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Localizing the nitroxide group of fatty acid and voltage-sensitive spin-labels in phospholipid bilayers

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The intramembrane locations of several spin labeled probes in small egg phosphatidylcholine (egg PC) vesicles were determined from the enhancement of the ^{13}C nuclear spin lattice relaxation of the membrane phospholipid. Electron paramagnetic resonance (EPR) spectroscopy was also used to measure the relative environmental polarities of the spin labels in egg PC vesicles, ethanol and aqueous solution. The binding location of the spin label group was determined for a pair of hydrophobic ion spin labels, a pair of long chain amphiphiles, and three stearates containing doxyl groups at the 5, 10 and 16 positions. The nuclear relaxation results indicate that the spin label groups on the stearates are located nearer to the membrane exterior than the analogous positions of the unlabeled phospholipid acyl chains. In addition, the spin label groups of the hydrophobic ions and long chain amphiphiles are located near the acyl chain methylene immediately adjacent to the carboxyl group. The relative polarities, determined by the EPR technique, are consistent with the nuclear relaxation results. This information, when combined with information on their electrical properties, allows for an assessment of the conformation and position of these voltage sensitive probes in membranes.

Introduction

The electrostatic properties of biological membranes are of fundamental importance in determining membrane protein conformation, ion transport rates, and other membrane associated processes. As a result, a wide range of molecular probe techniques have been developed to examine these properties. Several types of paramagnetic molecules have been developed to characterize membrane electrical properties [1], and can roughly be divided into two categories. One group consists of molecules containing a charged group and a hydrocarbon chain, such as the alkylammonium nitroxide derivatives [2]. We will refer to these as long chain amphiphiles. These long chain amphiphiles do not readily move across membranes because of the high energy involved in placing their charged group into the membrane interior. The charged region of these molecules, when bound to a membrane, is expected to reside at or near the membrane surface and the phase partitioning of these probes can be used to monitor potentials at the membrane surface [2,3]. A second group of probes is made up of derivatives of hydrophobic ions, which are characterized by

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Abbreviations: TPB^-, tetraphenylboron; TPP^+, tetraphenylphosphonium; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl.

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hydrophobic moieties surrounding a partially delocalized charge. These molecules are relatively membrane permeable. When associated with membranes, they bind to a region close to the membrane interface termed the boundary region [3,4]. The phase partitioning of hydrophobic ions can be used to measure the potential between the boundary region and the bulk aqueous solution [1].

Two of the most widely studied hydrophobic ions are tetraphenylphosphonium (TPP^+) and tetraphenylboron (TPB^-). TPB^- has a much stronger binding to phosphatidylcholine (PC) membranes than TPP^+ and the energy barrier to transmembrane ion movement is much lower for TPB^- than TPP^+ [5–7]. These observations have been accounted for by modeling the total energy profile for the PC membrane and including a dipole potential term [8]. The model predicts that the hydrophobic ions bind near the membrane aqueous-hydrocarbon interface, with TPB^- binding slightly further into the membrane than TPP^+. In addition, the model also puts limitations on the distance between the hydrophobic ion binding region and the atoms and/or molecules giving rise to the membrane dipole field. Unlike hydrophobic ions, the binding of the long chain amphiphiles does not appear to be sensitive to the dipole potential.

The electrical behavior of hydrophobic ions is reasonably well characterized; however, direct information on their location in membranes would be very useful. It would permit boundary potentials and dielectric constants to be assigned to a specific region in the membrane interface and would aid in defining the molecular source of the membrane dipole field. Information on the intramembrane location of the long chain amphiphiles would also contribute to a more complete electrostatic description of the interface of model and biological membranes.

In the present report, we used two methods to obtain direct information on the location of membrane bound spin labeled hydrophobic ions and long chain amphiphiles. First, we measured the enhancement of membrane egg PC ^{13}C spin lattice relaxation due to the unpaired electron of the spin label. This enhancement is highly dependent on the distance between the spin label and the ^{13}C nucleus observed. Second, we measured the field separation between the outer extrema of the EPR spectra for the spin labeled probes in egg PC membranes. Under the experimental conditions used, this spectral parameter is sensitive primarily to the environmental polarity of the labels. Three spin-labeled stearates were also studied by the above two techniques. The stearate data provided information on the intramembrane location of the attached spin label groups and aided in evaluating the intramembrane location of the hydrophobic ions and long chain amphiphiles.

