Determination of transmembrane pH gradients and membrane potentials in liposomes

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ABSTRACT Techniques for determining large transbilayer pH gradients (ΔpH) and membrane potentials ($\Delta\psi$) induced in response to ΔpH in large unilamellar vesicle liposomal systems by measuring the transbilayer redistribution of radiolabeled compounds have been examined. For liposomes with acidic interiors, it is shown that protocols using radiolabeled methylamine in conjunction with gel filtration procedures to remove untrapped methylamine provide accurate measures of ΔpH in most situations. Exceptions include gel state lipid systems, where transbilayer equilibration processes are slow, and situations where the interior buffering capacity is limited. These problems can be circumvented by incubation at elevated temperatures and by using probes with higher specific activities, respectively. Determination of ΔpH in vesicles with a basic interior using weak acid probes such as radiolabeled acetate in conjunction with gel filtration was found to be less reliable, and an alternative equilibrium centrifugation protocol is described. In the case of determinations of the membrane potentials induced in response to these pH gradients, probes such as tetraphenylphosphonium and thiocyanate provide relatively accurate measures of the $\Delta\psi$ induced. It is shown that the maximum transmembrane pH gradient that can be stably maintained by an egg phosphatidylcholine-cholesterol 100-nm-diam large unilamellar vesicle is ~3.7 units, corresponding to an induced $\Delta\psi$ of 220 mV or transbilayer electrical field of 5×10^5 V/cm.

INTRODUCTION

Recent work from this laboratory has been focused on the influence of transbilayer pH gradients in large unilamellar vesicle (LUV)[1] systems on the transbilayer distributions of lipophilic weak acids and bases, including a variety of drugs (1), ions (2), modified peptides (3), and lipids, including phospholipids (4, 5). An ability to measure the pH gradients (ΔpH) present across LUV membranes is clearly central to these and other investigations. A number of methods of measuring ΔpH in LUVs have been developed, based on early work demonstrating that the transmembrane ΔpH in organelles, such as chloroplasts and mitochondria, could be measured by determining the transmembrane distribution of weak bases such as ammonia (6). These methods employ nuclear magnetic resonance (7), electron paramagnetic resonance (8), or fluorescence (9, 10) techniques to determine the transbilayer distribution of appropriately labeled probes that are weak bases. Similar procedures can be used to measure membrane potentials ($\Delta\psi$) (11). In general, these procedures are tedious, require specialized equipment, and are usually restricted to ΔpH values of two units or less. As emphasized by Rottenberg (11), ΔpH values determined by probes that partition into the membrane can be misleading. Furthermore, the accuracy of specific probes, such as the fluorescent probe 9-aminoacridine as an indicator of ΔpH, is questionable (12).

In previous work, we have employed trace amounts of [^{14}C]methylamine to determine the ΔpH in LUVs. Due to the high permeability of the uncharged form of methylamine, rapid equilibration across the membrane is achieved (11). Protonation of the neutral form in the low pH environment of the vesicle interior results in a net accumulation of probe to achieve an equilibrium, where:

$$\frac{[MeNH_3^+]_{in}}{[MeNH_3^+]_{out}} = \frac{[H^+]_{in}}{[H^+]_{out}} \quad (1)$$

assuming that the interior and exterior proton concentrations are much larger than the dissociation constant of methylamine and that only the neutral form is membrane permeable. Separation of trapped probe from untrapped probe and the subsequent determination of entrapment allows the proton gradient to be measured. However, there are situations in which the accuracy of this technique can be compromised. First, as indicated above, to achieve equilibrium the neutral form of the probe must readily permeate the vesicle bilayer, leading to possible errors for relatively impermeable membranes. Second, the ratios of trapped to free [^{14}C]-$MeNH_3^+$ in liposomes are usually determined by removing exterior (untrapped) probe using centrifuged gel filtration minicolumns (7). However, once the vesicles enter the gel matrix, the system is not at equilibrium, resulting in possible efflux of the probe from the vesicles while the vesicles are on the column. Finally, the protonation of [^{14}C]$MeNH_2$ on arrival in the vesicle interior consumes a proton. Thus, the ΔpH is affected by the probe itself. In this work, we examine the extent to which these and other factors can compromise the accuracy of ΔpH measurements and describe procedures that avoid these difficulties. Furthermore, techniques to determine

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[1] *Abbreviations used in this paper:* CCCP, carbonyl cyanide mechlorophenyl hydrazone; CHES, 2-(*N*-cyclohexylamino)ethane-sulfonic acid; EPC, egg phosphatidylcholine; ΔpH, pH gradient; $\Delta\psi$, membrane potentials; DAPC, diarachidoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; LUV, large unilamellar vesicle; MES, 2-(*N*-morpholino)ethanesulfonic acid; TPP^+, tetraphenylphosphonium.

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TABLE 1 [^{14}C]-Glucose and [^{14}C]-citrate trapped volumes of 100-nm extruded vesicles

Lipid composition	Trapped volume	
	[^{14}C]-glucose	[^{14}C]-citrate
	liters/mol lipid	
EPC	1.78 ± 0.4 (8)	1.50 ± 0.1 (3)
EPC:cholesterol (55:45; mol/mol)	0.98 ± 0.28 (9)	0.841 ± 0.09 (3)

The amount of entrapped [^{14}C]-glucose (expressed as a volume equivalent) was determined after incubating vesicles at 45°C with 10 μCi/ml [^{14}C]-glucose. Citrate buffer space was determined by preparing vesicles in 300 mM citrate, pH 4.0, freezing in liquid nitrogen for 5-min cycles, and extruding through 100-nm filters. The number of experiments is given in parentheses.

the $\Delta\psi$ induced in response to these ΔpH's are also evaluated.

MATERIALS AND METHODS

All phospholipids were obtained from Avanti Polar Lipids, Inc. (Birmingham, AL), radiolabeled compounds from New England Nuclear (Mississauga, Ontario, Canada), and other chemicals and buffers from Sigma Chemical (St. Louis, MO). Cholesterol was incorporated in some lipid samples by colyophilization from benzene:methanol (70:30, vol/vol). Multilamellar vesicles of the stated lipid composition were prepared by adding the indicated buffer (generally 300 mM citrate buffer, pH 4.0, or 300 mM 2-(*N*-cyclohexylamino)ethane-sulfonic acid [CHES], pH 9.0) to the dry lipid powder at temperatures above the lipid gel-liquid crystalline phase transition temperature. Samples were vortexed for 15 min and then subjected to five cycles of freezing (>3 min in liquid nitrogen) and thawing to achieve equilibrium transbilayer solute equilibration (13). Large unilamellar vesicles were prepared by extruding these multilamellar vesicles 10 times through two stacked 100-nm filters as previously described (14). For saturated lipids, vesicle extrusion was performed above the gel-liquid crystalline phase transition temperature using a thermally jacketed extrusion apparatus (Lipex Biomembranes, Vancouver, British Columbia, Canada). Vesicles prepared by this method are unilamellar, with diameters of ~100 nm (14, 15).

