbound carriers, respectively, C_t is total carrier concentration, and the carrier pK = –log K is assumed to be identical on both sides of the membrane.

As shown in Figure 5 (curve labeled "one mobile carrier"), this model predicts that J_H is very sensitive to pH. This is the case if the unprotonated form is translocated much more rapidly than the unprotonated form or if the reverse is true. In fact, the relationship between transport and pH shown is

[4] The carrier model in eq 4 is defined by equilibrium constant $K = [H^+_{in}][C^-_{in}]/[CH_{in}] = [H^+_{out}][C^-_{out}]/[CH_{out}]$ and unimolecular rate constants k_c and k_c', which describe the rate of movement of bound and unbound carriers. Conservation of total carriers requires that $C_t = [C^-_{in}] + [C^-_{out}] + [CH_{in}] + [CH_{out}]$. The H^+ flux, J_H, is equal to the net rate of translocation of the loaded carrier, $J_H = k_c'([CH_{out}] - [CH_{in}])$. Equation 5 follows from these relations in the limit $[H^+_{in}] - [H^+_{out}]$ approaches zero.

valid even if protonated and unprotonated forms are translocated at equal rates. Therefore, this model cannot explain the observations.

The mobile carrier model could be modified to give an almost constant relationship between J_H and pH if several carriers with heterogeneous pK's exist in the membrane. Figure 5 shows that if six carriers are chosen with a range of pK's surrounding the pH range of interest, a relatively flat curve can be obtained. This model could explain the data, but it seems unlikely to be operative in pure lipid bilayers where Gutknecht (1984) finds little dependence of H^+/OH^- conductance on pH over the range 1.6–10.5. A mobile carrier model would require multiple carriers with at least a 9-unit range in pK. Such a pK heterogeneity is not likely to be found in a pure phospholipid bilayer.

A third model for passive H^+ transport in membranes is a fixed H^+/OH^- binding site in the membrane that is capable of simultaneously sampling both sides of the membrane. If this site is operating near its saturation point for H^+/OH^-, the H^+ conductance would be nearly independent of pH. Net flux of H^+ would occur because of a difference in binding rates to the carrier from the two sides of the membrane. If S^- is an immobile binding site that can bind H^+ from either side of the membrane, then the model becomes quite simple:

$$S^- \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} SH \qquad (7)$$

H^+_{in} or H^+_{out}

where k_1 and k_{-1} are forward and reverse rate constants for binding with equilibrium constant $K_1 = k_{-1}/k_1$. The total site stoichiometry, S_t, is given by $S_t = [S^-] + [SH]$. J_H is calculated from the difference in binding rates to both sites of the membrane, $J_H = (k_1/2)[S^-]([H^+_{out}] - [H^+_{in}])$. Since unbinding occurs randomly from either side of the membrane, the factor of $^1/_2$ has been included. In the limit of a small pH gradient ($\Delta[H^+]/[H^+] \ll 1$)

$$J_H(pH) = \frac{k_1 S_t \Delta[H^+]}{2(K_1 + 10^{-pH})} \qquad (8)$$

As shown in Figure 5, this fixed carrier model predicts a fairly constant H^+/OH^- transport rate over a very wide range in pH. Although we have no independent evidence that this model accounts for H^+ transport in biological or phospholipid membranes, it is consistent with the data in Figue 3 over the pH range examined.

Several other mechanisms of H^+/OH^- transport in lipid bilayers have been proposed to explain the anomalous relationship between H^+/OH^- flux and driving force. It has been suggested that protons traverse a lipid bilayer by passage along strands of hydrogen-bonded water. If the rate of formation of the strands or the rate of contact of short strands were rate-limiting compared with H^+/OH^- translocation rates, then H^+/OH^- flux might be relatively independent of pH. We have shown previously (Ives & Verkman, 1985) that membrane fluidizing agents, which increase brush border H^+/OH^- permeability 5-fold, have no effect on osmotic water permeability. This finding suggests that the pathways for water and H^+/OH^- flows are independent or at least that the enhanced rate of proton movement along strands is independent of the predominant pathway for osmotic water transport.

It has been proposed that protons may traverse a membrane by movement into and out of hydrated defects within the membrane. This model is unlikely since the permeabilities of non-H^+ ions are many orders or magnitude less than that for H^+/OH^-. In addition, a hydrated defect model would predict a strong dependence of H^+/OH^- flux on pH, contrary to the data in Figure 3.

