

Proton flux in large unilamellar vesicles in response to membrane potentials and pH gradients

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ABSTRACT The transport of protons across liposomes composed of phosphatidylcholine in response to electrical potentials or pH gradients has been investigated. The results support three major conclusions. The first of these concerns the need for reliable measurements of electrical potentials and pH gradients. It is shown that the potential probe tetraphenylphosphonium and the pH probe methylamine provide

accurate and self consistent measures of electrical potentials and pH gradients respectively in these systems. Second, it is shown by two independent techniques that the pH gradients induced in response to valinomycin and potassium dependent electrical potentials are significantly smaller than would be expected for electrochemical equilibrium. The pH gradients observed are stable over an 8 h time course and are

sensitive to the ionic composition of the buffers employed, where the presence of external sodium results in the smallest induced pH gradients. These results are discussed in terms of current models of proton conductance across membranes. In a final area of investigation, it is shown that valinomycin and carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) can transport sodium ions in a synergistic manner.

INTRODUCTION

Recent work in this laboratory has been concerned with large unilamellar vesicle (LUV) systems exhibiting electrical potentials and the influence of these potentials on the transmembrane distributions of lipophilic cations. It has been shown that stable electrical potentials in excess of 150 mV (inside negative) can be readily generated in LUV systems containing a K⁺ buffer by the addition of the potassium ionophore valinomycin (Hope et al., 1985). Such systems rapidly accumulate lipophilic cations such as safranin o (Bally et al., 1985) as well as amine containing drugs which are weak bases, such as chlorpromazine (Bally et al., 1985), doxorubicin (Mayer et al., 1985a), and dibucaine (Mayer et al., 1985b).

A detailed understanding of the mechanisms involved in such accumulation process requires a thorough characterization of the relation between electrical potentials and induced transmembrane proton gradients. In particular, due to the high proton permeability of membranes (Nichols et al., 1980; Clement and Gould, 1981; Deamer and Bramhall, 1986) the presence of an electrical potential (inside negative) could be expected to induce proton transport leading to acidification of the vesicle interior and electrochemical equilibrium where $[H^+]_{in}/[H^+]_{out} = [K^+]_{in}/[K^+]_{out}$. These proton gradients can drive accumulation of lipophilic cations that are weak bases due to the high permeability of neutral form (Nichols and Deamer, 1976).

The relationship between electrical potentials and induced pH gradients in vesicle systems is not clear, however. Induced pH gradients corresponding to electrochemical equilibrium have been observed in sonicated small unilamellar vesicles (SUV) systems (Cafiso and Hubbell, 1983). However, other authors (Garcia, 1984; Konishi, 1986) have reported that electrical potentials induce much smaller pH gradients than would be expected for electrochemical equilibrium.

Here we have examined in detail the relationship between electrical potentials and pH gradients in vesicle systems. We employ LUVs generated by extrusion procedures (Hope et al., 1985) that have the advantage that organic solvents or detergents are not required during the vesicle preparation. Particular attention is paid to establishing the reliability of measure of electrical potential and pH gradients provided by the probes triphenylphosphonium (TPP⁺) and methylamine (MeAm). It is shown by two independent techniques for egg phosphatidylcholine (EPC) LUV systems exhibiting a valinomycin induced electrical potential that electrochemical equilibrium is not achieved over an 8 h time course. Rather, a small, stable, quasi-equilibrium pH gradient is established and the magnitude of this gradient is sensitive to the ionic composition of the external buffer. The lack of equilibrium is dramatically revealed by the addition of the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which results in larger pH gradients corresponding to electrochemical equilibrium. In addition, an instability in the electrochemical gradients noted

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in the presence of valinomycin and CCCP together is attributed to a synergistic ability of valinomycin and CCCP to facilitate transport of Na^+ ions.

MATERIALS AND METHODS

Tetraphenylphosphonium bromide (TPP^+), valinomycin, CCCP, and all buffers were purchased from Sigma Chemical Co., St. Louis, MO. All radiochemicals were supplied by New England Nuclear, Canada. EPC was purified by standard procedures or purchased from Avanti Polar Lipids, Inc., Birmingham, AL. Phospholipid was >99% pure as ascertained by thin layer chromatography.

Vesicle preparation

Multilamellar vesicles (MLVs) were prepared by vortexing of an EPC lipid film in the presence of the appropriate buffer (50 mg/ml; wt/vol) for 15 min. The lipid dispersion was then frozen and thawed five times to obtain equilibrium transmembrane solute distributions (Mayer et al., 1986). The resulting frozen and thawed MLVs were then repeatedly ($10\times$) extruded through two (stacked) polycarbonate filters of 100 nm pore size using an extrusion device (Lipex Biomembranes, Vancouver, BC). The resulting large unilamellar vesicles (LUVs) exhibit trapped volumes of $1.5\ \mu\text{L}$ per μmol phospholipid and an average diameter of 90 nm (Hope et al., 1985).