The ΔpH was imposed by passing 300 μl of the vesicles down a 1-ml G-50 column equilibrated with the appropriate external buffer, usually 150 mM NaCl, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (Hepes), pH 7.0. Subsequently, these vesicles were diluted into the same buffer containing 1 μCi [^{14}C]$MeNH_3^+$/ml (or other radiolabel) to achieve a final lipid concentration of ~2 mM. The samples were incubated at the indicated temperatures, and at appropriate times 100-μl aliquots were removed and passed down 1-ml Sephadex G-50 minicolumns that were centrifuged for 3 min at 2,000 *g*. Entrapped probe was determined using liquid scintillation counting and phospholipid concentrations were determined using a phosphate assay (16). The ratios of entrapped to free concentrations of probe were determined using the measured internal aqueous volumes indicated in Table 1 for 100-nm vesicles. For samples containing significant amounts of tetraphenylphosphonium (TPP^+), lipid concentrations were determined by cholesterol assay (17).

For some samples, transmembrane probe distributions were determined using a "centrifree" equilibrium binding analysis apparatus (Amicon, Beverly, MA). After a 30-min incubation in the presence of the radiolabel under the conditions indicated above, 1-ml samples were placed in the upper filter chamber and the apparatus was centrifuged at 1,500 *g* for 5 min. Probe distributions were then determined.

The determination of ΔpH requires estimates of the interior (trapped) volume of the vesicle and the amount of trapped buffer. The interior volume was determined by preparing vesicles in 300 mM citrate, pH 4.0, exchanging the external buffer for 150 mM NaCl, 20 mM Hepes, 0.2% azide containing 10 μCi/ml [^{14}C]glucose and incubating the preparations for 24 h at 45°C. The half-time for glucose permeation through the membranes of 100 nm egg phosphatidylcholine (EPC): cholesterol (55:45; mol/mol) LUVs is ~1 h at 45°C (Mui, B., unpublished data). Entrapped [^{14}C]glucose was determined using gel filtration to separate free from entrapped material. The amount of trapped citrate buffer was established by adding 10 μCi/ml [^{14}C]citrate to the 300 mM citrate buffer used for hydration and determining citrate entrapment by gel filtration after freeze–thaw and extrusion through 100-nm pore size filters. As seen in Table 1, vesicles extruded through 100-nm filters have maximal aqueous trapped volumes as reflected by [^{14}C]-glucose trapping of 1.8 liters/mol lipid for EPC and 1.0 l/mol for EPC:cholesterol (55:45, mol/mol). The amount of citrate buffer entrapped corresponds to 1.5 and 0.84 liters/mol lipid, respectively. These values for aqueous volumes and buffer entrapment were used in the simulations presented here. It should be noted that the citrate buffer leaks very slowly from these vesicles, even in the presence of the imposed pH gradient (>90% retention after 2 h at 60°C for EPC vesicles).

Measures of membrane potential using [^{3}H]TPP^+ or ^{14}C-labeled thiocyanate were obtained in a similar manner as with [^{14}C]$MeNH_3^+$. For comparative purposes, electrical potentials are expressed as log ($[\text{probe}]_{in}/[\text{probe}]_{out}$). No corrections for probe binding were made (see Results).

Theoretical considerations

The pH of the LUV interior can be influenced by the influx of probe (which consumes a proton) and by proton efflux to set up a $\Delta\psi$ in electrochemical equilibrium with the ΔpH. We first consider the influence of amine accumulation on the interior proton concentration. For a buffer such as citrate with three titratable groups, it is straightforward to show that the concentration of the buffer in the neutral, fully protonated form ([B]), in the singly deprotonated form ([B^-]), and so on are related to the total buffer concentration $[B]^{tot}$ via the relations

$$[B] = \frac{[B]^{tot}}{f(H^+)} \quad (2)$$

$$[B^-] = \frac{K_1[B]^{tot}}{[H^+]f(H^+)} \quad (3)$$

$$[B^{2-}] = \frac{K_1K_2[B]^{tot}}{[H^+]^2f(H^+)} \quad (4)$$

$$[B^{3-}] = \frac{K_1K_2K_3[B]^{tot}}{[H^+]^3f(H^+)} \quad (5)$$

where

$$f(H^+) = (1 + K_1/[H^+] + K_1K_2/[H^+]^2 + K_1K_2K_3/[H^+]^3) \quad (6)$$

and K_1, K_2, and K_3 are the dissociation constants of the titratable groups. The proton concentration is indicated by $[H^+]$, and activity coefficients were ignored.

Each (neutral) methylamine that moves across the vesicle membrane consumes a proton as it is reprotonated in the vesicle interior. By charge balance, the final concentration of charged amine in the vesicle interior ($[AH^+]_i^f$) can thus be expressed as

$$[AH^+]_i^f = ([B^-]^b - [B^-]^a) + 2([B^{2-}]^b - [B^{2-}]^a) + 3([B^{3-}]^b - [B^{3-}]^a) \quad (7)$$

where the superscripts *a* and *b* indicate the original and final concentrations of the charged forms of the buffer, respectively.

At equilibrium, assuming that the charged form of methylamine does not partition appreciably into the vesicle bilayer, the final methylamine inside/outside concentration gradient must obey the relation

$$\frac{[AH^+]_i^f}{[AH^+]_o^f} = \frac{[H^+]_i^f}{[H^+]_o^f} \quad (8)$$

where the subscripts i and o refer to the inner and outer environments, respectively. Furthermore, writing K_a as the dissociation constant of methylamine (pK_a = 10.6), it is obvious that

$$[AH^+]_o^f = [A]^{tot} - V_i/V_o[AH^+]_i^f \quad (9)$$

where $[A]^{tot}$ is the total (initial) exterior concentration of methylamine, V_i is the interior (trapped) volume of the LUVs, and V_o is the external untrapped volume. Thus, combining Eqs. 8 and 9 and assuming that the exterior proton concentration does not vary significantly during probe uptake, we obtain

$$[H^+]_i^f = [H^+]_o[AH^+]_i^f/([A]^{tot} - (V_i/V_o)[AH^+]_i^f) \quad (10)$$

where $[H^+]_o$ is the initial external proton concentration and V_o is the total volume of the solution. By substituting Eqs. 2–6 in Eq. 7, the value of $[H^+]_i^f$ for a measured value of $[AH^+]_i^f$ can be calculated by an iterative process using Eqs. 7 and 10.

A second factor that can reduce the internal proton concentration results from the initial efflux of protons from the acidic interior to the exterior environment to set up an electrical potential ($\Delta\psi$). This equilibrium can be described by the Nernst relation for protons, assuming these are the only significantly permeant ions present:

$$\Delta\psi = \frac{RT}{F} \log \frac{[H^+]_i}{[H^+]_o} \quad (11)$$

where R, T, and F have their usual meanings. When it is necessary to ensure that the protons are the most permeant species, the proton ionophore carbonyl cyanide mechlorophenyl hydrazone (CCCP) can be employed. Under standard conditions RT/F = 59, where $\Delta\psi$ is expressed in millivolts. The number of proton equivalents required to set up this equilibrium can be estimated from the membrane capacitance relationship

$$Q = CA_m\Delta\psi \quad (12)$$

where Q is the charge in coulombs, A_m is the area of the membrane, and C is the membrane capacitance. The number of protons released is given by $N(H^+) = Q/e$, where e is the elementary unit of charge (1.6×10^{-19} C).

