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Potential-Dependent Phase Partitioning of Fluorescent Hydrophobic Ions in Phospholipid Vesicles

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Summary. Fluorescent, dansyl derivatives of triphenylalkylphosphonium ions have been synthesized and exhibit fluorescence intensities in small sonicated phospholipid vesicles that are dependent upon transmembrane potentials. The voltage-dependent fluorescence changes are a result of changes in quantum yield that accompany a voltage-dependent phase partitioning of the probe. This phase partitioning is easily quantitated by calibrating the intensities of totally membrane-associated and aqueous probe. The voltage-dependence is well accounted for by a simple thermodynamic model and allows an estimation of potentials from fluorescence intensities in small vesicle systems.

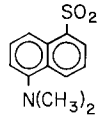
Key Words membrane potential · hydrophobic ions · fluorescent probes · lipid vesicles

Introduction

Molecular probes have become popular and important techniques for the measurement of membrane potentials in native and model systems. A number of techniques have been successfully applied and include radioactive (Bakeeva et al., 1970) and spin-labeled (Cafiso & Hubbell, 1981) derivatives of hydrophobic ions, ion-selective electrodes for hydrophobic ions (Kamo et al., 1979) and optical dyes. The optically detected dyes include the safranine dyes (Akerman & Saris, 1976; Akerman & Wikström, 1976), fluorescent derivatives of the merocyanine, oxanol and cyanine types (*see* Cohen & Salzberg, 1978, and Waggoner, 1979, for reviews) and the more recently developed stryl probes (Loew, Bonneville & Surow, 1978; Gupta et al., 1981; Loew & Simpson, 1981; Grinvald et al., 1982). The stryl probes apparently function by a 'charge-shift' mechanism, while other optical probes may respond to potential changes via a number of different mechanisms. The most common mechanism involves a change in the aggregation state of the probe following potential-dependent changes in probe environment (Waggoner, 1979). As a result, voltage-dependent fluorescence changes are dependent upon the probe-to-lipid ratio in these cases and an absolute measure of potential requires an empirical calibration, usually with ionophore-induced potentials.

Spin-labeled derivatives of hydrophobic ions allow an estimation of potentials in membrane vesicle systems using EPR[1], without an empirical calibration, given that the vesicle size and concentration is known (Cafiso & Hubbell, 1981). These hydrophobic ions function by a simple voltage-dependent phase partitioning and do not appear to directly interact with drugs and ionophores commonly used in transport studies. Additionally, the phosphonium spin-labels allow the measurement of interfacial potentials termed 'boundary potentials' which occur in a low-dielectric region inaccessible to salts in the aqueous phase (McLaughlin, 1977). Because there are circumstances where the use of spin labels is difficult, for example in the presence of strong reducing equivalents, a fluorescent derivative of the phosphonium could prove to be a useful addition to the paramagnetic methodology. It should be possible to interpret the fluorescence changes of such an analogue without the need for a voltage 'calibration' and it could therefore provide a useful complement to the current arsenal of optical probes.

In the present communication we describe the synthesis, binding and voltage-dependent behavior of the fluorescent dansyl-phosphonium ion I(n,m), shown below.

[1] Abbreviations used: EPR, electron paramagnetic resonance; EPC, egg phosphatidylcholine; $CHCl_3$, chloroform; MeOH, methanol, TLC, thin-layer chromatography; DMF, dimethylformamide: the dansyl fluorophore ≡ 

We demonstrate that its behavior, which is analogous to that seen for the spin-labeled analogue of this ion, is the result of a voltage-ependent phase partitioning into the vesicle membrane. This probe is apparently also sensitive to the structural asymmetry between the inner and outer monolayers of small vesicles, an observation previously seen for paramagnetic phosphoniums (Cafiso & Hubbell, 1982). A simple thermodynamic model adequately accounts for the voltage-dependent changes in fluorescence.

