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Characterization of CO_2/carbonic acid mediated proton flux through phosphatidylcholine vesicles as model membranes

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The apparent proton permeability coefficient for phospholipid vesicles measured in our laboratory (Norris, F.A. and Powell, G.L. (1990) Biochim. Biophys. Acta 1030, 165–171) for proton flux initiated by rapidly lowering of the external pH (acid jump) was a linear function of the reciprocal internal proton concentration. This behavior was ascribed to the presence of the weak acid carriers, carbonic acid/CO_2/bicarbonate. In the present work, a theoretical description, appropriate for proton transport by any weak acid carrier, has been developed which lends itself to novel graphical treatment permitting the separate estimation of the permeability coefficients for protons, hydroxide ions and bicarbonate. The proton permeability coefficient determined by this method was $1.8 \cdot 10^{-5}$ (S.E. $1.3 \cdot 10^{-5}$) cm/s; that for hydroxide ion was $3.8 \cdot 10^{-5}$ (S.E. $5.6 \cdot 10^{-6}$) cm/s and a lower limit for the permeability of bicarbonate ion, $4.3 \cdot 10^{-6}$ (S.E. $3.6 \cdot 10^{-7}$) cm/s, can be set. The presence of negative surface charge on the lipid bilayer increased the observed proton permeability coefficient in accordance with Gouy-Chapman theory. The charge was introduced by preparing vesicles containing increasing amounts of negatively charged dioleoylphosphatidylglycerol. The observed proton permeability coefficient increased and the observed permeability coefficients for hydroxide ion and bicarbonate decreased. The addition of the lipophilic cations, valinomycin-K^+ and tetrabutylammonium ion increased the slope of P vs. $1/[H_i^+]$. These changes are analogous to those reported for the permeant weak acid uncouplers FCCP and CCCP. These studies demonstrated that CO_2/carbonic acid was an effective carrier of protons across phospholipid model membranes.

Introduction

Proton gradients across biological membranes are an essential component of the energetics of living systems [1] driving such cellular processes as ATP synthesis [2,3], the active transport of metabolites [4] and flagellar motion [5,6]. In order for efficient coupling of proton gradients to bioenergetic processes to occur, the biological membrane must provide an effective barrier to the passive proton diffusion. However, it has been shown that the passive diffusion rate of proton/hydroxide ions across model phospholipid membranes is about six orders of magnitude faster than that of other monovalent cations such as sodium and potassium ions [7–12]. This difference in rate suggests a unique mechanism for the translocation of protons/hydroxide ions across phospholipid bilayers.

Two possible mechanisms have been proposed. Transiently formed chains of hydrogen bonded water molecules, referred to as 'water wires', may span the bilayer allowing protons to be translocated [7]. Alternatively, a carrier mechanism utilizing weak acids, c.f. fatty acids, may facilitate proton translocation by the diffusion of the protonated species across the bilayer followed by deprotonation [13].

Our laboratory has shown that the weak acid couple, CO_2/carbonic acid, can greatly enhance the dissipation of proton gradients across phospholipid liposomes [14]. Large unilamellar DOPC vesicles made by extrusion [15] were used as a the model membrane system for the study the kinetics of proton diffusion across phospholipid bilayers. The fluorescent pH probe, pyranine, was trapped inside the vesicles which allowed the internal proton concentration to be measured as a function of time after the external proton concentration was rapidly altered. The K^+ ionophore, valinomycin, was also present to abolish the electrical potential resulting from proton flux.

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If the net flux of protons, J, through a membrane were simple diffusion, the flux would be directly proportional to the proton gradient, G, across the membrane. The permeability, P, would be the proportionality constant: $J = P \cdot G$ (see Eqn. 1 below) However, we have observed that the permeability of vesicles perturbed by the external addition of acid (acid jump), was not constant but was a linear function of the reciprocal of the internal proton concentration (Ref. 14 and Fig. 1).

In the present work, a new theoretical description of the kinetics of CO_2/carbonic acid mediated proton flux is presented. The phenomenological equation is tested experimentally and by computer simulation. The theory provides graphical methods for estimating the permeability coefficients for protons, for hydroxide ions, and for bicarbonate. The rate-limiting step for proton transport facilitated by CO_2/carbonic acid is assumed to be the diffusion of the anionic form of the weak acid, A^-, through the membrane [16]. A negative surface potential can reduce the ability of weak acid uncouplers of oxidative phosphorylation to transport protons across model membranes while lipophilic cations such as the valinomycin-K^+ complex can enhance weak acid mediated proton transport [17] by ion pairing between the lipophilic cation and the anionic form of the weak acid facilitating the diffusion of A^- across the bilayer. These effects support the proposed mechanism and are appropriately described by the phenomenological equation.

Methods and Materials

Chemicals. L-α-Dioleoylphosphatidylcholine (DOPC) and L-α-dioleoylphosphatidylglycerol (DOPG) were a gift of Avanti Polar Lipids, Alabaster, AL. Pyranine (8-hydroxy-1,3,6-pyrene trisulfonate) (laser grade) was purchased from Eastman Kodak. Valinomycin, nigericin, p-nitrophenyl acetate and bovine erythrocyte carbonic anhydrase were obtained from Sigma, St. Louis, MO. Tetrabutylammonium hydroxide was purchased from Aldrich. Hepes (*N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid) was purchased from Research Organics, Cleveland, OH. Potassium sulfate and sodium bicarbonate (reagent grade) were obtained from J.T. Baker. EDTA was purchased from Matheson, Coleman, and Bell, Norwood, OH.

Preparation of vesicles. DOPC vesicles were prepared by extrusion (Mayer et al., [15]) in a buffer containing 25 mM Hepes, 50 mM K_2SO_4, 1 mM EDTA and 2 mM pyranine using a procedure described earlier [14]. Vesicle preparations were diluted to 0.5 mM phosphate and 1 μg of valinomycin was added per mg lipid unless otherwise stated. Vesicles containing 100% DOPC only, 20% (w/w) DOPG and 80% (w/w) DOPG in DOPC, and 100% DOPG prepared by this procedure, yielded a homogeneous population of vesicles with a mean diameter of 116 nm, 101 nm, 100 nm and 98 nm, respectively, as determined by quasi-elastic light scattering. For experiments involving carbonic anhydrase, vesicles were prepared in buffer containing 1 mg/ml enzyme. After vesicle preparation, carbonic anhydrase activity was verified by a p-nitrophenyl acetate hydrolysis assay [18].

