DEPENDENCY OF ΔpH-RELAXATION ACROSS VESICULAR MEMBRANES ON THE BUFFERING POWER OF BULK SOLUTIONS AND LIPIDS

STEPHAN GRZESIEK AND NORBERT A. DENCHER
Biophysics Group, Department of Physics, Freie Universität Berlin, Arnimallee 14, D-1000 Berlin 33, Federal Republic of Germany

ABSTRACT The dependency of ΔpH-relaxation kinetics across the membrane of sonicated small phospholipid vesicles on the concentration of internally entrapped buffer has been investigated by means of the pH-indicator dye pyranine. A very high contribution of lipid headgroups to the internal buffering power of the liposomes is observed, amounting to an equivalent phosphate buffer concentration of 110 mM. This localized two-dimensional proton/hydroxide ion reservoir must be considered in any determination of the H^+/OH^- permeability coefficient. Furthermore, it could have significance for energy-transduction across biological membranes. From the established linear relation between ΔpH-relaxation rates and buffering power, net H^+/OH^- permeabilities of $3 \cdot 10^{-3}$ cm/s for soybean phospholipid (SBPL) and $1 \cdot 10^{-4}$ cm/s for diphytanoyl phosphatidylcholine (diphytanoyl PC) vesicles at pH 7.2 as well as buffering powers per lipid molecule of $6 \cdot 10^{-2}$ (pH-unit)$^{-1}$ (SBPL) and $4 \cdot 10^{-2}$ (pH-unit)$^{-1}$ (diphytanoyl PC) are calculated. In the case of diphytanoyl PC vesicles, ΔpH-decay is accelerated by the presence of chloride ions.

INTRODUCTION

The lipid matrix of biological membranes is considered to act as a diffusion barrier for hydrophilic solutes. In accordance with this view, the permeability coefficient for inorganic ions such as sodium and potassium across pure lipid membranes was found to be in the range of 10^{-12} to 10^{-14} cm/s and is therefore extremely small. The chemiosmotic hypothesis of oxidative and photosynthetic phosphorylation (Mitchell, 1961, 1966) is based on a relatively low conductance for protons and hydroxide ions of the energy-transducing membranes in order to avoid short-circuiting of the electrochemical proton gradient. Since knowledge of the net H^+/OH^- permeability coefficient, P_{net}, is of importance for both the acceptance of Mitchell's chemiosmotic theory and membrane biology in general, numerous attempts have been made to determine this value for biological membranes and pure lipid bilayers. The results obtained are quite surprising in two respects. On the one hand, even for comparable systems such as the bilayer of lipid vesicles, the published permeability coefficients of the respective investigations differ by six orders of magnitude, ranging from 10^{-3} to 10^{-9} cm/s (Biegel and Gould, 1981; Cafiso and Hubbell, 1983; Deamer, 1982; Deamer and Nichols, 1983; Elamrani and Blume, 1983; Gutknecht and Walter, 1981; Gutknecht, 1984; Krishnamoorthy and Hinkle, 1984; Nichols et al., 1980; Nichols and Deamer, 1980; Nozaki and Tanford, 1981; O'Shea et al., 1984*a*; Pohl, 1982; Rossignol et al., 1982; Kell and Morris, 1980). On the other hand, the majority of the values cover the range between 10^{-3} to 10^{-6} cm/s indicating a rather high permeability of H^+/OH^- for pure lipid bilayers as compared to the inorganic ions K^+, Na^+, and Cl^-. To understand at least part of the possible reasons for the extreme variance in the recently determined P_{net} values we measured the H^+/OH^--permeability of small soybean phospholipid and diphytanoyl phosphatidylcholine vesicles. The vesicles were produced by sonication in order to obtain in a reproducible manner unilamellar vesicles lacking impurities such as organic solvents and detergents. The kinetics of the decay of a pH-gradient established in ≤5 ms across the lipid bilayer was monitored by means of the fluorescent pH-sensitive dye pyranine, entrapped in the aqueous internal volume of the vesicles. To test the validity of the flux equations applied, the concentration of the intravesicular phosphate buffer was varied between 10 and 100 mM. From the dependency of the decay time of the ΔpH on the internal phosphate buffering power, P_{net} values of $2 \cdot 10^{-3}$ cm/s for soybean phospholipid vesicles and $1 \cdot 10^{-4}$ cm/s for diphytanoyl phosphatidylcholine were calculated. In addition, from the measured dependency it has to be concluded that the lipid layer of the internal vesicle surface exhibits a very high buffering power, equivalent to a phosphate buffer concentration of up to 110 mM. This new observation, which has not been considered in previous investigations, obviously affects any determination of the apparent ΔpH decay kinetics and has therefore to be taken into consideration for the calculation of the true P_{net} value of a lipid bilayer. The strong buffering power of the lipid head groups, especially of net nega-

tively charged ones, which are present to a considerable proportion in biological membranes, should be of importance for energy-transduction across membranes as well. The lipid head group region at the membrane/bulk interface might represent a transient reservoir for at least part of the established proton gradient. Proton consuming systems, e.g. H^+-dependent ATP-synthases, could be supplied directly from this localized H^+-pool. In combination with the theoretically (Haines, 1983) and experimentally (Teissié et al., 1985) described proton-conducting pathway along the surface of membranes, the proton reservoir provided by the lipid head group layer will favor a localized or semilocalized chemiosmotic hypothesis and might explain some of the previously described deviations from Mitchell's delocalized chemiosmotic hypothesis.

MATERIALS AND METHODS

Soybean phospholipids (SBPL) (Sigma Chemical Co., St. Louis, MO) were purified according to the procedure of Kagawa and Racker (1971). Diphytanoyl phosphatidylcholine (diphytanoyl PC) (Avanti Biochemicals, Birmingham, AL), dimyristoyl phosphatidylcholine (DMPC) (Fluka, Neu-Ulm, FRG), 8-hydroxy-1,3,6-pyrenetrisulfonate (pyranine) (Eastman Kodak Co., Rochester, NY), carbonyl cyanide m-chlorophenyl hydrazone (CCCP) (Aldrich Chem. Co., Milwaukee) and valinomycin (Boehringer, Mannheim, FRG) were used without further purification.

For the preparation of vesicles the phospholipids were dissolved in a small volume of chloroform and spread homogeneously as a thin film on the wall of a glass tube by evaporation of the solvent ($\leq$1 Torr, $\geq$30 min). Solvent-free addition of valinomycin was achieved by adding the ionophore in ethanolic solution to the dissolved lipids prior to drying. Residual traces of solvent were removed under vacuum ($\leq 10^{-3}$ Torr, 2–20 h) at room temperature. The dried lipid film was rehydrated in aqueous buffer solutions of the desired pH and ionic composition containing 2.2 mM of the optical pH-indicator dye pyranine. This sample with a final lipid concentration of 10 mg/ml was sonicated in a bath-type sonicator (Sonorex RK 100 H, Bendelin electronic, FRG, 35 kHz) at room temperature (in the case of DMPC at 30°C) to clarity, i.e. 30 min. Dye molecules not trapped in the internal volume of the vesicle were removed by triple dialysis of the vesicle suspension at 4°C. Prior to the measurements the vesicle suspension was diluted to a final lipid concentration of 0.5 mg/ml. It is assumed that the concentration of pyranine entrapped in the vesicle interior maximally equals the concentration of pyranine prior to the sonication step, and that pyranine does not bind significantly to the vesicle membrane in the case of SBPL (Clement and Gould, 1981). Since this dye bears three to four negative charges the rate of leakage from anionic single compartment liposomes is <1% per day (Kano and Fendler, 1978).

The size distribution of vesicles was determined by negative-stain electron-microscopy with uranyl-acetate. A mean diameter of SBPL vesicles was also obtained by quasi-elastic light scattering.

