Diffusion of Ionizable Solutes Across Planar Lipid Bilayer Membranes: Boundary-Layer pH Gradients and the Effect of Buffers

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The diffusion of weak acids or bases across planar lipid bilayer membranes results in aqueous boundary layer pH gradients. If not properly taken into account, such pH gradients will lead to errors in estimated membrane permeability coefficients, P_m. The role of the permeant concentration, the buffer capacity, and the physicochemical properties of both permeant and buffer on the magnitude and impact of such pH gradients have been explored. A theoretical model has been developed to describe the diffusion of both permeant and buffer species. Significant pH gradients develop depending on solution pH and the pK_a's, concentrations, and P_m values of both permeant and buffer. The relative error in experimentally determined P_m values was calculated as the ratio, r, between apparent P_m values (obtained from flux measurements using an equation which neglected boundary layer pH gradients) and its true value. Simulated r values ranged from 1 (0% error) to <0.01 (>100% error) for weak acids, decreasing with decreasing buffer capacity and increasing solute flux. The buffer capacity required for an $r > 0.95$ was calculated versus pH for permeants varying in pK_a and P_m. Membrane-permeable buffers significantly reduce boundary layer pH gradients through a feedback effect due to buffer cotransport. Apparent P_m values of *p*-hydroxymethyl benzoic acid across lecithin bilayer membranes at 25°C were obtained as a function of permeant concentration in various buffers [glycolic, 2-(*N*-morpholino)ethanesulfonic, and formic acids]. Predictions agreed closely with experimental fluxes.

KEY WORDS: lipid bilayer membranes; bilayer permeability; unstirred boundary layers; pH effects; buffer effects.

INTRODUCTION

Fundamental knowledge of the basal permeability of various species across biological membranes can often be obtained in simpler model systems such as isolated lipid bilayers or related systems (1–3). Recent studies in these laboratories have been directed toward understanding the chemical nature of the rate determining barrier microenvironment within lipid bilayer membranes (4,5). One method of probing the polarity or hydrogen-bonding nature of the barrier region is by determining the contributions of various functional groups to permeant transport across lipid bilayer membranes. A novel approach for obtaining functional group contributions was recently described (4) which utilizes ionizable permeants coupled with pH adjustment to identify for each permeant a pH "window" in which transport is membrane, rather than unstirred water layer controlled.

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Even in well-stirred diffusion cells, aqueous boundary layers contribute an additional resistance and may become rate-limiting if the membrane transport of permeant is rapid (6). Boundary layer effects may also result in significant underestimates of the membrane permeability coefficients of weak acids and bases due to pH gradients in the unstirred layers arising from the preferential transport of either the conjugate acid or base across the lipid bilayer. Gutknecht and Tosteson (7) and Walter *et al.* (8) have analyzed these effects for weak acids undergoing transport across lipid bilayers. In the absence of boundary layer pH gradients, one can apply the following relationship:

$$\frac{1}{J_{HA}} = \frac{1}{P_m^{HA}[HA]} + \frac{2d}{D_{HA}[HA] + D_A[A^-]} \quad (1)$$

to determine the membrane permeability coefficient, P_m^{HA}, of a weak acid, HA, from the observed flux J_{HA} and the bulk solution concentrations of HA and A^- at a given bulk solution pH, assuming that only the neutral species, HA, crosses the membrane. The right-hand term in Eq. (1) accounts for the boundary layer resistances, where D_{HA} and D_A are the aqueous diffusion coefficients of the neutral and anionic species and d represents the thickness of the unstirred layers, assumed to be the same on either side of the membrane. Equation (1) does not account for the fact that rapid permeation of HA across the membrane may induce a proton ion concentration gradient within the unstirred layers depending on the buffer capacity of the bulk solution and the magnitude of the flux, J_{HA}. Neglecting pH gradients in the unstirred layers has been found to lead to significant errors in published permeability coefficients (8).

This study is aimed at examining, both theoretically and experimentally, the role of permeant concentration and buffer capacity and the physicochemical properties of both permeant and buffer on boundary layer pH gradients. A primary objective is to identify conditions under which Eq. (1) fails to provide a quantitatively accurate description of the relationship between permeant flux and bulk solution pH and to estimate the relative errors which may result from using Eq. (1). A theoretical model for the transport of ionizable solutes across planar lipid bilayer membranes is developed. The concentration profiles of the permeant and related species in the unstirred layers are generated numerically under various conditions. The model calculations are then compared with experimental data for the permeability of *p*-hydroxymethyl benzoic acid (HMBA) across planar lipid bilayer membranes made from egg lecithin in decane.

THEORY

In this section we develop a theoretical model to describe the diffusion of ionizable weak acid permeants across a planar lipid bilayer membrane in the presence and absence of buffers which may also be membrane permeable. Scheme I illustrates the diffusion processes in a typical transport experiment. The diffusion cell consists of two stirred compartments between which a bilayer membrane resides at position $z = 0$. The unstirred layers have a thickness of d, which can be obtained either by fitting the fluxes of a weak

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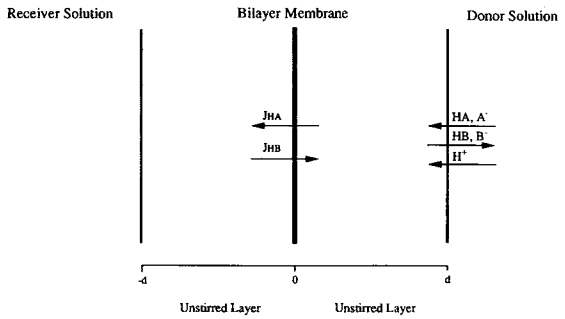

Scheme I

acid or base observed at different pH values using Eq. (1) or by measuring the flux of a lipophilic nonelectrolyte (e.g., butanol) which is sufficiently permeable to be unstirred layer controlled in its transport. Apart from a small amount of permeant in the donor solution, the solutions in both compartments are identical in chemical composition.

