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# A transmembrane potential does not affect the vertical location of charged lipid spin labels with respect to the surface of a phosphatidylcholine bilayer

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**Abstract**

The effect of a transmembrane potential on the vertical location of a charged lipid in a neutral phosphatidylcholine (PC) lipid bilayer has been investigated using negatively and positively charged spin-labeled lipids. A transmembrane potential was generated across extruded large unilamellar vesicles either by using a $K^+/Na^+$ ion gradient and a $K^+$ ionophore or by using a pH gradient. Since a transmembrane potential could have opposing effects on lipids in the inner and outer monolayer, some of the acidic spin labels were asymmetrically located in the inner monolayer as a result of a pH gradient. No significant effect on their order parameters was observed upon applying a transmembrane potential. The internal dipole potential of the bilayer was modified by using dialkyl-PC or by incorporating 10 mol% phloretin, or 6-ketocholestanol in the PC, but a transmembrane potential still had no detectable effect on the spin labeled lipids. Therefore, it is concluded that the electrochemical potential across membranes probably does not cause a significant change in the vertical location of charged lipids with respect to the surface of a PC bilayer. This suggests that polar interactions and/or van der Waals interactions between the spin probe and the surrounding lipids stabilize the overall structure of the membranes and these interactions are not disrupted by a selective effect of the transmembrane potential on the charged lipids.

*Keywords:* Transmembrane potential; Lipid bilayer; Spin label; EPR

## 1. Introduction

The transmembrane electrical potential which exists across various biological membranes might modulate membrane dynamics via effects on membrane proteins and/or the lipid bilayer. Studying the effect of a transmembrane potential on proteins or lipids will help to understand the molecular mechanism of various cellular processes which are regulated by membrane potential. Many studies have implicated the role of a transmembrane potential in regulation of protein function or conformation [1–4]. An inside negative transmembrane potential facilitates the translocation of positively charged signal peptides of mitochondrial proteins across a lipid bilayer [5]. The transmembrane potential may also change the conformation or depth of insertion of voltage-regulated ion channels in the lipid bilayer [6].

On the other hand, there has been a controversy as to whether the electrochemical potential modifies the properties of membrane lipids, such as bilayer fluidity and thickness and headgroup conformation [7–15]. Furthermore, the effect of a transmembrane potential on the vertical location of a charged lipid in a neutral lipid environment has not been specifically addressed. Therefore, we have used spin labeled charged lipids or lipid analogues in order to determine if a transmembrane potential can cause the electrophoretic vertical movement of the charged lipid probe with respect to the neutral lipid surrounding it. Since the lipid on one side of the bilayer will be affected by a membrane potential in the opposite way from the lipid on the other side of the bilayer, we have, where possible, used

Abbreviations: LUVs, large unilamellar vesicles; MLVs, multilamellar vesicles; DHPC, dihexadecylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; CBS, cerebroside sulfate; chol, cholesterol; 5-S-SL, 5-doxylstearic acid; 12-S-SL, 12-doxylstearic acid; 16-S-SL, 16-doxylstearic acid; HTEMPO, 4-(*N*,*N*-dimethyl-*N*-n-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl; EPR, electron paramagnetic resonance.

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asymmetric vesicles with the spin label probe located primarily in the inner monolayer.

Large unilamellar vesicles (LUVs) consisting of a charged lipid spin probe (1%) and phosphatidylcholine (PC) were produced by the extrusion technique, and a transmembrane potential across the bilayer was generated by using a $K^+/Na^+$ ion gradient and the $K^+$ ionophore valinomycin or by using a transmembrane pH gradient. A pH gradient with high pH inside and low pH outside the vesicles produces not only an inside-positive transmembrane potential but also asymmetric vesicles with the ionized form of acidic spin probes primarily on the inner leaflet [22]. If the positive potential induced inside the vesicles attracts the negatively charged spin probe in the inner layer toward it, a probe on the fatty acid chain would be located in a more ordered region of the bilayer, which would be reflected in its EPR spectrum [16].