Small unilamellar vesicles (SUVs) were prepared by sonication and exhibited a trapped volume of $0.16\ \mu\text{L}$ per μmol phospholipid. The SUVs exhibited a mean diameter of 28 nm as detected by quasi-elastic light scattering using a submicron particle sizer (model 270; Nicomp Instruments, Santa Barbara, CA). phospholipid concentrations were determined by analysis of lipid phosphorus as described previously (Fiske and Subbarow, 1925).

Uptake of probes into vesicles

Transmembrane ion gradients were created by preparing LUVs in the presence of the appropriate buffer and then exchanging the untrapped buffer for the buffer of choice employing Sephadex G-50 gel filtration columns. Experiments to monitor electrical potentials were initiated by adding a small volume of these liposomes (to achieve a final phospholipid concentration of $1\ \mu\text{mol}$ phospholipid per milliliter) to buffer solutions containing the appropriate membrane potential probes and ionophores. Where employed valinomycin was used at a concentration of $0.5\text{--}1.0\ \mu\text{g}$ per μmol of phospholipid and the ionophore CCCP was used at a concentration of $20\ \mu\text{M}$. Quantitation of vesicle associated probe was performed as previously described (Hope et al., 1985).

Calculation of membrane potentials and pH gradients

The membrane potentials were calculated assuming that the vesicle-associated probe was associated with the internal trapped volume and did not partition into the vesicle membrane. The electrical potential can then be calculated (Rottenberg, 1979) according to:

$$\Delta\Psi(\text{mV}) = -60 \log (C_{\text{in}}/C_{\text{out}}), \quad (1)$$

where C_{in} and C_{out} represents interior and exterior TPP^+ concentrations. The pH gradients may be measured employing methylamine according

to the equation:

$$\Delta\text{pH} = \log ([\text{HA}^+]_{\text{in}}/[\text{HA}^+]_{\text{out}}), \quad (2)$$

where HA^+ represents the vesicle associated (charged) methylamine (Rottenberg, 1979). For the purpose of discussion it should be noted that the proton motive force can be calculated according to:

$$\Delta p = \Delta\Psi - 60 \cdot \Delta\text{pH}. \quad (3)$$

Nuclear magnetic resonance

^{31}P nuclear magnetic resonance (NMR) studies employed a WP-200 (Bruker Instruments, Inc., Billerica, MA) spectrometer operating at 81 MHz. A free induction decay (256 transients) were obtained using a $11\ \mu\text{s}$ 47° pulse, a 1 s interpulse delay and a 10 KHz sweepwidth. An exponential multiplication corresponding to 5 Hz line broadening was applied to the free induction decay before Fourier transformation. The pH of the vesicle interior was determined by monitoring the chemical shift of Pi entrapped inside the vesicle and relating the chemical shift to that obtained for standard Pi solutions of known pH (Mayer et al., 1988).

RESULTS

Membrane potentials may be measured across liposome membranes by determining the transmembrane distributions of radiolabeled probes (Rottenberg, 1979; Hope et al., 1985), electron spin resonance (ESR) probes (Cafiso and Hubbell, 1978a) or fluorescent probes (Rottenberg, 1979). In the case of the radiolabeled probes employed here, it is important to show that correct measures of the membrane potential are obtained. We have shown previously that the equilibrium distribution of radio labeled methyltriphenylphosphonium (MTPP^+) across liposomal systems provides an accurate measurement of the actual transmembrane electrical potential (inside negative) as determined from K^+ ($^{42}\text{K}^+$) transmembrane distributions (Hope et al., 1985). Similar results are also observed for ^{14}C labeled TPP^+ except that the approach to equilibrium for tetraphenylphosphonium (TPP^+) is considerably faster than that for MTPP^+ . The ability of ^{14}C TPP^+ transmembrane distributions to accurately measure transmembrane electrical gradients was further examined here employing LUV systems with transmembrane pH gradients (inside acidic). As shown in Fig. 1 A, these systems exhibit near theoretical electrical potentials when incubated in the presence of the proton ionophore CCCP, which facilitates electrogenic proton ion movement. It should be noted that for large electrical potentials ($\Delta\Psi > 150\ \text{mV}$) the TPP^+ redistribution slightly underestimates the size of the electrical potential. However, at lower values the TPP^+ response appears to accurately reflect the transmembrane electrical potential.