In transport experiments conducted in the authors' laboratories, the thickness of the unstirred aqueous layers ($\approx$50–100 μm) is much smaller than the membrane diameter ($\approx$1 mm). Therefore, the concentration profiles in any plane parallel to the bilayer normal can be assumed to be uniform and diffusion can be treated as a one-dimensional problem. Fick's law for the diffusion of each solution component within the unstirred layers can be written as illustrated below for HA:

$$\frac{\partial[\mathrm{HA}]}{\partial t} = D_{\mathrm{HA}} \frac{\partial^2[\mathrm{HA}]}{\partial z^2} + \eta_{\mathrm{HA}} \tag{2}$$

where t is time, z is distance from the bilayer surface, and D_{HA} is the aqueous diffusion coefficient of HA. η_{HA} is a source function representing chemical reactions between interacting species. In a solution containing a weak acid permeant, a single monoprotic buffer, and added salt, there are eight components which should be considered (HA, A^-, HB, B^-, H^+, OH^-, Cl^-, and Na^+) and therefore eight such equations.

Assuming that the fluxes of all species are at steady state (i.e., time independent), the derivatives of all species concentrations at any point within the unstirred layer with respect to time are equal to zero. Mass balance considerations require that any change in the flux of HA or HB in the unstirred layers due to chemical reaction reflects corresponding changes in flux of their conjugate bases, A^- and B^-. Thus,

$$D_{\mathrm{HA}} \frac{\partial^2[\mathrm{HA}]}{\partial z^2} + D_{\mathrm{A}} \frac{\partial^2[\mathrm{A}^-]}{\partial z^2} = 0 \tag{3}$$

$$D_{\mathrm{HB}} \frac{\partial^2[\mathrm{HB}]}{\partial z^2} + D_{\mathrm{B}} \frac{\partial^2[\mathrm{B}^-]}{\partial z^2} = 0 \tag{4}$$

Integrating both (3) and (4) and substituting the boundary conditions of the net fluxes of HA and HB at $z = 0$ (the bilayer membrane/water interface), J_{HA} and J_{HB}, respectively, into the integrated equations, one has

$$D_{\mathrm{HA}} \frac{\partial[\mathrm{HA}]}{\partial z} + D_{\mathrm{A}} \frac{\partial[\mathrm{A}^-]}{\partial z} = J_{\mathrm{HA}} \tag{5}$$

$$D_{\mathrm{HB}} \frac{\partial[\mathrm{HB}]}{\partial z} + D_{\mathrm{B}} \frac{\partial[\mathrm{B}^-]}{\partial z} = J_{\mathrm{HB}} \tag{6}$$

The condition of charge neutrality requires that

$$D_{\mathrm{Cl}} \frac{\partial[\mathrm{Cl}^-]}{\partial z} + D_{\mathrm{OH}} \frac{\partial[\mathrm{OH}^-]}{\partial z} + D_{\mathrm{A}} \frac{\partial[\mathrm{A}^-]}{\partial z} + D_{\mathrm{B}} \frac{\partial[\mathrm{B}^-]}{\partial z} = D_{\mathrm{H}} \frac{\partial[\mathrm{H}^+]}{\partial z} + D_{\mathrm{Na}} \frac{\partial[\mathrm{Na}^+]}{\partial z} \tag{7}$$

The membrane permeability coefficients of Na^+ and Cl^- as determined from the ionic transference number and the membrane conductance are of the order of $(0.1-10) \times 10^{-10}$ cm/sec (9,10). Thus, it is appropriate for the purposes of this study to assume that these ionic species are impermeable through lipid bilayer membranes. The potential contribution of HCl, which has a bilayer membrane permeability coefficient several orders of magnitude higher than those of H^+, OH^-, or Cl^- (11), but is present at extremely low concentrations, having a pK_a of -6 to -7 (12,13), has also been ignored. This leads to zero fluxes of Na^+ and Cl^- at any given position within the unstirred layers. As a result, the only effect of NaCl on the transport process is to change the activity coefficients of the relevant ionic species in the solution.

Integrating Eqs. (5)–(7) over z to $z \pm d$ yields

$$J_{\mathrm{HA}}(z \mp d) = D_{\mathrm{HA}}\{[\mathrm{HA}]_z + [\mathrm{HA}]_{\pm d}\} + D_{\mathrm{A}}\{[\mathrm{A}^-]_z + [\mathrm{A}^-]_{\pm d}\} \tag{8}$$

$$J_{\mathrm{HB}}(z \mp d) = D_{\mathrm{HB}}\{[\mathrm{HB}]_z + [\mathrm{HB}]_{\pm d}\} + D_{\mathrm{B}}\{[\mathrm{B}^-]_z + [\mathrm{B}^-]_{\pm d}\} \tag{9}$$

$$D_{\mathrm{H}}\{[\mathrm{H}^+]_z + [\mathrm{H}^+]_{\pm d}\} = D_{\mathrm{A}}\{[\mathrm{A}^-]_z + [\mathrm{A}^-]_{\pm d}\} + D_{\mathrm{B}}\{[\mathrm{B}^-]_z + [\mathrm{B}^-]_{\pm d}\} + D_{\mathrm{OH}}\{[\mathrm{OH}^-]_z + [\mathrm{OH}^-]_{\pm d}\} \tag{10}$$

where the upper signs are used for the concentration distributions in the donor side unstirred layer and the lower signs in the receiver side unstirred layer.