Materials and Methods

Egg phosphatidylcholine was purified from fresh hen eggs according to the procedure of Singleton et al. [9] and stored in chloroform under an argon atmosphere at −20°C. The spin-labeled molecules we examined are shown in Fig. 1. The stearic acids, I(m,n) were obtained from Molecular Probes (Eugene, OR). The long chain amphiphiles II and III were synthesized as described by Castle and Hubbell [2] and Hartsel and Cafiso [10], respectively. The hydrophobic ion IV was synthesized as described previously [11], and the hydrophobic ion V was the generous gift of Wayne Hubbell. Small unilamellar phospholipid vesicles were prepared as described previously [2]; the phospholipid concentration was 300 mg/ml. Spin labeled molecules were added to the sonicated vesicle preparation so that the membrane-bound concentration of label was 1 mol%. Spin label IV was added as a concentrated aqueous solution to the vesicles. Ethanol solutions of all other labels were prepared, an appropriate aliquot was dried and vesicles were added. ^{13}C-NMR resonance assignments were taken from Brainard and Cordes [12]. ^{13}C spin-lattice relaxation times were obtained with a Nicolet NT-360 spectrometer as described previously [13]. Relaxation rate enhancements due to the presence of the spin labeled probes were obtained by calculating the difference between relaxation rates with and without probe.

The EPR spectra of labels I–V were obtained at −114°C with a modified Varian 4500 series spectrometer equipped with a Varian temperature controller. A modulation amplitude of 1 gauss and

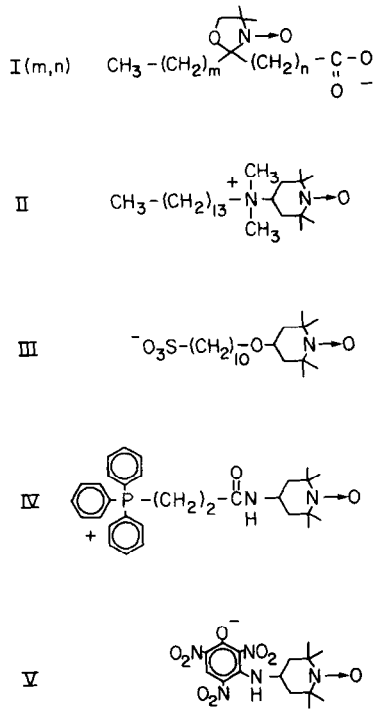


Fig. 1. Structure of spin-labeled probes examined in this study. Probes I are a series of spin-labeled stearates. Probes II and III are long chain amphiphiles used to measure membrane surface potentials. Probes IV and V are hydrophobic ions that are used to estimate transmembrane and interfacial potentials.

a microwave power 10 mW was used. The partitioning of the spin labeled probes between aqueous and membrane phases was measured as described previously [1].

Results and Discussion

Theoretical considerations in the interpretation of membrane ^{13}C spin-lattice relaxation due to spin labels