If the spin labels are in both monolayers, the charged spin label in the outer monolayer will be affected in the opposite way from the one in the inner monolayer upon applying a transmembrane electrical potential, resulting in a composite spectrum which is the sum of spectra characteristic of greater order and greater disorder. This would result in broadening of the spectrum.

## 2. Materials and methods

### 2.1. Lipids and spin label

Egg phosphatidylcholine (egg PC) and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma (St. Louis, MO). Dihexadecylphosphatidylcholine (DHPC) was from Fluka (Switzerland). Cholesterol (chol) was obtained from Supelco (Bellefonte, PA). 5-Doxylstearic acid (5-S-SL), 12-doxylstearic acid (12-S-SL), and 4-(*N*,*N*-dimethyl-*N*-n-hexadecylammonio)-2,2,6,6-tetramethylpiperidine-1-oxyl iodide (HTEMPO) were purchased from Aldrich (Milwaukee, WI). 16-Doxylstearic acid (16-S-SL) was from Syva (Palo Alto, CA). 1-Palmitoyl-2-(16-doxylstearoyl)-*sn*-glycerol-3-phosphoglycerol (PG-16-S-SL) was generously supplied by Dr. A. Watts (University of Oxford). 5-Doxylstearoyl cerebroside sulfate (CBS-5-S-SL) and 12-doxylstearoyl cerebroside sulfate (CBS-12-S-SL) were synthesized as described [17]. Phloretin and 6-ketocholestanol were purchased from Sigma (St. Louis, MO).

### 2.2. Preparation of multilamellar vesicles (MLVs)

Chloroform/methanol (2:1, v/v) solutions of egg PC, cholesterol, and spin probe, were combined to give a PC/probe molar ratio of 100:1, or a PC/cholesterol/probe molar ratio of 100:95:1, were dried under nitrogen gas, and were evacuated in a lyophilizer for 2 h. When phloretin or 6-ketocholestanol was incorporated, the molar ratio of PC/phloretin (or 6-ketocholestanol)/probe was 100:10:1. The dry lipid film was dissolved in 1–2 ml of benzene, and the lipid solution was frozen and lyophilized overnight. Large multilamellar vesicles were prepared by hydrating the dry lipids in an appropriate buffer and vortexing them for 15 min. The final concentration of PC was 25–100 mM. When DPPC (or DHPC)/cholesterol was used, the lipid dispersion was incubated at 50°C (8°C above the phase transition temperature) before and during vortexing.

### 2.3. Preparation of large unilamellar vesicles (LUVs)

The MLV dispersion was freeze-thawed five times using solid $CO_2$ and warm water in order to promote equilibrium transmembrane solute distributions [18]. The dispersion (1.5 ml) was then transferred into the extruder (produced by Lipex Biomembranes, Vancouver, B.C.) which allowed the extrusion of the MLVs through polycarbonate filters with 0.2 $\mu$m pore size under nitrogen pressure [19,20]. After passing the dispersion through two stacked filters ten times, the vesicles were extruded again through new filters with 0.1 $\mu$m pore size.

### 2.4. Generation of transmembrane electrical potential using an ion gradient

0.3–0.6 ml of LUVs, containing HTEMPO, prepared in 150 mM $K_2SO_4$/100 mM Hepes/2 mM EDTA (pH 7.4) were passed through two consecutive Sephadex G-50 columns (3 cc) eluted with 150 mM $Na_2SO_4$/100 mM Hepes/2 mM EDTA (pH 7.4) by centrifugation at 2000 rpm for 3 min in order to replace the external $K^+$ with $Na^+$. When phloretin ($pK \approx 9.0$) or 6-ketocholestanol was incorporated, 50 mM Mes/100 mM $K_2SO_4$ (pH 5.8) and 50 mM Mes/100 mM $Na_2SO_4$ (pH 5.8) were used. 1 ml of the $K^+$-ionophore, valinomycin (Sigma, St. Louis, MO), dissolved in DMSO (1 mg/ml) was added to 100 ml of LUVs (final valinomycin concentration was $10^{-5}$ M, lipid/valinomycin = $10^4$:1, molar ratio). The resulting LUVs had not only a transmembrane potential (negative inside) but also a pH gradient (acidic inside) due to an anti-transport of protons [21]. However, this will have no effect on the PC or HTEMPO used in these vesicles. In order to chemically reduce the HTEMPO in the outer layer, 40 $\mu$l of 4 mM ascorbic acid were added to 40 $\mu$l of LUVs on ice, so that the spectrum of only HTEMPO on the inner monolayer is observed.