Estimation of internal pH and permeability coefficients. The external proton concentration was rapidly altered by the addition of a desired volume (μl) of 0.5 M sulfuric acid (acid jump) or 0.5 M sodium hydroxide (base jump) and mixed by inversion. The fluorescent pH probe, pyranine, was excited at 450 nm and the emission intensity at 520 nm was observed using a Perkin-Elmer 650–40 fluorescence spectrophotometer equipped with a cell holder thermostated at 25°C. The fluorescence intensity was digitized and stored on a computer disk using a Zenith computer equipped with an Keithley Metrabyte DAS-8 A/D converter. The 520 nm emission of pyranine upon excitation at 416 nm (isosbestic point) was measured at the end of each experiment. The ratio of the emission at 520 nm when excited at 450 nm (function of the concentration of pyranine anion) and 416 nm (function of total pyranine concentration) was linearly correlated with pH (glass electrode) between pH 7 and 8 using nigericin to ensure equilibration of pH across the bilayer. The permeability coefficients for small intervals of the data were calculated using the numerical integration technique developed previously [14]. The standard error (S.E.) of the permeability coefficients was calculated using Data Desk® 3.0 software purchased from Data Description, Northbrook, IL.

The calculation of the proton concentration at the surface of negatively charged bilayer was estimated using Gouy-Chapman theory [16,19] as shown in the equations below.

$$[H_s] = [H_b] \exp(e\Psi/kT)$$

where $[H_s]$ is the proton concentration at the surface of the membrane, $[H_b]$ is the bulk phase concentration of protons, and Ψ is the surface potential which is estimated by:

$$\Psi = (2kT/Ze)\sinh^{-1}(\sigma/(8N_A C \epsilon\epsilon_0 kT)^{1/2})$$

where σ is the surface charge density (charge/Å^2), and C is the counterion concentration (mol/l) and Z is the valence of the counterion. The other symbols have their standard meanings [19]. At 25°C Ψ is given in millivolts by

$$\Psi = (51.38/Z)\sinh^{-1}(136.6\,\sigma/(C)^{1/2})$$

Theory

Kinetic model

The equation for diffusion of a molecular species, X, through a membrane is given by

$$J_X = P_X \Delta X \tag{1}$$

where J_X is the flux, P_X is the permeability coefficient *, and ΔX is concentration difference across the membrane of species X. The convention that influx is positive will be used.

Protons can be carried by permeant weak acids. If only one weak acid, HA, is present, the apparent flux of protons can be expressed as

$$J = J_H + D_{HA}J_{HA} - D_A J_A - D_{OH}J_{OH} \tag{2}$$

where J_H, J_{HA}, J_A, and J_{OH} are the fluxes of protons, the protonated form of the weak acid, the anionic form of the weak acid, and hydroxide ions, respectively, and D_{HA}, D_A and D_{OH} are the derivatives for protons with respect to the protonated form of the weak acid, the anionic form of the weak acid, and hydroxide ions, respectively **. These derivatives represent the fraction of flux for that species that results in a change in the internal proton concentration, i.e., because the transport of a mole of hydroxide per min is equivalent to the transport (in the opposite direction) of a mole of protons per min, $D_{OH} = -1$.

The apparent proton permeability coefficient, P, can then be expressed as the the apparent flux per unit molar proton concentration across the bilayer.

$$P = J/\Delta H = P_H + D_{HA}P_{HA}\Delta HA/\Delta H - D_A P_A \Delta A/\Delta H - D_{OH}P_{OH}\Delta OH/\Delta H \tag{3}$$

where $\Delta H = [H_0] - [H_i]$ is the difference between the proton concentration outside and inside the vesicle, respectively.

Since $[OH] = K_w/[H]$, where K_w is the ion-product constant for water, ΔOH can be expressed as

$$\Delta OH = K_w(1/[H_0] - 1/[H_i]) = K_W([H_i] - [H_0])/[H_0][H_i] \tag{4}$$

In an analogous manner ΔA can be expressed as

$$\Delta A = K[HA_0]/[H_0] - K[HA_i]/[H_i] \tag{5}$$

where K is the dissociation constant for the weak acid, $[HA_0]$ and $[H_0]$ are the protonated weak acid concentration and proton concentration outside of the vesicle, respectively, and $[HA_i]$ and $[H_i]$ are the protonated weak acid concentration and proton concentration inside of the vesicle, respectively. If the permeability coefficient for HA is much larger than the permeability coefficients of the other diffusing species, then the concentration difference, ΔHA, across the bilayer for HA quickly becomes small relative to concentration difference for H^+, making $[HA_0]$ nearly equal to $[HA_i]$. Then ΔA can be approximated as

$$\Delta A = K[HA_0](1/[H_0] - 1/[H_i]) = K[HA_0]([H_i] - [H_0])/[H_0][H_i] \tag{6}$$

Substituting Eqns. 4 and 6 into Eqn. 3 allows the expression for P to be simplified to

$$P = P_H + D_{HA}P_{HA}\Delta HA/\Delta H - (D_A P_A K[HA_0] + D_{OH}P_{OH}K_w)/[H_0][H_i] \tag{7}$$

Eqn. 7 predicts a linear relationship between P and $1/[H_i]$ with a plot of P vs. $1/H_i$ yielding

$$\text{slope} = [(-D_A)P_A K[HA_0] + (-D_{OH})P_{OH}K_w]/[H_0] \tag{8}$$

$$\text{intercept} = P_H + D_{HA}P_{HA}\Delta HA/\Delta H \tag{9}$$

Eqns. 8 and 9 give the physical significance of the slope and intercept of P vs. $1/[H_i]$. These slopes, when replotted vs. $[HA_0]$ provide a secondary plot with a slope equal to $D_A P_A K/[H_0]$ and an intercept of $D_{OH}P_{OH}K_w/[H_0]$. Positive slopes result consistent with the assignment of negative values to D_A and D_{OH}. When the intercepts from graphs of P vs. $1/[H_i]$ are replotted vs. $[HA_0]$, the values should approach P_H as $[HA_0]$ approaches zero. These properties provide graphical methods to experimentally determine P_H, P_{OH} and P_A.