Assuming that all reacting species are in chemical equilibrium throughout the unstirred layers, the above equations [(8)–(10)], along with equations for the ionization of HA, HB, and H_2O expressed in terms of the ionization constants K_{A} and K_{B}, and the ion product of water, K_{W}, constitute six conditional equations from which the six unknowns (i.e., the concentrations of HA, A^-, HB, B^-, H^+, and OH^- at position z) can be uniquely determined once the bulk concentrations and the fluxes J_{HA} and J_{HB} across the bilayer membrane are known.

Solving for $[\mathrm{HA}]_z$ and $[\mathrm{HB}]_z$, we obtain

$$[\mathrm{HA}]_z = \frac{J_{\mathrm{HA}}(z \mp d) + \{D_{\mathrm{HA}} + D_{\mathrm{A}}K_{\mathrm{A}}/[\mathrm{H}^+]_{\pm d}\}[\mathrm{HA}]_{\pm d}}{D_{\mathrm{HA}} + D_{\mathrm{A}}K_{\mathrm{A}}/[\mathrm{H}^+]_z} \tag{11}$$

$$[\mathrm{HB}]_z = \frac{J_{\mathrm{HB}}(z \mp d) + \{D_{\mathrm{HB}} + D_{\mathrm{B}}K_{\mathrm{B}}/[\mathrm{H}^+]_{\pm d}\}[\mathrm{HB}]_{\pm d}}{D_{\mathrm{HB}} + D_{\mathrm{B}}K_{\mathrm{B}}/[\mathrm{H}^+]_z} \tag{12}$$

Solving for H^+ by making all final substitutions into Eq. (10) yields the following implicit equation for $[\mathrm{H}^+]_z$:

$$[H^+]_z^4 + P[H^+]_z^3 + Q[H^+]_z^2 + R[H^+]_z + S = 0 \quad (13)$$

where

$$P = K_B D_B/D_{HB} + K_A D_A/D_{HA} - C/D_H \quad (14)$$

$$Q = \frac{D_A D_B K_A K_B}{D_{HA} D_{HA}} - \frac{1}{D_H}\{(A + C)K_A D_A/D_{HA} + (B + C)K_B D_B/D_{HB} + K_W D_{OH}\} \quad (15)$$

$$R = -\frac{1}{D_H}\left(\frac{K_A K_B D_A D_B (A + B + 1)}{D_{HA} D_{HB}} + K_W D_{OH}(K_A D_A/D_{HA} + K_B D_B/D_{HB})\right) \quad (16)$$

$$S = \frac{K_W D_{OH} K_A K_B D_A D_B}{D_H D_{HA} D_{HB}} \quad (17)$$

and

$$A = J_{HA}(z \mp d) + \{D_{HA} + D_A K_A/[HA^+]_{\pm d}\}[HA]_{\pm d} \quad (18)$$

$$B = J_{HB}(z \mp d) + \{D_{HB} + D_B K_B/[H^+]_{\pm d}\}[HB]_{\pm d} \quad (19)$$

$$C = \frac{D_H[H^+]_{\pm d}^2 - K_A D_A[HA]_{\pm d} - K_B D_B[HB]_{\pm d} - K_W D_{OH}}{[H^+]_{\pm d}} \quad (20)$$

Equations (14)–(20) were further simplified by assuming that the diffusion coefficients for the neutral and ionic species are equal (i.e., $D_{HA} = D_A$ and $D_{HB} = D_B$). It can readily be shown that, in the limiting case, where the buffer concentration in the bulk solution is infinitely large, Eq. (1) is recovered from the above equations.

The correct root among the four possible roots in Eq. (13) was found numerically by the Newton–Raphson method. $[OH^-]_z$, $[HA]_z$, $[A^-]_z$, $[HB]_z$, and $[B^-]_z$ can be determined once $[H^+]_z$ is calculated from Eq. (13).

MATERIALS AND METHODS

Materials

Egg lecithin in chloroform (20 mg/mL), obtained from Avanti Polar-Lipids, Inc. (Pelham, AL), was dried under nitrogen gas and dispersed (2%, w/v) in decane (99+%; Sigma Chemical Co., St. Louis, MO) which had been passed through an alumina column before use. *p*-Hydroxymethyl benzoic acid (HMBA) (Sigma), formic acid (≈99%; Sigma), glycolic acid (99%; Aldrich Chemical Co., Milwaukee, WI), and 2-(4-morpholino)ethanesulfonic acid (MES) (>98%, Eastman Kodak Co., Rochester, NY) were used without further purification.

Apparent Permeability Coefficient Determinations

Lipid membranes were made by applying a lipid solution (2%, w/v, egg lecithin in decane) across a 1-mm-diameter hole in a Teflon sheet separating two water-jacketed chambers (14). The temperature in both chambers was maintained at 25.0 ± 0.05°C by a combined refrigerated water bath/circulator (Haake A81, Haake Inc., NJ) and flow system (Masterfex, Cole-Parmer Instruments Co., Chicago, IL). Both chambers contained ≈4.5 mL aqueous buffer, which was stirred continuously with magnetic fleas during the course of the experiment. The ionic strength was held at $I = 0.1$ with NaCl. The pH in both chambers was monitored with a microcombination pH probe (MI-412, Microelectrodes, Inc., Londonderry, NH) and was found to be stable (±0.01) throughout each experimental run.