### 2.5. Generation of vesicles with a pH gradient and asymmetric lipid distribution

0.3–0.6 ml of LUVs, containing doxylstearic acid, prepared in borate buffer at pH 10 (150 mM $H_3BO_3$ adjusted to pH 10 with NaOH) were passed through two consecutive Sephadex G-50 columns (3 cc) eluted with either Hepes buffer at pH 7 (150 mM NaCl/20 mM Hepes) or

Mes buffer at pH 6.5 (150 mM NaCl/20 mM Mes). In the case of LUVs containing doxyl-PG, 0.3–0.6 ml of LUVs prepared in buffer at pH 9 (50 mM $Na_2B_4O_7 \cdot 10\ H_2O$ adjusted to pH 9 with $H_3BO_3$) were passed through two Sephadex G-50 columns eluted with buffer at pH 4 (37.5 mM sodium citrate adjusted to pH 4 with citric acid). As spin-labeled stearic acids or PG in the outer leaflet are protonated at low pH, they translocate into the inner leaflet where they are ionized at high pH and remain, resulting in asymmetric vesicles with an ionized form of the spin labels only in the inner leaflet [22]. These vesicles develop an inside-positive potential as protons move into the vesicles.

### 2.6. Electron paramagnetic resonance (EPR) spectra of spin-labeled vesicles

A Varian E-104 B EPR Spectrometer fitted with a variable temperature controller was used. EPR spectra of the MLVs containing 1% spin probes at various pH values were measured in order to study the effect of ionization on the vertical location of the lipid spin probe in the bilayer. EPR spectra of the LUVs with and without a transmembrane potential were measured to study the effect of a membrane potential on the vertical location of the charged lipid spin probe. The order parameter $S$ or motional parameter $\tau_0$ was measured as described earlier [23]. The order parameter, $S$, varies between 0 (in a highly disordered environment) and 1 (in a highly ordered environment) and increases as the spin label moves from the center of the bilayer to the carbonyl region [16]. The motional parameter, $\tau_0$, is inversely proportional to the rotational frequency of the spin label; the larger $\tau_0$ is, the less fluid the environment surrounding the spin label is.

### 2.7. Measurement of transmembrane potential

The negative-inside potential was measured by using a lipophilic cation, $^3$H-labeled methyltriphenylphosphonium ion ($MTPP^+$). 2 ml of [$^3$H]MTPPI (New England Nuclear, 0.2 $\mu$Ci/$\mu$l) in 20% ethanol were added to 0.3–0.6 ml of LUV dispersion. The mixture was left at room temperature for 15 min to allow $MTPP^+$ to diffuse into LUVs according to $\Delta\Psi$ [24]. 100 $\mu$l of the mixture was passed through two consecutive Sephadex G-50 minicolumns (1 cc) to remove untrapped [$^3$H]$MTPP^+$. An aliquot (50 $\mu$l) of the eluate was taken and counted for the amount of [$^3$H]$MTPP^+$ inside the vesicles. Using the concentrations of $MTPP^+$ inside and outside the vesicles, the potential was calculated using the Nernst equation as follows.

$$\Delta\Psi(\mathrm{mV}) = -59\log([\mathrm{MTPP}^+]_{in}/[\mathrm{MTPP}^+]_{out}) \quad (1)$$

The positive-inside potential was measured by using a membrane-permeable anion, $^{14}$C-labeled thiocyanate ion ($SCN^-$) [22]. 2 $\mu$l of $KS^{14}CN$ (Amersham, 0.2 $\mu$Ci/$\mu$l) in double-distilled water were added to 0.3–0.6 ml of LUVs, and the potential was calculated using the Nernst equation as follows.

$$\Delta\Psi(\mathrm{mV}) = 59\log([\mathrm{SCN}^-]_{in}/[\mathrm{SCN}^-]_{out}) \quad (2)$$

The membrane potential developed using either an ion gradient or a pH gradient was measured at various times and found to be very stable for hours as reported by others [19,21,24]. The EPR spectra were measured at a time when the potential was maximal and before significant dissipation had occurred. The potential was measured on a separate aliquot of the same LUV preparation immediately after measurement of the EPR spectrum.