In the case of CO_2/carbonic acid mediated proton flux, the P_{HA} term corresponds with the permeability coefficient of CO_2/carbonic acid; the P_A term is the permeability coefficient for bicarbonate. The contribu-

* The permeability coefficient, P, with no subscripts, is the apparent permeability. The permeability coefficients calculated for each ion are indicated by corresponding subscripts, i.e., for proton permeability, P_H. The concentration of hydrogen ion within vesicles, frequently given as the reciprocal, is indicated with subscript i: $1/H_i$; the concentration external to the vesicles is indicated with the subscript, o: $1/H_0$. DOPC is dioleoylphosphatidylcholine. DOPG is dioleoylphosphatidylglycerol. S.E. is the standard error of the reported values.

** Because the efflux of hydroxide ion into the vesicle is indistinguishable from the influx of a proton when near neutral pH, $D_{OH} = -1$. But exact analytical expressions for D_{HA} and D_A under these conditions are unavailable. D_{HA}, which is positive because carbonic acid moves in the same direction as protons under the conditions employed, appears in the intercept (Eqn. 9) and is eliminated by the extrapolation to zero bicarbonate concentration (Fig. 4). D_A, which is negative, appears in the slope term (Eqn. 8) which provides the product $D_A P_A$ from the slope of the secondary plot. D_A, is the fraction of bicarbonate (anion) molecules that associate with protons upon transfer of a bicarbonate molecule into the vesicle interior: $0 < D_A \leq -1$. The values for the permeability coefficients for bicarbonate derived from $D_A P_A$ (Fig. 3 and in Table I) are, then, lower limits.

tion of carbonate flux is considered to be negligible and not to contribute significantly to proton flux due to its divalent charge and its relatively low concentration within the pH range employed (pK = 10.36 for carbonate).

For the general case of n weak acids facilitating proton flux, Eqn. 7 can be expanded to include the sum of the of the contributions of each weak acid.

$$P = P_H + \sum D_{HA} P_{HA} \Delta HA / \Delta H$$

$$- \sum (D_A P_A K [HA_o] + D_{OH} P_{OH} K_w) / [H_o][H_i] \qquad (10)$$

Results

Proton flux across phospholipid vesicles

Proton flux should follow the same flux Eqn. as for other ions, i.e., eqn. 1. Using phospholipid vesicles of defined size containing the fluorescent pH indicator, pyranine, trapped inside, we are able to observe as a function of time the change in fluorescence when the pH external to the vesicles is suddenly decreased. The pH was calculated from the fluorescence, based on titration of vesicle trapped pyranine in the presence of nigericin as described in Methods, providing data like