Fast alterations of pH in the external medium of the vesicle suspension were induced by addition (1:1) of appropriate buffer solutions in a thermostated (±0.1°C) stopped-flow unit (Sigma Instrumente, Berlin) ($2 \times 2 \times 16$ mm^3 fluorescence and absorption cuvette). Except during the short piston movements, this system works almost without pressure. The mixing and overall dead time of the instrument as determined by the method of Paul et al. (1980) was found to be <5 ms.

For monitoring the kinetics of pH-change in the vesicle interior the optical pH-indicator pyranine was used (Kano and Fendler, 1978). Unless otherwise stated, pyranine fluorescence was excited and detected in a dual-wavelength photometer (Sigma Instrumente, Berlin, ZWS II) where the exciting light was chopped at a frequency of 1 kHz between 400 nm and 460 nm. Fluorescence ($\lambda \geq 500$ nm) was separated from scattered light by a KV 500 glass filter (Schott, FRG). The ratio of both fluorescence intensities, I_{460}/I_{400}, was calculated and its time-dependent value finally stored in an Apple II microcomputer for further analysis. One or two exponentials could be fitted to the data points by a Marquardt procedure (Bevington, 1969). According to Kano and Fendler (1978) the intensity ratio I_{460}/I_{400} should provide still greater linearity with respect to the vesicle internal pH than the sole intensity I_{460}. For pH measurements of the vesicle external medium a Beckmann Model 4500 pH-meter connected to a combined pH-electrode was used.

Additional details concerning the methodologies applied, i.e. preparation of the vesicles, measurement of internal pH-changes by pyranine, and instrumentation are described elsewhere (Dencher et al., 1986).

THEORY

Flux Equations for Ion and Proton/Hydroxide Ion Diffusion

In the absence of external forces, temperature and pressure gradients nonequilibrium situations in solutions are due to local differences in the chemical potential $\tilde{\mu}_i(\vec{x}, t)$ for the different solutes i and give rise to a diffusion flux $\vec{j}_i$ which tends to restore equilibrium. If the deviation from equilibrium is small, the flux $\vec{j}_i$ is assumed to be proportional to grad $\tilde{\mu}_i$ and the concentration c_i of the species i (Koryta et al., 1966)

$$\vec{j}_i(\vec{x}, t) = -\frac{c_i(\vec{x}, t) D_i(\vec{x}, t)}{RT} \operatorname{grad} \tilde{\mu}_i(\vec{x}, t), \qquad (1)$$

where D_i is the corresponding diffusion coefficient, R the universal gas constant and T the absolute temperature. Eq. 1 is the most general expression one can establish for particle transport due to a nonconstant chemical potential. For charged particles, which do not undergo chemical reactions, this chemical potential is usually split into two parts: a pure electrical potential energy, which is required to take an idealized point charge from infinity to a position $\vec{x}$, and a second term. This latter term should include concentration dependencies and all other energies besides those connected with the pure electrical potential energy, i.e., electrostatical contributions due to the solvation shell and the ionic atmosphere around charged particles. Within this framework the situation of a lipid membrane system, where the dielectric constant in the membrane differs from the bulk value of the aqueous phases, was treated by Neumcke and Läuger (1969). This concept leads to generalized Nernst-Planck equations which cannot be solved in a closed form. Under the assumption of very low membrane conductance, these authors derived steady-state solutions, which are characterized by a nonlinear current-voltage relationship due to image forces at the membrane/bulk interface. Recently the validity of such generalized Nernst-Planck equations for the description of passive proton/hydroxide ion transport across lipid bilayers has been seriously challenged (Cafiso and Hubbell, 1983;

Gutknecht, 1984; Krishnamoorthy and Hinkle, 1984). However, no other transport model, which allows interpretation of measured rate or transport constants in terms of microscopic parameters, was given. (See Discussion.)

Commonly, further simplifications of Nernst-Planck flux equations are made, which lead to closed solutions for special experimental set-ups. Thus for pH-jump experiments in vesicular systems a calculation of at least phenomenological proton/hydroxide ion permeabilities becomes possible. The simplifications are based on the following assumptions, whose physical validity must be carefully considered:

(*a*) at any time there is chemical equilibrium in the bulk phases on both sides of the membrane as well as between the lipid-water interfaces and the bulk phases. The concentration of any solute at the lipid-water interface will then be given by the bulk concentration of the corresponding membrane side and a partition coefficient which accounts for possible concentration differences between these two locations.

(*b*) the activities of the solutes are equal to their corresponding concentrations.

(*c*) there is no electrical potential difference between both sides of the membrane. Such a difference could be due to a diffusion potential or an asymmetric charge composition of the membrane. Abolishment of transmembrane potential difference can be achieved, for example, by ion-selective ionophores and an excess concentration of corresponding ions.

(*d*) the concentration of the solutes varies linearly across the membrane.

(*e*) H^+ and OH^- fluxes are independent.

These assumptions lead to the following equations for proton and hydroxide ion fluxes, which are commonly used:

$$j_H = -P_H([H^+]_o^i - [H^+]_i^i) \tag{2a}$$

$$j_{OH} = -P_{OH}([OH^-]_o^i - [OH^-]_i^i), \tag{2b}$$

where $P_H = D_H/d$, $P_{OH} = D_{OH}/d$ are the permeability coefficients for protons and hydroxide ions, d the membrane thickness, $[H^+]^i$ and $[OH^-]^i$ the concentrations at the membrane/water interface (superscript i). The subscripts i and o denote inside and outside of the vesicles and positive fluxes are in outward direction.

The net flux, j_{net}, responsible for any pH-change, is

$$j_{net} = j_H - j_{OH} = -P_H[H^+]_o^i\left(1 - \frac{[H^+]_i^i}{[H^+]_o^i}\right) + P_{OH}[OH^-]_o^i\left(1 - \frac{[OH^-]_i^i}{[OH^-]_o^i}\right). \tag{3}$$

According to assumption (*a*), the interface concentrations are proportional to the bulk concentrations and one can use $[H^+] \cdot [OH^-] = 10^{-14}\ M^2$ to rearrange Eq. 3

$$j_{net} = -P_H[H^+]_o^i\left(1 - \frac{[H^+]_i^b}{[H^+]_o^b}\right) + P_{OH}[OH^-]_o^i\left(1 - \frac{[H^+]_o^b}{[H^+]_i^b}\right), \tag{4}$$

where the superscript b denotes the bulk concentrations. The entire concentration change of protons per unit time averaged over the vesicle interior, which results from transmembrane flux, can be expressed as

$$\frac{d[H^+]}{dt} = -j_{net} \cdot \frac{A}{V}, \tag{5}$$

where A is the vesicle surface area (e.g., calculated for the middle of the bilayer thickness) and V is the vesicle internal volume. Taking into account interactions of these protons with all buffering substances of the internal vesicle system (i.e., added buffer, pH-sensitive dye molecules, and the head groups of the lipids), the following equation is obtained:

$$\frac{dpH_i}{dt} = \frac{dpH_i}{d[H^+]_i} \cdot \frac{d[H^+]_i}{dt} = -\frac{1}{B}\frac{d[H^+]_i}{dt} = j_{net}\frac{A}{VB}, \tag{6}$$

where $B = -(d[H^+]/dpH)$ is the overall buffering power ($d[H^+]$ is the added concentration of protons and dpH the resulting alteration of the pH-value) and pH values refer to the bulk pH. The buffering power of the buffer, the dye molecules, and the lipid head groups can either be determined from separate acid or base titration experiments, or calculated according to

$$B = \ln 10\left([H^+] + [OH^-] + \sum \frac{c_b k[H^+]}{(k + [H^+])^2}\right) \tag{7}$$

(Σ over all buffering substances), where k is the equilibrium constant of the protonation reaction $P + H^+ \rightleftharpoons PH^+$, and $c_b = [P] + [PH^+]$ the total concentration of the buffering molecules.