The pH gradients across liposomal systems can be measured by following the redistribution of weak bases or

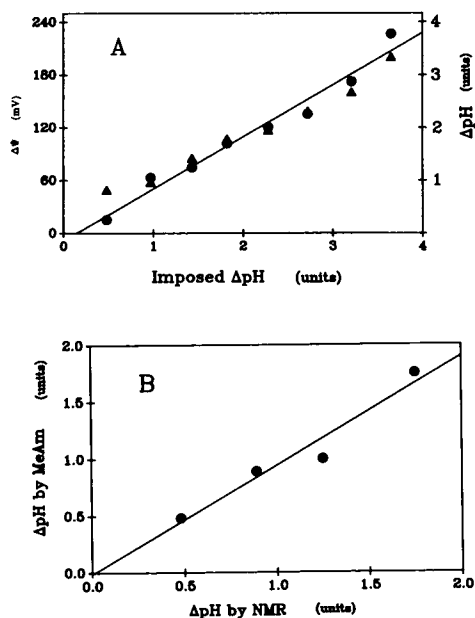


FIGURE 1 (A) $\Delta\Psi$ (●) and ΔpH (▲) as a function of the transmembrane proton gradient. EPC LUVs (1 mM phospholipid) were prepared in the presence of 10 mM glutamic acid, 10 mM Mes, 10 mM Hepes, 125 mM Na_2SO_4 where the pH of this buffer (which reflects the pH of the vesicle interior) was varied from pH 4 to pH 7.5 employing NaOH. Subsequent to the preparation of the LUVs the (exterior) pH was raised to pH 7.5. Then 20 μM CCCP was added and ^{14}C TPP⁺ or ^{14}C MeAm (0.5 $\mu\text{Ci}/\text{ml}$) introduced to allow determination of $\Delta\psi$ or ΔpH , respectively. The external buffer consisted of 125 mM Na_2SO_4 , 10 mM glutamic acid and 10 mM Mes (7.5). (B) Relation between intravesicular pH values determined employing methylamine or ^{31}P NMR. EPC LUVs (13 mM phospholipid) were prepared in 200 mM Na_2SO_4 , 50 mM Mes (pH with H_2SO_4 to 5.5, 6.0, 6.5, 7.0, 7.5) in the internal aqueous space. The external buffer consisted of 250 mM NaCl, 50 mM Hepes (pH 7.5). The pH gradient was determined by following the redistribution of ^{14}C MeAm or by following the chemical shift of the entrapped phosphate (see Methods).

acids that are either radiolabeled (Rottenberg et al., 1979), spin labeled (Cafiso and Hubbell, 1978b), or fluorescent (Deamer, 1982) or by following the ^{31}P chemical shift of entrapped phosphate (Mayer et al., 1988). Fig. 1 A demonstrates that redistribution of ^{14}C MeAm provides an accurate measure of pH gradients imposed across liposomal membranes. As would be expected, the inclusion of the proton ionophore CCCP results in a reduction of the proton motive force (Δp) to zero as the pH gradient measured by MeAm is equal in magnitude to the electrical potential detected by TPP⁺.

In order to further demonstrate that the MeAm redistributions accurately reflect the actual pH gradient across the vesicle bilayer we examined the MeAm response in vesicles that contained entrapped phosphate (Mayer et al., 1988). This allowed us to determine the pH gradient by following the chemical shift of the entrapped P_i . Fig. 1

B clearly indicates a close agreement between the interior pH detected by the ^{31}P NMR technique and the MeAm redistribution. The correlation between these two independent methods provides further evidence that the MeAm response accurately reflects pH gradients between the interior and exterior aqueous media.

The results to this stage show that the measured transmembrane distributions of TPP⁺ and MeAm provide accurate measures of electrical potentials and pH gradients in the LUV systems employed here. The next set of experiments were designed to measure the electrical potential and induced pH gradients obtained when LUVs containing a K^+ buffer (Na^+ exterior buffer) were incubated in the presence of valinomycin. As shown in Fig. 2, the addition of the potassium ionophore leads to the generation of an electrical potential of >160 mV (inside negative). This potential is stable over the 4 h time course (data not shown). As indicated in the Introduction, it would be expected that the presence of an electrical potential would drive proton or proton equivalent transport such that Δp would approach zero during the time course of the experiment. This did not prove to be the case. The pH gradient formed over the time course of the experiment reaches a plateau (Fig. 2) at ~1 pH unit (or 60 mV). That this plateau does not represent electrochemical equilibrium is demonstrated by the effects of the addition of the proton ionophore CCCP that causes a marked and rapid acidification of the vesicle interior. It is also clear that the presence of the proton ionophore and valinomycin together causes a destabilization of the electrical potential and the pH gradient such that a time dependent dissipation of $\Delta\Psi$ and ΔpH occurs after the addition of CCCP.