Our data also demonstrate an unexpected dependence of J_H on ΔpH. If transport occurred by simple diffusion of H^+, then J_H should be a linear function of $\Delta[H^+]$, as predicted by eq 4. $\Delta[H^+]$ would be much greater for pH gradients from 5 to 8 compared with those from 6 to 8, 7 to 8, etc. However, $\Delta[H^+]$ would increase little as the pH gradient was changed from 5–6 to 5–7 or 5–8. Our data show a unique relationship between H^+ flux and ΔpH, but not with $\Delta[H^+]$. The relationship applies for both inwardly and outwardly directed pH gradients and is independent of the initial vesicle pH. Therefore, the H^+/OH^- permeability coefficient ($P_H = J_H/\Delta[H^+]$) is not a constant.

The dependence of H^+/OH^- transport rates on ΔpH has been examined in mitochondrial and asolectin liposomes by using pH- and K^+-sensitive electrodes to follow the time course of H^+/OH^- transport (Krishnamoorthy & Hinkle, 1984). H^+/OH^- flux was linear with ΔpH for alkaline pH jumps from pH 5 to pH 8. In the brush border membrane, we find that H^+/OH^- flux is markedly nonlinear with ΔpH at 1.5 pH units. Either dependence is not consistent with mobile carrier models or fixed carrier models. The presence of multiple fixed binding sites in series, with varying pK's, could explain the complex dependence in brush border membranes. The fixed sites may reside on transmembrane proteins in the brush border membrane.

In some biological, liposome, and planar lipid membranes, the endogenous H^+ transport pathway exhibits non-ohmic current–voltage relationships for $\Delta\psi > 50$–200 mV (Benz & McLaughlin, 1983; Clark et al., 1983; Krishnamoorthy & Hinkle, 1984). In these systems H^+ conductance increases with increased transmembrane potential, consistent with a trapazoidal electrical potential barrier within the membrane. In the renal BBMV, we find an ohmic dependence of J_H on $\Delta\psi$ in the range 21–83 mV. The data are consistent with a simple H^+ conductance in which J_H induced by a 1-unit pH gradient at 0 mV is approximately equal to that induced by a 59-mV potential.

The nonideal dependences of BBMV J_H on pH and ΔpH indicate that the mechanism of H^+/OH^- transport in these membranes is very different from that of other monovalent cations. The lack of dependence of J_H on pH for small induced pH gradients can be interpreted by fixed carrier models or models in which H^+ is carried along transient formations of linear H_2O strands. On the basis of the data presented, it appears that ΔpH, rather than $\Delta[H^+]$ or $\Delta[OH^-]$, is the most appropriate driving force for H^+/OH^- transport across BBMV. An examination of the differential form of eq 3 is helpful to give further insight into the mechanism of H^+/OH^- transport:

$$d[H^+] = P_{H^+}[H^+][d\ (\ln [H^+]) + (F/RT)d\psi] \qquad (9)$$

$$d[OH^-] = P_{OH^-}[OH^-][d\ (\ln [OH^-]) - (F/RT)d\psi] \qquad (10)$$

where $[H^+]$ and $[OH^-]$ are average concentrations of H^+ and OH^- in the membrane and P_{H^+} and P_{OH^-} are constant permeability coefficients. The net H^+/OH^- flux is equal to $(d[H^+] - d[OH^-])/dt$.

Equations 9 and 10 predict little dependence of J_H on pH for a constant pH gradient if the average concentrations of H^+ and OH^- in the membrane were relatively independent of the external pH. This is possible for the linear H_2O strand model where dissociation of H_2O in the membrane is not directly coupled to external pH. Under these conditions, eq 9 and 10 predict that J_H is linearly related to ΔpH and to $\Delta\psi$. Such a model would fit our data extremely well for gradients

up to 1 pH unit and to 83 mV. The deviations of the data from the model at high gradients may be due to nonlinear potential barriers in the membrane or to differences in the pK for H_2O dissociation in the membrane at the membrane interfaces.