Substitution of Eq. 4 into Eq. 6 leads to

$$\frac{dpH_i}{dt} = \frac{A}{VB}\left[-P_H[H^+]_o^i\left(1 - \frac{[H^+]_i^b}{[H^+]_o^b}\right) + P_{OH}[OH^-]_o^i\left(1 - \frac{[H^+]_o^b}{[H^+]_i^b}\right)\right]. \tag{8}$$

In order to linearize the nonlinear differential equation with respect to $\Delta pH = pH_i - pH_o$, one can expand $[H^+]_i^b/[H^+]_o^b$ for small ΔpH (i.e., $\ln 10 \cdot \Delta pH \ll 1$) into a Taylor series

$$\frac{[H^+]_i^b}{[H^+]_o^b} = 10^{-\Delta pH} = \exp[\ln 10 \cdot (-\Delta pH)] \approx 1 - \ln 10 \cdot \Delta pH.$$

Since pH_o remains constant during the experiment

$$\frac{dpH_i}{dt} = \frac{d\Delta pH}{dt} = -\frac{A \cdot \ln 10}{VB} \cdot (P_H[H^+]_o^i + P_{OH}[OH^-]_o^i)\Delta pH. \quad (9)$$

Any small pH-gradient across the bilayer of a single vesicle should decay according to

$$\Delta pH(t) = \Delta pH(t = 0) \cdot \exp(-t/\tau) \quad (10)$$

with a lifetime

$$\tau = \frac{BV}{A \ln 10(P_H[H^+]_o^i + P_{OH}[OH^-]_o^i)}. \quad (11)$$

Relation 11 could be used, in principle, to separate P_H from P_{OH} by variation of pH (see Discussion). Concentration differences between bulk and interface are now neglected and for small pH-changes around pH 7.0 it is further assumed that

$$[H^+]_o^b \approx [H^+]_o^i \approx [OH^-]_o^i. \quad (12)$$

Only for this crude approximation it is justified to define the widely used net permeability coefficient

$$P_{net} = P_H + P_{OH}. \quad (13)$$

For a population of vesicles with a size distribution an averaged lifetime $\langle\tau\rangle$ is used

$$\langle\tau\rangle = \left\langle\frac{VB}{A}\right\rangle \frac{1}{P_{net}[H^+]_o^b \ln 10}, \quad (14)$$

which allows calculation of P_{net} from the experimental data.

We have tried to assess the linear relation between the buffering power in the vesicle interior and the decay time τ. Such a linear relation is already implied in Eq. 6, which does not refer to a specific flux mechanism. Indeed any proton/hydroxide ion flux, which decays in an exponential manner, should therefore exhibit such proportionality between decay time and buffering power. This allows determination of the influence of the lipid headgroups on the buffering power of the whole liposome interior by time-dependent flux measurements without worrying about the specific ΔpH-decay mechanism involved.

RESULTS

Determination of Vesicle Sizes

Fig. 1 *a* and *b* show the size distributions of SBPL and diphytanoyl PC vesicles as determined by electron-microscopy. Outer mean radii are 148 ± 74 Å for SBPC and 322 ± 157 Å for diphytanoyl PC vesicles, respectively. The mean hydrodynamic radius of 120 Å determined by quasi-elastic light-scattering for SBPL vesicles is in good agreement with the electron-microscopic data. Mean diameters for both vesicle species correspond also well to the mean

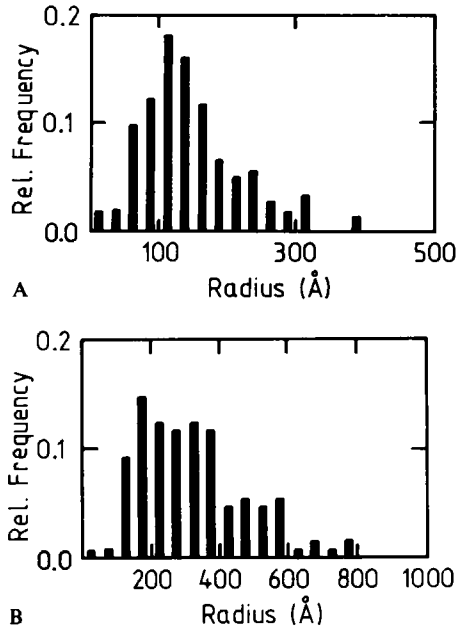

FIGURE 1 Size distribution of sonicated SBPL (*a*) and diphytanoyl PC (*b*) vesicles as determined by negative-stain electron-microscopy.

diameters of sonicated egg-phosphatidylcholine vesicles (300 Å) and sonicated diphytanoyl PC vesicles (550 Å) observed by Cafiso and Hubbell (1983). For calculation of geometrical parameters needed in Eq. 14 we assumed a membrane thickness of 55 Å. Means of several geometrical parameters as determined by the size distribution depicted in Fig. 1 *a* and *b* are listed in Table I.

Determination of Buffering Power

To determine the buffering power B of the phospholipids in vesicular form, titrations of vesicle solutions were performed in the presence of KCl and small amounts of valinomycin and CCCP (Fig. 2 *a*, *b*, and *c*). The joint action of both ionophores should ensure rapid equilibration of a pH-gradient across the vesicular membrane (Mitchell and Moyle, 1967). For SBPL vesicles addition of KOH

TABLE I
MEAN PARAMETERS OF VESICLE GEOMETRY AS DETERMINED BY THE RADII DISTRIBUTION (FIG. 1)

Parameter	SBPL	Diphytanoyl PC
$\langle r_0\rangle$*	148	322
$\langle V_i/A_m\rangle$*	21	75
$\langle V_i\rangle$‡	$1.23 \cdot 10^8$	$1.71 \cdot 10^9$
$\langle A_i\rangle$§	$1.82 \cdot 10^5$	$1.19 \cdot 10^6$
$\langle A_i/A_m\rangle$	0.514	0.773

r_0 is the outer radius, V_i is the internal volume, A_m is the surface area at midpoint, and A_i is the inner surface area. A membrane thickness of 55 Å was assumed.
*In Angstroms.
‡In Angstroms cubed.
§In Angstroms squared.

(Fig. 2 *a*) or HCl resulted in the same titration curves and was highly reproducible. Titration experiments of diphytanoyl PC vesicles (Fig. 2 *b*), however, were far less reproducible in the range pH 5–pH 11. This seems mainly due to a very slow pH-exchange (>15 min at 30°C) between vesicle inside and outside even in the presence of high amounts of the ionophores, so that determination of stable pH-values proved difficult. This is also reflected in the hysteresis-like curve for titration with KOH and HCl (Fig. 2 *b*). pH-equilibration below pH 5 and above pH 11 was very fast (<3 min). One possible explanation for this behavior is that the branched phytanoyl chains severely hinder the diffusion of the ionophores. For both lipids the titration curve in the range pH 6.8–pH 7.4 was highly linear in all experiments and allowed calculation of a constant buffering power B by straight line fits. To obtain an independent estimation for the buffering power of the PC headgroups of diphytanoyl PC, suspensions of DMPC vesicles were titrated in a similar manner above the phase transition temperature, i.e. at 30°C (Fig. 2 *c*). The buffering power of the pH-indicator dye pyranine (Fig. 2 *d*) and of the phosphate buffer used (data not shown) was also determined by titration.