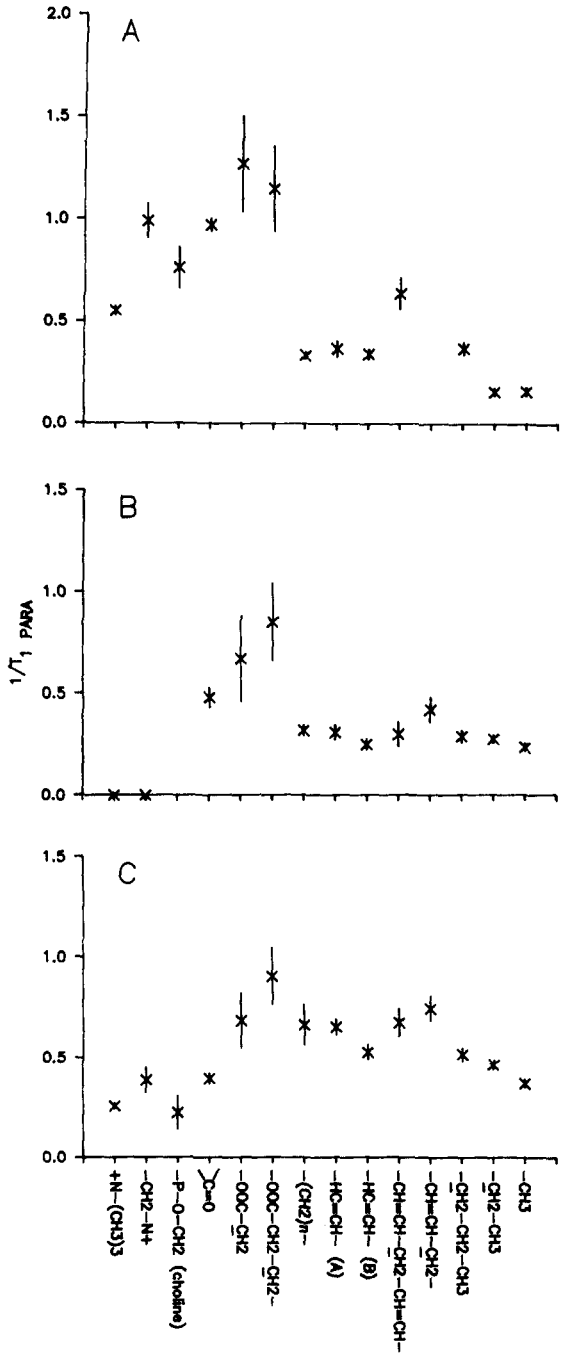
An expression describing the nuclear spin lattice relaxation due to spin labels in small membrane vesicles was derived previously by Brulet and McConnell [14]. In this expression the dominant factor determining the enhancement in relaxation along the phospholipid molecule is the average distance between the unpaired electron of the spin label and the relaxing nucleus. Recent work on intramolecular nuclear spin-lattice relaxation in membranes indicates that molecular order is important in determining the differences in relaxation among different phospholipid positions [15,16]. However, the relative importance of molecular order is different for intramolecular and intermolecular cases. The distance between interacting dipoles is variable for the intermolecular case whereas it is fixed for the intramolecular case. In addition, the vectors describing the interaction between the measured nucleus and the dipole causing relaxation have greater degrees of freedom in the intermolecular case due to molecular diffusion. As a result, the order of the dipolar interaction vectors is lower for the intermolecular case. A recent study of the quenching of chain labeled fluorescent phospholipids by spin labeled phospholipids in membranes indicated that molecular motion was sufficient to isotropically average the interactions between fluorophores and quenchers [17]. Effects due to phospholipid orientation or order were not observed. Thus, we expect that the positional dependence of the nuclear relaxation due to the intermolecular dipolar interaction between the nucleus and spin label will primarily reflect the spin label-nuclear distances. For these reasons, we will assume that positional variations in the order parameter are of secondary importance in the present study.

Enhancement of membrane ^{13}C spin-lattice relaxation by spin labeled stearates

Figs. 2A–C shows the enhancement of membrane egg phosphatidylcholine ^{13}C spin-lattice relaxation rates due to the presence of 1 mol% stearate, spin labeled in the 5, 10 and 16 positions. The order of the carbon positions listed on the abscissa reflects, in general, that found in the phospholipid as one proceeds from the headgroup to the methyl end of the acyl chains. As expected, the relaxation enhancement for the acyl chain positions near the methyl end increases (relative to the positions near the carboxyl end) as the spin label is moved from the carboxyl to the methyl end of stearic acid. This is in general agreement with an earlier more limited study [18].