Materials and Methods

Materials

Egg phosphatidyl choline was prepared according to the procedure of Singleton et al. (1965) and stored in chloroform under argon at −20°C at a concentration of 100 mg/ml. Valinomycin was obtained from Sigma Chemical Company, St. Louis, Mo. 5-Carboxylpentyltriphenylphosphonium bromide was synthesized following the procedure of Denny and Smith (1962).

Synthesis of 1-Dansyl-1,4-diaminobutane

Diaminobutane (350 mg; 0.004 mol) was dissolved in dry dichloromethane (approximately 15 ml) and cooled to 0°C in an ice bath. Triethylamine (190 mg; 0.0019 moles) was then added to this solution with stirring. Dansyl chloride (500 mg; 0.0019 moles) was dissolved in a minimal volume of dry dichloromethane, cooled to 0°C and added dropwise with stirring to the above mixture. After the addition of dansyl chloride, the reaction mixture was allowed to warm to room temperature and stand for 2 hr. Dichloromethane was removed from the reaction mixture by rotary evaporation, the products were dissolved in a minimum volume of $CHCl_3$/MeOH 95 : 5 (vol/vol), and then loaded onto a column of silica gel 60 (E. Merck Reagents). The undesired didansyl product (which was ninhydrin negative) eluted first with $CHCl_3$/MeOH, 9 : 1 (vol/vol), followed by a second fluorescent product, the dansylaminobutane, which was ninhydrin positive. The product gave a single spot on TLC when developed with $CHCl_3$/MeOH, 9 : 1 ($R_f \simeq 0.4$).

Synthesis of I(4,4)

5-Carboxypentyltriphenylphosphonium bromide (440 mg, 0.001 moles) was dissolved in approximately 5 ml of dry DMF and triethylamine (110 mg; 0.0011 moles) was added with stirring on ice. To the mixture, *i*-butylchloroformate (150 mg; 0.0011 moles) was added and allowed to stir for approximately 15 min. 1-Dansyl-1,4-diaminobutane (320 mg, 0.001 moles) was added with stirring and the reaction mixture was allowed to warm to room temperature and stand for 16 hr. The mixture was then filtered and diluted into 40 ml of ether. The ether-insoluble product was isolated by centrifugation, dissolved in a minimal volume of $CHCl_3$/MeOH, 95 : 5 (vol/vol) and run on a column of silica gel 60 with this solvent. A small fluorescent band which eluted first was followed by a larger band containing the desired product. The isolated product, which was fluorescent, showed a single spot on TLC in MeOH/$CHCl_3$, 9 : 1 (vol/vol), ($R_f \simeq 0.85$) and was ninhydrin negative. The hygroscopic fluorescent product was water soluble and precipitated with sodium tetraphenylborate, a characteristic behavior of phosphonium salts.

Preparation of Phospholipid Vesicles

Aliquots of the stock lipid in chloroform were dried under a stream of nitrogen and vacuum desiccated for approximately 15 hr. The lipid was suspended at a concentration of approximately 0.1 M in a buffer containing 0.125 M K_2SO_4 and 0.050 M MOPS, pH = 7.0 and sonicated to form vesicles as previously described (Castle & Hubbell, 1976). The final concentration of lipid in the suspension was determined by phosphate analysis according to the procedure of Bartlett (1959). For binding experiments, vesicles were simply diluted into the K_2SO_4, MOPS buffer to obtain the desired concentration. For potential-dependent measurements, the working concentration of the vesicles was sufficiently low so that the sonicated vesicle suspension could be diluted into a buffer with K_2SO_4, Na_2SO_4 and MOPS, pH = 7.0 that produced the desired ratio of K^+_{in}/K^+_{out} (here $[K_2SO_4] + [Na_2SO_4] = 0.125$ M). To create transmembrane potentials, valinomycin (1 mM in ethanol) was added to the suspension to a concentration of 1 μM. The expected equilibrium potentials were estimated as previously described (Cafiso & Hubbell, 1978) and included small corrections for the vesicle capacitance and the transmembrane equilibration of protons. The fluorescent phosphonium was used in the range of 0.5 to 2.0 μM as indicated and was usually present at a probe/lipid ratio of 1 : 1000 or smaller.