As a measure of the buffering power of one single buffer molecule, the buffering power B of the solution is normalized with respect to the buffer concentration c_b. The ratio B/c_b gives the number of protons which must be removed from one buffer molecule in the solution to obtain a pH-change of 1. If the equilibrium constant k of the protonation reaction is known, one can also calculate the ratio B/c_b according to Eq. (8). Table II summarizes experimental and calculated values of the ratio B/c_b in the range pH 6.8–pH 7.4 for the constituents of the vesicle interior. Since HCl, HSO_4^- and H_2SO_4 are very strong acids and KOH is a very strong base, their buffering powers have not to be taken into account at pH 7. For phosphate and pyranine good agreement between calculated and measured ratios B/c_b is observed.

ΔpH-Relaxation Measurements

Fig. 3 depicts a typical time-dependent fluorescence signal I_{460}/I_{400} of pyranine loaded SBPL vesicles after inducing a pH-jump in the vesicle outside medium from pH 7.0 to pH 7.2. The intensity ratio I_{460}/I_{400} depends in a linear way on the pH in the vesicle interior (Kano and Fendler, 1978; Clement and Gould, 1981; Biegel and Gould, 1981). Fig. 3 shows also a one-exponential fit to the data points and resulting residuals. As can be seen from the residuals a slight deviation exists from the one-exponential decay. This deviation is always observable both in samples containing valinomycin in order to destroy a diffusion potential (see

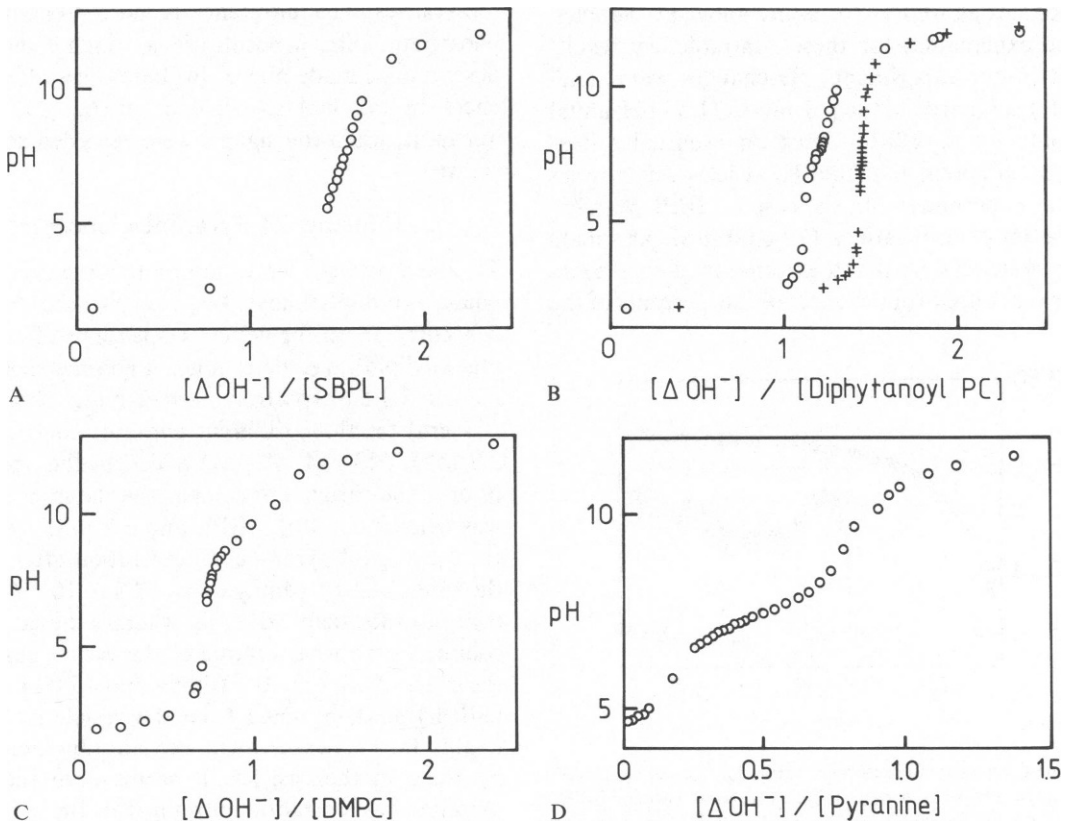

FIGURE 2 Titrations by KOH (O) and HCl (+) for determination of buffering power. (*a*) to (*c*) Vesicle suspensions containing 20 mg/ml lipid, 100 mM KCl, 25 μM CCCP, 10 μM valinomycin: (*a*) SBPL at 25°C, (*b*) diphytanoyl PC at 30°C and (*c*) DMPC at 30°C. (*d*) Titration of 7 mM pyranine at 25°C.

TABLE II
VALUES OF THE RATIO B/c_b IN THE RANGE pH 6.8–7.6

	B/c_b Titration	B/c_b Calculated at pH 7.0
SBPL	$6.0 \cdot 10^{-2}$	
diphytanoyl PC	$1.5 \cdot 10^{-2}$	
DMPC	$2.4 \cdot 10^{-2}$	
pyranine	$4.4 \cdot 10^{-1}$	$5.4 \cdot 10^{-1}$*
phosphate	$5.1 \cdot 10^{-1}$	$5.6 \cdot 10^{-1}$‡

*pK = 7.22 (Kano and Fendler, 1978).
‡pK = 7.12.

below) as well as in preparations containing no valinomycin (not shown). There is thus no indication for a biphasic decay due to limiting counterion fluxes (Clement and Gould, 1981; Biegel and Gould, 1981). We neither observed any very fast component of the intravesicular pyranine signal up to the instrumentally limited observation time of 5 ms. Biegel and Gould (1981) found that the stopped-flow mixing device itself had an influence on the decay kinetics of ΔpH monitored by pyranine in SBPL vesicles, resulting in a monophasic decay observed in the stopped-flow device and a biphasic decay upon manual mixing in a cuvette. This possibility was ruled out for our experiments by comparative measurements of samples mixed in a normal fluorescence cuvette. No differences with respect to the stopped-flow experiments were noticed for SBPL vesicles as well as for diphytanoyl PC vesicles. One possible explanation for these contradictory results may be that in our experiments pH-changes were small (0.2 pH-units) compared to the pH-jumps (1.55 pH-units) of Biegel and Gould (1981). Thus an eventually flux-limiting diffusion potential should be at least seven times smaller in our experiments. In the case of SBPL vesicles, for higher buffer concentrations (70–100 mM phosphate buffer in the vesicle internal and external medium), there was a more pronounced (in the order of few percents of the total signal), very slow (τ > 10,000 s) increase of the fluorescence intensity ratio I_{460}/I_{400}. With diphytanoyl PC vesicles the additional signal increase was even more accentuated. There can be several factors contributing to deviations from an ideal one-exponential decay. At first the flux mechanism itself may not exhibit exponential characteristics. In the case of normal diffusion flux described by Eq. 7, the approximation ΔpH · ln10 ≪ 1 was made in order to obtain a linear differential equation. This condition is certainly not completely fulfilled (ΔpH = 0.2). Secondly as there are vesicles of different sizes one has to average solutions of the flux equation over the whole size distribution of vesicles in order to describe the experimental situation. This will lead to a decay function that differs from the simple solution of the flux equation. Finally, the intensity ratio I_{460}/I_{400} is not independent of light scattering changes as light scattering is proportional to λ^{-3}. Indeed the solutions cannot be stirred in the small stopped-flow fluorescence cuvette so that there may be artefacts from sedimentation of the vesicles during long-time measurements. There is also a slight difference of solute concentration across the membrane (vesicle interior pH 7.0: 100 mM phosphate + sodium counterions + 50 mM K_2SO_4: total concentration: 411 mM; outside bulk solution pH 7.2: 100 mM phosphate + sodium counterions + 50 mM K_2SO_4: total concentration: 423 mM) resulting in an osmotic pressure difference and maybe subsequent compression of the vesicles. This difference is more pronounced for high phosphate buffer concentrations, which coincides with the observations made above. In these cases also slight differences in the kinetic behavior of I_{460} and I_{400} became apparent, when the signals were recorded separately (not shown).