RESULTS

Influence of lipid composition and temperature

The first set of experiments was directed toward examining the influence of lipid composition and temperature on the ΔpH reported by the methylamine spin column technique. Vesicles composed of EPC, dipalmitoylphosphatidylcholine (DPPC) (16:0/16:0 PC), distearoylphosphatidylcholine (DSPC) (18:0/18:0 PC), and diarachidoylphosphatidylcholine (DAPC) (20:0/20:0 PC) alone and in combination with cholesterol (PC/cholesterol, 55:45, mol/mol) were therefore prepared by extrusion (200-nm pore-size filters) to exhibit a ΔpH of three units (inside acidic; pH_i 4.0, pH_o 7.0) as indicated

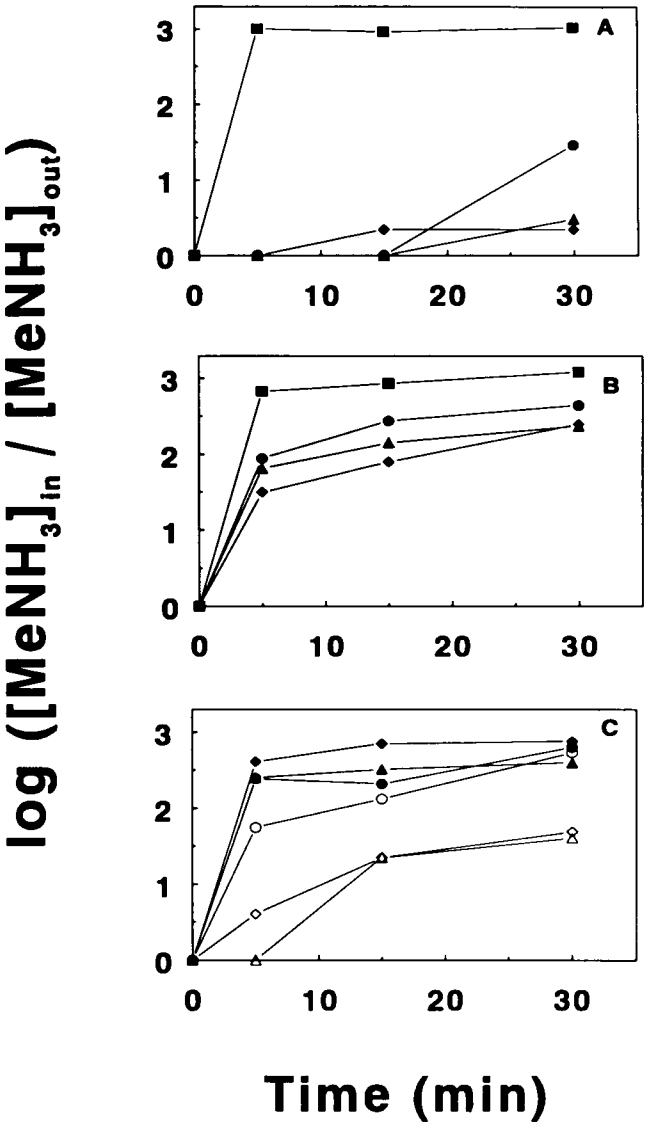

FIGURE 1 (*A*) Apparent ΔpH as determined by [^{14}C]$MeNH_3^+$ distributions for vesicles with various lipid compositions in response to an imposed transmembrane pH gradient (inside acidic). Methylamine distributions were determined as described in Materials and Methods for vesicles containing 300 mM citrate (pH 4.0) extruded through 200-nm filters and subsequently incubated in 150 mM NaCl, 20 mM Hepes, pH 7.0 at 21°C. Vesicles were composed of EPC (■), DPPC (●), DSPC (▲), and DAPC (♦). (*B*) Methylamine response determined as in *A*, but where vesicles contained 45 mol% cholesterol. (*C*) Methylamine response for vesicles (in the absence of cholesterol) incubated at 37°C (*open symbols*) or 60°C (*closed symbols*). The symbols have the same meaning as in *A*.

under Materials and Methods. As shown in Fig. 1 *A*, lipid composition can have a profound effect on the ΔpH reported. Specifically, whereas the ΔpH reported for the EPC vesicles accurately reflects the applied ΔpH, little or no pH gradient is detected in the vesicles composed of the saturated lipids DPPC, DSPC, or DAPC. In the case of DAPC, the half-time for methylamine accumulation is >24 h at 20°C. The lack of the methylamine response may be attributed initially to the impermeable,

gel state nature of those vesicles at the incubation temperature (21°C), which would be expected to reduce the permeability of the neutral form of methylamine. This interpretation is supported by the results presented in Fig. 1, *B* and *C*. The presence of 45 mol% cholesterol, which eliminates the gel-to-liquid crystalline transition and increases the motion available to previously gel state phospholipids, results in equilibration of methylamine that is nearly complete by 30 min for the DPPC, DSPC, and DAPC systems (Fig. 1 *B*). Alternatively, heating the vesicles to temperatures above their gel-to-liquid crystalline transition temperature (T_c) should also result in rapid equilibration. As shown in Fig. 1 *C*, this is the case for the DPPC ($T_c \simeq 42$°C) and DSPC ($T_c \simeq 58$°C) vesicles when incubated at 60°C, where rapid transbilayer equilibration of methylamine is observed within 5 min. It is interesting to note that the presence of gel state lipid per se does not prevent methylamine equilibration, as methylamine accurately reports the ΔpH in the DAPC vesicles at 60°C, some 15°C below the T_c of this phospholipid (15). Furthermore, the [^{14}C]methylamine distributions report a three-unit ΔpH for all vesicle types at 60°C and indicate that this gradient is stable for ≥1 h at this temperature for all lipid compositions tested.

Influence of probe concentration

It is straightforward to show that the percentage of the probe that is accumulated for a given ΔpH obeys the relation:

$$\% \text{ entrapped} = \frac{10^{\Delta pH}[PL]V_i}{1 + 10^{\Delta pH}[PL]V_i} \times 100\% \qquad (13)$$

where [PL] is the phospholipid concentration and V_i is the trapped volume (per mole of lipid). Thus, under the experimental conditions employed here (2 mM lipid, 1 μCi/ml methylamine), detection of a three-unit ΔpH involves the accumulation of 67% of the probe, assuming a trap volume of 1 liter/mol lipid. Given the initial external concentration of the radiolabeled $MeNH_3^+$ as 1 μCi/ml or 21 μM (specific activity = 48 mCi/mmol), this indicates a final interior probe concentration of 7 mM. As each methylamine accumulated consumes a proton on arrival in the vesicle interior, it is clear that the vesicle interior must be reasonably well buffered in order that radiolabeled methylamine provides an accurate measure of the initial ΔpH. This effect is illustrated in Fig. 2 *A*, where it is found that at interior citrate concentrations < 50 mM (under isoosmotic conditions), the accuracy of the ΔpH reported by methylamine is increasingly compromised for a ΔpH that was initially three units.