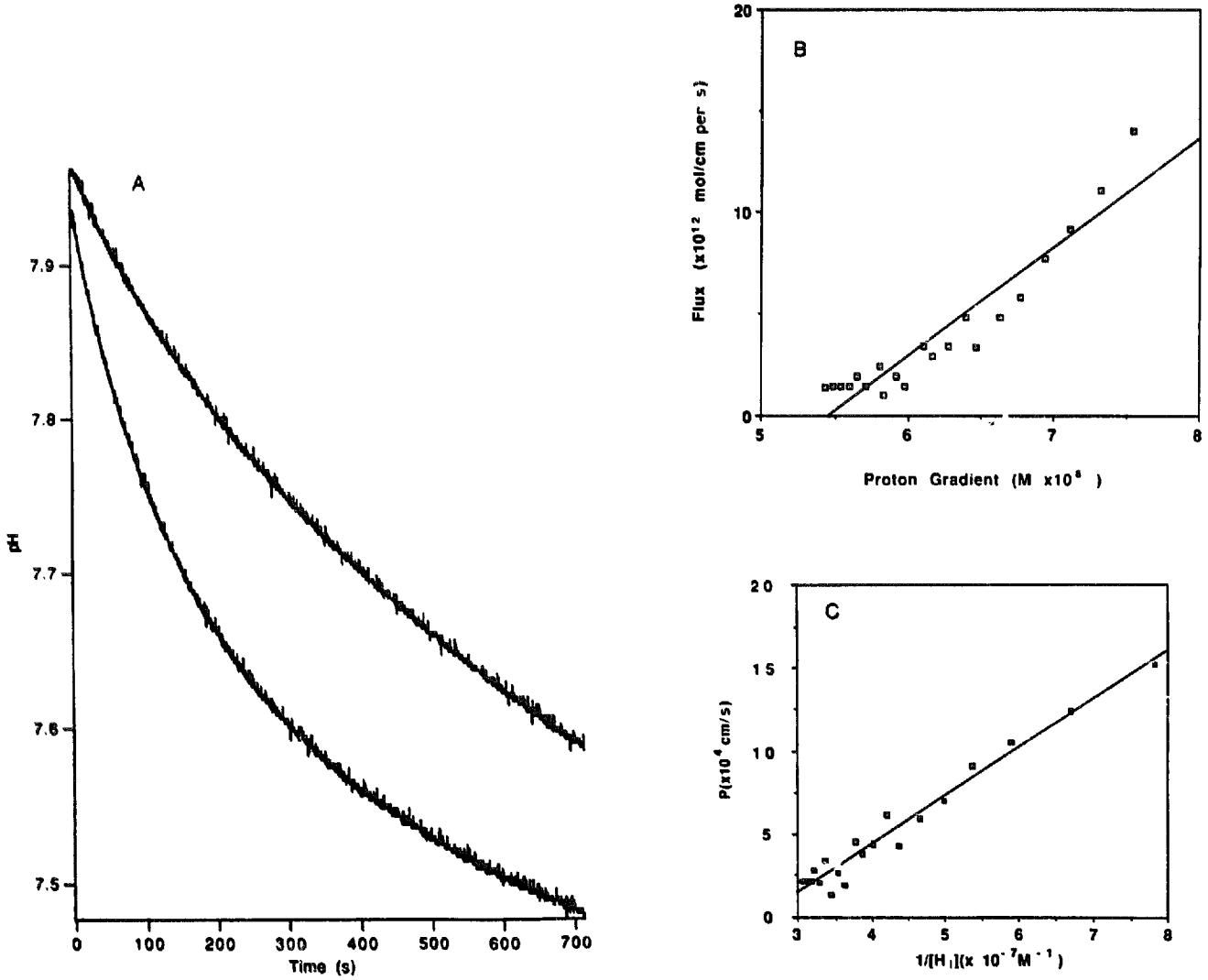

Fig. 1. Analysis of proton flux in vesicles. (A) Time course of proton influx. DOPC vesicles containing trapped pyranine are prepared in pH 8.0 buffer with valinomycin and 0.5 M sulfuric acid was added to obtain a final pH value of 7.06. Sodium bicarbonate was added to a total concentration of 2 mM in the lower curve. Vesicles of known surface area and whose buffer capacity had been determined are used. Each curve contains 2850 data points collected every 0.25 s. (B) Proton flux vs. proton gradient. The data from 1A (2 mM bicarbonate) were used to calculate the net proton flux over 140 points using Eqn. 1 from Ref. 14 and graphed vs. the proton gradient, i.e., the difference between the external hydrogen ion concentration and the average hydrogen ion concentration between the initial and the final points of this interval. The line is a linear least-squares fit to the data points. Systematic deviations from linear behavior are apparent (r^2 = 0.86). (C) Permeability coefficient vs. reciprocal internal hydrogen ion concentration. Numerical integration of the data from 1A (2 mM bicarbonate) over each 140 points was used to estimate the permeability coefficient using Eqn. 2 from Ref. 14. The average internal hydrogen ion concentration for each 140 points was estimated from the first and last points of the interval. The linear least squares fit for these data points is good (r^2 = 0.96).

shown in Fig. 1A. The upper curve is the standard system buffered with Hepes at an initial pH of 8.0 in the presence of valinomycin and potassium ion to provide rapid cation counterflux to compensate for the movement of protons. The external pH was rapidly decreased to 7.06 by the addition of 0.5 M sulfuric acid. The lower (faster) curve is the standard system to which 2 mM bicarbonate has been added. This concentration is considerably less than the 10–22 mM bicarbonate present within mitochondria [27]. Bicarbonate addition clearly accelerated the proton flux. Using the known values for the surface area of the vesicles and the internal buffer capacity, the net proton flux can be calculated for discrete time intervals (see Eqn. 1 in Norris and Powell [14]) from the data of Fig. 1A. When the flux in the presence of 2 mM bicarbonate was graphed vs. the proton gradient (the difference in the external and the internal molar hydrogen ion concentrations at the beginning and the end of these time intervals), a curvilinear plot was obtained (Fig. 1B) rather than the linear result expected from Eqn. 1. This behavior was an important clue to the presence of the weak acid carrier present in this system [14]. The instantaneous permeability coefficient was estimated from the data of Fig. 1A (2 mM bicarbonate) using numerical integration (see Eqn. 2 in Norris and Powell [14]). When the apparent instantaneous permeability coefficients are graphed vs. the reciprocal of the internal hydrogen ion concentration, linear behavior was obtained (Fig. 1C). We present below a theoretical explanation for the observed non-linear behavior based on Eqn. 1 but using the sum of the fluxes for protons, hydroxide ions, carbonic acid (and/or carbon dioxide) and bicarbonate, extract values for some of these permeability coefficients and otherwise test the derived

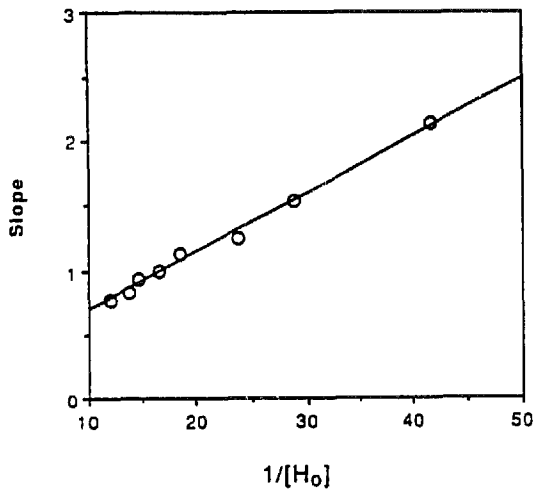

Fig. 2. The slope of P vs. $1/[H_i]$ as a function of the reciprocal external proton concentration for acid jumped DOPC vesicles. DOPC vesicles prepared in pH 8.0 buffer. Vesicle samples were then jumped to different values by the addition of sulfuric acid. The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

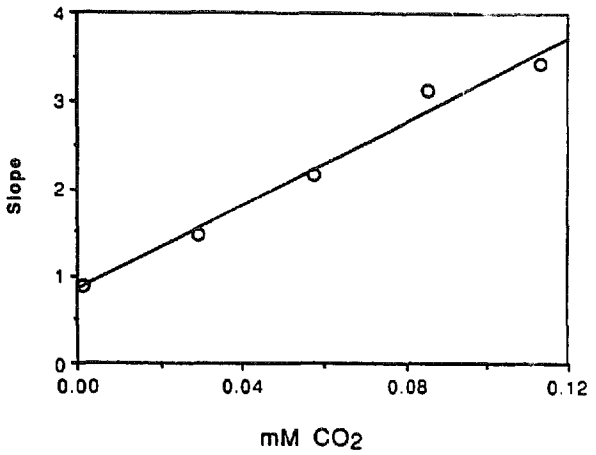

Fig. 3. Linear relationship between CO_2 concentration and the slope of P vs. $1/[H_i]$ internal for acid jumped DOPC vesicles. Eqn. 7 predicts the intercept for this secondary plot to be equal to $D_{OH}P_{OH}K_w/[H_0]$ and the slope to be $D_AP_AK/[H_0]$.

Eqn. which contains $1/[H]$. For example, Eqn. 7 describes these properties and in addition predicts that the slope of P vs. $1/[H_i]$ should also be a linear function of $1/[H_0]$. This relationship was experimentally verified by the data shown in Fig. 2 for DOPC vesicles.

Determination of P_{OH} and P_A by graphical analysis

The data shown in Fig. 3 indicate a linear relationship between the slope parameter given by Eqn. 8 and the concentration of CO_2. The concentration of CO_2 at 25°C was calculated from the amount of bicarbonate added using the equilibrium relationship $[CO_2] = [HCO_3]/10^{(pH-6.1)}$ [20]. The slope and intercept of this plot should be equal to $D_AP_AK/[H_0]$ and $D_{OH}P_{OH}K_w/[H_0]$, respectively. Since K_w and $[H_0]$ are known and the value of D_{OH} is approximately equal to -1, the permeability coefficient for hydroxide ion determined from the intercept shown in Fig. 3 was $3.8 \cdot 10^{-5}$ (S.E. $5.6 \cdot 10^{-6}$) cm/s. The intercept of Fig. 3 provides D_AP_A which, because of the uncertainty in the value of D_A (see footnote ** on p. 19), is an estimate of the lower limit of the permeability coefficient for bicarbonate: $4.3 \cdot 10^{-6}$ (S.E. $3.6 \cdot 10^{-7}$) cm/s.