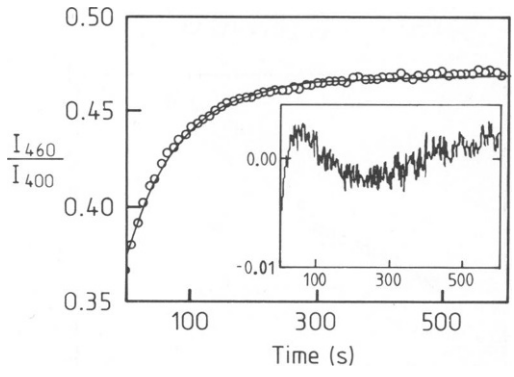

FIGURE 3 Time-dependent fluorescence ratio I_{460}/I_{400} of 2.2 mM pyranine enclosed in vesicles after rapid mixing (1:1) of 0.5 mg/ml SBPL vesicles, $3.2 \cdot 10^{-3}$ mol valinomycin/mol lipid, 10 mM phosphate, 50 mM K_2SO_4, pH 7.0 with 10 mM phosphate, 50 mM K_2SO_4, pH 7.5 (25°C). Solid line shows one-exponential fit to data points (O). Deviations from one-exponential behavior are depicted in the inset.

Influence of Pyranine Concentration

To assess the concentration of pyranine entrapped in or bound to diphytanoyl PC vesicles, the total pyranine concentration in the vesicle suspensions after dialysis (0.5 mg/ml lipid) was determined fluorometrically in a Shimadzu RS 540 spectrofluorometer (λ_{ex} = 460 nm, λ_{em} = 508 nm) for three different pyranine concentrations (i.e., 2.2 mM, 440 μM, 88 μM) added to the lipid suspension prior to sonication. For comparison the same measurement was carried out with SBPL and 2.2 mM pyranine. In all cases the total pyranine concentration after dialysis is in the range of 10^{-4} (diphytanoyl PC) to 10^{-5} (SBPL) of the concentration before dialysis, whereas the ratio of the inner volume to the total volume of the vesicle suspension is in the order of 10^{-3} to 10^{-2} (diphytanoyl PC) or 10^{-4} to 10^{-3} (SBPL) as determined from the vesicle size distribution (Table I). This experiment excludes any accumulation of pyranine in the vesicles. It seems even that the actual pyranine concentration entrapped in the vesicles is lower than the initial one, possibly due to dye leakage during vesicle preparation and prolonged dialysis.

The amount of external pyranine was assayed by com-

paring fluorescence intensities (λ_{ex} = 460 nm) of the dialysed vesicle suspensions measured before and directly after (5 ms) a pH-change was induced in the outside medium, as well as after complete decay of the pH-gradient. For all three pyranine concentrations and for both lipids, after triple dialysis ~40% of the signal change occurred faster than 5 ms, thus indicating that this percentage of dye may be bound to the outer vesicle surface or is free in the outer bulk phase. However, this fraction can be reduced to 10–20% by further dialysis so that significant binding of the dye to the lipid surface seems rather improbable. This assumption was supported by the determination of fluorescence polarisation (λ_{ex} = 460 nm, λ_{em} = 508 nm), yielding values of $1.5 \cdot 10^{-2}$ in the case of SBPL and $2.5 \cdot 10^{-2}$ for diphytanoyl PC, respectively. This result is in reasonable agreement with the data of Kano and Fendler (1978) for anionic and neutral single compartment vesicles. In any way, ΔpH-decay times, as measured by signals slower than 5 ms, which refer to pyranine inside the vesicles, were the same for all three pyranine concentrations. It can thus be concluded that pyranine concentration had no effect on ΔpH-relaxation rates.

It is worthwhile to mention, that even for a completely surface bound internal pH-indicator dye the determination of the H^+/OH^- permeability and the buffer capacity according to Eq. 14 would not be affected as long as monitored signal changes are proportional to internal bulk pH-changes—a condition which should be always met for sufficiently small pH-gradients.

Abolishment of Diffusion Potential by Valinomycin

To ensure that no diffusion potential was limiting the ΔpH-decay, it was first attempted to titrate SBPL vesicle solutions with the potassium carrier valinomycin by simply adding valinomycin in ethanol to the potassium containing samples before mixing. In these experiments the ΔpH-decay time decreased drastically, but a limiting value was only reached at valinomycin concentrations as high as 1 mmol/g lipid (i.e., ≈1 molecule valinomycin/1 molecule lipid). Biegel and Gould (1981) found similar high valinomycin concentrations and ascribed the resulting decay time to the intrinsic membrane proton permeability. It is obvious, however, that such high ionophore to lipid ratios will result in a completely different behavior of the membrane. The fact that only such high valinomycin concentrations yielded limiting time constants for the ΔpH-decay may be due to the following reasons: Addition of ethanolic solutions to the samples always leads to contamination by organic solvent, the effect of which is not precisely known (Cafiso and Hubbell, 1983; Gutknecht, 1984). Furthermore it cannot be completely excluded that valinomycin acts itself to a small extent as a protonophore (Nichols and Deamer, 1980; Lev and Buzhinsky, 1967) and that even very low concentrations of valinomycin in the membrane may disturb the lipid order (see below). We have performed similar titration experiments of SBPL vesicles with the lipophilic ions tetraphenylphosphonium and tetraphenylborate in the presence of 100 mM KCl. In these experiments also very high concentrations of the lipophilic cation or anion (≈5 mmol/g lipid) were required to reach saturation values of the ΔpH-decay constants. Probably the same arguments as given for valinomycin account for these high saturation concentrations, and we do not ascribe the limiting time constants reached to the intrinsic membrane conductance for protons or hydroxide ions.

If one assumes that valinomycin shows the same behavior in liposomes as in planar lipid bilayers, one can roughly estimate the time needed to collapse a given diffusion potential. Due to its high partition coefficient (lipid/water ≈50,000, P. Läuger, private communication), almost all valinomycin present in liposome solutions will reside in the lipid membrane. The turnover rate of valinomycin has a value of $10^4 - 10^5\ s^{-1}$ (Stark et al., 1971). Starting from this turnover rate and assuming conservative values for the

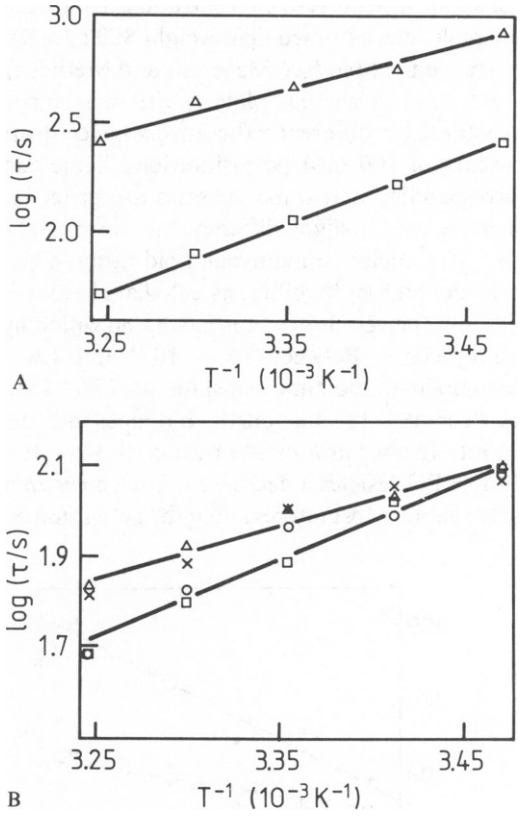

FIGURE 4 Arrhenius plots of one-exponential ΔpH-decay constants as monitored by pyranine for different concentrations of valinomycin added solvent-free to the lipids. Vesicle suspensions: 0.5 mg/ml lipid, 10 mM phosphate, 100 mM KCl, pH 7.0, molar valinomycin/lipid ratios of (△) 0, (x) $1.6 \cdot 10^{-4}$, (□) $1.6 \cdot 10^{-3}$ and (○) $3.2 \cdot 10^{-3}$. Mixing buffer solution: 10 mM phosphate, 100 mM KCl, pH 7.5. Data points represent means of at least three measurements. Straight-line fits are given for molar valinomycin/lipid ratios of 0 and $3.2 \cdot 10^{-3}$. (*a*) SBPL and (*b*) diphytanoyl PC.