As indicated under Materials and Methods, the presence of a ΔpH (inside acidic) across the vesicle bilayer will also induce a membrane potential (Δψ; inside negative) due to the outward efflux of H^+ ions. The effective interior concentration of protons $[N(H^+)]_i^{eff}$ that will be

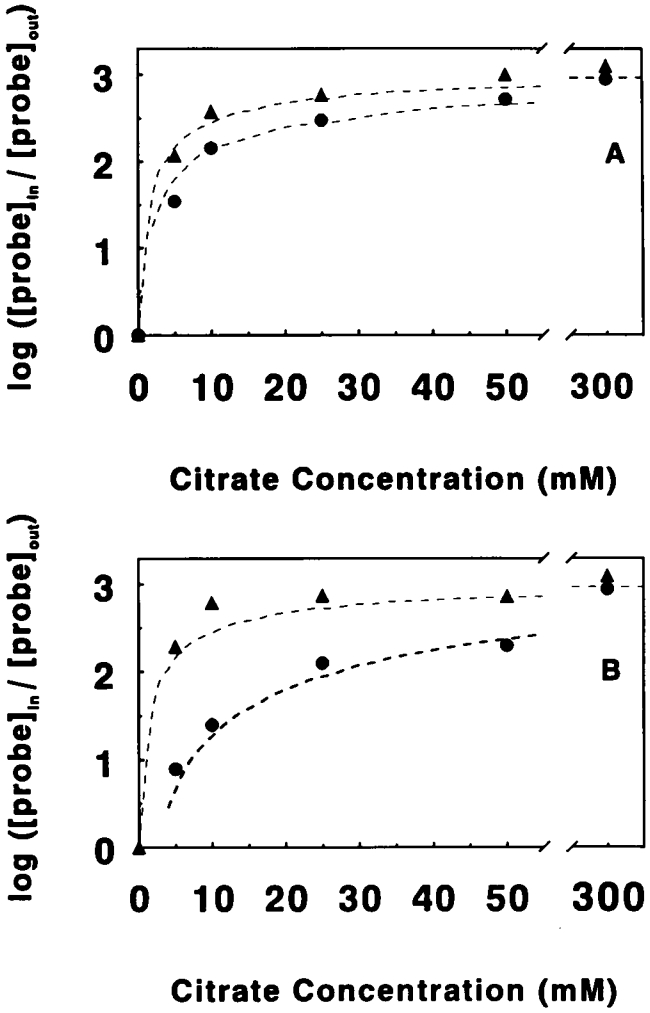

FIGURE 2 The effects of internal buffering capacity (*A*) and osmotic gradients (*B*) on the apparent transmembrane pH gradients and membrane potentials. EPC/cholesterol (55:45; mol/mol) vesicles (2 mM, 100 nm diam) were prepared in the indicated concentration of citrate, pH 4.0. These vesicles were incubated for 30 min with (*A*) an isoosmotic NaCl–Hepes-buffered saline buffer (pH 7.0) or (*B*) 150 mM NaCl, 20 mM Hepes, pH 7.0. These buffers contained 1.0 μCi/ml [^{14}C]methylamine (21 μM $MeNH_3^+$) to determine ΔpH (●) or 1.0 μCi/ml ^{3}H-TPP$^+$ (26 nM TPP$^+$) to determine Δψ (▲). Transmembrane distributions of the probes were determined as described in Materials and Methods. The solutions also contained 5.0 μM CCCP to speed development of Δψ. The dotted lines represent theoretical curves derived from the model described in Materials and Methods.

lost to set up electrochemical equilibrium can be written as $[N(H^+)]_i^{eff} = 3.7 \times 10^{-9}\ \Delta pH/d$, for a membrane capacitance $C = 1\ \mu F/cm^2$, where d is the vesicle diameter in centimeters and ΔpH is the pH gradient at equilibrium. For a 100-nm-diam LUV with a ΔpH of 3.0, this corresponds to a loss of 1.1 mM proton equivalents of buffering capacity, and thus the loss of buffering capacity to set up Δψ would not be expected to compromise the measurement of ΔpH to the extent observed for the radiolabeled methylamine. This conclusion can be tested by measuring the induced Δψ employing ^{3}H-TPP$^+$ as a

probe for $\Delta\psi$. In this regard, as each TPP^+ moves into an LUV in response to $\Delta\psi$, an H^+ ion is released to reestablish $\Delta\psi$, and thus there is a 1:1 stoichiometry between TPP^+ accumulation and internal protons "consumed," as for $MeNH_3^+$. However, ^{3}H-TPP^+ is available at specific activities (39 Ci/mmol) that are nearly 1,000-fold higher than those of [^{14}C]$MeNH_3^+$. As a result, under the standard initial conditions of 1 μCi/ml TPP^+ to measure $\Delta\psi$, the final interior TPP^+ concentration (and thus the concentration of proton equivalents consumed) is only 8.5 μM. Thus, as shown in Fig. 2 *A*, measurement of the ΔpH-induced $\Delta\psi$ using ^{3}H-TPP^+ can be a more accurate method of measuring ΔpH than the methylamine procedure for low ($\leq$50 mM citrate) internal buffering capacities.

Influence of osmotic effects

It should be noted that relatively small errors in determining the amount of entrapped $MeNH_3^+$ or in determining the aqueous trapped volume can result in large errors in the estimates of ΔpH. An example of this is given by the ΔpH data of Fig. 2 *B*, where the exterior aqueous buffer is maintained as 150 mM NaCl, 20 mM Hepes, whereas the interior citrate concentration is varied. At lower interior citrate concentrations, the LUVs will shrink due to the osmotic imbalances to achieve an equilibrium volume $V_i^{eq} = V_i^i\pi_i^i/\pi_o$, where V_i^i and π_i^i indicate the initial interior volume and osmolarity, respectively, and π_o indicates the osmolarity of the exterior medium. As a result, less $MeNH_3^+$ will be accumulated to satisfy the relationship $[MeNH_3^+]_i/[MeNH_3^+]_o = [H^+]_i/[H^+]_o$. If no correction is made for the change in volume, an apparent ΔpH (ΔpH^{app}) will be measured that is less than the actual ΔpH according to the relation.

$$\Delta pH^{app} = \Delta pH - \log\left(\frac{\pi_o}{\pi_i}\right) \qquad (14)$$

This correction applies only to hyperosmotic gradients since these vesicles do not swell beyond the maximal values seen in Table 1. It is interesting to note that the measured TPP^+ distribution is less affected by vesicle shrinkage. This is probably related to the ability of this probe to partition in the vesicle membrane (see below).

Influence of internal citrate concentration

The influences of the internal citrate concentration and external (initial) $MeNH_3^+$ concentration on the levels of internalized $MeNH_3^+$ in 100-nm-diam EPC/cholesterol LUVs at equilibrium and the derived ΔpH values are illustrated in Fig. 3, *A* and *B*, respectively. At high initial concentrations of external $MeNH_3^+$ (10 mM), extremely high levels of internalized $MeNH_3^+$ can be achieved (300 nmol/μmol lipid) for internal citrate concentrations of 300 mM. The solid lines indicate the theoretical behavior expected on the basis of the analysis presented in Materials and Methods, employing the measured trapped buffer of 0.84 liters/mol (Table 1) with no adjustable parameters. An important ramification of the observed agreement with theory is that the uptake of any simple weak base can be predicted on the basis of the buffering capacity or, alternatively, that the buffering capacity can be measured by determining methylamine distributions at higher methylamine concentrations.