## 3. Results

### 3.1. Effect of change in ionization of stearic acids on EPR spectrum

It has been demonstrated that ionization of spin-labeled stearic acids affects their spectra. This is thought to be due to location of the ionized form closer to the lipid headgroup region than the neutral form [25,26]. Therefore, the spectra of 5-S-SL, 12-S-SL, and 16-S-SL in phosphatidylcholine bilayers at pH 10 were compared with those at pH 6–7 in order to determine the magnitude of the effect of this change in location on the order parameter ($S$) and the motional parameter ($\tau_0$).

Table 1 shows changes in $S$ or $\tau_0$ upon changing pH. $S$ (or $\tau_0$) was larger for 5-S-SL, 12-S-SL, and 16-S-SL at pH 10 than at pH 6–7 with the exception of 16-S-SL in DPPC/chol. 12-S-SL was affected most (Table 1). This can be explained by $^2$H-NMR studies [27], which show that the order parameter of the lipid bilayer in the fluid phase changes more with carbon number for carbons 9–18 than for carbons 1–8. Therefore, the order parameter of spin labels should be more sensitive to their vertical

Table 1
Effect of ionization of stearic acids and acidic lipids on their order or motional parameters[a]

| Spin probe | Surrounding lipids | $S_{high\ pH}$[b] or $((\tau_0)_{high\ pH})$ | $S_{low\ pH}$[b] or $((\tau_0)_{low\ pH})$ | $\Delta S$[c] or $(\Delta\tau_0)$ |
|---|---|---|---|---|
| 5-S-SL | egg PC | 0.650 | 0.642 | 0.008 |
| | DPPC/chol | 0.721 | 0.714 | 0.007 |
| 12-S-SL | DPPC/chol | 0.644 | 0.616 | 0.028 |
| | DHPC/chol | 0.657 | 0.635 | 0.022 |
| 16-S-SL | egg PC | (1.17) | (1.06) | (0.11) |
| | DPPC/chol | (1.73) | (1.78) | (−0.05) |
| PG-16-S-SL | egg PC | (1.80) | (1.77) | (0.03) |
| CBS-5-S-SL | egg PC | 0.723 | 0.727 | −0.004 |

[a] All EPR spectra were measured at 22.5°C. Values in brackets are $(\tau_0)$ values in ns.
[b] High pH was pH 10 for fatty acid spin labels and pH 9 for PG-SL and CBS-SL. Low pH was pH 6–7 for fatty acid spin labels and pH 4 for PG-SL and CBS-SL.
[c] $\Delta S = S_{high\ pH} - S_{low\ pH}$; $\Delta\tau_0 = (\tau_0)_{high\ pH} - (\tau_0)_{low\ pH}$. $\Delta S$ values of 0.005 and $\Delta\tau_0$ values of 0.06 ns are considered significant.

location in the middle of the bilayer than near the ester linkage. PG-16-S-SL in egg PC was not affected by pH. The negatively charged CBS-5-S-SL was not expected to show any change in its order parameter upon changing pH, since the sulfate group would not be protonated even at pH 4. To conclude, the EPR spectrum is sensitive to a change in the vertical location of doxylstearic acids, especially for 12-S-SL.

The locations of the carboxyl group of the ionized and protonated forms of the fatty acid in the PC bilayer are not known. However, it is reasonable to suggest that the ionized carboxyl interacts electrostatically with the choline moiety, while the protonated species may hydrogen bond with either glycerol oxygens or with the ester carbonyl oxygens. Using X-ray and neutron diffraction, it has been shown that the distance between the choline and the glycerol carbons in dioleoylphosphatidylcholine is about 4 Å and between the choline and the carbon of the ester carbonyl is about 6 Å [35]. Thus, on ionization the fatty acid may move toward the surface of the bilayer by 4–6 Å, resulting in an increase in order parameter of 12-S-SL of 0.028 (Table 1). Since a difference of 0.005 or greater can be detected, this indicates that the minimum vertical movement of 12-S-SL which could be detected is 0.7–1.1 Å.