TABLE III
ONE EXPONENTIAL ΔpH-DECAY CONSTANTS τ AT 25°C AND ACTIVATION ENERGIES E AS DEPICTED IN FIG. 4

[valinomycin]/[lipid]	0	$1.6 \cdot 10^{-4}$	$1.6 \cdot 10^{-3}$	$3.2 \cdot 10^{-3}$
τ_{SBPL}	466.5 ± 13.1	426.4 ± 20.1	129.4 ± 4.6	112.9 ± 1.9
E_{SBPL}	9.4	—	—	13.9
E_{SBPL}*	—	—	—	16.6
$\tau_{diphytanoyl\ PC}$	101.0 ± 2.3	101.9 ± 3.6	76.6 ± 0.7	91.8 ± 1.3
$E_{diphytanoyl\ PC}$	5.2	5.6	8.5	7.8
$E_{diphytanoyl\ PC}$*	—	—	—	12.6

Time constants in seconds, energies in kilocalories per mole.
*Same conditions as in Fig. 4 except that 100 mM KCl was replaced by 50 mM K_2SO_4.

coefficients in our experiments (B = 150 mM, ΔpH = 0.25, r = 500 Å and a molar valinomycin/lipid ratio of $3.2 \cdot 10^{-3}$), valinomycin should ensure that an equilibrium according to the Goldman-Hodgkin-Katz equation with very high permeabilities and nearly equal concentrations on both sides of the membrane for potassium ions is reached within 3 ms. This means that the diffusion potential arising from proton/hydroxide ion flux should collapse almost completely within 3 ms.

For the reasons given above, we attempted to assess the effect of small valinomycin concentrations (0 to $3.2 \cdot 10^{-3}$ per lipid molecule, assumed lipid weight 800 g/mol) added solvent-free to the lipid (see Materials and Methods). Figs. 4 *a* and *b* show Arrhenius plots of the monoexponential time constants for different valinomycin concentrations in the presence of 100 mM potassium ions. Time constants and corresponding activation energies are given in Table III. There is only a slight difference in values between 0 and $1.6 \cdot 10^{-4}$ molar valinomycin/lipid ratios. This corresponds to the high probability, as calculated from Poisson statistics and the vesicle size, for having no valinomycin at all in a liposome. Between $1.6 \cdot 10^{-4}$ and $1.6 \cdot 10^{-3}$ valinomycin/lipid the time constant at 25°C for SBPL vesicles decreases to one fourth, but does not diminish significantly further at a concentration of $3.2 \cdot 10^{-3}$. For diphytanoyl PC vesicles a decrease in time constants is not as clearly visible. Most interestingly the activation energies

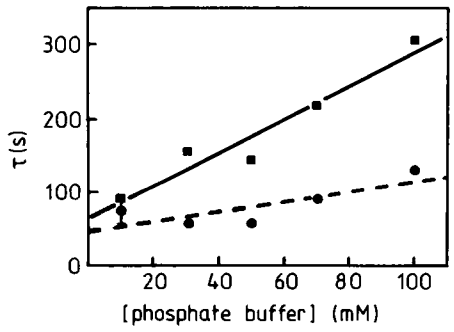

FIGURE 5 ΔpH-decay time constants for varying amounts of phosphate buffer entrapped in vesicles at 25°C. Vesicle suspensions: 0.5 mg/ml lipid, $3.2 \cdot 10^{-3}$ mol valinomycin/mol lipid, 100 mM KCl, x mM phosphate, pH 7.0. Mixing buffer: 100 mM KCl, x mM phosphate, pH 7.5. (●) SBPL and (■) diphytanoyl PC.

rise for both lipids upon addition of valinomycin. We have no obvious explanation for this effect, but even at low concentrations valinomycin may induce a more rigid or/and ordered lipid phase which in turn hinders proton/hydroxide ion flux. From these experimental results and the rough estimate of $\Delta\psi$-decay time we conclude that a molar valinomycin/lipid ratio of $3.2 \cdot 10^{-3}$ in the presence of 100 mM potassium ions is sufficient to prevent limitation by counterion flux to the decay of small pH-gradients of 0.2 units (corresponding to a Nernst-potential of 12 mV). In all further experiments described, this valinomycin concentration was used and added solvent free to the lipid.

Dependence of ΔpH-Relaxation Time Constants on Buffering Power

To test the linear relationship between the relaxation time constant τ of a pH-gradient and the buffering power B of the vesicle interior stated in Eq. 14 and in the more general Eq. 6, the phosphate buffer concentration entrapped in the vesicles (pH 7.0) as well as the phosphate buffer concentration of the mixing solution (pH 7.5) was varied from 10 mM to 100 mM. Experiments were carried out with SBPL and diphytanoyl PC in the presence of 100 mM KCl or 50 mM K_2SO_4 (Fig. 5 and 6). In the latter case ionic strength, which varies due to the variation of phosphate concentration, was kept constant at a value of 208 mM by addition of changing amounts of Na_2SO_4. We observed, however, no significant difference whether Na_2SO_4 was present or not (Fig. 6 *a*). Figs. 5 and 6 depict straight line fits to all data points; values for their slope and interception with the buffer concentration axis are listed in Table IV. The errors indicated for SBPL vesicles (Fig. 5 and 6 *a*) were obtained as standard deviations from the measurements of three independently prepared samples. Different preparations of diphytanoyl PC show lower variations of time constants, and also reproducibility for different measurements of the same samples is by far higher (Table III). We ascribe the relatively large variations of time constants in the case of SBPL vesicles to possible oxidation of unsaturated hydrocarbon chains as well as to general inhomogeneities of this lipid mixture. It was observed by Cafiso and Hubbell (1983) that small traces of hydrocarbon solvent could

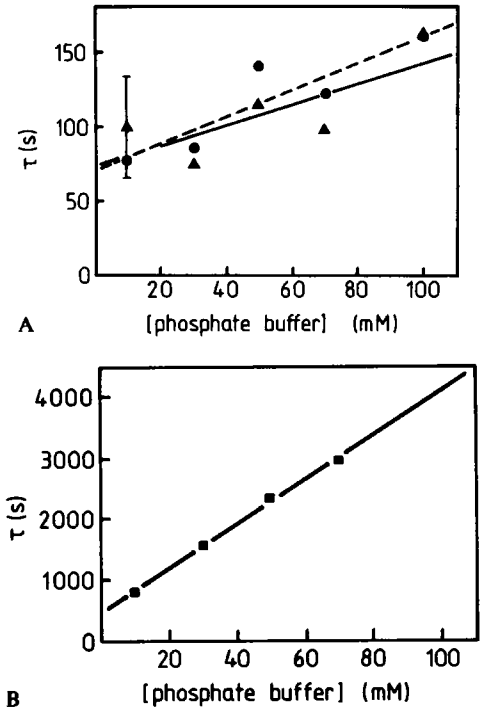

FIGURE 6 (*a*) SBPL: (▲) same as Fig. 5 (SBPL) except that 100 mM KCl was replaced by 50 mM K_2SO_4. (●) same as (▲) except that ionic strength was kept constant at 208 mM by varying amounts of Na_2SO_4. (*b*) Diphytanoyl PC: same conditions as *a* with ionic strength kept constant.

enhance electrogenic H^+/OH^- movement detected by paramagnetic hydrophobic ions. In experiments with SBPL vesicles in the presence of 50 mM K_2SO_4, however, no difference in time constants was found whether the valinomycin containing lipid had been dried for only 1 h at 1 Torr or additionally for 20 h at 10^{-3} Torr (room temperature).