The lack of electrochemical equilibrium between the

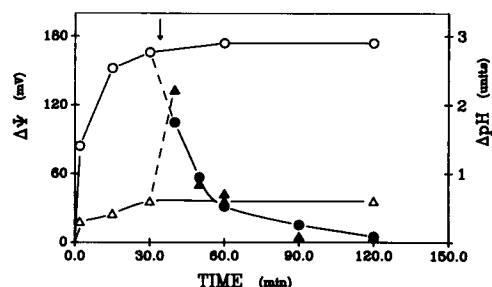


FIGURE 2 Transmembrane pH gradients induced in response to electrical potential as monitored employing methylamine. EPC LUVs were prepared in 169 mM potassium glutamate, 20 mM Hepes (pH 7.5) and the external buffer was exchanged for 150 mM NaCl, 20 mM Hepes (pH 7.5). Transmembrane electrical gradients and pH gradients were measured as outlined in Methods. ΔpH (Δ) and $\Delta\Psi$ (○) were measured after the addition of valinomycin (0.5 $\mu\text{g}/\mu\text{mol}$ phospholipid). At 38 min CCCP was added to the vesicle systems and ΔpH (▲) and $\Delta\Psi$ (●) were monitored.

electrical potential and the induced pH gradient was further demonstrated by an independent technique as shown in Fig. 3. In this experiment the ^{31}P NMR chemical shift of entrapped phosphate was monitored after the establishment of an electrical potential. The time course for the establishment of the pH gradient is similar to that observed employing MeAm (see Fig. 2). After 8 h the pH gradient was only 60 mV whereas the electrical potential was still >140 mV.

Cafiso and Hubbell (1983) have demonstrated employing spin labeled probes that the presence of an electrical potential (inside negative) across small (sonicated) unilamellar vesicles resulted in the establishment of a pH gradient (inside acidic) that reached electrochemical equilibrium with the electrical potential within 100 min. Proton or proton equivalent transport driven by imposed pH gradients was also shown to reach electrochemical equilibrium across SUVs (Cafiso and Hubbell, 1982). This observation was later extended to include other liposomal systems including LUVs of different lipid composition (Perkins and Cafiso, 1986). These observations contrast with the results presented in Figs. 2 and 3. We therefore examined whether the discrepancy could arise from differences in buffers or liposome preparations. Sonicated SUVs were prepared that exhibited a similar size (28 nm diam v.s. 30 nm diam) to those employed by Cafiso and Hubbell (1983). A K^+ chemical gradient of two orders of magnitude was employed. As shown in Fig. 4, this system exhibited a lack of electrochemical equilibrium that was even more pronounced than observed for the LUV system. An imposed electrical gradient of 118 mV did not drive formation of a bulk pH gradient as detected by methylamine accumulation. This could be attributed to a lower proton permeability in SUVs as

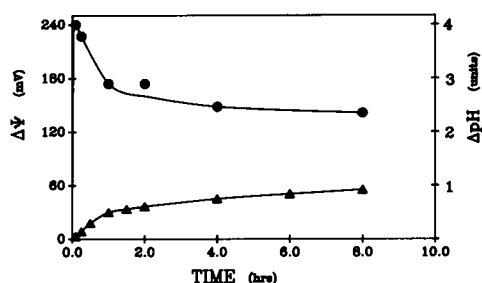


FIGURE 3 Transmembrane pH gradients induced in response to electrical potential as monitored employing ^{31}P NMR of entrapped Pi. Vesicles were prepared in 100 mM KGlu, 50 mM K_2HPI (pH 7.5) and subsequently the external buffer was exchanged for 150 mM NaCl, 20 mM Hepes (7.5). The electrical potential (●) was measured employing the radiolabeled probe TPP $^+$ (see Methods). The pH gradient (▲) was assayed by monitoring the ^{31}P NMR chemical shift of the entrapped Pi (see Methods). Valinomycin (0.5 $\mu\text{g}/\mu\text{mol}$ phospholipid) was added to the vesicles at time zero.

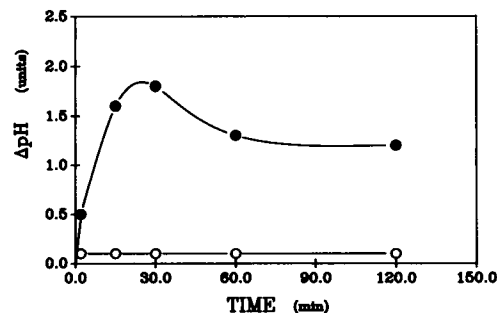


FIGURE 4 Time course for the development of pH gradients induced in response to electrical potentials in SUVs. Vesicles were prepared by sonication in 250 mM K_2SO_4 , 50 mM NaPi (pH 6.8). The external buffer was subsequently exchanged for 247.5 mM Na_2SO_4 , 2.5 mM K_2SO_4 (pH 6.8) by gel filtration. The detection of pH gradients by MeAm is outlined in Methods. The SUVs (1 mM EPC) were incubated in the presence of (○) 1.0 μg valinomycin or (▲) 1.0 μg valinomycin and 20 μM CCCP.

compared with LUVs as has been observed by others (Perkins and Cafiso, 1986). The subsequent addition of CCCP led to a rapid and marked acidification of the SUV interior, graphically illustrating the lack of electrochemical equilibrium. It may be noted that the instability of the electrochemical gradient in the presence of both valinomycin and CCCP was not as marked as observed for the LUVs.