The thinning of the lipid membrane into a bilayer of ≈50 Å in thickness was monitored by capacitance measurements as described previously (4). After the lipid bilayer was formed and a constant capacitance across the membrane was attained, a small amount (≈50 μL) of solution containing permeant at a known concentration was injected into the donor solution and a corresponding volume of buffer was added to the receiver solution. Samples were withdrawn from both chambers at various time intervals. The samples from the donor solution were appropriately diluted to bring the solute concentrations within a linear absorbance range and samples taken from both chambers were then analyzed by HPLC as described in a later section.

Apparent permeability coefficients were calculated from the slopes of plots of receiver HA concentration versus time interval Δt according to the following equation

$$P_{app} = \frac{J_{HA}}{C_{donor}} = \text{slope } x \frac{V_{cell}}{S_m C_{donor}} \quad (21)$$

where P_{app} is the apparent permeability coefficient and V_{cell} is the volume of the aqueous solution in each chamber. Point-by-point areas, S_m, were monitored continuously during the experiments either microscopically or by the capacitance method described previously (4). Apparent permeability coefficients were calculated using the average S_m over each time interval.

Aqueous Diffusion Coefficient Determinations

The aqueous diffusion coefficient for HMBA was determined using an open-ended capillary method (15). The capillaries used were about 0.6 mm in internal diameter and 5 cm in length (l). At the start of an experiment four capillaries were filled with a solution of known solute concentration. They were then immersed vertically into a reservoir (≈1 L) containing blank solvent. The reservoir was placed in a large circulating bath at a temperature of 25 ± 0.05°C. After diffusion had taken place for a certain length of time (t), the capillaries were withdrawn from the reservoir and the solution in each capillary was removed with a 10-μL syringe, the capillaries were rinsed several times, and the pooled aliquots were diluted to 1 mL with water for HPLC analysis. The diffusion time was selected such that $D_s t/l^2 > 0.2$. Under these conditions, a simple relationship exists between D_s and C_t,

$$D_s = \frac{4l^2}{\pi^2 t} \ln\left[\left(\frac{8}{\pi^2}\right)\left(\frac{C_o}{C_t}\right)\right] \quad (22)$$

where C_o is the initial concentration of the solute in the capillaries.

HPLC Analyses

An HPLC system (4) employing a reversed-phase column packed with 5-μm Spheri-5 RP-18 (Brownlee OD-MP,

4.6-mm ID × 10-cm L; Rainin) was used at ambient temperature for the analyses of HMBA in samples taken from the transport and diffusion experiments. The mobile phase contained acetonitrile (15%) in deionized water and was buffered to a pH of 3.0 using 0.02 *M* phosphate buffer. Peak heights were linear with respect to the concentration of known standards over a concentration range which included the concentrations of the most dilute samples analyzed. Coefficients of variation on repeated injections of standards were ≤3% at all concentrations.

RESULTS AND DISCUSSION

Physicochemical Properties of Permeants and Buffers Used in Flux Predictions

Shown in Table I are values for various physicochemical parameters used in model calculations or in experimental tests of the theoretical predictions. The lipid bilayer membrane permeability of the model permeant HMBA was calculated from its previously published apparent permeability–pH profile (4) according to Eq. (1). HMBA's apparent permeability coefficient is pH independent at low pH, reflecting aqueous boundary layer controlled transport. With increasing pH, the fraction of neutral species decreases and the transport of HMBA becomes membrane controlled, establishing that it is the neutral species which accounts for bilayer flux. P_m ($=1.6 \times 10^{-3}$ cm/sec) was determined from the apparent permeability coefficients in the pH-dependent region, while the pK_a of HMBA (4.18 ± 0.01) was determined by titration (4). The aqueous diffusion coefficient for HMBA (Table I) was determined by the open-ended capillary method as described in this paper.

Buffers chosen for model calculations or experimental studies varied in pK_a and in their membrane permeabilities. Glycolic acid (pK_a = 3.83), MES (pK_a = 6.20), and HEPES (pK_a = 7.59) were assumed to have negligible bilayer membrane permeability coefficients due to their highly polar or zwitterionic nature. Formic acid (pK_a = 3.75) is quite similar to glycolic acid in its pK_a but exhibits a much higher membrane permeability due to its small molecular size and higher lipophilicity. Buffer aqueous diffusion coefficients listed in Table I were taken from the literature or, in the case of MES and HEPES, estimated from the modified Wilke-Chang correlation (16), $D_w = 1.11 \times 10^{-7} M_w^{0.5} T/\eta_w V^{0.6}$ cm²/sec, where M_w and η_w are the molecular weight and viscosity (cp) of the solvent water, T is the absolute temperature, and V is the molar volume (cm³/mol) of solute. The molar volumes for MES and HEPES were estimated by the atomic incremental method (17). Literature values for the aqueous diffusion coefficients and lipid bilayer membrane permeability coefficients for H^+ and OH^- are also shown.