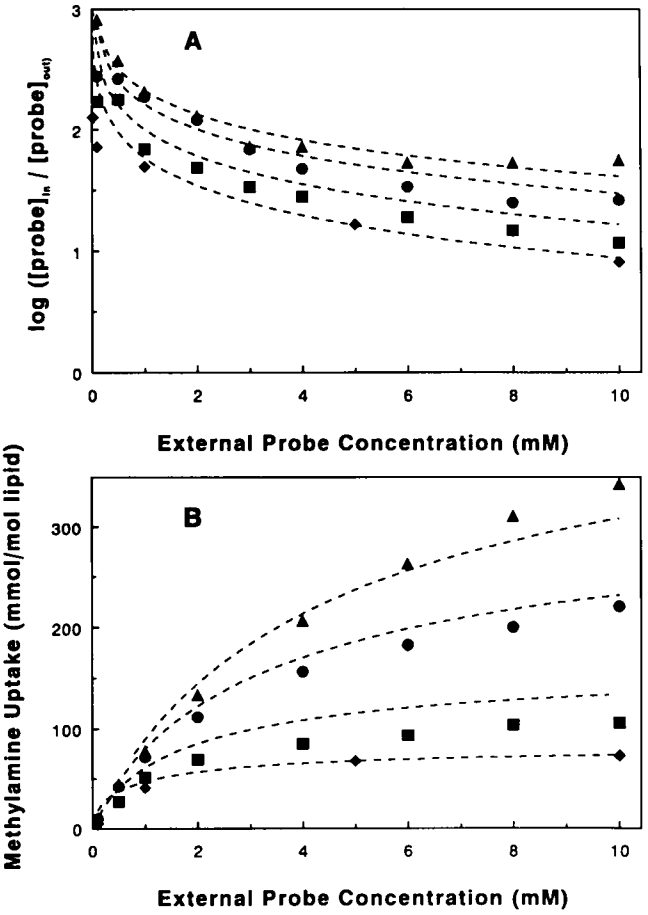

FIGURE 3 Effect of external methylamine concentration on methylamine uptake into EPC/cholesterol (55:45; mol/mol) LUVs exhibiting a three-unit transmembrane pH gradient (inside acidic) LUVs (100 nm diam, 4 nm lipid) were prepared in 50 (♦), 100 (▲), 200 (■), or 300 mM citrate (●) (see Materials and Methods) and the vesicles incubated in the indicated concentration of methylamine, containing 0.5 μCi/ml [^{14}C]methylamine. Methylamine incorporation into vesicles was determined after passing 100-μl aliquots through 1.0-ml spun G-50 minicolumns. *A* indicates the amount of accumulated $MeNH_3$; *B* shows the effect of probe accumulation on the apparent ΔpH. The dotted lines represent the theoretical behavior predicted by the model described in Materials and Methods, assuming an internal volume of 0.84 liters/mol lipid for the trapped citrate buffer, with no adjustable parameters.

Influence of absolute value of internal and external pH

A final situation that would be expected to compromise the accuracy of the [^{14}C]$MeNH_3^+$ technique for measur-

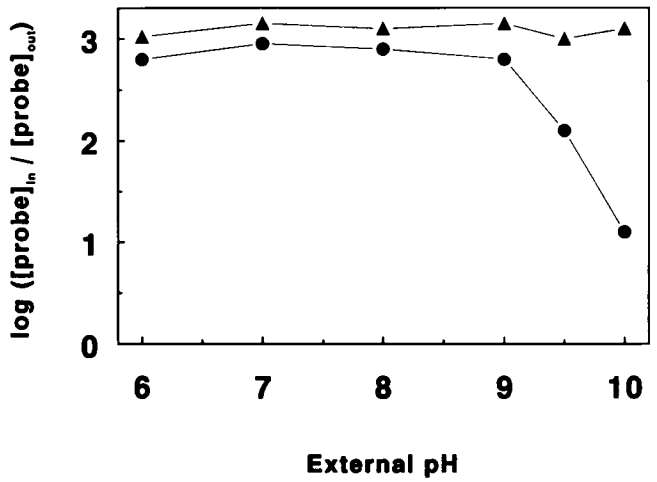

FIGURE 4 Determination of pH gradients (ΔpH = 3 units) over a range of internal and external pH values. EPC LUVs (100 nm) were prepared in 200 mM citrate, 200 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), or 200 mM Hepes, at pH 3.0, 4.0, 5.0, 6.0, or 7.0 (*closed symbols*). This buffer was exchanged for an external buffer of 150 mM NaCl, 20 mM MES, 20 mM Hepes, and 20 mM CHES with the appropriate pH required to maintain a 3 pH unit difference between the internal and external buffers. Transmembrane distributions of $[^{14}C]MeNH_3^+$ were determined after 30-min incubations at room temperature (●); transbilayer distributions of ^3H-TPP$^+$ (▲) were determined after 60-min incubations at room temperature, to allow the generation of the $\Delta\psi$.

ing ΔpH concerns the influence of higher internal and external pH values. The equilibrium relation $[H^+]_i/[H^+]_o = [MeNH_3^+]_i/[MeNH_3^+]_o$ only holds for $[H^+]_i$, $[H^+]_o \ll K_d$, where K_d is the dissociation constant of the weak base. As the exterior and interior pH approach K_d, the apparent ΔpH (ΔpH^{app}) is related to the actual ΔpH (ΔpH^{real}) by the relation $\Delta pH^{app} \simeq \Delta pH^{real} - \log(1 + K_d/[H]_o)$, leading to a decrease in the measured ΔpH as $[H^+]_o$ approaches K_d. More importantly, as the interior pH is raised, the proportion of internalized amine that is in the neutral, membrane permeable form will be increased, leading to the probability of increased leakage during the spin column separation. This will also lead to lower measures of ΔpH. As shown in Fig. 4, the influence of these effects becomes noticeable at exterior pH values of 9.0 or higher for 100-nm EPC LUVs exhibiting a ΔpH of three units. As may be expected, detection of the induced $\Delta\psi$ employing ^3H-TPP$^+$ is not subject to such limitations and provides an accurate measure of $\Delta\psi$ (and thus, ΔpH) at exterior pH values up to 9.5.

The results to this point indicate that the $[^{14}C]$-$MeNH_3^+$ probe, in combination with the spin column procedure, provides a convenient and accurate measure of ΔpH (up to three units), assuming that conditions allowing the equilibrium transbilayer equilibration of the neutral form are observed and that the interior environment is sufficiently well buffered. A further point of interest concerns the magnitude of the ΔpH that can be generated and measured. In this regard, it is experimentally convenient to work in a region where the maximum probe entrapment, at the maximum ΔpH, corresponds to 75% or less than the total amount of probe originally present in solution. For larger pH gradients, this in turn limits the amount of phospholipid that can be used. To detect a pH gradient of five units while only accumulating 75% of the probe would require using only 0.03 mM phospholipid, which could result in phospholipid phosphorus assay errors after the spin column. An alternative technique, which becomes progressively more accurate at high ΔpH values, involves equilibrium filtration to separate the vesicles from the external buffer (see Materials and Methods). As shown in Fig. 5, both the equilibrium filtration and the spin column procedure are in excellent agreement for imposed ΔpH gradients from zero to five units and indicate that the maximum pH gradient that 100-nm-diam EPC:cholesterol (55:45; mol/mol) LUVs can maintain is ~3.7 units. This is also indicated by the transmembrane distributions of ^3H-TPP$^+$.