In summary, the following results were obtained from these "titration" experiments:

(*a*) a linear relationship between buffering power B inside the vesicles and ΔpH-relaxation time τ is demonstrated within experimental error. This is in agreement with the general flux equation (Eq. 6) and also with Eq. 14 derived for a simple combined diffusion flux of protons and hydroxide ions. Eq. 14 allows calculation of net combined permeability for protons and hydroxide ions from the slope of the straight line fits in Figs. 5 and 6 according to

$$P_{net} = \left\langle \frac{V}{A} \right\rangle \frac{1}{[H^+]_o \ln 10 \dfrac{d\langle\tau\rangle}{dB}}. \qquad (15)$$

The change in buffering power dB is proportional to the change in phosphate concentration dc_p and can be expressed as $dB = 0.51\ dc_p$, where the experimental value of B/c_b for phosphate in Table II has been used. Values for P_{net} calculated from the slope $d\langle\tau\rangle/dB$ are independent of the buffering power of the lipid headgroups (see below) which is, in general, unknown. Table V lists values for P_{net} as derived by Eq. 15 for the experiments in which K_2SO_4 has been used.

(*b*) From the data listed in Table IV, there is clear evidence that the presence of chloride ions accelerates the decay of ΔpH in diphytanoyl PC vesicles. A result, which has also been obtained for planar lipid bilayers of egg phosphatidylcholine/decane by Gutknecht and Walter (1981*a*). Within experimental error the ΔpH-decay across the membrane of SBPL vesicles is not significantly accelerated by the presence of chloride ions at 25°C (Fig. 5 and 6 *a*). However, the activation energy for the transport process is lowered for both vesicle species by 3 to 5 kcal/mol in the presence of chloride (Table III).

(*c*) Obviously the relaxation times plotted in Fig. 5 and 6 have a nonzero value for extrapolation to zero phosphate buffer concentrations. This can be explained by the residual buffering power inside the vesicles, i.e., the buffering power of the used dye pyranine and of the lipid headgroups. In this way the buffering power of the lipid headgroups within closed vesicles can be determined and compared to the buffering power derived from normal titration experiments (Table II). For the calculation again Eq. 14 is used, assuming that the total buffering power B inside the vesicles is given by

$$B = B_{lipid} + B_{pyranine} + B_{phosphate}. \qquad (16)$$

According to Eq. 14 the decay time $\langle\tau\rangle$ will have a zero value when $\langle BV/A\rangle$ becomes zero. Therefore B_{lipid} can be obtained from the interception of the straight line fits with the abscissa (Table IV). It was assumed that the lipid headgroup occupies an area of 60 Å^2 in the inner vesicle surface, and the experimental buffering powers for pyranine and phosphate listed in Table II were substituted into

TABLE IV
STRAIGHT-LINE FITS TO DATA POINTS IN FIGS. 5 AND 6

Figure	5(●)	5(■)	6*a*(▲)	6*a*(●)	6*b*
$\frac{d\tau}{d[\text{phosphate}]}$*	0.79	2.3	0.68	0.89	36
Interception‡ with abscissa	−68	−29	−110	−79	−14

*In s/mM.
‡In mM.

TABLE V
NET PROTON/HYDROXIDE ION PERMEABILITIES, P_{net}

Electrolyte	SBPL	diphytanoyl PC
K_2SO_4 (Fig. 6 *a*[▲])	$2.1 \cdot 10^{-3}$	—
$K_2SO_4 + Na_2SO_4$ (Fig. 6 *a*[●] and 6 *b*)	$2.7 \cdot 10^{-3}$	$1.4 \cdot 10^{-4}$

Permeabilities are given in cm/s.

Eq. 16. As there is only a maximum concentration of 2.2 mM inside the vesicles, its buffering power contributes, however, only to a very small extent in Eq. 16. This was confirmed by control experiments with reduced pyranine concentrations. Special care was taken to use the correct mean geometrical parameters in order to calculate $\langle BV/A \rangle$. All mean values needed for this calculation are listed in Table I. The relative buffering powers B/c_b for the lipid headgroups as determined by this method are listed in Table VI.

DISCUSSION

Intrinsic Buffering Power of Lipid Headgroups in the Vesicle Interior

We have described a method to determine the buffering power of lipid headgroups in the vesicle interior by the measurement of ΔpH-kinetics. This method should be independent of the specific proton/hydroxide ion flux mechanism involved, if the pH-gradient is sufficiently small (Eq. 6). For the correct evaluation of proton/hydroxide ion fluxes across vesicular membranes, knowledge of the buffering power of the internal vesicular volume is, however, essential. Several authors (Nichols et al., 1980; Nozaki and Tanford, 1981; Rossignol et al., 1982; Cafiso and Hubbell, 1983) have used, sometimes implicitly, this value, in order to calculate H^+/OH^- permeabilities, but no reference to the contribution of lipid headgroups was made.

Our method requires the independent preparation of several vesicle suspensions containing varying amounts of internal bulk buffer. Due to relatively large variations of ΔpH-decay times for independently prepared samples, some statistical error may have been introduced. Therefore we do not believe that the values for the relative buffering powers of the lipid headgroups as stated in Table VI significantly depend on the different electrolyte solutions (potassium chloride, potassium sulphate, or sodium and potassium sulphate) used. However, this possibility may not be completely ruled out. The mean values of the relative buffering powers per single lipid molecule for the different electrolytes ($6.3 \cdot 10^{-2}$ for SBPL vesicles and $3.5 \cdot 10^{-2}$ for diphytanoyl PC vesicles in the range pH 7.0–pH 7.2) agree surprisingly well with the relative buffering powers found from simple titration of vesicle suspensions in the presence of uncouplers ($6.0 \cdot 10^{-2}$ for SBPL, $1.5 \cdot 10^{-2}$ for diphytanoyl PC and $2.4 \cdot 10^{-2}$ for DMPC vesicles, respectively). In fact the only assumption necessary to derive Eqs. 6 and 16 is that chemical equilibrium between bulk and interface as well as within the bulk solutions on both sides of the membrane is established significantly faster than between both sides of the membrane. The good agreement between the "dynamic" and "static" titration method is thus in support of the idea that the rate limiting step for H^+/OH^- transport across the membrane is really the transition through the hydrophobic membrane core, and not the exchange between lipid bulk interface and the bulk solutions. This statement is, however, not in contradiction to the result of Teissié et al. (1985) that the proton conduction within the interface between lipid headgroups and water may be substantially higher than within the water itself.