Table I. Lipid Bilayer Membrane Permeability Coefficients (P_m), Aqueous Diffusion Coefficients (D_w), and Ionization Constants (K_a) in Water at 25°C for Various Agents Used in Flux Determinations

Compound	P_m (cm/sec)	D_w (cm₂/sec)	K_a
p-Hydroxymethyl benzoic acid	1.6×10^{-3}[a] ($\pm 0.4 \times 10^{-3}$)	7.2×10^{-6}[b] ($\pm 0.2 \times 10^{-6}$)	6.31×10^{-5}[a]
Formic acid	7.3×10^{-3}[c]	1.52×10^{-5}[d]	1.78×10^{-4}[e]
Glycolic acid	—	9.8×10^{-6}[f]	1.48×10^{-4}[e]
MES	—	9.0×10^{-6}[g]	6.24×10^{-7}[e]
HEPES	—	7.9×10^{-6}[g]	2.57×10^{-8}[e]
H^+	$<10^{-8}$[h]	9.31×10^{-5}[i]	—
OH^-	$<10^{-8}$[h]	5.30×10^{-5}[i]	—

[a] From our previous study, Ref. 4.
[b] From the present work using the open-ended capillary method.
[c] From Ref. 19.
[d] From Ref. 20.
[e] From Ref. 18.
[f] From Ref. 21.
[g] Estimated using the modified Wilke–Chang relationship (16).
[h] From Ref. 11.
[i] From Refs. 22 and 23.

Boundary Layer pH Gradients and Potential Errors in P_m Estimated from Flux Measurements of Weak Acid Permeants with and Without Added Buffers

Numerous studies have shown that the transport of weak acids through lipid bilayer membranes may be accounted for by the membrane permeability of the nonionized species (i.e., the membrane permeability of the anionic species is negligible) (4,7,8). The membrane permeability coefficient P_m^{HA} is determined from the observed net flux J_{HA} for HA and the concentration difference of HA at both interfaces, $[HA]_{-0}$ and $[HA]_{+0}$,

$$P_m^{HA} = \frac{J_{HA}}{[HA]_{+0} - [HA]_{-0}} \tag{23}$$

If the hydrogen ion concentration remains constant throughout the unstirred layers, the HA concentration gradients in the unstirred layers are linear and can be expressed analytically by

$$[HA]_z = [HA]_{\pm d} \mp \frac{dJ_{HA}}{D_{HA} + D_A K_A/[H^+]_{\pm d}} \tag{24}$$

As pointed out by Walter *et al.* (8), however, the transfer of HA across the membrane causes a depletion of H^+ in the unstirred layer on the donor side of the membrane and an enrichment on the receiver side, which causes the pH in the interfacial region to increase on the donor side, thus suppressing $[HA]_{+0}$ and to decrease on the receiver side, which increases $[HA]_{-0}$. In cases where the hydrogen ion concentration varies within the unstirred layers due to insufficient solution buffer capacity, the actual concentration gradients of HA, A^-, and other relevant species become nonlinear and can be determined only by numerically integrating the appropriate diffusion equations over the unstirred layer thickness as described under Theory. As shown in the simulations that follow, boundary layer pH gradients may result in significant underestimates of membrane permeability coefficients.

The unstirred aqueous layer thickness in the diffusion apparatus employed has been previously determined to be 170 (±8) μm (4). Unstirred layer thicknesses on either side of the membrane were therefore assumed to be 85 μm for these simulations.

Shown in Fig. 1 is the calculated H^+ concentration profile across the unstirred layers in a simulated diffusion ex-

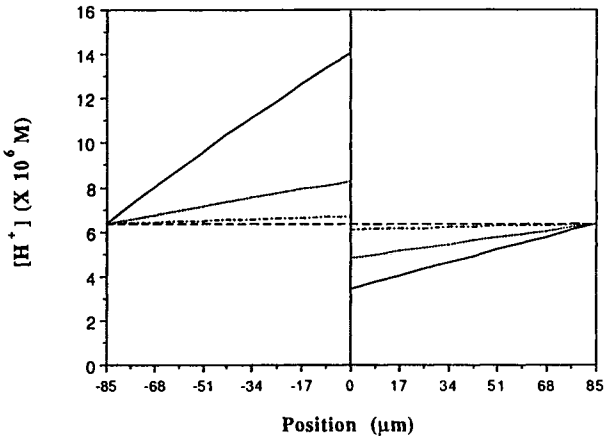

Fig. 1. Theoretical hydrogen ion concentration profiles within the unstirred aqueous layers in the absence of buffer (——), in a 0.01 *M* glycolate buffer (· · ·), in a 0.01 *M* formate buffer (- · -), and in a buffer having infinite buffer capacity (---). The permeant concentrations in the donor and receiver solutions were assumed to be 0.002 and 0 *M*. respectively, and a solution pH of 5.2 was assumed.

periment conducted at an HMBA donor concentration of 0.002 *M* and a pH of 5.2 (I = 0.1) in the absence of buffer, in 0.01 *M* glycolate or formate buffers, and in a buffer having infinite buffer capacity. Because egg lecithin is zwitterionic over a wide pH range, its barrier properties are pH independent over the pH range 2–9 (4). Thus, the predominant effect of the significant pH gradients developing as the buffer capacity of the bulk solution decreases is the suppression of $[HA]_{+0}$–$[HA]_{-0}$ which is accompanied by a reduction in permeant flux, as illustrated in Fig. 2.

The error in the estimated membrane permeability coefficient calculated from flux measurements when pH gradients exist can be estimated from the ratio, r, which is the ratio between the permeability coefficient obtained by applying Eq. (1) to the experimentally determined flux of HA and the true permeability coefficient.