The acyl chain portion of the profile is relatively flat for membranes containing I(1,14). However, for I(7,8) and I(12,3) the enhancement near the carbonyl end of the chain (at the C=O, OOC-$\underline{C}H_2$ and OOC-CH_2-$\underline{C}H_2$ groups) increases relative to those further down the chain. The ratio of

$N(CH_3)_3$ enhancement to acyl chain $(CH_2)_n$ enhancement has the following order: I(12,3) > I(7,8) ≈ I(1,14). This is consistent with the doxyl group at the 5 position occupying a position considerably closer to the membrane exterior than the doxyl groups at the 10 and 16 positions.



Several intriguing features are apparent in the relaxation enhancement profiles for the spin labeled stearates. It is well established that the dynamics of the acyl chain establishes a distribution of intramembrane locations for each position along the chain. Fig. 2 shows that the width of the distribution of spin labeled stearates has the following order: I(1,14) > I(7,8) ≥ I(12,3). The wide distribution found for I(1,14) is in agreement with statistical mechanical calculations showing the widest distribution of locations for the chain terminus [19]. It was observed previously that position 16 had the greatest relaxation enhancement, among the resonances examined, in the presence of I(1,14) [18]. The same was not true of our results. We found that the enhancement at several positions exceeded that at the 16 position for this label. Our data suggests that the labeled position of the spin-labeled stearates does not reside at the same depth in the membrane interior as the analogous positions on the phospholipid acyl chains. The spin labeled positions appear to lie closer to the exterior of the membrane than the analogous phospholipid acyl chain positions. This is probably, in part, a result of the fact that the spin label groups are considerably more polar than the phospholipid acyl chains and therefore prefer a more polar environment than the membrane hydrocarbon interior.

Enhancement of membrane ^{13}C spin-lattice relaxation by spin-labeled long chain amphiphiles and hydrophobic ions

Fig. 3 shows the EPC membrane ^{13}C spin lattice relaxation enhancement due to two long chain

Fig. 2. Enhancement of the ^{13}C spin-lattice relaxation in small egg PC vesicles promoted by 1 mol% of the following stearate spin labels: (A) I(12,3); (B) I(7,8); (C) I(1,14). $1/T_1$para is the difference in spin-lattice relaxation rate between the membranes with and without spin label. Phospholipid resonance positions shown on the abscissa are arranged according to their spatial location in the molecule, starting at the choline methyl groups and proceeding to the acyl chain methyls. The order is somewhat arbitrary because some resonances arise from several positions. In Figs. 2 and 3, the relaxation rates in the presence of spin labels are the average of two determinations. Relaxation rates for membranes without spin labels are the average of three determinations.

amphiphiles and two hydrophobic ion spin labels. A rather striking result is the similarity in the ^{13}C relaxation enhancements promoted by these labels, in spite of their dissimilar structures. For example, the enhancement for all these probes is largest at the acyl OOC-$\underline{C}H_2$ position, indicating that the spin label moieties of the hydrophobic ions are located near this position. This is close to the aqueous-hydrocarbon interface of the membrane [20].

Some minor differences in the enhancements produced by labels II, III and IV, V (Figs. 3A, B, C, D, respectively) are seen. Label IV promotes a larger relaxation of the C=O position than seen by the other labels. In the alkyl chain region, a modest level of enhancement is induced by all probes except label II, the alkylammonium. The lack of an effect in the hydrocarbon interior for this probe is consistent with the close proximity of the spin label to a charge center that has a high energy in the membrane interior. The high Born-image energy for this ion may prevent it from entering the membrane hydrocarbon. It is interesting to note that in the case of the alkylsulfonate label, the nitroxide apparently occupies an interfacial location. Since the negative charge for III must also reside at the interface, this probe likely has a 'hairpin' configuration.

Polarities of spin labeled stearate and hydrophobic ion environments in membranes

Many studies have established that as one proceeds from the membrane exterior to the interior, the polarity (or dielectric constant) decreases [8,21].