Fluorescence Measurements

Fluorescence measurements were recorded on an SLM-4000 fluorometer (SLM instruments) using an excitation wavelength of 342 nm. In some experiments a 3-75 Corning glass cutoff filter was included in the detected 90° emission beam to reduce artifacts from strong reflected light at the excitation wavelength. Scattering at the excitation wavelength was quantitated by measuring the absorbance at 342 nm in a standard spectrophotometer. The absorbance did not exceed 0.04 OD units at 342 nm in a suspension of sonicated EPC vesicles at 12 mM lipid. Integrated fluorescence intensities were typically obtained by integrating the emission curves from 540 to 610 nm.

Results

Potential-Dependent Fluorescence of I

Shown in Fig. 1 are emission spectra for the dansylphosphonium label I(4,4) in the presence of suspensions of sonicated EPC vesicles of different lipid concentration. Increasing the vesicle concentration results in an increase in the fluorescence emission and a slight blue shift in the spectrum. As detailed

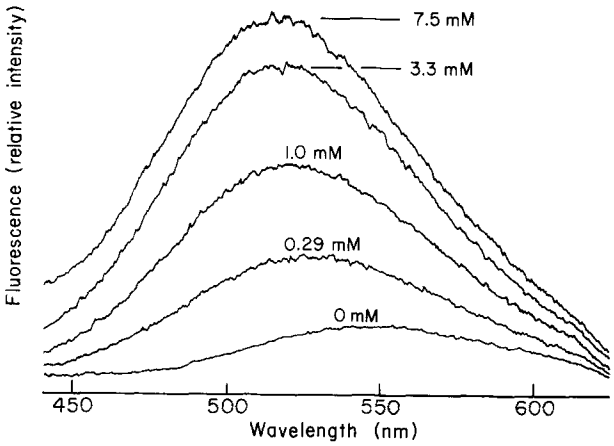


Fig. 1. Emission spectra are shown for 0.5 μM I(4,4) in the presence of sonicated phospholipid vesicles (with $\Delta\psi = 0$) in a buffer of 250 mM K_2SO_4 and 50 mM MOPS, pH = 7.0. The excitation wavelength was 342 nm and the spectra were taken at 25°C. The concentrations indicated in mM lipid were obtained by phosphate analysis

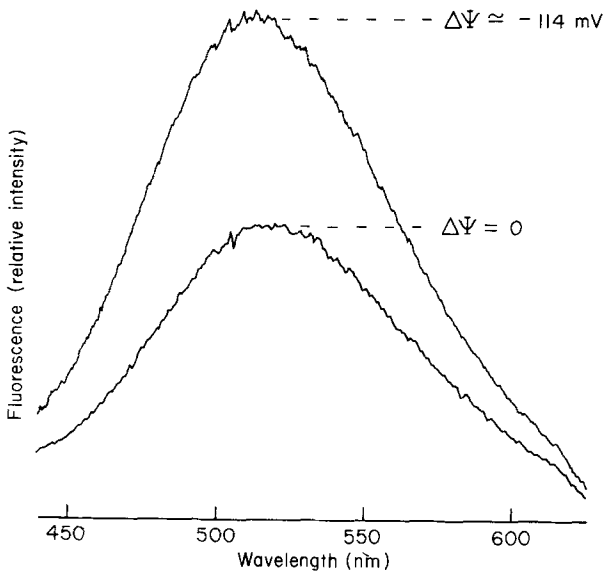


Fig. 2. Two emission spectra for 0.5 μM I(4,4) in the presence of vesicles with and without an inside-negative transmembrane potential are shown. Both samples contain 1 μM valinomycin and 1.39 mg/ml of EPC. For $\Delta\psi = 0$, $[K^+]_i/[K^+]_o = 1$ and for $\Delta\psi \simeq -114$ mV, $[K^+]_i/[K^+]_o = 90$. The high potassium gradient was obtained by diluting the vesicles, which were sonicated in a K_2SO_4 buffer solution, into a buffer containing K_2SO_4 and Na_2SO_4. The excitation wavelength was 342 nm