The influence of the ionic composition of the exterior buffer on electrical potentials and induced pH gradients is illustrated in Fig. 5. These results demonstrate that the external buffer composition does, in fact, have a marked influence on the induced pH gradient. Vesicles prepared in a K^+ buffer with low buffering capacity exhibited quite different induced pH gradients when incubated in a

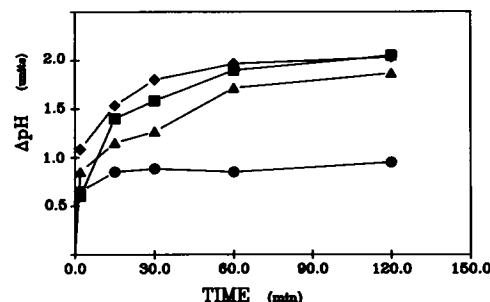


FIGURE 5 Influence of external buffer composition on the pH gradients induced in response to electrical potentials in EPC LUVs. Vesicles were prepared in 100 mM K_2SO_4 , 1 mM Hepes (pH 7.0) and the external buffer was exchanged for a buffer containing 20 mM histidine, 10 μM K_2SO_4 (pH 7.0) as well as the following salts: (●) 150 mM NaCl, (▲) 150 mM LiCl, (■) 150 mM Choline Cl, (◆) 200 mM sucrose. The pH gradient was detected by following the redistribution of the radiolabeled probe MeAm as described in Methods.

variety of external buffers. As expected, the vesicles exhibited similar electrical potentials regardless of the choice of external buffer (data not shown). However vesicles with external buffer containing NaCl exhibited the lowest induced pH gradient (60 mV). In contrast, vesicles with exterior LiCl, Choline Cl, or sucrose exhibit larger pH gradients that approach 120 mV at 2 h. Addition of the proton ionophore CCCP to these vesicle systems led to an additional increase in the pH gradient (data not shown) indicating that complete electrochemical equilibrium was not achieved. An interesting feature of these systems was the fact that the electrochemical gradient was stable in the presence of both valinomycin and CCCP, in contrast to the instability noted in the presence of external NaCl. The external presence of anions such as SO_4^{2-} or Glu^- for Cl^- did not effect the magnitude of the induced pH gradient when Na^+ ions were present (data not shown).

The lack of electrochemical equilibrium between the electrical potential and induced pH gradient in the presence of external Na^+ led us to examine the relation between imposed pH gradients and the induced electrical potentials. Points of interest concern whether electrochemical equilibrium is observed and whether Na^+ ions influence such equilibrium. The results of Fig. 6, *A* and *B* demonstrate that proton equivalent transport can be followed by monitoring the appearance of an electrical potential (inside negative) in an LUV after establishing a pH gradient (inside acidic). In Fig. 6 *A* the proton equivalent transport in the absence of Na^+ ions is presented. As would be expected, the proton chemical potential across the membrane drives electrogenic proton transport resulting in electrical potentials which approach equilibrium values. The generation of the electrical potential is time dependent and complete electrochemical equilibrium is not reached even at 2 h. This is in contrast to results obtained for vesicles prepared by reverse phase techniques that indicated electrochemical equilibrium at 20 min (Perkins and Cafiso, 1986). Fig. 6 *A* also indicates the effect of ionophores on proton equivalent transport. The addition of valinomycin increases proton transport significantly as has also been observed elsewhere (Rossignol et al., 1982). The addition of the proton ionophore CCCP leads to a stable electrochemical equilibrium. The presence of both CCCP and valinomycin does not affect this stability.