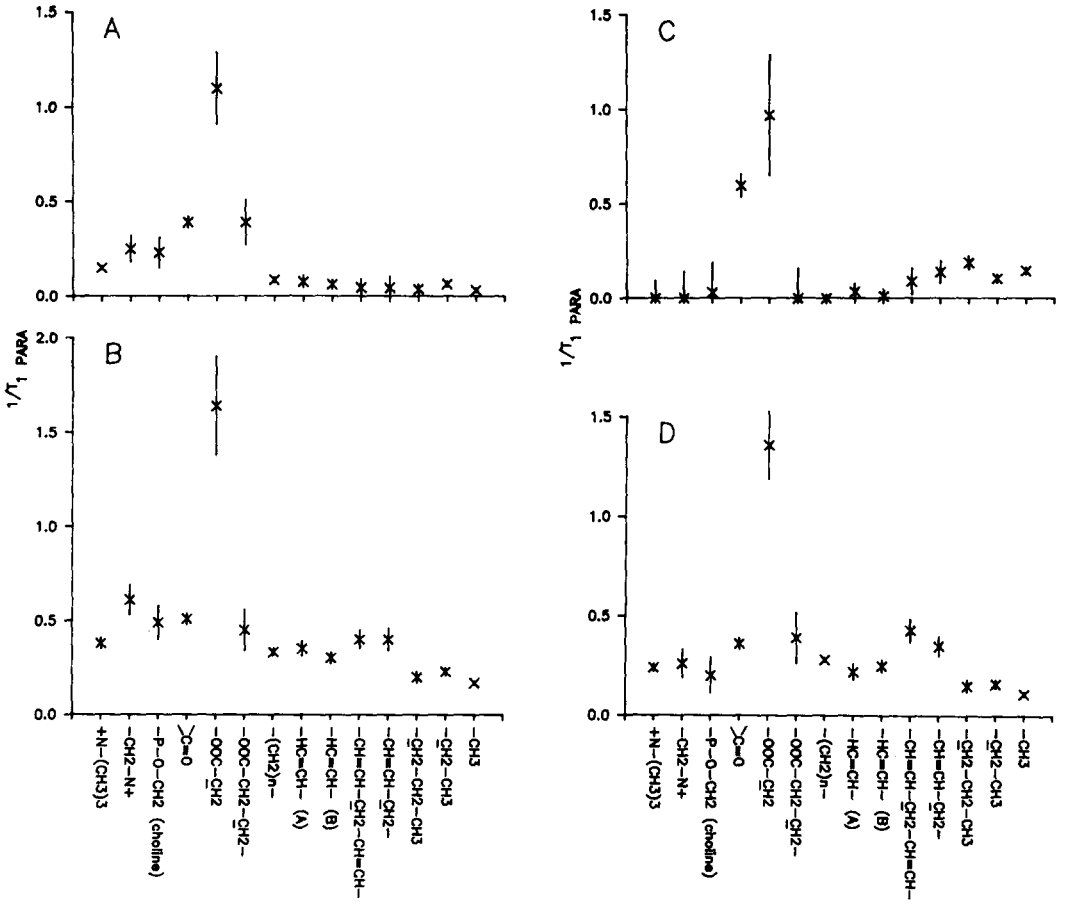


Fig. 3. Enhancement of ^{13}C spin-lattice relaxation in small egg PC vesicles induced by 1 mol% of: (A) II; (B) III; (C) IV and (D) V. The phospholipid resonances are arranged in approximate order from the choline methyl to the chain terminus as in Fig. 2.

Under conditions where motional effects on EPR spin label spectra are constant, the spectral splittings decrease as the polarity of the label environment become less polar [21]. This result has been used to measure membrane polarity as a function of position or depth. Table I shows the splitting between the outer peaks of the EPR spectra for the spin-labeled stearates in EPC membranes and ethanol at −114°C. A representative spectrum and the splitting measurement is shown in Fig. 4. Spectra were taken at low temperature so that motional differences would not be present to obscure the polarity dependence of the splittings. The spectra of the spin labels in a given medium had the same or very similar lineshapes. The polarity differences among the membrane-bound, labeled stearates are indicated in Table I where differences in splitting between the membrane-bound and ethanol-solubilized labels are listed. The results are very similar to those obtained in a previous study [21]. They indicate that membrane-bound I(12,3) resides in an environment with a polarity very similar to that of ethanol and as the label is moved from the carboxyl to the methyl end of the chain it experiences a less polar environment.