Estimation of P_H by graphical analysis

Eqn. 7 also predicts that as the CO_2/carbonic acid concentration approaches zero, secondary plots of the intercepts plotted vs. $[CO_2]$ should approach the value of P_H, the permeability coefficient for protons. P_H can be obtained by measuring the intercept as a function of bicarbonate concentration and extrapolating to zero bicarbonate concentration (Fig. 4). The value for P_H was determined by this method was $1.8 \cdot 10^{-5}$ (S.E. $1.3 \cdot 10^{-5}$) cm/s. An interesting characteristic of the P vs. $1/H_i$ plots is that they frequently give a negative intercept. From Eqn. 7 the intercept is composed of

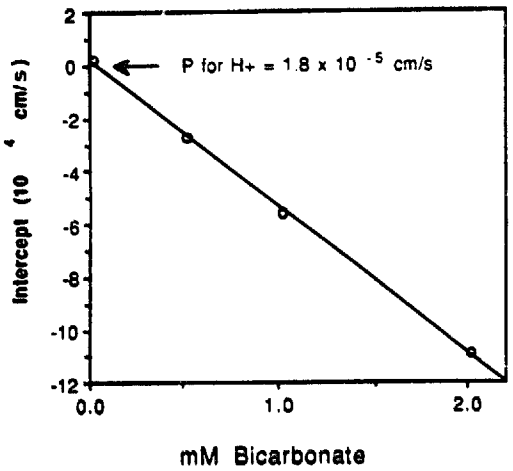

Fig. 4. The intercept of P vs. $1/[H_i]$ as a function of added bicarbonate for acid jumped DOPC vesicles. Vesicles were prepared at pH 7.0 and were jumped to pH 7.35. Eqn. 7 predicts that this plot yields an intercept equal to P_H.

$P_H + D_{HA}P_{HA}\Delta HA/\Delta H$. Since P_H, P_{HA}, and ΔH have positive values, ΔHA must be able to take on negative values. Computer simulation of weak acid mediated proton flux based on Eqn. 7 was performed using an iterative program. If the assumption is made that P_{HA} is much greater than P_H, P_A, and P_{OH}, which is the key assumption in the development of Eqn. 7, then the values during the steady state for ΔHA obtained from the computer simulation are negative. The rapid influx of HA causes the total concentration of HA and A inside the vesicle to be greater than the total concentration outside. As the internal pH is decreased by the continued influx of protons and efflux of hydroxide ions, the concentration of internal HA increases and can exceed the extenal HA concentration yielding a negative ΔHA. As expected, the computer simulation

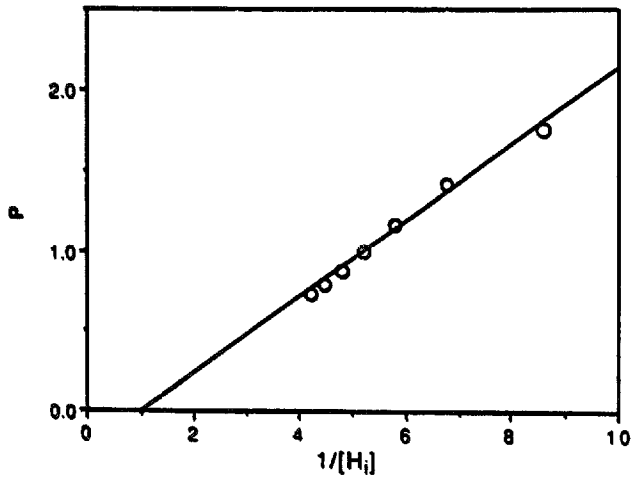

Fig. 5. Computer simulation of the relationship between P and reciprocal internal proton concentration. An iterative computer program was used to simulate proton flux mediated in part by a weak acid. The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

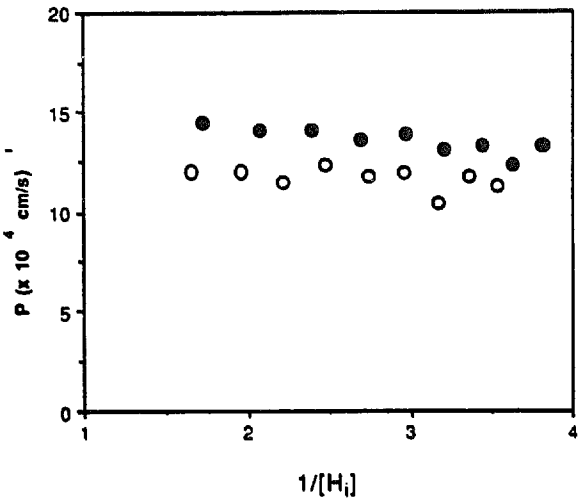

Fig. 6. The effect of carbonic anhydrase on P vs. $1/[H^+]$ for base jump conditions. DOPC vesicles were prepared in pH 7 in the presence (●) and absence (○) of 1 mg/ml carbonic anhydrase. The pH was jumped to 7.8 by the addition of NaOH. The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

yields linear P vs. $1/[H_i]$ plots that possess negative intercepts (Fig. 5).

Applicability of the phenomenological equation to other weak acids and base jump conditions.

The general characteristics of CO_2/carbonic acid mediated proton flux for acid jumped vesicles are common to proton flux facilitated by other weak acids. For example, the addition of acetic acid to DOPC vesicle samples resulted in a linear relationship between P and $1/[H_i]$ with the slope of these plots increasing with increasing concentration (data not shown). This behavior supports the generality of the expression derived for transport of protons by weak acids. When the external pH was increased by the addition of sodium hydroxide (base jump), P was constant [14]. However, the kinetic model and the assumptions that lead to Eqn. 7 do not provide a constant P. Vesicles prepared in the presence of carbonic anhydrase to ensure the equilibration of CO_2 with carbonic acid during the time scale of the experiment gave slightly increased values of P but the characteristic feature of a constant P was unaltered (Fig. 6). The presence of carbonic anhydrase did not alter the linear dependence of P vs. $1/[H_i]$ characteristic of the acid jump condition (data not shown).