Shown in Table II are r values for a wide range of weak

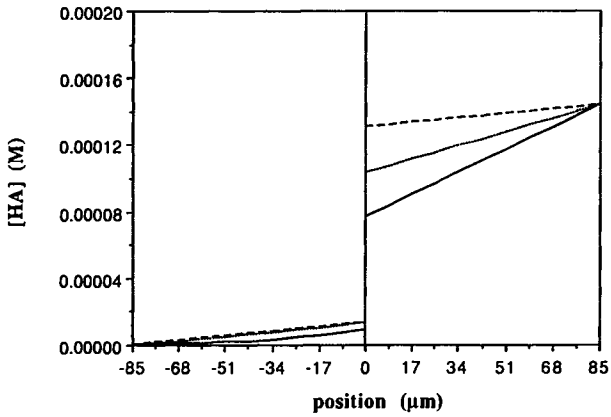

Fig. 2. Theoretical concentration gradients of the neutral form of the permeant within the unstirred aqueous layers in the absence of buffer (——), in a 0.01 *M* glycolate buffer (· · ·), and in a buffer having infinite buffer capacity (---). The permeant concentrations in the donor and receiver solutions were assumed to be 0.002 and 0 *M*, respectively, and a solution pH of 5.2 was assumed.

acid permeants (0.002 *M*; pK_a = 4.2) varying in membrane permeability coefficient as a function of pH in the absence of buffer and in the presence of 0.01 *M* glycolic acid, MES, and HEPES, respectively. A wide range of membrane permeability coefficients would be expected in molecules with different lipophilicity due to various functional group substitutions. Consistent with this expectation and with the P_m values chosen in Table II, a recent study in the authors' laboratories on lipid bilayer transport of *p*-toluic acid and six α-substituted derivatives obtained permeability coefficients, ranging from 1.1 cm/sec for *p*-toluic acid to 4.1×10^{-5} cm/sec for the α-amido-substituted permeant (5).

Several trends are evident in Table II. Most significant is the finding that r values ranged from 1.0, reflecting 0% error, to <0.01, reflecting >100% error in the experimentally determined membrane permeability coefficients, showing the important influence of pH gradients in the unstirred layers. At the permeant concentration assumed (0.002 *M*), errors due to pH gradients are negligible over the entire pH range when P_m is less than 10^{-4} cm/sec, but at any given pH the error increases (r decreases) as P_m increases. The presence of a buffer at a concentration of 0.01 *M* greatly diminishes the error (increases r) but in some cases the need for either higher buffer capacities or lower permeant concentration is evident. As expected, the efficiency of a given buffer is highest near its pK_a, where its buffer capacity is highest. Thus, glycolic acid (pK_a = 3.83) is the most effective in increasing r values near a pH of 4, MES (pK_a = 6.20) is most effective at ≈pH 6, and HEPES (pK_a = 7.59) is most effective between pH 7 and pH 8. For a given permeant, the r values vary with solution pH in a complex manner, exhibiting minima at intermediate pH values. This is attributed mainly to two competing factors. At lower pH values, the permeant (HA) exists largely in its neutral form and ionization reactions in the unstirred layers are less significant. At higher pH values, the fraction of permeant in its neutral form becomes very small, and so the overall permeant flux decreases until reactions in the unstirred layers have little effect on unstirred layer pH gradients. In both extremes, deviation from the predictions of Eq. (1) are diminished.

The dramatic effects of buffers described in Table II are consistent with experimental observations. Walter *et al.* (8) have shown that the net flux of butyric acid at pH 7.2 decreases by 15-fold (from 5.6×10^{-10} to 3.7×10^{-11} mol/cm²/sec) when a 0.05 *M* HEPES buffer is replaced with water on the receiver side in a lipid bilayer transport study. Our model calculations, which assumed a membrane permeability coefficient of 0.095 cm/s, an aqueous diffusion coefficient for butyric acid of 8.67×10^{-6} cm²/sec, and an unstirred layer thickness of 206 μm, as reported by Walter *et al.* (8) predicted a decrease of 14-fold under the same conditions (from 5.2×10^{-10} to 3.7×10^{-11} mol/cm²/sec), remarkably close to that observed.

Experimental Approaches to Minimize Errors Due to Unstirred Layer pH Gradients

For a given weak acid or weak base permeant, errors in the estimated membrane permeability coefficient due to the development of pH gradients in the aqueous boundary layers may be minimized by appropriate selection of (a) permeant

Table II. Values of r for Weak Acid Permeants[a] Varying in Permeability Coefficient (P_m) as a Function of pH in Unbuffered and Buffered Solutions