below, we interpret the increase in fluorescence as an increase in the membrane-associated population of I(4,4) and we quantitatively interpret this increase in terms of the phase-partitioning (ratio of membrane associated-to-aqueous probe) of I. As illustrated in Fig. 2, this fluorescence emission is also dependent upon the transmembrane electrical potential difference $\Delta\psi$ across the phospholipid vesicles. When an inside negative potential is established by the addition of valinomycin (in the presence of a K^+ gradient, $[K^+]$ internal > $[K^+]$ external) the fluorescence intensity of I increases. This change is interpreted (*see below*) as an increase in the membrane-associated population of I. As shown below, this voltage-dependent fluorescence is accounted for by the predicted voltage-dependent phase partitioning of I in membrane vesicle systems.

Thermodynamic Analysis of the Binding of I to Phospholipid Vesicles

The behavior of paramagnetic derivatives of hydrophobic ions has been well characterized (Cafiso & Hubbell, 1978, 1982). These ions are membrane permeable and in equilibrium in four distinct regions: the vesicle interior and exterior aqueous spaces and the internal and external boundary regions associated with the vesicle interface (regions labeled i, o, m_i and m_o, respectively). Here, we assume a similar behavior for probes such as I. Since the quantum yield of fluorophores such as dansyl is strongly dependent upon the probe environment, we expect the quantum yield of I to be dependent upon its phase partitioning. If the phosphonium I has fluorescence intensities I_f and I_b when located in the aqueous and membrane phases, respectively, and λ (the phase partitioning) is given as N_b/N_f (the ratio of membrane associated-to-aqueous probe), then the observed fluorescence I_o in the presence of vesicles is given by:

$$I_o = \left(\frac{\lambda}{\lambda + 1}\right) I_b + \left(\frac{1}{\lambda + 1}\right) I_f - S \qquad (1)$$

where S is a factor, dependent upon the vesicle concentration, that accounts for scattering at the excitation wavelength. S is small at the vesicle concentrations used here and accounts for ~4% of the fluorescence intensity at a lipid concentration of 10 mg/ml.

As shown previously, we can relate λ to the lipid concentration, m_l/V_t (in grams lipid/ml of solution), the binding constant K and the volumes V_i, V_o, V_{m_o} and V_{m_i} which are the effective thermodynamic volumes occupied by I in each of the regions i, o, m_o and m_i, respectively. If $\bar{V}_i$ and $\bar{V}_l$ are the volumes of the internal vesicle space and hydrated lipid bilayer per gram lipid, then we can write:

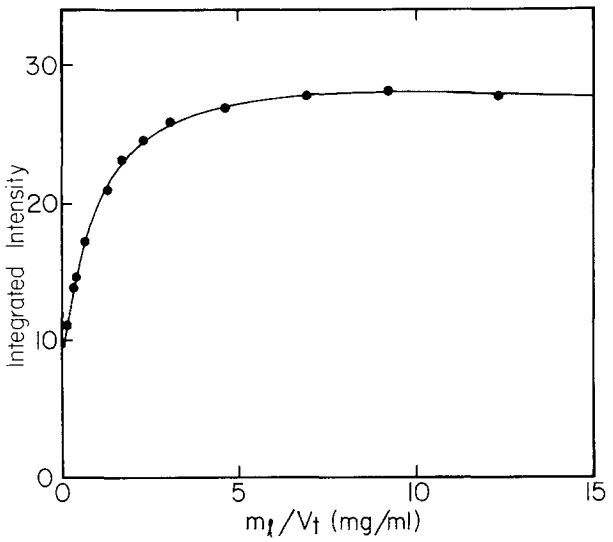


Fig. 3. A plot of the integrated intensities (points ●) of the emission spectra of 0.5 μM I(4,4) in the presence of sonicated EPC vesicles as a function of the concentration of lipid, m_l/V_t, in mg/ml. The excitation wavelength was 342 nm and the integration range was between 540 and 610 nm. The line is a fit to this data using Eq. (1) (*see text*). From this fit we obtain values for I_b and I_f of 32.7 and 7.80, respectively.