Effects of DOPG on the permeabilities of protons, hydroxide ions and bicarbonate

For the kinetic model that has been proposed (see theory) for CO_2/carbonic acid mediated proton flux across phospholipid bilayers, the rate-limiting step is considered to be the translocation of the anion, bicarbonate. Negative surface charge should inhibit the

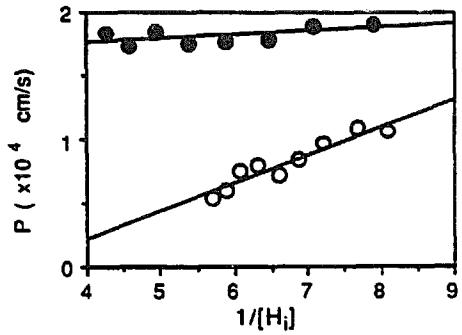

Fig. 7. The apparent proton permeability coefficient as a function of $1/[H_i]$ for DOPC and DOPG vesicles. DOPC (○) and DOPG (●) vesicles were prepared at pH 8.0. The external pH was rapidly altered to pH 7.35 by the addition of microliters of 0.5 M sulfuric acid. The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

proposed process by reducing the concentration of bicarbonate at the membrane interface by electrostatic repulsion. In Fig. 7 the effect of increasing amounts of the negatively charged phospholipid DOPG on plots of P vs. $1/[H_i]$ increased the values for P and decreased the values for the slope of the lines. The negative surface charge causes an increase in the concentration of protons near the surface of the membrane which increases the interfacial proton concentration gradient resulting in an increase in the observed values of P. When the proton concentration at the surface of DOPG vesicles was estimated using Gouy-Chapman theory (see Methods and Materials), and the corrected values for the proton gradient used to estimate P, the values of P approached those of DOPC vesicles (Fig. 8). Secondary plots of the slope parameter as a function of CO_2 were linear ($r^2 > 0.98$) and were used to calculate the permeability coefficients for hydroxide ion and

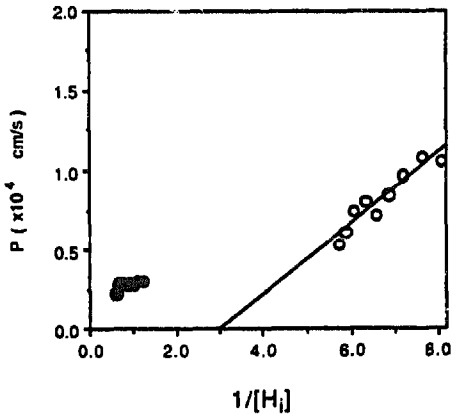

Fig. 8. The effect of Gouy-Chapman estimation of the interfacial proton concentration on P vs. $1/[H_i]$ for DOPG vesicles. The proton concentration at surface of DOPG vesicles was estimated using Gouy-Chapman theory. The apparent proton permeability coefficient was calculated using the adjusted values for the proton concentration difference across the bilayer. P calculated for DOPG vesicles (●) are compared to DOPC vesicles (○). The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

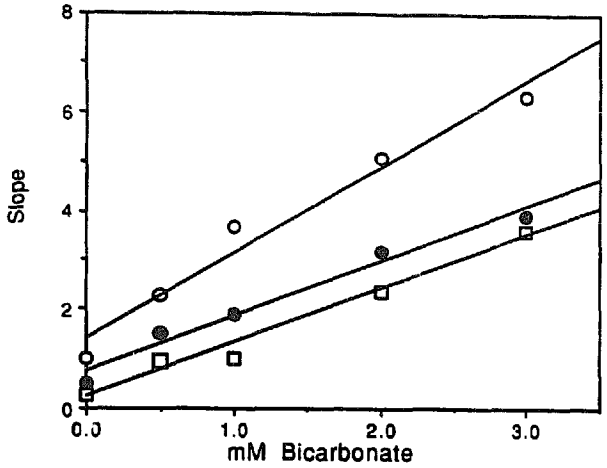

Fig. 9. The effect of DOPG on the slope parameter as a function of bicarbonate. The slope of P vs. $1/[H_i]$ for vesicles prepared at pH 8.0 and jumped to pH 7.35. Vesicles were composed of DOPC (○), 20% DOPG (●) and 80% DOPG (□).

TABLE I

The effect of increasing DOPG content on the observed permeability coefficients for protons, hydroxide ions and bicarbonate

Vesicle composition	Permeability coefficient (10^{-5} cm/s)		
	protons	hydroxide ions	bicarbonate [a]
DOPC	1.8 (1.3)	3.0 (0.58)	0.59 (0.033)
20% DOPG	20 (1.2)	2.8 (0.53)	0.40 (0.022)
80% DOPG	78 (1.0)	0.72 (0.51)	0.33 (0.022)

[a] The bicarbonate permeability coefficient is estimated from $D_A P_A$ and represents a lower limit for these values (see Eqn. 8 and footnote ** on p. 19). The values in parenthesis are the standard errors.

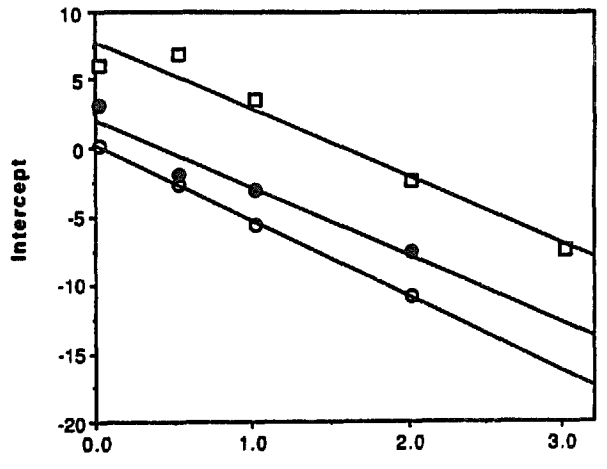

Fig. 10. The effect of increasing bicarbonate concentration on the intercept parameter. The intercept parameter was measured as a function of bicarbonate concentration for acid jumped vesicles composed of DOPC (□), 20% DOPG (●) and 80% DOPG (○).