Buffer	P_m (cm/sec)	Solution pH					
		3	4	5	6	7	8
No buffer	1	—[b]	—	0.0014	0.0014	0.0048	0.15
	10^{-1}	—	—	0.014	0.014	0.040	0.34
	10^{-2}	—	—	0.13	0.13	0.21	0.77
	10^{-3}	—	0.86	0.61	0.59	0.65	0.97
	10^{-4}	1.0	0.99	0.95	0.94	0.95	1.0
	10^{-5}	1.0	1.0	0.99	0.99	0.99	1.0
Glycolic acid (0.01 *M*)	1	—	—	0.0044	0.0039	0.0087	0.078
	10^{-1}	—	—	0.042	0.038	0.063	0.38
	10^{-2}	—	—	0.31	0.28	0.32	0.78
	10^{-3}	—	—	0.82	0.80	0.81	0.97
	10^{-4}	1.0	1.0	0.98	0.98	0.98	1.0
	10^{-5}	1.0	1.0	1.0	1.0	1.0	1.0
MES (0.01 *M*)	1	—	—	—	—	0.31	0.37
	10^{-1}	—	—	—	—	0.81	0.85
	10^{-2}	—	—	0.37	0.92	0.98	0.98
	10^{-3}	—	0.87	0.86	0.99	1.0	1.0
	10^{-4}	1.0	0.99	0.98	1.0	1.0	1.0
	10^{-5}	1.0	1.0	1.0	1.0	1.0	1.0
HEPES (0.01 *M*)	1	—	0.0059	0.0018	0.014	0.43	0.87
	10^{-1}	—	0.056	0.017	0.13	0.88	0.99
	10^{-2}	—	0.37	0.15	0.63	0.99	1.0
	10^{-3}	1.0	0.86	0.64	0.94	1.0	1.0
	10^{-4}	1.0	0.99	0.95	0.99	1.0	1.0
	10^{-5}	1.0	1.0	1.0	1.0	1.0	1.0

[a] Calculations assume a permeant concentration of 0.002 *M* and permeant pK_a of 4.2.
[b] P_{app} is within 50% of its unstirred layer controlled value.

concentration; (b) pH, (c) buffer pK_a and concentration (i.e., buffer capacity), and (d) buffer permeability characteristics. Being the result of chemical reactions, the pH gradients which are established in the unstirred layers during transport of weak acids or bases are amplified with increasing permeant concentration in the donor compartment. Thus, the experimental permeability coefficient decreases with increasing permeant concentration, resulting in larger errors in determinations of P_m using Eq. (1) at high permeant concentrations. This is illustrated in Fig. 3 (middle curve), where the apparent permeability coefficient for HMBA in a glycolic acid buffered system ([HB] = 0.004 *M*, pH 5.20), is plotted versus the donor concentration of HA. The curve represents the theoretical prediction using a model which accounts for pH gradients within the unstirred layers and parameters independently determined from separate experiments.

As suggested by the sensitivity of r values (Table II) to the permeant pK_a and P_m values, pH, and buffer pK_a, errors in the estimation of P_m at high permeant concentrations can be minimized by appropriate selection of pH and buffer. As illustrated in Fig. 3 (lower curve), P_{app} is nearly independent of permeant concentration (i.e., $r \approx 1$) when the flux of HMBA is determined at a pH of 6.15 using an MES buffer (0.01 *M*) having a pK_a of 6.2. At this pH the flux of HMBA is low ($\sim 1.5 \times 10^{-11}$ mol/cm^2 · sec) and the buffer capacity of the 0.01 *M* MES buffer is near its maximum value, thus minimizing the effects of increasing permeant concentration.

For a given experiment, knowledge of the minimum buffer capacity required, β_{min}, to achieve an r value greater than 0.95 (i.e., less than 5% error in the experimental permeability measurement due to pH gradients across the bilayer membrane) would be quite helpful. Buffer capacity is defined as

$$\beta = 2.303K_a'C_{buffer}[H^+]/(K_a' + [H^+])^2 \qquad (25)$$

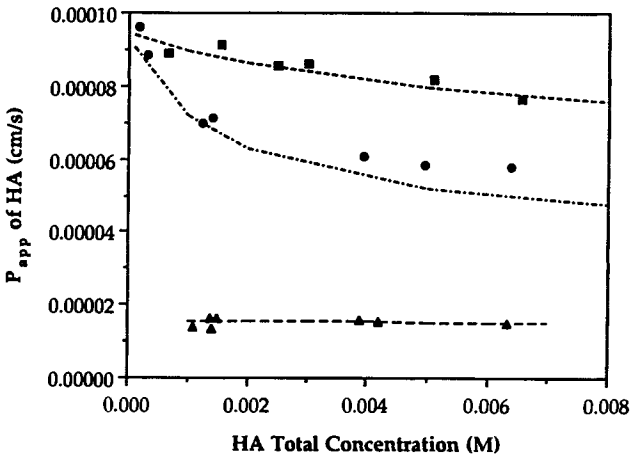

Fig. 3. Experimental apparent permeability coefficients of *p*-hydroxymethyl benzoic acid (HA) at 25°C as a function of the permeant concentration in the donor solution. (●) pH 5.2 with 0.004 *M* glycolic acid buffer; (■) pH 5.2 with 0.004 *M* formic acid buffer; (▲) pH 6.15 with 0.01 M MES buffer. Curves represent predictions from the diffusion model derived under Theory.

where K'_a is the buffer ionization constant corrected for ionic strength (18). Estimates of β_{min} for permeants varying in P_m and pK_a were obtained from simulations in which buffer concentration (pK_a fixed) was varied until an r value of 0.95 was obtained. This concentration (C_{buffer}) was substituted into Eq. (6) to obtain the corresponding β_{min}. Values of β_{min} obtained in this manner are listed in Table III. These estimates assumed a buffer aqueous diffusion coefficient of 9.8×10^{-6} cm^2/sec. Several trends are apparent in Table III. First, at a given solution pH, β_{min} decreases with decreasing permeant permeability coefficient because reduced permeant flux results in smaller pH gradients within the unstirred layers. Second, for a given permeant, β_{min} varies with pH, reaching a maximum near the permeant pK_a due to two principal factors: (a) the net flux is relatively high in this region because the fraction of permeant in unionized form is significant, and (b) a significant fraction of the total HA crossing the bilayer membrane dissociates within the unstirred layer when bulk solution pH is at or above the permeant pK_a.