$$\frac{1}{\lambda} = \left(\frac{V_t}{m_l}\right)\frac{1}{\bar{V}_i\beta} - \frac{1}{\beta}\cdot\frac{\bar{V}_l}{\bar{V}_i} \tag{2}$$

where $\beta = KV_{m_i}/V_i(1 + V_{m_o}/V_{m_i})$. For the EPC vesicles, we use a value of 0.9885 ml/g for $\bar{V}_l$ (Huang, 1969) and a value of 0.50 ml/g for $\bar{V}_i$ (Cafiso & Hubbell, 1978). In the more general case, the binding constants of I to the internal and external membrane interfaces, K_i and K_o, respectively, will not be equal. In this case $\beta = V_{m_i}/V_i(K_i + K_oV_{m_o}/V_{m_i})$. Because of the low vesicle concentrations being used here. $V_t/m_l \gg \bar{V}_l$ and we use the approximate expression for Eq. (2) below.

$$\lambda \simeq \beta\bar{V}_i(m_l/V_t). \tag{3}$$

To quantitatively determine the voltage-dependent fluorescence of I(4,4) (*see below*), we will need to determine I_b and I_f (so that λ may be estimated from I_o). The quantity β will also be useful. We have determined I_b, I_f and β by measuring I_o at various vesicle concentration ($m_l/\bar{V}_t$) and analyzing the data using Eq. (1) and (3). Shown in Fig. 3 are points for the integrated intensity of the emission spectra of I(4,4) plotted as a function of the lipid concentration (m_l/V_t) in mg/ml. The solid line represents a least-squares fit to the data using Eq. (1).[2] In Fig. 4 this data is expressed in terms of the partitioning λ, and displayed as a double reciprocal plot, λ^{-1} *vs.* $(m_l/V_t)^{-1}$. Here this fit yields a straight line with a slope of $(\bar{V}_i\beta)^{-1}$, *see* Eq. (3). From this data we obtain values for the ratio $I_b/I_f = 4.19 \pm 0.1$ and $\beta = 1900 \pm 100$.

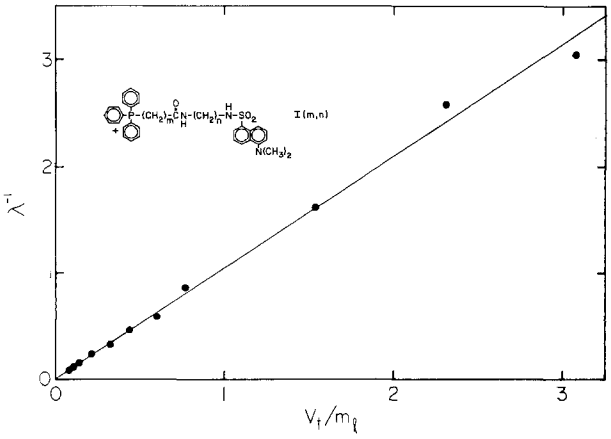


Fig. 4. A reciprocal plot of the phase partitioning λ *vs.* the vesicle concentration (m_l/V_t) for I(4,4). The points (●) were obtained by converting the intensity data in Fig. 3 into values of λ using Eq. (1). The solid line represents the least-squares fit to this data (*see* Eq. 3). The slope of this line is $1/\bar{V}_i\beta$ and yields a value for β of 1900

Analysis of Potential-Dependent Fluorescence

The fluorescent phosphonium I is expected to undergo a voltage-dependent phase partitioning, identical to that seen previously for other hydrophobic ions (Cafiso & Hubbell, 1978). The voltage-dependence of λ is described by Eq. (4), shown below, where $\phi = ZF\Delta\psi/RT$ and $\alpha \equiv w/d$.