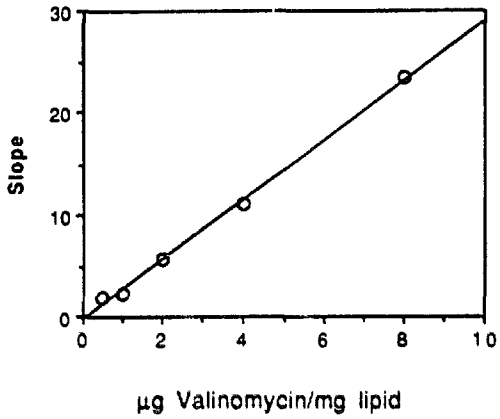

Fig. 11. The effect of valinomycin on the slope parameter for DOPC vesicles. The slope of P vs. $1/[H_i]$ for vesicles prepared at pH 8.0 in buffer containing 50 mM K_2SO_4. The pH was jumped to pH 7.3 with H_2SO_4.

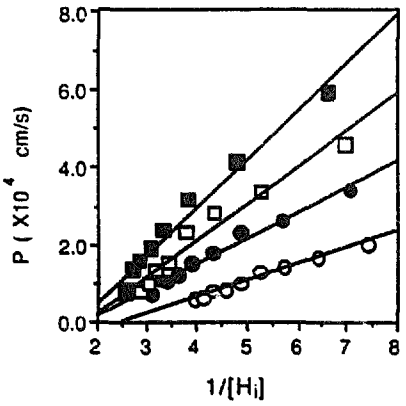

Fig. 13. The effect of tetrabutylammonium ion on P vs. $1/[H_i]$ for DOPC vesicles. Tetrabutylammonium ion was added to DOPC vesicles to give a concentration of 0 μM (○), 22 μM (●), 44 μM (□), 88 μM. The unit for the abscissa is M^{-1} ($\times 10^{-7}$).

bicarbonate of vesicles containing differing amounts of DOPG (Fig. 9). The hydroxide ion and bicarbonate permeability coefficients calculated from these data show a systematic decrease with increasing DOPG (Table 1). The effect on increasing the amount of DOPG contained within the vesicles on the intercept parameter as a function of bicarbonate concentration is shown in Fig. 10. The proton permeability coefficients calculated from these data systematically increased as the DOPG content was increased (Table 1).

Valinomycin and tetrabutylammonium ion enhances bicarbonate permeability by ion pairing

Lipophilic cations have recently been shown to enhance the rate of proton flux across phospholipid vesicles from the formation of an ion pair between the unprotonated form of the uncoupler and the lipophilic cation which increases the permeability (Ahmed and Krishnamoorthy, 1990 [17]). Theoretical analysis of the kinetic model for CO_2/carbonic acid mediated proton flux has shown that the slope parameter contains a contribution from the permeability coefficient of bicarbonate. Increasing the permeability coefficient of bicarbonate by using lipophilic cations also should result in an increase in the slope parameter. Fig. 11 shows that increasing the valinomycin concentration increases the value of the slope parameter for DOPC vesicles. In order to determine if the increase in the slope parameter results from uncomplexed valinomycin or valinomycin complexed with potassium ion, the slope parameter was determined as a function of valinomycin concentration at different concentrations of potassium ion (Fig. 12). The lipophilic cation tetrabutylammonium ion also was shown to enhance the value of the slope parameter for DOPC vesicles (Fig. 13).

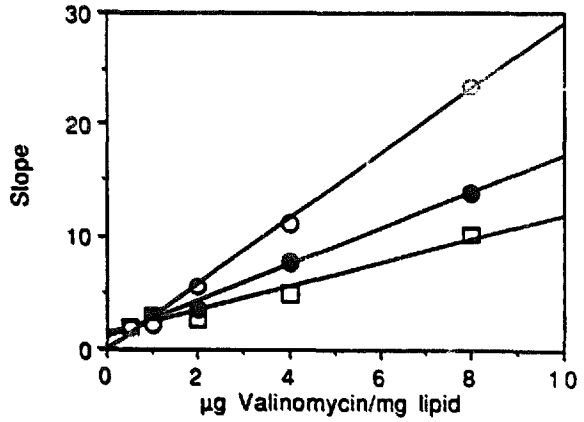

Fig. 12. The effect of valinomycin on the slope parameter for DOPC vesicles at different concentrations of K^+. Vesicles were at pH 8.0 in buffer containing 40 mM Na_2SO_4 and 10 mM K_2SO_4 (□), 25 mM Na_2SO_4 and 25 mM K_2SO_4 (●) and 50 mM K_2SO_4 (○). The pH was jumped to pH 7.3 with H_2SO_4.

Discussion

The key assumption for the development of Eqn. 7 is that the concentration difference of HA rapidly comes nearly to equilibrium across the membrane and that the rate-limiting step for the process of CO_2/carbonic acid mediated proton translocation is movement of A^-, i.e., bicarbonate, across the membrane. The assumptions made in the derivation of Eqn. 7 are essentially those usually made for the proton transport mechanism of weak acid uncouplers like FCCP and CCCP [21–23]. The values for P_H and P_{OH} determined by our graphical analysis are $1.8 \cdot 10^{-5}$ (S.E. $1.3 \cdot 10^{-5}$) cm/s and $3.8 \cdot 10^{-5}$ (S.E. $5.6 \cdot 10^{-6}$) cm/s respectively. These values of P_H and P_{OH} are in the range of the apparent proton permeability coefficients that have been reported [7–14]. However, this is the first report to our knowledge of the resolution of both P_H and P_{OH} from the apparent proton permeability. The similarity of the values of P_H and P_{OH} suggests a common mechanism for the translocation of both protons and

hydroxide ions across a bilayer. A 'water wire' would be such a common mechanism.

Since the proton and hydroxide ion permeability coefficients are determined by extrapolating the intercept parameter and slope parameter, respectively, to zero concentration of CO_2, the residual amount of CO_2/carbonic acid/bicarbonate present in the vesicle sample is a source of error. Because of the nature of the plots (Figs. 3 and 4) used to determine the permeability coefficients, the presence of a residual amount of CO_2 will result in an underestimate of the proton permeability coefficient and an overestimate of the hydroxide ion permeability coefficient. It should also be pointed out that because weak acids other than CO_2/carbonic acid can facilitate the diffusion of protons across the bilayer, the P_H and P_{OH} values determined from the extrapolation to zero CO_2/carbonic acid concentration also would be in error if other weak acid contaminants were present.