The EPR outer splittings of the membrane-bound and ethanol-solubilized long chain amphiphile and hydrophobic ion spin labels at −114°C are also shown in Table I. When the splittings of the membrane-bound labels II–V are compared with those of the stearate labels, the nitroxides of these probes are found in an environment more polar than that seen by any of the stearate labels. The data in Table I shows that the environments of labels II–V are more polar than ethanol. Label IV has the lowest membrane binding constant of the labels examined and it has a modest solubility in aqueous solution. The outer splitting for this label in aqueous solution was substantially larger than all other splittings. This larger splitting indicates that the low temperatures used to record these spectra do not simply exclude the labels from the membrane.

TABLE I

HYPERFINE SPLITTINGS FOR NITROXIDE PROBES I–V

The error in the measured values of 2 A_{max} is ±0.3 gauss, EPC, egg PC.

Probe	2 A_{max} (gauss)				
	1% in EPC membranes	500 μM in EtOH	100 μM in buffer	EPC-EtOH	EPC-buffer
I(12,3)	68.7	68.6	–	0.1	–
I(7,8)	67.9	69.0	–	−1.1	–
I(1,14)	66.2	67.6	–	−1.4	–
II	71.7	69.1	–	2.6	–
III	69.5	68.6	–	0.9	–
IV	71.4	70.0	75.5	1.4	−4.1
V	71.7	70.9	–	0.8	–

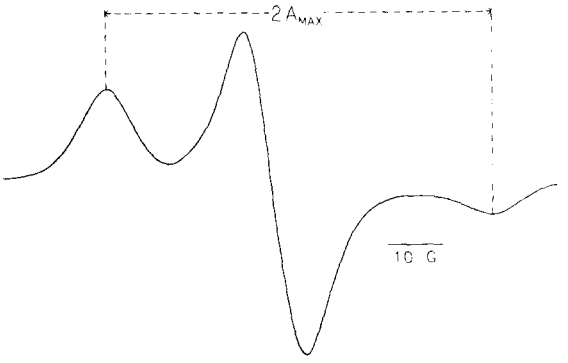


Fig. 4. EPR spectrum of 1 mol% I(12,3) in EPC membranes at −114°C. The measurement 2 A_{max} is shown and was used to obtain the values given in Table I.

The above results indicate that the amphipathic labels II–V in egg PC membranes experience an environment that has a polarity intermediate between water and ethanol. The spin label groups of II–V must be in a position between the membrane exterior and the position occupied by the stearate label I(12,3).

Location of amphipathic spin labeled molecules in membranes

The EPR and ^{13}C spin-lattice relaxation data shown above for the spin-labeled stearates extends previous studies and provides a basis for determining the intramembrane location of voltage-sensitive spin labels. As stated above, our NMR results are in general agreement with previous results [18]; however, the location of the stearate labels is in need of some revision. Our ^{13}C relaxation enhancement profile for the stearate labels is skewed toward the membrane surface compared to the expected profile of the corresponding phospholipid acyl chain methylene segments (i.e. the profile

expected for an unlabeled acyl chain attached to a phospholipid [19]). For example, when I(1,14) was present in membranes, we did not find the relaxation enhancement maximum at or near the 16-methylene of the acyl chains as was found previously. Fig. 2C indicates that the spin label group attached to the 16 position of stearic acid is located at a wide distribution of positions. The distance between the aqueous-hydrocarbon interface and the most likely position of the 16 doxyl group is 2/3–1/2 times the distance between the interface and the 16 position of an unlabeled chain. Figs. 2A and 2B suggest that spin labels attached at the 5 and 10 positions are also located at distances that are closer to the membrane surface than the analogous acyl chain methylene positions. This is not necessarily a surprising result, since micelle and fatty acid monolayer studies have shown that spin label groups, even when attached to very non-polar moieties, often prefer to be located near the aqueous-hydrocarbon interface [22,23]. This result contrasts to that found for the position of some steroid labels in membranes. For the fluorescent probe 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD), and some spin labels attached to the hydrocarbon chain of steroids, the fluorescent and spin probes are found to reside deep in the hydrocarbon interior [17,24].