The induced electrical potentials observed when Na^+ ions are present in the external medium are shown in Fig. 6 *B*. Given the previous results indicating that Na^+ reduces the induced ΔpH observed in response to $\Delta\Psi$, it is perhaps surprising that the presence of Na^+ ions does not affect the rate of proton equivalent transport as detected by the induced electrical potential. Similar results were also obtained when Na^+ ions were present in the interior

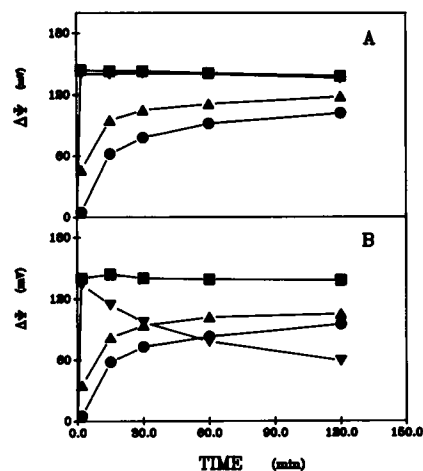


FIGURE 6 Time course for the development of an electrical potential (inside negative) generated in response to transmembrane pH gradients in EPC LUVs. Vesicles were prepared in 50 mM citric acid (pH with histidine to 4.0) and the external buffer was exchanged for a buffer (*A*) containing 200 mM sucrose, 20 mM histidine (pH 7.0) or (*B*) an external buffer containing 150 mM NaCl, 20 mM histidine (pH 7.0). The electrical potential was detected by following the redistribution of the radiolabelled probe TPP^+ as described in Methods. The LUVs (1 mM EPC) were incubated in the presence of (●) no ionophores, (▲) 1.0 μg valinomycin, (■) 20 μM CCCP or (▼) 1.0 μg valinomycin, and 20 μM CCCP.

buffer (data not shown). Again, complete electrochemical equilibrium is not reached in this system at 2 h. Addition of valinomycin causes a slight increase in the transport of proton equivalents but there is no evidence that components of the buffer are transported in a manner that leads to decay of the electrochemical gradients. Addition of the proton ionophore CCCP results in the generation of a stable electrochemical equilibrium. The addition of both valinomycin and CCCP also leads to electrochemical equilibrium, however, a slow time dependent dissipation of the electrochemical gradient is subsequently observed. This dissipation is not seen in Fig. 6 *A* indicating that it is caused by the NaCl buffer.

We now return to two observations made in the course of this work which indicate that, in the presence of exterior Na^+ , the presence of both CCCP and valinomycin causes dissipation of the electrochemical gradients. In the case of valinomycin induced electrical potentials observed in LUVs with interior K^+ and exterior Na^+ buffers, the addition of CCCP results not only in electrochemical equilibrium but also in a rapid subsequent decrease of $\Delta\Psi$ and ΔpH . This behavior is not observed in the presence of exterior ions such as choline or Li^+ . Alternatively, in the systems where an imposed ΔpH leads to an induced $\Delta\Psi$, the presence of valinomycin and CCCP also results in a rapid decrease in ΔpH and $\Delta\Psi$ when Na^+

is present, but not when other external buffers are employed (Fig 6). These observations suggest that CCCP and valinomycin may act synergistically to transport Na^+ ions, thus dissipating electrochemical gradients. In order to test this possibility, the ability of these ionophores to induce electrical potentials in systems exhibiting Na^+ chemical gradients was investigated. LUVs were prepared in the presence of a Na^+ containing buffer and the external buffer was then exchanged for a sucrose containing buffer. The flux of Na^+ can be followed by measuring the generation of an electrical potential or pH gradient (as detected by TPP^+ or MeAm, respectively). As would be expected, in the absence of any ionophores there is no net movement of Na^+ and consequently no net generation of an electrical potential. As shown in Fig. 7, the presence of valinomycin or CCCP alone does not increase the flux of Na^+ ions as detected by the establishment of a membrane potential. However, the presence of both valinomycin and CCCP causes the establishment of an electrical potential (inside negative) and pH gradient (inside acidic). This experiment demonstrates that valinomycin and CCCP can act synergistically to transport Na^+ ions. It is not clear whether or not this is electrogenic or electroneutral transport.

DISCUSSION

The results presented here provide new information regarding the transport of protons or proton equivalents across membranes. There are three major points of interest. The first concerns the accuracy of the electrical potentials and pH gradients reported by the radiolabeled probes TPP^+ and MeAm. The second point concerns the lack of electrochemical equilibrium between $\Delta\Psi$ and the induced ΔpH , particularly in the presence of external Na^+ . Finally, the observations that CCCP and valinomycin together can facilitate Na^+ transport are discussed.