It was shown previously that the apparent proton permeability coefficient, P, under base jump conditions, was a function of the bicarbonate concentration and was a linear function of the reciprocal external proton concentration. However, in contrast to the acid jump case, P was not a function of the reciprocal internal proton concentration, $1/[H_i]$ [14]. The behavior of P under base jump conditions can not be explained using the assumptions used to derive the kinetic model developed for the acid jump case. The presence of carbonic anhydrase did not alter the independence of P on $1/[H_i]$. Thus, the independence was not a result of a non-equilibrium condition in which the conversion of carbonic acid to CO_2 and water was the rate-limiting step rather than the diffusion of bicarbonate across the bilayer proposed for the acid jump case. Attempts to simulate the base jump using the assumptions that successfully model the acid jump case does not yield values for P that are independent of $1/[H_i]$. Differences in the observed values of P between acid and base jumped vesicles have also been reported by others [9].

Electrostatic potential resulting from the presence of charged lipids can greatly influence the transport properties of lipid bilayers [16]. The presence of the negatively charged phospholipid, DOPG, in vesicles is shown in Fig. 7 to increase the observed value of P. This can be explained by an increase in the concentration of protons at the surface of the membrane. When the values of P are calculated using interfacial proton concentrations estimated by Gouy-Chapman theory the values are reduced to values similar to those obtained for zwitterionic DOPC vesicles (Fig. 8). Although the observed values of P were increased for vesicles with a negative surface charge, the slope of P vs. $1/[H_i]$ was markedly reduced (Figs. 7–9). The slope of P vs. $1/[H_i]$ has been shown to contain the permeability coefficients for bicarbonate and hydroxide ion (Eqns. 7 and 8). A reduction in the slope parameter as a function of bicarbonate concentration indicates the permeability of bicarbonate is reduced. As the concentration of DOPG was increased the observed proton permeability coefficient was systematically increased and the observed permeability coefficients for hydroxide ion and bicarbonate were systematically decreased (see Table I). Since bicarbonate flux is the rate-limiting step in the proposed mechanism of CO_2/carbonic acid mediated proton transport, the reduction in the bicarbonate permeability coefficient reduces the efficiency of this mechanism of passive proton flux.

These results are in agreement with those previously reported for planar phospholipid membranes with other weak acid proton carriers. The conductance of planar phospholipid membranes composed of bacterial phosphatidylglycerol in the presence of the weak acid uncoupler DTFB (5,6-dichloro-2-trifluoro-methylbenzimidazole) has been shown to be 1.8 orders of magnitude less than those composed of bacterial phosphatidylcholine [24]. Also, planar membranes composed of lipid isolated from *Staphyloccoccus aureus* exhibited conductance in the presence of the uncouplers S13 and DNP (dinitrophenolate) that was 10-fold smaller in the case of phosphatidylglycerol relative to the neutral lipid diglucosyldiglyceride [25]. It has been proposed that the high acidic lipid content in the bacterial membrane was responsible for the reduction in the effectiveness of DNP to uncouple electron transport and ATP synthesis in bacteria relative to mitochondria [25].

The K^+ ionophore, valinomycin has used extensively to modify the membrane potential, $\Delta\Psi$, often in conjunction with weak acid proton ionophores such as FCCP and CCCP to probe the contribution of $\Delta\Psi$ and ΔpH in energy transduction systems [21,26]. In the study of passive proton permeability using the phospholipid vesicle model system, valinomycin has been used extensively to abolish the development of a proton diffusion potential by transporting K^+ in the opposite direction of proton flux (c.f. Refs. 7 and 10). Recently, It has been shown that valinomycin-K^+ can greatly enhance weak acid mediated proton transport by forming a complex with weak acid anion [17]. In order to understand further contributions of valinomycin-K^+ on CO_2/carbonic acid mediated proton flux through DOPC vesicles and to test the equation that has been developed to describe the kinetic behavior of the CO_2/carbonic acid mediated process under acid jump conditions, experiments were performed at different concentrations of valinomycin-K^+ and tetrabutylammonium ion.

The slope of P vs. $1/[H_i]$ is shown in Fig. 11 to be a linear function of valinomycin concentration. As shown in Fig. 12, the slope was systematically increased as the K^+ concentration was increased relative to the Na^+

concentration indicating that the K^+ complexed form of valinomycin was responsible for the increase in slope. An increase in the slope in the presence of valinomycin K^+ can be explained by an increase in the permeability coefficient for bicarbonate. This is the expected result if ion pairing occurs between valinomycin-K^+ and bicarbonate in an analogous manner to that proposed for valinomycin-K^+ and the weak acid uncouplers, FCCP, CCCP and SF6847 [17]. If the change is a result of an increase in the bicarbonate permeability coefficient via an ion pairing mechanism, then lipophilic cations other than valinomycin-K^+ should also increase the slope value. As shown in Fig. 13, the addition of increasing amounts of tetrabutylammonium ion results in a increase in slope. The concentration of tetrabutylammonium ion required to increase the slope value is significantly higher than the concentration of valinomycin. This can perhaps be explained by a difference in the partition coefficients for the phospholipid bilayer of valinomycin-K^+ and tetrabutylammonium ion. Similar results in the difference between the ability of valinomycin-K^+ and tetraphenylphosphonium ion to enhance the rate of FCCP, CCCP, SF6847 mediated proton transport have been reported [17].

CO_2 and carbonic acid are essential components of the living system and are present in cells and organelles that must maintain large pH gradients. In bovine heart mitochondria the bicarbonate concentration has been measured to be 10–22 mM [27] which is much greater than the concentration required to significantly increase proton flux though phospholipid vesicles. The proton flux through biological membranes mediated by CO_2/carbonic acid would reduce the efficiency of system by dissipating the pH gradient in a non-productive process. Biological membranes commonly contain large amounts of negatively charged lipids and negative surface potential could be an important factor in reducing CO_2/carbonic acid mediated proton flux in vivo. For example, the inner mitochondrial membrane contains appreciable amounts of the negatively charged lipid cardiolipin [28]. The negative surface charge conferred by lipids could inhibit proton flux mediated by CO_2/carbonic acid and thereby increase the efficiency of the coupling of electron transport and ATP synthesis.

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