### 3.2. *Effect of transmembrane electrical potential on the vertical location of stearic acids*

Since the order parameter was sensitive to a change in the vertical location of stearic acids caused by ionization of the carboxyl group, the effect of a transmembrane electrical potential on the vertical location of stearic acids was examined by measuring the order parameter.

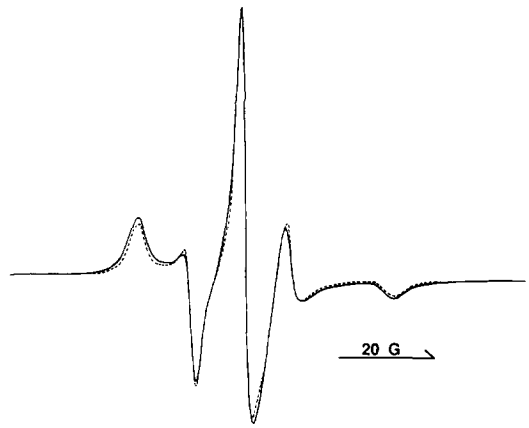


Fig. 1. EPR spectra of 5-S-SL in egg PC vesicles in the presence of a positive inside potential ($\Psi = +81$ mV) (—) due to a pH gradient, and in the absence of a transmembrane potential at pH 10 (- - -). The spin label is asymmetrically distributed on the inner monolayer of the vesicles in the presence of the pH gradient (pH 10 inside, pH 7 outside).

First of all, the effect of an inside-positive membrane potential, induced by a pH gradient, on 5-S-SL asymmetrically located in the inner monolayer of egg PC vesicles was determined. As shown in Table 2, the order parameter was not affected significantly by a potential for three different experiments. However, changes in the line shape were observed. The height of the outer hyperfine peaks was greater, and the height of the inner peaks was smaller in the presence of a potential in all three experiments (Fig. 1). Symmetric vesicles with a valinomycin-induced inside-negative potential showed the same phenomenon.

It was suspected that the shape of LUVs might be changed upon inducing a potential, and this might affect either the packing of the lipid bilayer or the orientation of the vesicles in the capillary tube. It has been reported that LUVs prepared by the extrusion technique have an oblong tubular shape. A spherical shape can be subsequently induced by generating an osmotic gradient across the bilayer [28]. This was done by replacing the external buffer of vesicles prepared in 150 mM $K_2SO_4$ with a buffer containing 75 mM $K_2SO_4$. The effect of reducing the ionic strength outside the vesicles on the spectrum was determined. There was no effect on the spectrum relative to that of LUVs under iso-osmotic conditions in the absence of a potential indicating that the vesicular shape itself does not affect the line shape of the spectrum. Furthermore the spectrum was similar in MLVs.

The observed change in height of the outer hyperfine peaks of the spectrum suggests an increase in the number of spin label molecules oriented parallel to the magnetic field. This could be caused by an increase in the number of the oblong vesicles oriented perpendicular to the magnetic field, and a decrease in the number oriented parallel to the

Table 2
Effect of transmembrane electrical potential on the order and motional parameters of stearic acids [a]

| Spin probe | Surrounding lipids | $\Delta\Psi$ [b] | $S(0)$ or ($\tau_0(0)$) | $S(\Psi)$ or ($\tau_0(\Psi)$) | $\Delta S(0-\Psi)$ [c] or ($\Delta\tau_0(0-\Psi)$) |
|---|---|---|---|---|---|
| 5-S-SL | egg PC | +81 mV | 0.633 | 0.635 | −0.002 |
| | | +96 mV | 0.624 | 0.627 | −0.003 |
| | | +100 mV | 0.631 | 0.629 | 0.002 |
| | | −145 mV | 0.629 | 0.627 | 0.002 |
| 12-S-SL | DPPC/chol | +106 mV | 0.640 | 0.637 | 0.003 |
| | DHPC/chol | +105 mV | 0.655 | 0.655 | 0.000 |
| 16-S-SL | egg PC | +150 mV | (1.58) | (1.48) | (0.10) |
| | DPPC/chol | +116 mV | (1.78) | (1.80) | (−0.02) |