$$\lambda = \left(\frac{V_{m_i}}{V_i}\right)\left[\frac{K_ie^{\alpha\phi} + K_oV_{m_o}/V_{m_i}e^{(1-\alpha)\phi}}{1 + V_o/V_ie^{\phi}}\right]. \tag{4}$$

Here, d is the bilayer thickness and w is the distance from the boundary region to the corresponding membrane-solution interface. The potentials at the

[2] We have not included the intensity for $m_l/V_t = 0$ in our calculation due to the large blue shift in the spectrum and our fixed integration range. Excluding this data point made little difference in the fit and we find a value for I_f (I_0 at $m_l/V_t = 0$) which is very close to the experimental value.

inner and outer boundary regions are $(1 - \alpha)\Delta\psi$ and $\alpha\Delta\psi$, respectively (assuming a constant field approximation). To compare the behavior of the phosphonium I with that predicted by Eq. (4) we must simply convert the voltage dependence of λ into a voltage-dependent intensity.

We have investigated the voltage-dependent intensity of I(4,4) in sonicated EPC vesicles by measuring the integrated emission intensity as a function of K^+-valinomycin-induced potentials. These intensities are represented by the points in Fig. 5 plotted as a function of the transmembrane potential $\Delta\psi$. By taking the value of I_b/I_f found for I(4,4) along with the value for β, we have generated curves for the expected voltage-dependent intensity using Eqs. (4) and (1). In our initial calculation here, we set $\alpha = 0$ and $K_i = K_o = K$, as previously found for paramagnetic phosphoniums (Cafiso & Hubbell, 1978). The fit shown in Fig. 5 with $K_i/K_o = 1$ is plotted from Eq. (4) with no adjustable parameters. This fit, although tolerable, is systematically lower than found experimentally and results in errors as great as 10 mV. Varying α does not correct this condition and α is expected in any case to be small. However, we can obtain an improved fit if we allow the ratio K_i/K_o to vary. In the present case a value of $K_i/K_o = 2.25$ gives an excellent fit to the data. In three different experiments at different lipid concentrations we have obtained an excellent agreement between Eq. (4) and the data with $K_i/K_o \sim 2$. As discussed below, there is a precedent for this result.

As the concentration of vesicles is increased so that I(4,4) becomes totally membrane associated (this occurs at lipid concentrations of approximately 10 mg/ml), the fluorescence intensity becomes independent of the transmembrane potential. This is expected since a voltage-dependent phase-partitioning is no longer possible. It also indicates that the fluorescence of membrane-bound I is not altered by $\Delta\psi$.