TABLE VI
RELATIVE BUFFERING POWERS OF LIPID HEADGROUPS, B/c_b, AS DETERMINED BY ΔpH-RELAXATION

Electrolyte	SBPL	diphytanoyl PC
KCl (Fig. 5)	$5.0 \cdot 10^{-2}$	$4.9 \cdot 10^{-2}$
K_2SO_4 (Fig. 6 *a* [▲])	$8.1 \cdot 10^{-2}$	—
$K_2SO_4 + Na_2SO_4$ (Fig. 6 *a* [●] and 6 *b*)	$5.8 \cdot 10^{-2}$	$2.1 \cdot 10^{-2}$
Mean	$(6.3 \pm 1.6) \cdot 10^{-2}$	$3.5 \pm 2.0 \cdot 10^{-2}$

In any way, the values of the relative buffering power of lipid headgroups are quite large, if one considers their contribution to the overall buffering power of the vesicular interior. They correspond to an additional phosphate buffer concentration of 10–30 mM for diphytanoyl PC vesicles and of even 70–110 mM in the case of SBPL vesicles. A comparable value for SBPL has been determined recently by Sarti et al. (1985) and a theoretical explanation for the high buffering power of lipid headgroups in the neutral pH-range was discussed by Haines (1983). These results may have implications for the discussion between localized, semilocalized, and delocalized chemiosmotic theories. Another effect of this large contribution to the internal buffering power of vesicles is that its neglect may introduce some underestimation of transmembrane proton/hydroxide ion fluxes and therefore on the calculation of proton/hydroxide ion permeabilities P_{net}. A determination of P_{net} from the slope $d\langle\tau\rangle/dB$ excludes this error.

The Net Proton/Hydroxide Ion Permeability

Our values for the net H^+/OH^- permeability at pH 7.0 through the bilayer of lipid vesicles ($2.7 \cdot 10^{-3}$ cm/s and $1.4 \cdot 10^{-4}$ cm/s for SBPL and diphytanoyl PC, respectively) are in accordance with the previously published data of 10^{-3} to 10^{-6} cm/s for a variety of lipids in very different membrane systems (Nichols et al., 1980; Nichols and Deamer, 1980; Deamer and Nichols; 1983; Deamer, 1982; Cafiso and Hubbell, 1983; Rossignol et al., 1982; Gutknecht, 1984). The permeability of SBPL vesicles exceeds the value of diphytanoyl PC vesicles by a factor of ~20. In terms of the simple flux mechanism outlined in Eqs. 2 this behavior can be attributed to several reasons. For the calculation of $[H^+]_o^i$ in Eq. 11 the bulk value (pH_o 7.2) was used. In the case of the negatively charged lipid headgroups of SBPL the pH value at the lipid-water interface will, however, be lower. If protons (hydronium

ions) actually contribute more to the net permeability than hydroxide ions, this will lead to an overestimation of P_{net} as the proton concentration is underestimated at the interface. On the other hand, intrinsic effects such as a higher membrane tension due to the small size of SBPL vesicles as compared with diphytanoyl PC vesicles or a higher mobility of the unsaturated fatty acid chains of SBPL in comparison to the saturated phytanoyl hydrocarbon chains could contribute to the observed higher apparent permeability.

The ΔpH-decay for diphytanoyl PC vesicles is accelerated and energetically facilitated (ΔE = 4.8 kcal/mol, Table III) by the presence of chloride ions. A rather high permeability for molecular HCl has been determined recently (Gutknecht and Walter, 1981*a*), and it would be easy to incorporate a simple hydrochloric acid diffusion ($J_{HCl} = -P_{HCl}([HCl]_o^i - [HCl]_i^i)$) into the flux equations (Eqs. 2). However, actual calculation of a permeability for hydrochloric acid would require the knowledge of the dissociation constant of hydrochloric acid at the membrane/water interface and within the membrane. Due to the low dielectric constant attractive forces between protons (hydronium ions) and chloride ions will be many times greater in the hydrophobic membrane interior than in an aqueous solution (Walz et al., 1969). Therefore it is not justified to use the high dissociation constant of hydrochloric acid (*pK* of about −6; Perrin, 1969) in aqueous solutions for this calculation. Within experimental error the ΔpH-decay for SBPL vesicles is not accelerated by the presence of chloride ions at 25°C. Nevertheless, as in the case of diphytanoyl PC the activation energy for the transport process is reduced (ΔE = 2.7 kcal/mol, Table III). One possible explanation for the weaker influence of chloride is the net negative charge of headgroups in this lipid mixture, which will reduce the concentration of chloride ions at the membrane/water interface.

Since the exact nature of proton/hydroxide ion transport mechanism is not known, it is not possible to interpret our values of the activation energy on a molecular level. Even in the case of the simple diffusion flux which leads to Eqs. 11 and 14, the activation energy will depend at least on two different contributions, i.e., the temperature behavior of the frictional forces within the membrane and the temperature behavior of the partition coefficient between bulk and membrane/bulk interface for the permeating molecules.

Although still large variations in the observed H^+/OH^- permeabilities through lipid bilayers exist, it is now widely accepted that its value is substantially higher than the permeability for other monovalent ions. Proton/hydroxide ion permeabilities are almost in the range of the permeability of water (e.g., 10^{-3}–10^{-2} cm/s across the membrane of erythrocyte ghosts, Barton and Brown, 1964; Lawaczek, 1984). Therefore a mechanism of transport different from the simple diffusion of other ions as described in (generalized) Nernst-Planck equations by means of water strands through the membrane has been proposed by Deamer and Nichols (1980). Several variations of this mechanism have been discussed recently (Gutknecht, 1984). Apart from this order of magnitude argument, there has been some experimental evidence that Nernst-Planck equations may not describe the proton/hydroxide ion flux in a correct way. A nonlinear current-voltage relationship has been found by several authors (Krishnamoorthy and Hinkle, 1984; O'Shea et al., 1984*b*; Gutknecht, 1984). Though this finding could be explained by image forces in the generalized Nernst-Planck formalism (Neumcke and Läuger, 1969), also a voltage induced opening of single "channels" as reported by Kaufmann and Silman (1983) should be taken into consideration. In contrast to this non-ohmic current-voltage behavior, Cafiso and Hubbell (1983) derived a linear relation. These authors have measured time-dependent transmembrane voltages induced by pH-jumps and calculated the corresponding current by differentiation of the voltage with respect to time, assuming RC-characteristics of the membrane. The linear current-voltage relation was taken as an argument against the validity of generalized Nernst-Planck equations. However, the nonlinear current-voltage characteristics were derived by Neumcke and Läuger (1969) for steady-state conditions, where the voltage difference across the membrane is maintained externally. Thus the linear current-voltage relation for the non steady-state pH-jump only reflects an exponential approach of the transmembrane diffusion potential to Nernst equilibrium. Another and very serious challenge to the validity of Nernst-Planck flux equations comes from the observed proportionality of flux to ΔpH (Krishnamoorthy and Hinkle, 1984) and the apparent independency of $\Delta\psi$-induced flux from pH (Gutknecht, 1984). In Nernst-Planck equations fluxes must always be proportional to the concentration of diffusing charges. Because of its great generality, however, Eq. 1 should be a correct description of proton/hydroxide ion fluxes. The most essential restriction necessary to derive Nernst-Planck equations from Eq. 1 is the imposition that the diffusing species do not undergo chemical reactions during their transport. As pointed out by Nichols and Gutknecht (Gutknecht, 1984) recombination of protons and hydroxide ions within the membrane could explain, at least in part, the observed independency of permeabilities from pH. Numerous alternative flux mechanisms based on this recombination and formally described by Eq. 1 are conceivable.

Albeit the exact proton/hydroxide ion exchange mechanism is not known, the value of the buffering power of the lipid headgroup region observed in our investigation does not depend on the nature of the transport process. In addition to the importance for the calculation of the true net proton/hydroxide ion permeability, the high buffering power of the lipids might be of relevance for the understanding of energy-transducing processes across biological membranes. The lipid headgroup region could represent a

two-dimensional reservoir capable of storage and rapid transfer (Teissié et al., 1985) of protons from proton delivering reactions to proton consuming ones (e.g., ATP-synthase). Further work will be necessary to assay the contribution of the lipid headgroup region to chemiosmotic theories.

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