There are three lines of evidence supporting the reliability of the electrical potential and pH gradients reported by TPP^+ and MeAm. First, the electrical potential reported by TPP^+ for LUV systems exhibiting imposed pH gradients (in the presence of CCCP) correlates well to the theoretical values expected for electrochemical equilibrium. This also supports previous work on valinomycin induced electrical potentials in LUV systems containing K^+ ions where it was found that the electrical potential reported by MTTP^+ (Hope et al., 1985) and TPP^+ (Mayer et al., 1988) correlated well with theoretical values calculated from the K^+ ($^{42}\text{K}^+$) transmembrane concentration gradients. Second, in the case of MeAm, the pH gradient reported correlates well with imposed transmembrane pH gradients. Further, the pH gradients measured by MeAm agree closely with those measured by

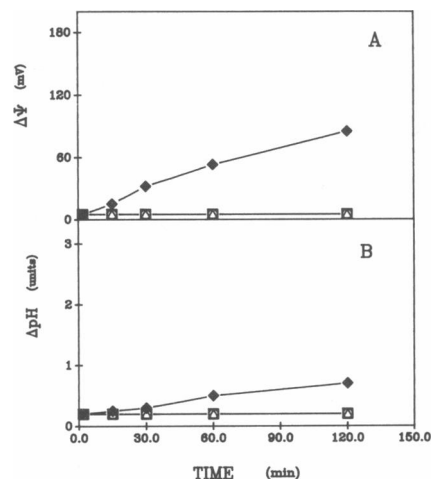


FIGURE 7 Time course for the development of an electrical potential (inside negative) or a pH gradient (inside acidic) for vesicles experiencing a Na^+ diffusion potential in the presence of a variety of ionophores. Vesicles were prepared in 100 mM Na_2SO_4 , 1 mM HEPES (pH 7.0) and the external buffer was exchanged for a buffer containing 20 mM histidine, (pH 7.0). The electrical potential (A) and pH gradient (B) were detected as outlined in Methods. The LUVs (1 mM EPC) were incubated in the presence of (O) no ionophores, (Δ) 1.0 μg valinomycin, (\square) 20 μM CCCP or (\blacklozenge) 1.0 μg valinomycin, and 20 μM CCCP.

monitoring the ^{31}P NMR chemical shift of entrapped Pi. A final point is that the electrochemical gradient reported by MeAm and TPP^+ are internally self consistent. Thus in systems where electrochemical equilibrium is expected, the pH gradient and electrical potentials obtained are consistent with a proton motive force of zero. This is observed in LUVs with imposed pH gradient in the presence of CCCP (Fig. 6 A) and in LUVs with a K^+ ion gradients in the presence of valinomycin and CCCP (Fig. 2).

The observed accuracy of electrochemical gradients reported by TPP^+ and MeAm would not be expected *a priori* for the LUV systems employed here. This is mainly due to their small size and the lipophilic character of the probe molecule. For example, LUV of diameter 90 nm would be expected to have an internal aqueous volume to lipid volume 2.3, assuming a bilayer thickness of 5 nm. Given the lipophilic character of membrane potential probes (Demura et al., 1985; Demura et al., 1987; Lee and Forte, 1978; Cools and Janssen, 1986) and pH probes, a large proportion of the LUV-associated probes would be expected to be associated with the bilayer rather than contained in the interior aqueous compartment. In the case of the membrane potential probes this would be expected to lead to overestimation of the actual electrical potential. As detailed here, such deviations are not observed. The reasons for this are not understood, but may be related to a greater ability to remove the lipid

associated as opposed to the entrapped probe during the chromatographic procedure used here to separate LUVs from untrapped material.

We now turn to the lack of electrochemical equilibrium between membrane potentials developed by valinomycin in LUVs containing K^+ buffer and the induced pH gradients. As detailed here, this effect is particularly obvious when the external buffer contains Na^+ and the lack of equilibrium is unambiguously demonstrated by the much larger pH gradients induced in the presence of CCCP. Particular points of interest concern the discrepancy between these results and previous studies by other workers, the possible mechanisms that could result in such a lack of equilibrium and the role of nominally nonpermeating ions in influencing the magnitude of induced pH gradients.

Previous work employing SUVs (Cafiso and Hubbell, 1983) and spin labeled probes for the detection of electrical potentials and pH gradients indicated that pH gradients induced in response to membrane potentials come to electrochemical equilibrium within 100 min. In our hands, SUVs with a similar buffer composition did not exhibit a detectable induced ΔpH after 2 h. This discrepancy cannot be attributed to the small size of the vesicles or the buffer composition. Similarly, Perkins and Cafiso (1986) have shown for LUVs produced by the reverse phase technique that an electrical potential corresponding to electrochemical equilibrium is observed within 20 min after establishing a transmembrane pH gradient. This contrasts with the observations presented here where complete equilibrium is not reached even after 2 h. Possible reasons for these discrepancies could include an influence of the membrane associated spin labeled probes on proton or proton equivalent permeability or some small differences in the properties of the lipid or composition of the bilayer. The LUVs produced by extrusion have the important advantage that organic solvents or detergents are not employed for their synthesis. The possibility that residual organic solvents or lipid oxidation products are present that increase proton permeability (Cafiso and Hubbell, 1982; Gutknecht, 1987) should not be easily dismissed. It should also be noted that other workers (Garcia, 1984; Konishi et al., 1986) noted a lack of electrically driven proton transport, which they attributed to a threshold effect where an electrical potential of at least 60 mV is required to drive proton or proton equivalent transport.