Several membrane studies have demonstrated that it may be inappropriate to extrapolate the behavior of membrane-bound spin labeled and fluorescent probes to analogous unlabeled membrane components [17,25,26]. The results obtained here indicate that assumptions regarding the location of the nitroxide on stearate labels should also be made with caution. For example, a recently introduced method for determining the intramembrane location of fluorescent probes is based on quenching these probes by phospholipids containing spin-labeled fatty acids [17]. In that work it was assumed, based mainly on internal consistency and previous work [18], that the spin label groups attached to phospholipid acyl chains resided at a position equivalent to that of the analogous unlabeled acyl chain methylene positions. Based on our results, this assumption is not rigorously correct; in addition, we expect a similar location whether the acyl chain is free, or part of a phospholipid [26,27]. The error introduced by this assumption is in any case not large. We estimate that a 30% decrease in the distance between any two of the spin label groups used for the determination of fluorophore depth would have resulted in a ≤ 2.5 Å change in calculated depth. This change is near the resolution limit of the method.

In previous work, we used ^1H nuclear Overhauser effects to examine the position of the hydrophobic ions tetraphenylphosphonium and tetraphenylboron when present in membranes at low concentrations (≤ 10 mol%). These ions were found to be located near the aqueous-hydrocarbon interface slightly below the level of the carbonyl group [13]. The location found in the present study for the spin label groups of the two hydrophobic ions, IV and V, is consistent with this finding. However, the position of the nitroxides attached to the two long chain amphiphiles is somewhat unexpected. Unlike the hydrophobic ions (IV and V) the alkylammonium is relatively membrane impermeable. It also measures surface potentials [2] and responds to potentials that are clearly distinct from those measured by the hydrophobic ions [1]. When membrane bound, the charge on the alkylammonium can apparently be screened by increasing ionic strength, whereas the charge associated with probes IV and V can not [11]. Therefore, based on these electrical differences, we expect the charge center for II to be located closer to the headgroup than the charge on probes IV or V. Surprisingly, the nitroxide of probe II resides in virtually an identical position to that for the nitroxides attached to IV or V. There are several ways of rationalizing these results. It is important to keep in mind that the nitroxide position does not represent the position of the amphiphile charge (this is obvious in the case of probe III). A bent-over conformation for the nitroxide moiety of the alkyammonium probe could leave the charge near the headgroup and place the nitroxide below the carbonyl. The nitroxide does appear to have an affinity for this region as is apparent from the nitroxide position for the sulfonate probe III (Fig. 3B). It is also possible that significant changes in the electrical behavior of these probes represent rather small changes in the position of their charges. That is, the electrical properties of the interface may

change dramatically over very short distances, leaving these probes roughly in the same position.

In conclusion, the ^{13}C spin-lattice relaxation technique used here appears to resolve the position of the nitroxide groups extremely well. This is due to the relative simplicity and magnitude of the interaction involved, and the large dispersion of the ^{13}C chemical shifts. The EPR hyperfine coupling constants provide a rough indication of polarity, and are entirely consistent with the NMR results. For stearic acid spin-labels, the nitroxide groups reside at distances significantly closer to the membrane solution interface than is expected for the unlabeled alkyl chains. The distribution of positions for the nitroxide group is also largest when it is attached to the end of the alkyl chain, consistent with statistical mechanical predictions. The positions of the nitroxide groups of two long chain amphiphilic probes and two hydrophobic ion probes were studied. Surprisingly, these nitroxides are in virtually identical locations just below the level of the carbonyl groups. This position is not inconsistent with other electrical and positional information obtained on these probes, but indicates that the nitroxide group may have a preference for this region of the membrane interface.

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