Measurement of ΔpH for vesicles with a basic interior

The techniques discussed above relate to measurement of pH gradients in vesicles with an acidic interior. It is of interest to determine whether similar spin column procedures can be applied to determine ΔpH in vesicles with

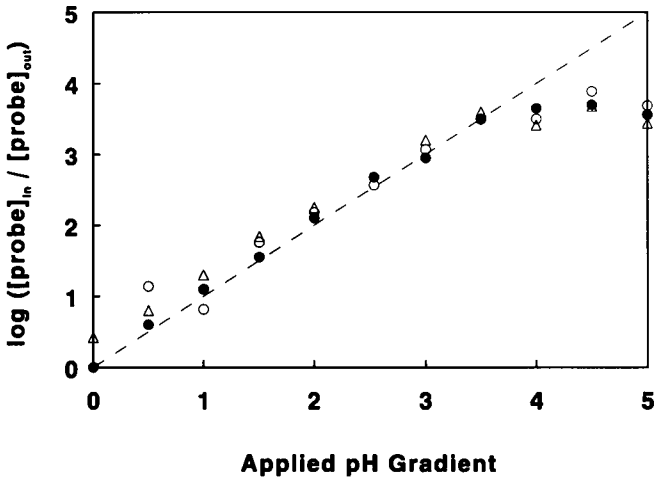

FIGURE 5 The ΔpH measured employing $[^{14}C]MeNH_3^+$ using gel filtration and an equilibrium method of $MeNH_3^+$ separation for 100 nm EPC/cholesterol (55:45; mol/mol) LUVs. Vesicles were prepared in 300 mM citrate, pH 4.0, and then were placed in 150 mM NaCl, and 20 mM of citrate, MES, Hepes, N-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid, or CHES buffer with pH values in the range 4.5–9.0. The ΔpH was determined by the transmembrane distribution of $[^{14}C]$-methylamine using spun minicolumns (●) or "centrifree" filters (○) after a 30-min incubation at room temperature. ^3H-TPP distributions (▲) were determined in the presence of 5 μM CCCP, as indicated in Materials and Methods. The dotted line represents the size of the imposed gradient.

a basic interior using radiolabeled weak acids as the ΔpH probes. Such studies were pursued for 100-nm EPC vesicles experiencing a three-unit pH gradient (pH_i 9.0; pH_o 6.0), utilizing as probes ^{14}C-labeled benzoic, acetysalicylic, acetic, and mevalonic acid. As shown in Fig. 6, the measured transmembrane distributions of benzoic and acetylsalicylic acid do not reflect the imposed ΔpH, whereas the transbilayer distribution of acetic acid significantly underestimates the pH gradient. Mevalonic acid appears a useful indicator of ΔpH for vesicles with basic interiors; however, a long (2 h) incubation time to achieve equilibrium is required at 20°C. The most rapid and accurate indication of ΔpH is given by the membrane potential indicator [^{14}C]thiocyanate, which gives a transmembrane distribution commensurate with the induced $\Delta\psi$ (inside positive) expected for a three-unit pH gradient.

The response of [^{14}C]acetate was further examined to understand the basis of the behavior exhibited in Fig. 6. As for $MeNH_3^+$, a logical possibility is that entrapped acetate is released during the spin column procedure. The inclusion of cholesterol or the substitution of DSPC for EPC would be expected to reduce such leakage. As shown in Fig. 7 *A*, [^{14}C]acetate provided a much improved measure of ΔpH for vesicles with these lipid compositions. Alternatively, as shown in Fig. 7 *B*, the equilibrium filtration procedure can be usefully applied to achieve accurate measures of ΔpH even for the EPC system.

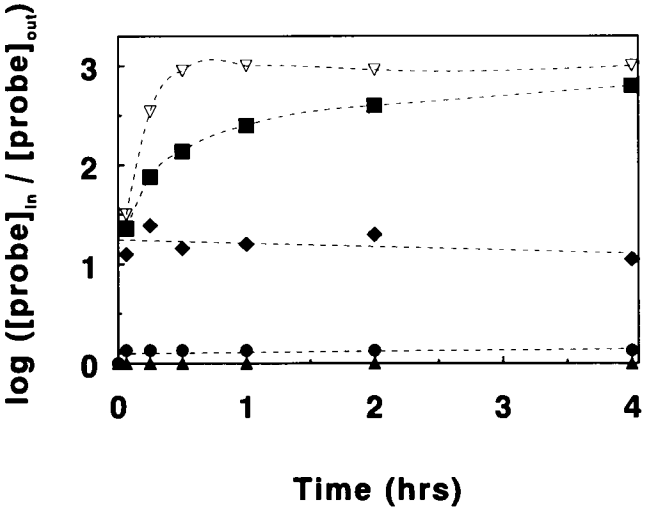

FIGURE 6 Transmembrane distributions of radiolabeled probes in EPC LUVs (100 nm diam) as determined by gel filtration for vesicles with a basic interior. The apparent transmembrane distributions of radiolabeled benzoic acid (▲), acetylsalicylic acid (●), acetic acid (◆), and mevalonic acid (■) using gel filtration was determined in vesicles containing 300 mM CHES (pH 9.0) incubated in 150 mM NaCl, 20 mM MES (pH 6.0) with 0.5 μCi/ml of the indicated probe. Positive (interior) membrane potentials induced in response to these pH gradients were determined by the redistribution of [^{14}C]thiocyanate in EPC vesicles using the gel filtration separation procedure.

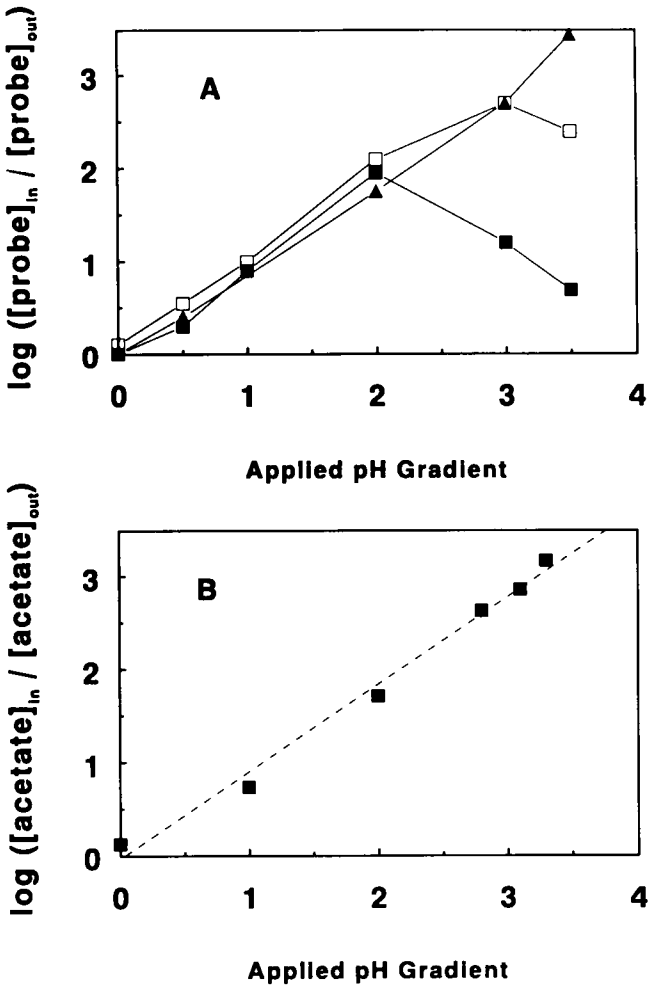

FIGURE 7 Determination of pH gradients (interior basic) using [^{14}C]-acetate. (*A*) Transmembrane distributions of labeled acetate were determined as in Fig. 6 for 100-nm-diam LUVs composed of EPC (■), EPC/cholesterol (55:45; mol/mol) (□), or DSPC/cholesterol (55:45; mol%) (▲). (*B*) The transmembrane distribution of [^{14}C]acetate as determined by equilibrium centrifugation (■) employing EPC (100 nm) LUVs. These vesicles were incubated in 150 mM NaCl, 20 mM CHES, 20 mM Hepes, and 20 mM MES, with the pH adjusted to values between 9.0 and 6.0 and the transmembrane distribution of 0.5 μCi/ml [^{14}C]acetate measured by "centrifree" filters as indicated in Materials and Methods. The dotted line is a linear regression, with a slope of 0.94.