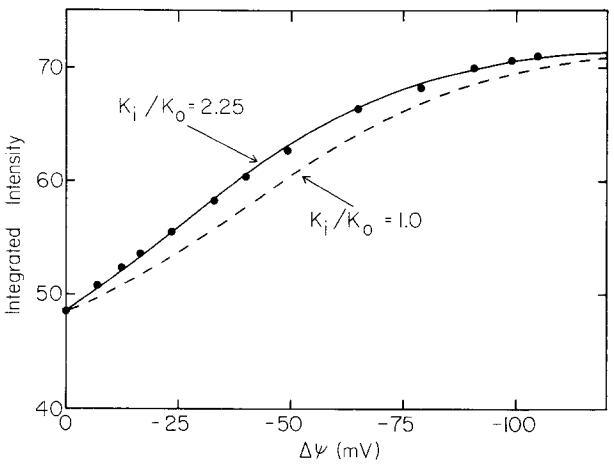


Fig. 5. A plot of the voltage-dependent fluorescent intensity of I as a function of the transmembrane potential $\Delta\psi$. The points (●) represent the experimentally determined integrated intensities for 1 μM I(4,4) as a function of $\Delta\psi$ in sonicated EPC vesicles. Here the lipid concentration is 1.38 mg/ml as determined by phosphate assay. The solid (—) and broken lines (---) represent the predicted $\Delta\psi$ dependence using Eq. (4) for two different values of K_i/K_o using the expression $V_o/V_i = (V_t/m_l) \cdot (\bar{V}_l)^{-1} - (\bar{V}_l/\bar{V}_i + 1)$ (Cafiso & Hubbell 1978). Since a calibration of the absolute fluorescence intensity was not always maintained between runs, we used the intensity of $\Delta\psi = 0$ (either no valinomycin or no K^+ gradient) to establish the absolute values for I_f or I_b. The value of V_{m_o}/V_{m_i}, determined from the vesicle size, was 2.3. For the case where $K_i/K_o = 1$, $K_iV_{m_i}/V_i = K_oV_{m_i}/V_i = 576$, as determined directly from β. With $K_i/K_o = 2.25$ the values for $K_iV_{m_i}/V_i$ and $K_oV_{m_i}/V_i$ were 940 and 418, respectively

Discussion

The behavior of paramagnetic phosphoniums in small sonicated vesicles has been well characterized. They bind to membrane interfacial regions and exhibit a transmembrane equilibrium; properties which readily account for their voltage-dependent phase-partitioning (Cafiso & Hubbell, 1981). The fluorescent dansylphosphonium I was synthesized with the anticipation that it too should demonstrate a potential-dependent phase-partitioning, rendering the quantum-yield of the dansyl fluorescence potential-dependent. This probe would then provide an alternative to the use of paramagnetic probes and because of its simple well-characterized properties would also be a complement to other optical techniques.

We have found that the fluorescence of the phosphonium I(4,4) in sonicated vesicles is dependent upon the transmembrane electrical potential $\Delta\psi$ and that this voltage-dependence is readily predicted by the simple thermodynamic model previously developed for paramagnetic hydrophobic ions. To quantitate the behavior of I, it was first necessary to quantitate the phase partitioning λ from the integrated fluorescence intensities. To do this we assumed that the probe, in a vesicle suspension, would have two characteristic quantum yields: one associated with aqueous probe and the other with membrane-bound probe. This, of course, presumes that the membrane-bound fluorescence of I is independent of $\Delta\psi$; for example, it presumes that the fluorescence is independent of whether I is

located on the internal or external boundary region. Here, we demonstrated the validity of this assumption by demonstrating that the intensity of membrane-bound label is independent of $\Delta\psi$ (*see above*). The excellent fits to the binding data for I(4,4) that are obtained using Eqs. (1) and (3) are further justification for this assumption.

Light scattering was not a problem with our samples since the high binding constant of I(4,4) to the membrane necessitated the use of dilute vesicle suspensions. In fact, at the vesicle concentrations used for $\Delta\psi$ measurements here, scattering losses (which were due to a scattering of the excitation beam) amounted to less than 1% of the total fluorescence intensity. The binding experiments such as that shown in Fig. 3 were clearly affected by scattering at higher lipid concentrations (above about 10 mg/ml). Because of the low lipid concentrations used here we were careful to maintain the lipid/probe ratio above 1000 : 1. This is above the level at which electrostatic saturation in the boundary region occurs (Andersen & Fuchs, 1975; Andersen et al., 1978).