[a] All spectra were measured at 22.5°C except the spectrum of 16-S-SL in egg PC, which was measured at 6.5°C. $\tau_0$ values in ns are given in brackets. All vesicles contain the fatty acid spin label asymmetrically distributed in the inner monolayer except those with a negative inside potential which have a symmetric distribution.

[b] $\Delta\Psi$ is the electrical potential inside relative to the outside of the vesicles. Negative $\Delta\Psi$ was obtained by using a $K^+/Na^+$ gradient and valinomycin. All other positive potentials were obtained by using a pH gradient (pH 10/pH 6.5–7).

[c] $\Delta S$ (or $\Delta\tau_0$) was obtained by subtracting $S(\Psi)$ (or $\tau_0(\Psi)$) in the presence of potential from $S(0)$ (or $\tau_0(0)$) in the absence of potential. For comparison with a positive potential, pH 10 was used for both the inside and outside of vesicles in the absence of a potential. For comparison with a negative potential, vesicles in the absence of a potential had $K^+$ inside and outside.

field. Cylindrical orientation of smectic liquid crystals in very small diameter glass capillaries results in similar changes in the spectrum recorded with the magnetic field perpendicular to the cylinder axis from that observed for a random distribution of smectic liquid crystals [36]. Therefore, the observed changes in the line shape are probably not due to changes of the vertical location of stearic acids but due to changes in orientation of the vesicles in the magnetic field.

12-S-SL in DPPC/chol vesicles was also not significantly affected by the presence of the electrical potential (Table 2). 16-S-SL in egg PC vesicles became more mobile upon applying an inside-positive electrical potential, contrary to the expectation (Table 2). On the other hand, the same spin probe in DPPC/chol vesicles did not show a detectable change in its spectrum. These results with 16-S-SL could have been caused by a decrease in pH inside the vesicles (Table 1) as the pH gradient decreases. Thus the electrical potential does not affect the vertical location of stearic acids significantly.

### *3.3. Effect of electrical potential on the vertical location of PG and CBS*

The effect of a transmembrane potential on the vertical location of negatively charged PG and CBS spin labels was examined. PG-16-S-SL incorporated in egg PC LUVs was expected to be in the ionized form at pH 9, and it was induced to translocate into the inner layer of the bilayer in a protonated form by changing the external buffer to pH 4. However, it has been reported that PG cannot translocate completely even at 45°C, and the maximum ratio obtained was 3:1 (inner layer/outer layer) [29]. Therefore, a composite spectrum was expected due to contributions from both the negatively charged PG-SL in the inner layer and the neutral (protonated) PG-SL in the outer layer. However, the neutral form in the outer layer should not be as responsive to a membrane potential. Since pH did not affect the spectrum of PG-SL, any changes which occur can be attributed to an effect of membrane potential (Table 1). However, the motional parameter of PG-16-S-SL was not significantly affected by an inside-positive membrane potential ($\Delta\tau_0(0 - \Psi) = 0.05$ ns).

A transmembrane potential was alternatively generated by using a $K^+/Na^+$ ion gradient and valinomycin. An effect of a membrane potential in this case might have resulted in broadening of the spectrum due to opposing effects on lipids on both sides of the bilayer. However, neither a positive-inside potential nor a negative-inside potential caused a dramatic change in the motional parameter ($\Delta\tau_0(0 - \Psi) = -0.01$ ns and 0.07 ns, respectively) nor any broadening of the spectrum.

Negatively charged CBS-5-S-SL symmetrically distributed across the bilayer of egg PC vesicles was also not affected by an inside-positive transmembrane potential generated by a pH gradient ($\Delta S(0 - \Psi) = -0.004$).