Relation between $\Delta\psi$ and ΔpH measurements

A final point of investigation concerned the relation between ΔpH as measured by probes such as methylamine and the induced $\Delta\psi$ measured by probes such as TPP^+. Clearly, in the absence of other factors, the transbilayer concentration gradients detected by methylamine and TPP^+ resulting from a given ΔpH and induced $\Delta\psi$, respectively, should be the same at equilibrium. However, other authors (18) have reported that TPP^+ exhibits a significant membrane-water partition coefficient. This would be expected to increase the inside-outside concen-

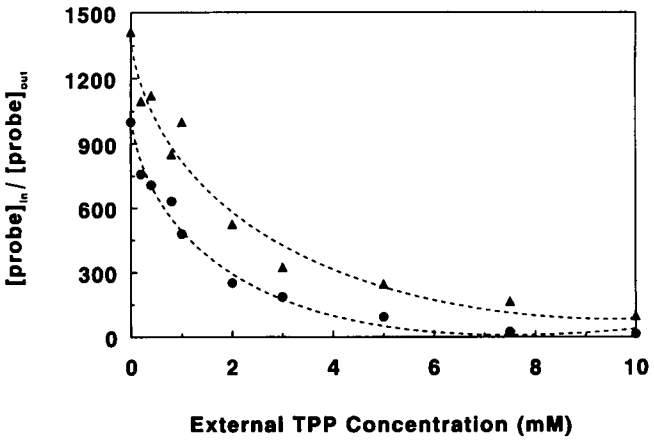

FIGURE 8 The relationship between transmembrane TPP^+ gradients and $MeNH_3^+$ gradients achieved in response to a 3-unit pH gradient (interior acidic) in EPC/cholesterol (55:45; mol/mol) LUVs (100 nm diam) containing 300 mM citrate (pH 4.0) in the presence of 5 μM CCCP. Transmembrane TPP^+ (▲) and $MeNH_3^+$ (●) gradients were determined as a function of the external TPP concentration after a 30-min incubation at 25°C as indicated in Materials and Methods. The solid lines represent best fits to the data.

tration gradient for TPP^+, for a given $\Delta\psi$, due to the small aqueous volume to membrane volume ratio in the vesicle interior. As shown in Fig. 8, behavior corresponding to such effects can be observed in 100-nm EPC LUVs exhibiting a ΔpH of three units (inside acidic). The measured ratios $[TPP^+]_i/[TPP^+]_o$ are consistently larger than the $[MeNH_3^+]_i/[MeNH_3^+]_o$ ratios over a wide range of external TPP^+ concentrations. However, the increased inside/outside ratios lead to a relatively small overestimate of $\Delta\psi$ ($\lesssim$7 mV), indicating that the effects of TPP^+ partition can be neglected for the large pH gradients examined in this work.

DISCUSSION

The results presented here give insight into factors influencing the validity of measurements of transmembrane pH gradients and membrane potentials in liposomal systems and techniques that result in improved accuracy. The major factors considered were lipid composition, interior buffering capacity, osmotic gradients, the absolute magnitude of the ΔpH, the measurement of ΔpH in vesicles with a basic interior and, with respect to measuring the induced membrane potential, the influence of probe partitioning into the lipid bilayer. These aspects are discussed in turn.

The lipid composition can strongly influence the measured ΔpH determined by weak bases such as $MeNH_3^+$. As illustrated here, liposomes composed of saturated, gel state lipids can exhibit apparent ΔpH values that are substantially less than the actual gradient. Clearly, some minimum level of permeability of the neutral form of the probe through the membrane is required to allow equilibrium to be achieved within a reasonable time frame. As indicated elsewhere (reference 3), amine uptake in response to ΔpH can be treated as a simple first order process, described by the relation $[AH^+(t)]_i = [AH(eq)]_i(1 - e^{-kt})$, where $[AH^+(t)]_i$ is the interior concentration of the amine at time t and $[AH(eq)]_i$ is the equilibrium interior amine concentration at long incubation times. Uptake data of the type presented in Fig. 1 can be utilized to obtain the rate constant (k) associated with the uptake process. Thus, an approximate measure of the minimum permeability coefficient for $MeNH_3^+$ required can be determined from the rate constant determined from the data of Fig. 1 *B* for DPPC:cholesterol (55:45) at 20°C. This yields a value $k = 2 \times 10^{-3}$ s^{-1}. It is straightforward to show that for unilamellar vesicles, this rate constant is related to the permeability coefficient P of the neutral form of methylamine via the relation $P = kV_o[H^+]_o/(A_mK_d)$, where V_o is the total external aqueous volume, A_m is the membrane area, and K_d is the dissociation constant of $MeNH_3^+$ ($pK_d = 10.6$). Assuming an area per phospholipid of 60 $Å^2$, this indicates a permeability coefficient for the neutral form of 7×10^{-4} cm/s or larger is required. As indicated in Results, a brief incubation at an elevated temperature (e.g., 60°C) increases P for all the systems studied to the extent that equilibrium is achieved within 5 min, without compromising the ΔpH. A related point concerns the fact that the rate constant associated with probe efflux may be slower than the rate constant associated with uptake for vesicles with an acidic interior, as the proportion of the probe in the permeant (neutral) form decreases as the proton concentration increases.

The accuracy of the ΔpH detected across LUV membranes by radiolabeled probes such as $MeNH_3^+$ is a sensitive function of both the interior buffering capacity of the vesicles and measures of the interior trapped volume. As detailed here, interior citrate concentrations of 20 mM or higher are necessary to accurately detect pH gradients of two units or higher for ^{14}C-labeled $MeNH_3^+$ (specific activity 48 mCi/mmol). The need for such high interior buffering capacities can be reduced by using probes with higher specific activity. In this regard, it is often convenient to use probes of $\Delta\psi$ with higher specific activity (such as TPP^+) to detect the $\Delta\psi$ induced in response to ΔpH as a more accurate measure of ΔpH. As indicated here, the ability of TPP^+ to partition into the lipid bilayer does introduce a slight overestimate of $\Delta\psi$ and therefore ΔpH. This is relatively minor under the conditions employed here, ~0.1 pH units. An important general point is that for pH gradients of three units or more in 100-nm vesicle systems, interior citrate buffering concentrations in excess of ~20 mM are required in order that the pH gradient is not significantly dissipated by proton efflux required to form $\Delta\psi$. Furthermore, the presence of osmotic gradients that lead to vesicle shrinkage can cause significant underestimates of the ΔpH present.

The fourth point of discussion concerns the maximum pH gradients that can be achieved. A major thrust of this work has concerned the accurate measurement of relatively large pH gradients of three units or more. The results presented here for EPC/cholesterol (55:45) indicate a maximum ΔpH of $\sim$3.7 units, corresponding to a $\Delta\psi$ of 220 mV. An inability to generate larger pH gradients and induced membrane potentials is probably due to electrical breakdown of the bilayer (19). The filtration centrifugation procedure is clearly a sensitive technique for measuring large ΔpH values, and the close agreement demonstrated here between this technique and the spin column approach is gratifying.