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REFERENCES

Benz, R., & McLaughlin, S. (1983) *Biophys. J. 41*, 381–398.
Booth, A. G., & Kenny, A. J. (1974) *Biochem. J. 142*, 575–581.
Cafiso, D. S., & Hubbell, W. L. (1983) *Biophys. J. 44*, 49–57.
Clark, A. J., Cotton, M. P. J., & Jackson, J. B. (1983) *Biochim. Biophys. Acta 723*, 440–453.
Deamer, D. W., & Barchfield, G. L. (1984) in *Hydrogen Ion Transport in Epithelia* (Forte, J. G., Warnock, D. G., & Rector, F. C., Eds.) pp 13–19, Wiley, New York.
Gutknecht, J. (1984) *J. Membr. Biol. 82*, 105–112.
Ives, H. E. (1985) *Am. J. Physiol. 248*, F76–F86.
Ives, H. E., & Verkman, A. S. (1985) *Am. J. Physiol. 249*, F933–F940.
Ives, H. E., Yee, V. J., & Warnock, D. G. (1983) *J. Biol. Chem. 258*, 13513–13516.
Krishnamoorthy, G., & Hinkel, P. C. (1984) *Biochemistry 23*, 1640–1645.
Moolenaar, W. H., Tsien, R. Y., der Saag, V., & de Laat, S. W. (1983) *Nature (London) 304*, 645–648.
Nichols, J. W., & Deamer, D. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2038–2042.
Reenstra, W. W., Warnock, D. G., Yee, V. J., & Forte, J. G. (1981) *J. Biol. Chem. 256*, 11663–11666.
Rossignol, M., Thomas, P., & Grignon, C. (1982) *Biochim. Biophys. Acta 684*, 195–199.
Warnock, D. G., Reenstra, W. W., & Yee, V. J. (1982) *Am. J. Physiol. 242*, F733–F739.
Wright, E. M., Schell, R. E., & Gunther, R. D. (1984) in *Hydrogen Ion Transport in Epithelia* (Forte, J. G., Warnock, D. G., & Rector, F. C., Eds.) pp 21–33, Wiley, New York.

Modification by Diacylglycerol of the Structure and Interaction of Various Phospholipid Bilayer Membranes†

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ABSTRACT: The effects of incorporating diacylglycerol (DG) derived from egg phosphatidylcholine (PC) into PC, egg phosphatidylethanolamine (PE), and bovine phosphatidylserine (PS) have been measured. In excess solution DG induces a multilamellar-to-hexagonal (L–H) structural transition in PE and PC that is temperature dependent. At 37 °C it begins at about 3 and 30 mol %, respectively. In PC at lower DG concentrations a modified lamellar phase is formed; at about 70 mol % DG a single primitive cubic phase forms. An L–H transition induced by 20–30 mol % DG in PS is dependent on ionic strength and degree of lipid hydration, with the appearance of crystalline acyl chains at the higher DG levels. Calcium precipitates of DG/PS (1/1) mixtures have melted chains. Structural parameters were derived for the lamellar phases at subtransition levels of DG in PE and PC. The area per polar group is increased, but by contrast with cholesterol, the polar group spreading is not accompanied by an increase in bilayer thickness. DG does not affect the equilibrium separation of PC or PE bilayers. Measured interbilayer forces as they vary with bilayer separation show that DG at 20 mol % does not effect closer apposition of PC bilayers at any separation. Spreading the polar groups may effect the binding of protein kinase C or the activation of phospholipases; the nonlamellar phases may be linked to the biochemical production of DG in cellular processes involving membrane fusion.

Rapid turnover of a membrane phospholipid, phosphatidylinositol, and its phosphorylated forms is known to occur in a large variety of cells in response to various extracellular stimuli (Hawthorne & Pickard, 1979; Michell, 1975; Nishizuka, 1983; Berridge, 1984; Berridge & Irvine, 1984). Diacylglycerol and inositol trisphosphate produced as a result of this turnover act as second messengers and trigger various cellular responses. The phosphoinositides are soluble in the cytosol and are likely responsible for many cellular responses by mobilization of calcium from intracellular stores (Berridge, 1984). The extremely nonpolar molecule diacylglycerol acts at the membrane level (Nishizuka, 1984). Diacylglycerol has been implicated in several cellular processes, for example, calcium-independent activation of cell secretion and shape changes in platelets (Hokin & Hokin, 1953; Rink et al., 1983), vesiculation and shape changes in erythrocytes (Allan & Michell, 1975; Allan et al., 1978), exocytosis in adrenal medulla chromaffin granules (Knight et al., 1982), intracellular pH changes (Moolenaar et al., 1984), fusion of myoblasts

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