### *3.4. Effect of electrical potential on the vertical location of a positively charged spin probe*

The HTEMPO probe differs from the doxyl probes in that the free radical nitroxide group is located at the terminus of a positively charged quaternary amine bearing a hexadecyl chain instead of on the hydrocarbon chain. In addition, HTEMPO is always positively charged regardless of pH, since the nitrogen atom linking the TEMPO group to the hexadecyl chain is dimethylated. Hence, HTEMPO is located on both sides of the bilayer and will be affected in opposing ways by a transmembrane potential. As a result, a composite spectrum was expected upon applying a transmembrane potential. However, neither a negative-inside potential nor a positive-inside potential caused a significant change in the order parameter or any broadening of the spectrum of HTEMPO in PC.

In an attempt to selectively study the behavior of HTEMPO in the inner layer of the bilayer and to eliminate the signal due to a small percentage of free (soluble) HTEMPO, the signal due to HTEMPO in the outer layer and that free in solution was reduced by adding ascorbic acid to the LUV suspension. However, the signal from the unreduced HTEMPO was then too low to determine the effect of a transmembrane potential.

### *3.5. Effect of transmembrane potential on lipid after modification of dipole potential*

In addition to the imposed transmembrane potential, lipid bilayers also possess an internal dipole potential. The magnitude of the dipole potential has been reported to be on the order of 300 mV, hydrocarbon positive, in PC vesicles [30]. Although the molecular source of the dipole potential has not been identified, carbonyl oxygens and water at the membrane interface appear to be the most likely sources. Therefore, it was considered possible that the dipole potential due to the carbonyl groups of the ester-linked DPPC might have had a greater effect on the vertical location of the spin label than the transmembrane potential. In order to decrease this effect, dihexadecyl-*sn*-glycero-3-phosphorylcholine (DHPC) was used [31]. In this lipid, the hydrocarbon chains are linked to glycerol through ether rather than ester bonds. However, there was no effect of a transmembrane potential on the spectrum of 12-S-SL in DHPC/chol vesicles, either (Table 2).

Alternatively, it has been reported that using phloretin and 6-ketocholestanol, the dipole potential can be altered by over 200 mV in PC vesicles [30]. Phloretin reduces the magnitude of the membrane dipole potential [32] whereas 6-ketocholestanol increases it. Therefore, 12-S-SL, CBS-12-S-SL, and HTEMPO in egg PC MLVs were examined in the absence and presence of phloretin or 6-ketocholestanol. The incorporation of phloretin was expected to change the order parameter ($S$) in an opposite direction to 6-ketocholestanol.

However, both molecules caused an increase of the order parameter of the charged spin probes in proportion to mol% (5 to 50%) of the molecules as cholesterol did. In addition, the order parameter of the uncharged spin probes at low pH was also increased in the presence of either phloretin or 6-ketocholestanol. These results imply that phloretin and 6-ketocholestanol not only modify the dipole potential but also decrease the fluidity of the lipid bilayer. Because of this, the effect of changes in dipole potential on the location of lipid spin labels could not be measured.

However, the effect of the transmembrane potential on the charged spin probes after modifying the dipole potential with these agents could be examined by imposing a transmembrane potential on vesicles containing phloretin or 6-ketocholestanol. A transmembrane potential still had no detectable effect on the spin labeled lipids, such as negatively charged CBS-12-S-SL and positively charged HTEMPO in the presence of 10 mol% phloretin or 6-ketocholestanol (results not shown). This indicates that the vertical position of the charged lipids with respect to the PC molecules does not change significantly upon applying a transmembrane potential even when the dipole potential of the bilayer is reduced.

## 4. Discussion

These results reveal that the electrochemical potential across membranes does not have a detectable effect on the vertical position of charged stearic acid, PG, CBS, and HTEMPO with respect to phosphatidylcholine (PC) in the bilayer. A minimum change in vertical position of about 1 Å should be detectable using spin labeled lipids. The transmembrane electrochemical potential is the 'macroscopic' potential, which comes from the difference in concentrations of cations and anions between two bulk solutions across membranes. There is another component of the membrane potential, which is the 'microscopic' potential [33]. This is the value that would be measured if electrodes could be placed at the surface of the membranes.