With regard to the measurement of ΔpH in vesicles with a basic interior, a suitable probe for use with the gel filtration procedure is not readily identified. Most of the probes investigated (acetic, benzoic, and acetylsalicylic acids) are poor indicators of the ΔpH in such situations, apparently because they leak from the vesicles during the separation procedure. Alternatively, although mevalonic acid does not leak from the vesicles during separation, long equilibration times are inconvenient. More accurate procedures are provided by the filtration centrifugation method or by measuring the $\Delta\psi$ induced in response to ΔpH.

The final point of discussion concerns the measurement of the $\Delta\psi$ induced in response to ΔpH using cationic probes such as TPP^+ and the influence of probe partitioning into the membrane. As indicated above, this partitioning does not introduce large errors ($\sim$0.11 pH units or 7 mV) if large pH gradients are examined; however, it is of interest to compare the value of the partition coefficient that may be calculated from this data with previous reports. Specifically, using the formalism developed by Cafiso and Hubbell (20) and Rottenberg (21), a partition coefficient $\beta \cong 1 \times 10^{-6}$ cm can be calculated for TPP^+ in this EPC/cholesterol LUV system. This is in reasonable agreement with previous values of 6×10^{-7} cm in POPC MLVs (22) and 4×10^{-6} cm for sonicated EPC vesicles (18). In passing, the underestimate of $\Delta\psi$ reported by Nakazato et al. (23) employing TPP^+ in cholesterol-containing systems may be due to a kinetic effect. The small amounts of the protein ionophore CCCP used to generate the data of Fig. 2 to ensure rapid development of the induced $\Delta\psi$ appear to increase the rate of TPP^+ equilibration, probably via an ion pairing mechanism as observed with TPB^- (24).

In summary, techniques for the accurate measurement of pH gradients in LUV systems have been described. For LUVs with an acidic interior, determination of the equilibrium transbilayer distributions of radiolabeled methylamine using the gel filtration procedure provides a reliable procedure, provided that transbilayer equilibration rates are sufficiently rapid and that interior buffering capacities are sufficiently high. In situations where this accuracy is compromised, equilibrium centrifugation techniques or techniques to measure membrane potentials induced by the pH gradient provide straightforward alternatives.

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REFERENCES

1. Madden, T. D., P. R. Harrigan, L. Tai, M. B. Bally, L. D. Mayer, T. Redelmeier, L. W. Tilcock, and P. R. Cullis. 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient. A survey. *Chem. Phys. Lipids* 53:37–46.
2. Viero, J. A., and P. R. Cullis. 1990. A novel method for the efficient entrapment of Ca^{+2} in large unilamellar vesicles. *Biochim. Biophys. Acta.* 1025:109–115.
3. Chakrabarti, A., I. Clark-Lewis, P. R. Harrigan, and P. R. Cullis. 1992. Uptake of basic amino acids and peptides into liposomes in response to transmembrane pH gradients. *Biophys. J.* 61:228–234.
4. Redelmeier, T. E., M. J. Hope, and P. R. Cullis. 1990. On the mechanism of transbilayer transport of phosphatidylglycerol in response to transmembrane pH gradients. *Biochemistry.* 29:3046–3053.
5. Eastman, S. J., M. J. Hope, and P. R. Cullis. 1991. Transbilayer transport of phosphatidic acid in response to transmembrane pH gradients. *Biochemistry.* 30:1740–1745.
6. Rottenberg, H. 1979. The measurement of membrane potential and delta pH in cells, organelles and vesicles. *Methods Enzymol.* 55:547–569.
7. Redelmeier, T. E., L. D. Mayer, K. F. Wong, M. B. Bally, and P. R. Cullis. 1989. Proton flux in large unilamellar vesicles induced by transmembrane ion gradients. *Biophys. J.* 56:385–393.
8. Cafiso, D. S., and W. L. Hubbell. 1978. Estimation of transmembrane pH gradients from phase equilibria of spin labelled amines. *Biochemistry.* 17:3871–3877.
9. Nichols, J. W., M. W. Hill, A. D. Bangham, and D. W. Deamer. 1980. Net proton-hydroxyl permeability of large unilamellar vesicles measured by an acid-base titration technique. *Biochim. Biophys. Acta.* 596:393–403.
10. Cools, A. A., and L. H. Janssen. 1986. Fluorescence response of acridine orange to changes in pH gradients across liposomal membranes. *Experientia (Basel).* 428:954–956.
11. Rottenberg, H. 1989. Proton electrochemical potential gradient in vesicles, organelles and prokaryotic cells. *Methods Enzymol.* 172:63–85.
12. Grzesiek, A., and N. A. Dencher. 1988. Delta pH-induced fluorescence quenching of 9-aminoacridine in lipid vesicles is due to excimer formation at the membrane. *Biochim. Biophys. Acta.* 938:411–424.
13. Mayer, L. D., M. J. Hope, P. R. Cullis, and A. S. Janoff. 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochim. Biophys. Acta.* 817: 193–196.
14. Hope, M. J., M. B. Bally, G. Webb, and P. R. Cullis. 1985. Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size, trapped volume and ability to maintain a membrane potential. *Biochim. Biophys. Acta.* 812:55–65.
15. Nayar, R., M. J. Hope, and P. R. Cullis. 1989. Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique. *Biochim. Biophys. Acta.* 986:200–206.

16. Fiske, C. H., and Y. Subbarow. 1925. The colorimetric determination of phosphorous. *J. Biol. Chem.* 66:375–400.

17. Rudel, L., and M. D. Morris. 1973. Determination of cholesterol using opthaldehyde. *J. Lipid Res.* 14:364–366.

18. Flewelling, R. F., and W. L. Hubbell. 1986. The membrane dipole potential in a total membrane potential model. Applications to hydrophobic ion interactions with membranes. *Biophys. J.* 49:531–540.

19. El-Mashak, E. M., and T. Y. Tsong. 1985. Ion selectivity of temperature induced and electric field induced pores in dipalmitoyl phosphatidylcholine vesicles. *Biochemistry.* 24:2884–2888.

20. Cafiso, D. S., and W. L. Hubbell. 1978. Estimation of transmembrane potentials from phase equilibria of spin labelled amines. *Biochemistry.* 17:187–195.

21. Rottenberg, H. 1984. Membrane potential and surface potential in mitochondria: uptake and binding of lipophilic cations. *J. Membr. Biol.* 81:127–138.

22. Altenbach, C., and J. Seelig. 1985. Binding of the lipophilic cation tetraphenyl-phosphorous to phosphatidylcholine membranes. *Biochim. Biophys. Acta.* 818:410–415.

23. Nakazato, K., N. Murakami, T. Konishi, and Y. Hatano. 1988. Membrane potential in liposomes measured by transmembrane distribution of $^{86}Rb^{+}$, tetraphenylphosphonium or triphenylmethylphosphonium: effect of cholesterol in the lipid bilayer. *Biochim. Biophys. Acta.* 946:143–150.

24. Cafiso, D. S., and W. L. Hubbell. 1982. Transmembrane electrical currents of spin-labeled hydrophobic amines. *Biophys. J.* 39:263–272.