The excellent fit of the binding data, Figs. 3 and 4 using Eqs. (1) and (3) indicates that the phase partitioning λ can be obtained directly from the fluorescence intensity using the intrinsic aqueous and membrane-bound intensities of I. The transmembrane potential $\Delta\psi$ can then be estimated using Eq. (4) if the vesicle concentration and geometry are known. When $K_i = K_o = K$, a value for KV_{m_i}/V_i is obtained directly from the binding data. The fit, using an average binding constant for I to the internal and external vesicle surfaces (*see* Fig. 5), indicates that inside-negative (and -positive) voltages would be overestimated in this case. As shown above, the fit to the data is dramatically improved if $K_i \neq K_o$; a ratio of $K_i/K_o = 2.25$ gives an excellent fit to the data shown here. An identical, but smaller effect has been observed for paramagnetic phosphoniums where $K_i/K_o \simeq 1.17$ (Cafiso & Hubbell, 1982); however, the large inequivalence between K_i and K_o here cannot be ignored when estimating $\Delta\psi$. In general, the binding constant may not be equivalent on each side of the membrane due to structural or electrical differences. In the case of native membrane vesicles, differences in surface charge due to protein or lipid asymmetry would result in nonequivalent values of K_o and K_i. In these cases K_i and K_o can be determined independently by measuring the phase-partitioning before and after the transmembrane equilibration of the phosphonium (Cafiso & Hubbell, 1982).

We believe that the inequivalence between K_i and K_o represents a binding difference which is structural in origin; probably due to the high curvature of the small sonicated vesicles used here. Other possibilities do not seem reasonable. For example, if the probe had a higher intrinsic fluorescence when bound to the internal vesicle interface, we could account for the discrepancy; however, we have directly demonstrated (*see above*) that the fluorescence of membrane-bound I(4,4) is independent of its location (i.e., on either the internal or external vesicle surface). The difference might also be imagined to be due to a change in the effective internal volume for I(4,4) when compared to other probes or molecules. A fraction of the internal water of a vesicle does not, for example, solvate sucrose (Katz & Diamond, 1974). However, a change in the effective internal volume $\bar{V}_i$ makes *no* difference in the behavior of I(4,4) simply because the effective probe concentration in the internal volume is always zero. This is a result of the strong binding of I(4,4) and the small internal volumes of these vesicles.

Due to their small radius of curvature, small sonicated vesicles are expected to have a higher headgroup density and higher chain disorder on the interior monolayer (*see* Dill & Flory, 1980, 1981). Since the higher packing would be expected to decrease the binding of a surface amphiphile to the interface, this property must not dominate the binding. The increased binding could reflect the more favorable entropy of mixing experienced by the label on the internal monolayer due to its greater alkyl chain disorder. The larger effect seen here for the dansyl-phosphonium, compared to other labels, is probably a result of its larger size and position in the membrane. The binding difference can be equated to an energy difference where $K_i/K_o = \exp(-\Delta\mu/RT)$ and $\Delta\mu \equiv \mu_{m_i} - \mu_{m_o}$. Here, $\Delta\mu$ represents the difference in the chemical potential of I between each interface in the absence of a transmembrane potential. For $K_i/K_o = 2.25$, $\Delta\mu \simeq 480$ cal/mole which although not large is almost the free energy of transfer per methylene found previously for phosphoniums from water into the membrane interface.

The determination of membrane potentials utilizing molecules such as I is best suited to model membrane systems where the complexities often found in native membrane systems are at a minimum. The additional complexity encountered in native membrane systems (such as surface charge asymmetry, heterogeneity in membrane composition, etc.) has been previously discussed for spin-labeled hydrophobic ions and will also apply to the fluorescent phosphonium described here (Cafiso & Hubbell, 1981). In principal many of the problems that may be encountered in native systems can be accounted for quantitatively.

Clearly the fluorescent group chosen here is not the only or the best fluorophore to pick. Any probe

demonstrating a strong dependence upon environment (in this case solvent polarity) could have been used. In the range of probe and vesicle concentrations we have examined this probe appears to have a simple behavior which is adequately explained by the present model. Additionally, these molecules are unaffected by specific interactions with ionophores and many membrane proteins. The transmembrane migration of I(4,4) is expected to be faster than that seen previously for some paramagnetic phosphoniums (Cafiso & Hubbell, 1982) and we are currently investigating its kinetic properties. It is hoped that molecules such as I will provide an alternative in cases where paramagnetic probes are not well suited. Their predictable behavior makes them ideal for the estimation of transmembrane and interfacial boundary potentials in model membrane systems.

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