Boundary-layer pH gradients developed during the transport of a weak acid or base across a lipid bilayer membrane are accompanied by concentration gradients of the buffer species, HB and B^-, within the unstirred layers. Even though the buffer concentrations are identical in both the donor and the receiver solutions, gradients in HB concentration across the lipid bilayer will result in the diffusion of buffer across the bilayer if the membrane permeability coefficient of the neutral buffer species, HB, is not negligible.

The transport of HMBA in formic acid buffers was compared to that in glycolic acid buffers to explore the effects of buffer cotransport. While the pK_a of formic acid (3.75) is very close to that of glycolic acid (3.83), the membrane permeability coefficient of glycolic acid is much smaller than that of formic acid (see Table I) due to its smaller molecular size and higher lipophilicity in comparison to glycolic acid (19). Buffer cotransport is in the opposite direction to that of the permeant, and since the buffer concentration used in a typical transport experiment is greater than the permeant concentration, a buffer flux comparable in magnitude to that of the permeant is possible, substantially reducing the proton concentration gradients within the unstirred layers (Fig. 1) and partially restoring the concentration gradient of the neutral form of the permeant toward that attained at infinite buffer capacity—a feedback effect resembling the one found in operation amplifier electric circuitry.

This feedback effect decreases the relative error resulting from using Eq. (1) to estimate the permeability coefficient for HA. The extent of the effect, however, depends on the concentrations of both permeant and buffer and the buffer's membrane permeability coefficient. The advantage afforded by a membrane permeable buffer in lipid bilayer transport experiments is illustrated in Fig. 3 (upper curve), which shows that the deviation of P_{app} for HMBA from its limiting value of one as a function of the permeant concentration is reduced when a membrane permeable formate buffer is used. Again, both the experimental results and the

Table III. Buffer Capacity Required to Achieve an $r > 0.95$ for Permeants Varying in Permeability Coefficient (P_m) and pK_a as a Function of Solution pH Using Either a Membrane-Permeable or an Impermeable Buffer[a]

Permeant			Solution pH				
pK_a	P_m (cm/sec)	Buffer	4.0	5.0	6.0	7.0	8.0
3.00	1	Glycolate	—[b]	—	—	7.6×10^{-2}	7.5×10^{-4}
	10^{-1}		—	—	7.5×10^{-3}	7.6×10^{-4}	6.3×10^{-5}
	10^{-2}		—	7.1×10^{-3}	7.3×10^{-4}	7.2×10^{-5}	0.0
	10^{-3}		3.2×10^{-3}	4.7×10^{-4}	2.3×10^{-5}	3.6×10^{-6}	0.0
	10^{-4}		0.0	0.0	0.0	0.0	0.0
4.00	1	Glycolate	—	—	—	—	9.3×10^{-3}
	10^{-1}		—	—	—	9.3×10^{-3}	9.1×10^{-4}
	10^{-2}		—	—	8.9×10^{-3}	9.1×10^{-4}	7.9×10^{-5}
	10^{-3}		—	6.0×10^{-3}	8.3×10^{-4}	8.5×10^{-5}	0.0
	10^{-4}		0.0	6.3×10^{-5}	1.9×10^{-5}	0.0	0.0
	1	Formate	—	—	—	—	6.2×10^{-4}
	10^{-1}		—	—	—	6.4×10^{-4}	6.3×10^{-5}
	10^{-2}		—	—	6.4×10^{-4}	6.3×10^{-5}	5.0×10^{-6}
	10^{-3}		—	5.1×10^{-4}	5.1×10^{-5}	5.0×10^{-6}	0.0
	10^{-4}		0.0	2.8×10^{-6}	7.7×10^{-7}	0.0	0.0
5.00	1	Glycolate	—	—	—	—	—
	10^{-1}		—	—	—	—	7.6×10^{-3}
	10^{-2}		—	—	—	7.2×10^{-3}	7.4×10^{-4}
	10^{-3}		—	8.7×10^{-3}	5.4×10^{-3}	7.0×10^{-4}	5.8×10^{-5}
	10^{-4}		0.0	0.0	2.3×10^{-4}	3.0×10^{-5}	0.0
6.00	1	Glycolate	—	—	—	—	—
	10^{-1}		—	—	—	—	—
	10^{-2}		—	—	—	—	7.3×10^{-3}
	10^{-3}		—	—	9.0×10^{-3}	5.4×10^{-3}	6.9×10^{-4}
	10^{-4}		0.0	0.0	1.3×10^{-4}	2.4×10^{-4}	2.2×10^{-5}

[a] Calculations assume a permeant aqueous diffusion coefficient of 8.5×10^{-6} cm^2/sec and a concentration of 0.002 M.
[b] P_{app} is within 50% of its unstirred layer controlled value.

model predictions are plotted and agree reasonably well with one another. A comparison with data generated in glycolic acid buffer under identical conditions (middle curve) shows that the membrane permeable formate buffer reduced the error in permeability coefficient at high permeant concentrations by a factor of >2.

The buffer capacity required to achieve an $r > 0.95$ for a given weak acid permeant is substantially reduced when the buffer is membrane permeable. This is illustrated in Table III for weak acid permeants with a pK_a of 4.0 having various membrane permeability coefficients and assuming the buffer to be formic acid.

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