The physical basis for an inequivalence between electrical potentials and induced transmembrane pH gradient is clearly of major interest. The small quasi-equilibrium induced pH gradient of 1 pH unit (60 mV) or less induced in response to an electrical potential of 150 mV or more suggests an inequivalence between proton fluxes induced in response to proton gradients and those induced in

response to electrical potentials. In particular these results suggest that a lower proton flux is obtained in response to electrical potentials than is obtained in response to chemical potentials. Such behavior has been reported previously. For example, Krishnamoorthy and Hinkle (1984) observed a proton equivalent flux of 1.6 nmol/min/mg phospholipid for a chemical driving force of 60 mV in asolectin vesicles. The corresponding flux for a 60 mV electrical potential was only 0.5 nmol/min/mg phospholipid, while the flux for a 160 mV electrical driving force was 2.2 nmol/min/mg phospholipid. This would suggest that a pseudo equilibrium condition could be achieved in these vesicles when an electrical potential of 160 mV was offset by a pH gradient of only 60 mV.

There is some theoretical basis for the suggestion that proton or proton equivalent flux in response to chemical gradients can be larger than for an electrical potential. In particular, among the models for transmembrane proton transport by transient hydrogen bonded water (Nagle, 1987) such observations could be consistent with a model where proton equivalent conduction is mediated by a transient hydrogen bonded chain (*tHBC*) which stretches across one phospholipid monolayer. This model, originally suggested by Deamer and Nichols (1983) appears to be the only model analyzed by Nagle (1987) that could account for the observed quasi-equilibrium condition. Other models suggest that proton equivalent transport in response to electrical potentials is equal to or greater than proton equivalent transport in response to chemical gradients.

However, this model does not provide a clear understanding of the smaller induced pH gradients observed when Na^+ ions are present in the external buffer. In this model the rate limiting step for proton translocation in response to electrical potentials is the recombination of *tHBC*, and it is difficult to see how the presence of Na^+ ions should slow down this recombination. One possibility is that the presence of Na^+ ions in the external buffer facilitate the movement of OH^- ions in response to the chemical driving force or that Na^+-H^+ exchange can occur due to some unidentified factor. However, one would then expect to see an effect of Na^+ ions on the stability of a pH gradient, which was not observed (see Fig. 6 B). This leaves open the possibility that Na^+ ions can alter the concentration of *tHBC*, or that Na^+ ions exert their effect on OH^- ion movement in response to electrical potentials only at neutral or basic pH values.

The final topic of discussion involves the striking observation that valinomycin and CCCP can catalyze the transport of Na^+ ions across a liposome in a synergistic manner. Ternary complexes have been described for a variety of uncouplers of oxidative phosphorylation (Yoshikawa and Terada, 1981; Blok et al., 1974; Castaing et al., 1986). Blok and co-workers first suggested that

valinomycin can form a ternary complex with K^+ and SCN^- anion (Blok et al., 1974). Since then Yoshikawa and Terada have suggested that valinomycin and K^+ can form ternary complexes (1:1:1 ratio) with a variety of weak acid uncouplers such as FCCP and 2,4-dinitrophenol (Yoshikawa and Terada, 1981). These complexes will accelerate the transport of both protons and K^+ ions in a synergistic manner. Finally, Castaing and co-workers (1986) have demonstrated that FCCP can accelerate the transport of both K^+ and Na^+ ions across LUVs by a macropolycyclic complexing agent (222) C_{10} -cryptand. These observations are consistent with the possibility that CCCP, valinomycin and Na^+ ions can form a ternary complex that can synergistically promote net Na^+ transport.

In summary, the results presented here show that radiolabeled probes of membrane potential (TPP⁺) and pH gradient (MeAm) provide surprisingly accurate measures of electrical potentials and pH gradients in LUV systems. These probes have been employed to demonstrate that, particularly in the presence of external Na^+ ions, the induced pH gradients observed in response to K^+ diffusion potentials are appreciably smaller than would be expected on the basis of electrochemical equilibrium. It is suggested that this reflects a higher proton or proton equivalent flux in response to proton chemical gradients than for nominally equivalent electrical potentials. Finally, it is demonstrated that the K^+ ionophore valinomycin and the proton ionophore CCCP can transport Na^+ ions in a synergistic manner. It is suggested that valinomycin, CCCP and Na^+ ions can form a ternary, membrane permeable complex.

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