The microscopic transmembrane potential includes the surface potential and the dipole potential. The surface potential arises from charges at the surface of the membrane. It falls off nearly exponentially from the membrane surface since the oppositely charged ions from the medium accumulate in the interfacial region and screen the charges. The surface potential is negligible beyond about 30 Å from the membrane and thus cannot be detected by electrodes. The dipole potential, which is thought to come from the ester linkages of the lipid, provides a positive potential in the interior of the bilayer and a negative potential at the surface of the bilayer [31,34]. Since these microscopic transmembrane potentials are symmetrical, they would not affect the macroscopic transmembrane potential.

However, the charged lipid probes in the bilayer might be affected by these more intimate electric fields more than the externally induced electric field. In other words, although the net charge of the surrounding lipids (PC) is zero, providing little surface potential, each PC molecule is zwitterionic, providing residues for electrostatic interaction. Electrostatic interaction and hydrogen bonding between charged lipids and polar headgroups of PC may be stronger than the external electric field. The interior-positive dipole potential, which exists not only in a PC bilayer but to a lesser extent also in a dialkyl-PC bilayer and a phloretin-containing PC bilayer, might interact with the negatively charged lipids, preventing them from being selectively affected by the external potential. In addition, van der Waals interactions between the non-polar acyl chains of spin probes and PC might stabilize the overall structure of the membranes so that the charged lipid cannot move vertically as a result of a selective effect of the transmembrane potential.

The electric field resulting from an ion gradient-induced transmembrane potential is felt primarily in the hydrophobic core of the bilayer and very little directly in the polar headgroup region [13]. If the headgroup of the charged lipid is anchored in this region and never makes transient excursions deeper into the bilayer, it will experience only a very small electric field and thus its vertical location will not be affected by a transmembrane potential.

These probes also did not detect any change in fluidity of the PC bilayer due to electrostriction of the bilayer caused by a transmembrane potential. Such effects have been detected using 1,6-diphenyl-1,3,5-hexatriene (DPH) in biological membranes and lipid vesicles [7–9,11] but not with other fluorescent probes [7,8] or with spin label probes (used in neutral protonated form) [12]. DPH may be more sensitive to these effects. Using $^{31}$P-NMR and $^{2}$H-NMR of PC deuterated in the headgroup, a transmembrane potential has been reported to have no detectable effect on the PC headgroup of lipid vesicles [10,13]. On the other hand, an electric field has been found to affect the PC headgroup in CHO cells [14] and in lipid bilayers [12] using $^{31}$P-NMR. However, an electric field imposed by electrodes at some distance from either side of the bilayer will be larger at the polar headgroup region than one induced by an ionic gradient. Modification of the dipole potential due to addition of phloretin was also found to affect the conformation of the deuterated PC headgroup [32]. Thus transmembrane and dipole potentials have some effects on lipids although they do not have any significant detectable effect on the vertical location of a charged lipid with respect to PC in the bilayer.

A transmembrane potential did have one interesting but unexpected effect on the spectrum of 5-S-SL in egg PC vesicles, however. It caused an increase in the number of spin label molecules oriented parallel to the magnetic field, suggesting increased orientation of the oblong tubular extruded vesicles perpendicular to the magnetic field. Orientation of lipid bilayers in the absence of a transmembrane

potential in a strong magnetic field, as found in NMR spectrometers, has been reported previously [37,38]. However, this orientation is due to the magnetic anisotropy of the lipid molecules and causes the bilayers to be oriented parallel to the magnetic field. In our case, they are oriented perpendicular to the magnetic field. The magnetic field found in an EPR spectrometer (about 3500 G) is not strong enough to cause orientation of these lipid bilayers in the absence of a transmembrane potential. While the exact mechanism of the effect observed in the presence of a transmembrane potential is not understood by us, the combination of the smaller magnetic field plus the ion flux through the bilayer results in a current flowing in a magnetic field. This gives rise to a force which is proportional to the vector cross product of the local ion flux and the magnetic field. This force may produce a torque on the oblong liposomes causing them to be preferentially oriented perpendicular to the magnetic field.

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